Technical University of Denmark

Investigations of equine alphaherpesvirus type 1 and 4 infections in a group of foals and their dams in Iceland

Master thesis in Veterinary Medicine

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Preface

This thesis is a final project of the Veterinary Medicine study at the Faculty of Health and Medical Science, University of Copenhagen. This project represents 30 ECTS points and is written from Technical University of Denmark (DTU), National Veterinary Institute, Section for Virology. The study was carried out at the Institute for Experimental Pathology, University of Iceland, Keldur (Keldur), Department of Virology and Molecular Biology, from September 2012 to March 2013. The project was financed by Keldur.

First of all I would like to thank my supervisors Vilhjálmur Svansson and Sigurbjörg Þorsteinsdóttir at Keldur for giving me the opportunity to work on this project. I am sincerely thankful to them for their excellent guidance, support, encouragement, patience and kindness. I would also like to thank my co-workers at Keldur for their advice and help. I especially thank Lilja Þorsteinsdóttir, Sigríður Jónsdóttir for their assistance, advices and endless patience, and Birkir Þór Bragason, Ívar Örn Árnason and Heiða Sigurðardóttir for their expert help with real-time PCR. They have all helped me to become a more independent scientist.
My supervisor Lars Erik Larsen at DTU I thank for his excellent guidance, support and motivation throughout the process.
My sincere gratitude goes to Technical University of Denmark to sponsor me by giving EHV-1 and EHV-4 DNA used as positive controls in the study. I would also like to thank Solvej Østergaard Breum at DTU for assistance with the real-time PCR.
Last but certainly not least I would like to thank my family and friends for all their patience and precious support through this work and throughout the years of my veterinary medicine study.

The thesis´ topic is the presence and prevalence of equine alphaherpesvirus type 1 and type 4 infections in a group of foals, during the first 20 months after birth, and their dams in Iceland. The topic was chosen on one hand on the basis of my interest in horses and horse diseases and on the other hand because the presence of these viruses has only been sporadically investigated with modern molecular techniques in the horse population in Iceland. The thesis appeals to veterinarians, virologists, veterinary students, horse owners in Iceland and other who are interested in the topic.

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Abstract

The horse population in Iceland is a special breed, isolated from other horse populations for at least 1000 years. This provides an exceptional opportunity to investigate old and new pathogens in an isolated horse population with few infectious diseases. Horses are hosts to three types of alphaherpesviruses, equine herpesvirus (EHV) 1, 3 and 4. Both EHV-1 and EHV-4 are endemic in horse population worldwide and are considered clinically, economically and epidemiologically among the most important pathogens in horses. Symptoms of EHV-1, as abort storms have never been observed and neurological symptoms are seldom seen in horses in Iceland and therefore it has been assumed that the country is free of EHV-1. EHV-4 cause respiratory disease, especially in young horses and is endemic in horses in Iceland.

Presence of EHV-1 and EHV-4 was determined using real-time PCR on 200 nasal swab samples, collected for 20 months from 10 foals and their dams in Iceland. The time kinetic of EHV-1 and EHV-4 infections in the foals was studied, i.e. when the foals became infected with the viruses for the first time and the infection of the dams followed at the same time. All samples in the study were negative for EHV-1. For EHV-4 6% of the 200 samples were positive. Three samples from three different mares were positive and nine samples from six different foals. The average age of first EHV-4 detection in the foals was 209.5 days or ca. seven month of age. The youngest foal was found positive at twelve days old and the oldest twenty months old. Three foals were found positive before and at one month of age.

Resumé

Heste populationen i Island er en special race, idet den har været isoleret fra andre heste populationer i mindst 1000 år. Dette giver en enestående mulighed for at undersøge gamle og nye patogener i en isoleret heste population med få sygdomme. Heste er værter for tre typer af alfaherpesvira, equine herpesvirus (EHV) 1, 3 og 4. Både EHV-1 og EHV-4 er endemiske i heste populationer over hele verden og det er antaget at de er klinisk, økonomisk og epidemiologisk blandt de vigtigiste patogener der findes i heste. EHV-4 er endemisk i den islandske hestabestand, hvor den forårsager lidelser i luftvejene hos især ungheste. Abortstorm og neurologiske forstyrrelser er typiske symptomer på infektion med EHV-1. Disse symptomer er ikke almindeligt forekommende blandt hestene i Island, hvorfor hestebestanden i landet antages at være fri for EHV-1.
Forekomsten af EHV-1 og EHV-4 blev bestemt ved brug af real-time PCR udført på 200 næsesvabre indsamlet fra 10 føl og deres mødre gennem en periode på 20 måneder.
EHV-1 og EHV-4 infektionernes forløb hos føllene blev undersøgt. Infektionsforløbet hos deres respektive mødre blev samtidig fulgt.
Alle prøverne i studiet var negative for EHV-1. For EHV-4 6% af de 200 prøver var fundet positive. Tre pøver fra tre forskellige hopper og ni prøver fra seks forskellige føl var positive. Gennemsnitalderen ved det første EHV-4 fund blandt føllene war 209,5 dage eller ved ca. syv månedes alderen. Det yngste føl, som blev fundet positiv for EHV-4, var 12 dage gammelt, og det ældste var 20 måneder gammelt Tre føl blev fundet positive før og ved ét måneds alderen.

Table of Contents

I. INTRODUCTION

1. The aims and background of the study

Equine alphaherpesviruses are endemic in horse population worldwide and are considered clinically, economically and epidemiologically among the most important pathogens in horses (Crabb & Studdert, 1995). They have only been sporadically investigated with modern molecular techniques in the horse population in Iceland. The examination of the EHV alpha-types is an interesting and useful task because of the isolation of the Icelandic horse population and their unique disease status.

The project was limited to examine the presence and prevalence of EHV-1 and EHV-4 infections in nasal swab samples from young horses in their first 20 months of age and their dams for six months after foaling. EHV-1 and EHV-4 real-time PCR was established. The time kinetic of EHV-1 and EHV-4 infections in the foals was studied, i.e. when the foals became infected with the viruses for the first time and the infection of the dams followed at the same time.

2. Hypotheses

Serological evidence for EHV-4 has been detected in blood samples from unweaned, unvaccinated foals in the first five weeks of life (Foote *et al*., 2006). In another study, where the aim was to investigate whether EHV-1 and EHV-4 could be detected in nasal swabs and blood samples from young foals from vaccinated mares, the youngest EHV-4 PCR positive foal was only 11 days of age (Foote *et al*. 2004). In the only publication on EHV-1 and EHV-4 in horses in Iceland, antibodies to both EHV-1 and EHV-4 in serum samples were found. EHV-4 specific antibodies were found in 99.5% of 207 tested horses and EHV-1 antibodies in 8% (Nordengrahn *et al*., 1998). However, no clinical signs of EHV-1, as abort storms and neurological symptoms have been seen indicating that EHV-1 is in the Icelandic horse population. Therefore Iceland has been assumed to be free of EHV-1. Because Nordengrahn *et al*. (1998) reported 8% prevalence rate of EHV-1 antibodies in horses in Iceland, it is a valid reason to examine it further. Inspite of that I did not expect to find EHV-1 in the nasal swab samples.

Based on this the following hypotheses were formulated and will be demonstrated in the project:

a. **EHV-4 will be detected in the nasal swabs samples from the foals around one month of age**
b. **EHV-1 will not be detected in the nasal swabs samples**

3. The outline of the master thesis

Background literature study is presented under the theoretical part, with focus on viruses in general and herpesviruses. Special focus is on Equine herpesvirus (EHV), especially the two types of alphaherpesviruses EHV-1 and EHV-4, their clinical symptoms, epidemiology, diagnosis, treatment and latency. At last is a historical chapter about the origin and diseases of the Icelandic horse.

The material and methods used in the study are described and the results of the study are presented. Finally is general discussion, conclusions and perspectives.

II. THEORETICAL PART

1. Viruses

1.1 Classification

Viruses infecting animals are small infectious agents, usually 20 to 300 nm. They are composed of nucleic acid that is surrounded by a protein coat, called capsid, and in addition some have a lipid membrane, called envelope. The nucleic acid, they contain, is either DNA or RNA or both. Unlike bacteria and fungi, viable host cells are required for viruses to replicate and some viruses have an affinity for particular cell types (Quinn *et al*., 2009). Four characteristic can be used to classify all viruses into orders, families, genera and species. These are: nature of the nucleic acid in the viron (DNA or RNA), symmetry of the capsid that can either be helical or icosahedral, presence or absence of an envelope and the size of the virion and capsid (Flint *et al*., 2004).

1.2 Genetic material

Viruses are categorized into three main groups on the basis of their nucleic acid composition: DNA viruses, RNA viruses and viruses which use both DNA and RNA for replication (Quinn *et al*., 2009). Genomes of viruses can be single-stranded or double-stranded. Most DNA viruses contain double-stranded genomes but most RNA contains single-stranded genomes (Flint *et al*., 2004; Quinn *et al*., 2009). The genome can be linear or circular, segmented, a single molecule, positive-, negative- or ambisense (refers to nucleic acid polarity) and it can be gapped. The type of the genetic material reflects how the virus replicates (Murphy, 1996; Quinn *et al*., 2009).

The central event in replication of viruses is the synthesis of viral proteins by the host cells and requires a production of viral mRNA from their nucleic acid. Some viruses replicate inside the host nucleus and can use the host cell´s transcriptase to synthesize their mRNA. Others use their own enzymes to generate mRNA. Then they can use the host cell´s ribosome to translate mRNA to proteins (Flint *et al*., 2004; Roizman & Palese, 1996; Quinn *et al*., 2009).

Baltimore classification is a system to classify viruses based on the nature and polarity of the genome and on the pathways viruses use to synthesize mRNA. The viruses can be grouped into seven classes after this classification, table 1 and figure 1. The theme is to generate +ssmRNA (positive-sense, single-stranded mRNA) (Flint *et al*., 2004; Roizman & Palese, 1996; Shors, 2011; Quinn *et al*., 2009).

Table 1: The Baltimore classification

I.	dsDNA viruses
II.	ssDNA viruses
III.	dsRNA viruses
IV.	+ssRNA viruses
V.	–ssRNA viruses
VI.	RNA reverse transcribing viruses
VII.	DNA reverse transcribing viruses

ds: double-stranded
ss: single-stranded
+: positive-sense
-: negative sense

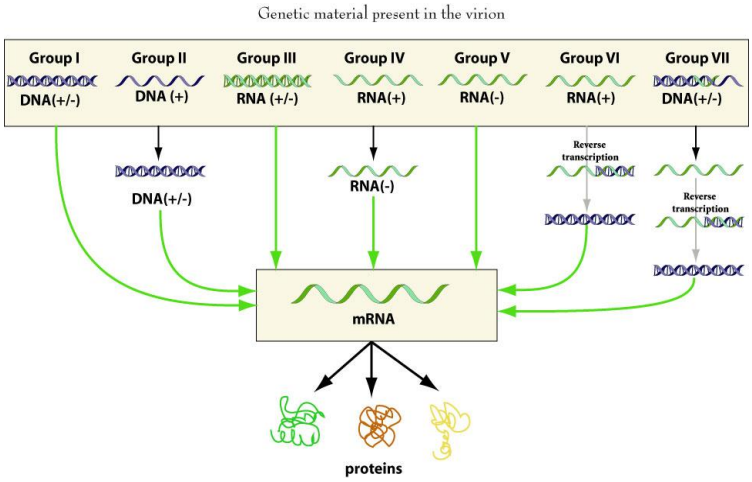

Figure 1: The Baltimore classification system
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1.3 Structure

Viruses come in a great variety of shapes and sizes, determined by requirements for the function of the virus. The size range from 20 nm to 300 nm in diameter. The genome of vertebrate viruses is enclosed with a shell of proteins, called a capsid. The capsid is made of viral structural protein units, called capsomers, arranged symmetrically around the viral genome and makes a very stable structure. Icosahedral or helical symmetries are the two dominant types of capsid symmetry in viruses, figure 2 (Shors, 2011, Quinn *et al*., 2009).

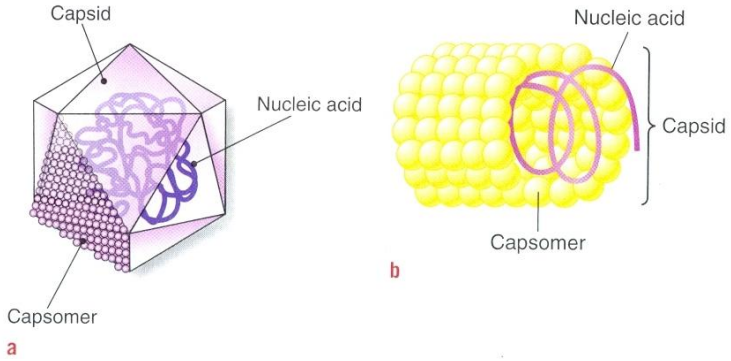

Figure 2: a) Icosahedral symmetry b) Helical symmetry
Shors, 2011

The icosahedral symmetry has 20 equilateral triangular faces and 12 vertices. At its simplest it has 60 identical structural units, three in each triangular face and forms a closed sphere like virus structure. The helical symmetry can have a rotation axis and forms open, two-dimensional cylindrical structure. Helical capsids may be rigid or flexible depending on the arrangement of the protein subunits (Flint *et al*., 2004; Harrison *et al*., 1996).

In many animal viruses the nucleocapsid, the packaged form of the genome in the capsid, is surrounded by an envelope. The envelope is generated when virus buds from the host cell and is composed of a lipid bilayer, in many cases from the membrane of the host cell, and viral proteins embedded in the lipid, figure 3. Most of the virus proteins that are embedded in the lipid membrane are glycoproteins, which are conjugated proteins that contain oligosaccharide

chains. These glycoproteins are associated with binding to receptors on the host cell causing membrane fusion and entry of the virus into the cell (Harrison *et al*., 1996; Shors, 2011; Quinn *et al*., 2009).

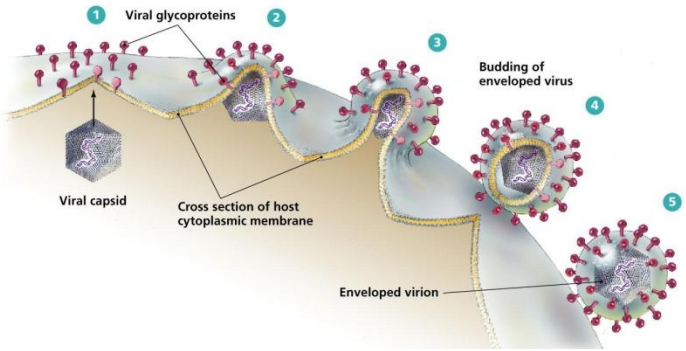

Figure 3: Virus budding from host cell and generating an envelope Copyright © 2006 Pearson Education Inc. Publishing as Benjamin Cummings

2. Herpesviruses

There are today approximately 100 species known of the order *Herpesvirales* according to the International Committee on Taxonomy of viruses (ICTV) (http://www.ictvonline.org). Herpesviruses have a wide spectrum of vertebrate animals as hosts and most animal species have at least one herpesvirus (Roizman, 1996). Fish, amphibians, reptiles, birds and mammals including humans are susceptible to herpesvirus infection (Davidson, 2009). Most herpesviruses are species specific i.e. are restricted to one species, but there are also known herpesviruses with broad host range (Flint *et al*., 2009).

2.1 Classification

The taxonomy of herpesviruses has recently been updated by the ICTV. The former family *Herpesviridae* has been made into the order *Herpesvirales*, which has been divided into three families; *Alloherpesviridae*, that infects fish and frogs, *Herpesviridae*, that contains the mammal, bird and reptile viruses and *Malacoherpesviridae* that contains a bivalve virus (Davidson *et al*, 2009). The family *Herpesviridae* is then divided into three subfamilies based on host range, reproductive cycles, cytopathology and genome structure (Reed & Toribio, 2004). These subfamilies are: *Alphaherpesvirinae* (α) with five genera and 37 species, *Betaherpesvirinae* (β) with four genera and 14 species and *Gammaherpesvirinae* (γ) with four genera and 32 species (http://www.ictvonline.org; Davidson *et al*, 2009). The viruses of the subfamily *Alphaherpesvirinae* have a wide and variable host range, a short reproductive cycle, spread rapidly, destroy host cells and often establish latent infections in sensory ganglia. The viruses of *Betaherpesvirinae* have restricted host range, a long reproductive cycle, spread slowly, cause infected cells to enlarge and can be in latent form in secretory glands, lymphoreticular cells, kidneys and other tissue. The viruses of *Gammaherpesvirinae* have a narrow host range, a slow reproduction cycle and are latent in T- and/or B-lymphocytes (Roizman, 1996; Quinn *et al*., 2009).

2.2 Genetic material

Herpesviruses contain linear double-stranded DNA (dsDNA), but it becomes circular when released from the capsids into the nuclei of infected cell (Roizman, 1996). The genomic size can vary from approximately 110 to 250 kbp. The viruses with smaller genome are found among the alphaherpesviruses, whereas the larger genomes are those of the betaherpesviruses. The guanine (G)-cytosine (C) base composition varies from 32% to 75 % (Davison, 1992; Roizman, 1996). An interesting feature of the herpesvirus genome is their sequence arrangement where many have repeated sequences at the terminal, internal or both. The five major types of herpesvirus genome structure are shown in figure 4. Group 1 has the simplest genome arrangement where large sequence from one terminal is directly repeated at the other terminal. The genome of Equine herpesvirus 2 (EHV-2) is that of group 1. The group 2 genome arrangement is similar to that of group 1 except a variable number of repeats are at the genome terminal (number of repeats can vary). Group 3 differs from group 2 by the internal repeats as well to the terminal repeats. Group 4 has a terminal repeat which is repeated in an inverted orientation internally and divides the genome into a long and short unique sequence. EHV-1, EHV-3 and EHV-4 have genomes in this group. In group 5 sequences from both terminals are repeated in an inverted orientation and juxtaposed internally, dividing the genome into two parts, each of which consists of unique sequences flanked by inverted repeats (Davison, 1992; Roizman, 1996).

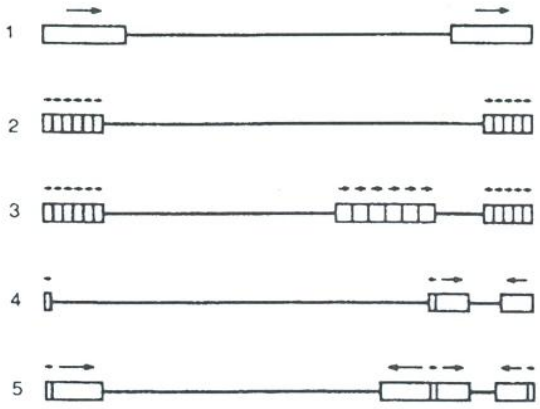

Figure 4: The five major types of herpesvirus genome structure
Unique regions are shown as horizontal lines. Repeats are shown as rectangles and their orienations are indicated by arrows. Davison, 1992

A hallmark of herpesvirus genomes is that they encode most of the enzymes required to increase the pool of nucleotides in a cell and to replicate the DNA genome. This feature allows the virus to replicate in slowly dividing or nondividing cells such as neurons (Davison, 1992).

2.3 Structure

The size of herpesviruses range from 120 nm to 200 nm in diameter. They contain their linear double-stranded DNA within an approximately 100 nm icosahedral capsid. A layer of amorphous material, the tegument, surrounds the nucleocapsid. The tegument consists of at

least eight different viral proteins. Outermost is the envelope that incorporates a large number of glycoproteins, figure 5 (Flint *et al*., 2004; Harrison *et al*., 1996; Quinn *et al*., 2009). The envelope must be intact for herpesviruses to be infectious. The envelope is extremely sensitive to damage and therefore the virus is usually transmitted through direct contact with mucosal surfaces or secretions, e.g. from the mouth, nose or genitals, of an infected creature (Shors, 2011).

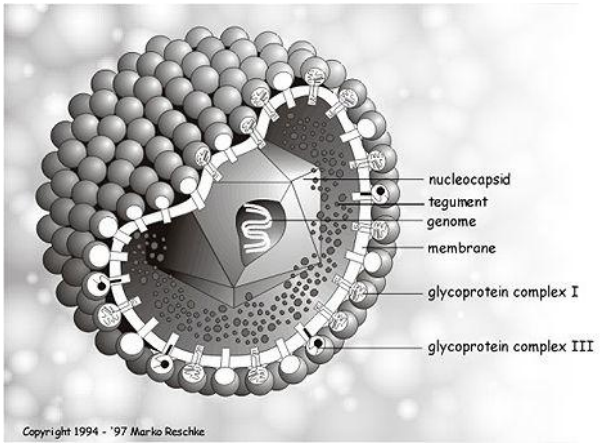

Figure 5: Structure of herpersvirus
Coptright © 1994-´97 Marko Reschke

2.4 Glycoprotein B

Glycoprotein B (gB) is the most abundant component of the virion envelope and the most highly conserved glycoprotein among the members of the *Herpesviridae* family (Dunowska *et al*., 2000; Pereira, 1994). Homologues of gB are encoded by the genomes of all the herpesviruses (Pereira, 1994). Herpesvirus gB is essential for virus growth and involved both in virus penetration, promoting fusion of the virion and plasma membrane, and spread between cells. It has also been shown to be a major target for the immune response (Dunowska *et al*., 2000; Neubauer *et al*., 1997; Pereira, 1994).

2.5 Replication cycle

The replication cycle of a herpesvirus can be divided into a number of stages: attachment and entry into the host cell, uncoating of the viral nucleic acid, replication of viral nucleic acid and synthesis of virus specific proteins, assembly and release of newly formed viruses from the host cell. This process can vary between herpesvirus species, the target cell and other factors.

2.5.1 Attachment and entry into the host cell

A virion, the infected form of virus, must first attach to cell surface receptors in order to produce infection (Quinn *et al*., 2009). Because herpesviruses are enveloped viruses they must fuse their envelope with the lipid membrane of the cell to enter. For this the herpesviruses use the glycoproteins, embedded in the envelope, to bind to receptors on the target cell. Herpesviruses require at least three glycoproteins for fusion and, in some cases, an additional receptor binding glycoprotein (Heldwein & Krummenacher, 2008).
The glycoproteins are glycoprotein B (gB), glycoprotein H (gH) and glycoprotein L (gL). Glycoprotein D (gD) serves as the receptor binding glycoprotein (Gianni *et al*., 2006).

In many cases attachment of viruses to cells leads to irreversible changes in the structure of the virion (Roizman & Palese, 1996). Penetration of the virus into the target cell occurs almost directly after the attachment (Roizman & Palese, 1996).

2.5.2 Uncoating of viral nucleic acid

Uncoating occurs after the penetration and is a process whereby the viral genome is released in a form suitable for transcription. In cells infected with herpesviruses some tegument proteins and the nucleocapsid are released into the cytoplasm as a result of membrane fusion. The nucleocapsid is transported by microtubules from the site of entry to nuclear pores. At the nuclear pores a virus specific function releases the viral DNA into the cell nucleus. The empty shell of capsid and tegument proteins disintegrates. When the viral genome enters the nucleus it circularizes (Roizman & Palese; 1996).

2.5.3 Replication of viral nucleic acid and synthesis of virus specific proteins

Herpesviruses replicate in the nucleus of the host cell. The viral DNA is transcribed by cellular DNA-dependent RNA polymerase (transcriptase) to form mRNA. The transcriptional program consists of at least three cycles of transcription (Roizman & Palese; 1996), in a specific order, where three classes of viral genes are expressed: Immediate-early (α), early (β) and late (γ) genes, and the mRNA is transported to the cytoplasm where it is translated to proteins. Then the proteins are transported to the nucleus, figure 6. The α genes encode DNA binding proteins that play a role in viral transcription of β genes. The β genes encode proteins that function in DNA replication and production of substrates for DNA synthesis and additional viral transcription factors. Some β proteins have function in the cytoplasm but others are transported to the

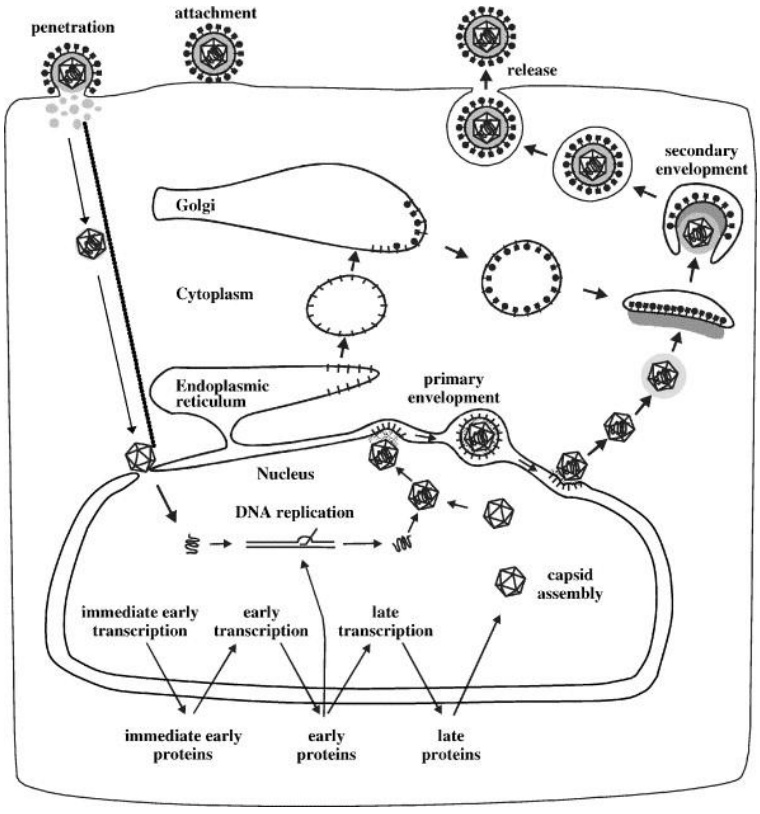

Figure 6: Replication cycle of herpesviruses
Mettenleiter, 2002.

nucleus. The γ genes encode the late structural proteins that are produced after viral genome replication has begun. They are needed for virus assembly and particle egress (Flint *et al*., 2004; Shors, 2011). After the γ proteins are synthesized, some are transported into the host cell nucleus, where capsid assembly occurs and DNA packaging. Other γ proteins are inserted into the membranes of the rough endoplasmic reticulum and become glycosylated. They are further modified and processed in the Golgi apparatus to become glycoproteins. Mature glycoproteins are then transported to the cell plasma membrane (Flint *et al*; 2004).

2.5.4 Assembly of newly formed virus particles and release from host cell

In the nucleus newly replicated viral DNA is packed into nucleocapsids, and some tegument proteins wrapped around it (Flint *et al*., 2004; Shors, 2011).

Viral membrane formation is a double envelopment process. The nuclear envelope is composed of inner and outer nuclear membrane separated by the perinuclear space and connected by nuclear pore complexes (Farnsworth *et al*., 2007). The nucleocapsids together with the tegument bud through the inner nuclear membrane into the perinuclear space and thereby become enveloped. Then they are deenveloped when fusing with the outer nuclear membrane. In the cytoplasm they acquire a second envelope in the trans-Golgi network and are then reenveloped (Farnsworth *et al*., 2007; Flint *et al*., 2004; Shors, 2011). As the nucleocapsids bud into the trans-Golgi network they acquire also a complete tegument layer. The enveloped virus particle then buds into a vesicle that is transported to the cell plasma membrane for release by exocytosis (Flint *et al*., 2004). Complete cell death is a result of cellular injuries that occur as a result of viral replication and cellular responses to infection (Shors, 2011).

2.6 Latent infection

Once an individual has become infected with a herpesvirus, the virus remains in the body for life. Herpesviruses have evolved so called latent infection, an efficient survival strategy in which the virus can remain dormant for periods of time, hiding within the cells of the body. By that they can evade the host´s immune system (Flint *et al*., 2004; Roizman, 1996; Shors, 2011; Studdert, 1996). Latent infection of alphaherpesviruses occurs primarily in the cell body of neurons in sensory ganglia (Flint *et al*., 2004; Rock, 1991) but beta- and gammaherpesviruses are latent in other cell types (Shors, 2011). Following infection of alphaherpesvirus, it replicates at a mucocutaneous surface before entry into nerve axons. Direct uptake into axons may occur if the infecting dose of virus is sufficiently high. Virus is then transported retrograde to cell bodies of neurons in sensory and autonomic nerve ganglia,

and in some cases to the central nervous system. In the cell body the viral DNA remains dormant within the cytoplasm or nucleus until reactivated (Rock, 1991).

Reactivation of a latent virus must occur so the virus can spread. The reactivation can be spontaneous or induced by stimulation changing neuronal physiology. This includes stress factors such as transportation, adverse weather condition, overcrowding, infection, trauma, hormonal changes, immunosuppression, ultra violet irradiation, pregnancy and lactation, excessive fatigue, malnutrition, etc. (Flint et al., 2004; Rock, 1991; Shors, 2011). How the reactivation process is launched is poorly understood, but during the reactivation the virus is transported along the neuron axon to an epithelial cell surface, of the infection site. There the replication is finished and the infection can be transmitted to other susceptible hosts. Recurrent clinical symptoms of infection can develop following viral reactivation (Rock, 1991).

Alphaherpesviruses are extremely lytic to their host cell, but regulate their lytic replication cycle in neurons and do not replicate there during latency (Rock, 1991). Most neurons neither replicate their DNA nor divide (Flint *et al*., 2004).

The viral genome of latent herpesviruses is usually in a circular form in neurons (Roizman, 1996; Roizman & Sears, 1996). Estimations using human herpes simplex virus type 1, suggest that most latent infected neurons contain 20-30 copies of the viral genome (Rock, 1991). A balance between regulators of viral and cellular gene expression is necessary for the stability of the genome (Flint *et al*., 2004). Over 70 gene products are transcribed in productive herpesvirus infection but only one gene is transcribed in latent infection, producing latency-associated transcripts (LATs) (Ahmed *et al*., 1996; Flint *et al*., 2004). The LAT expression is important during latency and possibly early reactivation. It has been hypothesized that LATs have an anti-apototic function (block apoptosis) in infected cells. Others suggest that the LATs play a role in maintaining viral latency by acting as antisense RNAs to immediate early gene (ICP0). ICP0 is a crucial transcription factor for blocking viral replication. Although a number of earlier studies showed that mutations in LAT region had no effect on the level of latent viral DNA, a small deletion in the LAT promoter alone can cause reduction in the number of latent infected cells. This indicates that the transcription of the LATs is crucial for establishing latency (Kent *et al*., 2003).

3. Equine herpesviruses (EHV)

Eight herpesviruses have been identified in equids: five of them belong to the subfamily *alphaherpesviriane* and belong to the genus *Vallaricellovirus,* and three to the *gammaherpesvirinae and* are members of the genus *Percavirus*. No *betaherpesviriane* have been described for equids (http://www.ictvonline.org; Davidson *et al*, 2009). The horse (*Equus caballus*) is the natural host to alphaherpesvirus types 1 (EHV-1, Equine abortion virus), 3 (EHV-3, Equine coital exanthemavirus), 4 (EHV-4, Equine rhinopneumonitis virus) and gammaherpesvirus types 2 (EHV-2) and 5 (EHV-5). EHV-1 has occasionally infected domestic cattle and captive camelids and cervids. Therefore it seems as EHV-1 has a wider and more variable host range (Allen *et al*., 2004).

The donkey (*Equus asinus*) is the host to asinine herpesvirus type 3 (AHV-3) that is homologue to EHV-1, asinine herpesvirus type 1 (AHV-1) homologue to EHV-3 and asinine gammaherpesvirus type 2 (AHV-2) homologue to EHV-2, see table 2 (Crabb & Studdert, 1995; Patel & Heldens, 2005; Reed & Toribio, 2004). Recently a virus serologically related to EHV- 1 was isolated from a *Gazella Thomsoni* in a Japanese zoological garden, (Gazelle herpesvirus 1, GHV-1 or EHV-9). This virus gave neurological symptoms such as spasms and rotation (Fukushi *et al*., 1997). In addition the virus was observed in guinea pigs and caused abortions and stillbirths (Wohlsein *et al*., 2011). Meningoencephalitis in a polar bear with neurological symptoms caused by EHV-9 has also been demonstrated by PCR (Donovan *et al*., 2009). This indicates the presently known equid herpesviruses are likely to expand (Fukushi *et al*., 1997).

Table 2: Herpesviruses of some members of the family Equidae

Virus	Common name	Subfamily
Equus caballus		
EHV-4	Equine rhinopneumonitis	α
EHV-1	Equine abortion virus	α
EHV-3	Equine coital exanthema virus	α
EHV-2	Slow-growing equine herpesvirus	γ
EHV-5	Slow-growing equine herpesvirus	γ
Equus asinus		
EHV-8/AHV-3	Related to EHV-1	α
EHV-6/AHV-1	Related to EHV-3	α
EHV-7/AHV-2	Related to EHV-2	γ
Other		
EHV-9/GHV-1	Related to EHV-1	α
Equus grevyi (Zebra)	Zebra herpesvirus isolates	

3.1 Alphaherpesviruses: EHV-1 and EHV-4

Both EHV-1 and EHV-4 are endemic in the horse population worldwide and are considered clinically, economically and epidemiologically among the most important pathogens in horses. Infections by the two herpesviruses have been recognized for over sixty years as significant and impediments to the breeding, competition and recreational horse industries. The economic losses and negative impact on equine welfare caused by these viruses are the results of morbidity or mortality from virus induced abortion, respiratory disease, neurological disorders or death of fulltermed new born foals.

EHV-1 and EHV-4 are closely related viruses and before 1981 were considered a single virus type, or two subtypes of the same virus, known as EHV-1. It was assumed that this "EHV-1" caused respiratory disease, established a latent infection and when reactivated caused abortion in pregnant mares. Examination of restriction endonuclease DNA fingerprints then showed that EHV-1 was comprised of two distinct viruses, EHV-1 and EHV-4 (Crabb & Studdert, 1995). These two viruses are antigenically and genetically highly cross reactive (Crabb &Studdert, 1995) and their genomes are similar in structure (Crabb & Studdert 1996). Data based on the nine genes sequenced for both viruses showed 65% to 85% homology in nucleotides and 58% to 89% homology in amino acids (Crabb & Studdert, 1996).

3.1.1 EHV-1

The alphaherpesvirus EHV-1, also known as equine abortion virus, has been identified in horses in all countries (Crabb & Studdert, 1996; Harless & Pusterla, 2006; Reed & Toribio, 2004). It has been assumed that Iceland is the only country in the world to be free of EHV-1.

EHV-1 causes abortion, respiratory disease and myeloencephalopathy. EHV-1 is the most common viral cause of abortion in mares and the cause of abortion "storms" (where many mares abort), resulting in major financial losses to the horse breeding industry. Over the last years, research into the role of EHV-1 in abortion revealed that the detection rate of the virus varies between 4.5% and 8.9% (data obtained in the UK and the USA) (Léon *et al*., 2008). EHV-1 is most commonly recognized as a cause of abortion in mares that do not show any other clinical sign, figure 7. They usually abort in late gestation, at sixth to eleventh months. EHV-1 infection is followed by latent

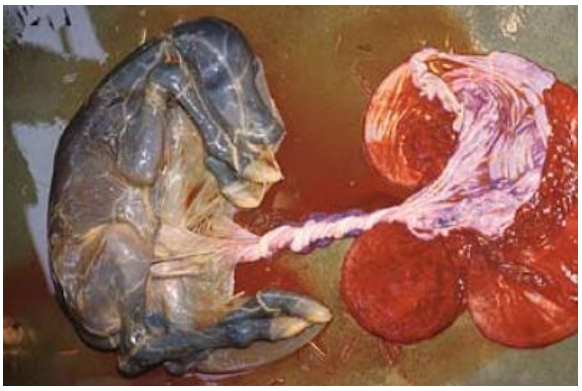

Figure 7: Aborted fetus and placenta
http://www.thehorse.com/free-reports

infection and a mare may therefore abort months or years after primary infection (Crabb & Studdert, 1996).

EHV-1 is also associated with mild, respiratory disease and fever in adult horses, while it causes more moderate to severe respiratory disease in the juvenile or immunological naive horses (Wilsterman *et al*., 2011).

EHV-1 can cause equine herpesvirus myeloencephalopathy (EHM) which can affect 10% of adult infected horses in outbreaks (Wilsterman *et al*., 2011). Although EHM is a sporadic and relatively uncommon manifestation of EHV-1 infection, it can cause devastating losses and have a severe impact on the horse industry (Pusterla *et al*., 2009). The spinal cord is the part of the central nervous system that becomes most affected during EHM. Resulting in ataxia, weakness and can progress to paresis requiring euthanasia (Pusterla *et al*., 2009; Wilsterman *et al*., 2011). In addition the manifestation can be urinary incontinence (Pusterla *et al*, 2009). Prevention is difficult because many horses are latently infected, allowing the virus to circulate silently in horse populations, and currently available vaccines do not confer protection against the neurological manifestations of the infection (Pusterla *et al*, 2009).

The entire EHV-1 genome has been sequenced and is about 150 kbp with 56.7% G+C base composition (Allen et al., 2004). It has been demonstrated that single nucleotide polymorphism resulting in variation in a single amino acid position of the viral DNA polymerase is responsible for differing pathogenic effect. Molecular epidemiology studies have determined differences between neuropathogenic strain and a non-neuropathogenic strain of EHV-1 indicating occurrence of distinct strains of EHV-1 that circulate in the field. There is evidence that certain strain groups are geographically restricted, being recovered predominantly from outbreaks occurring in either North America or Europe. Variation of a single amino acid of the DNA polymerase is strongly associated with neurological versus non-neurological disease outbreaks (Goodman *et al*., 2007).

3.1.2 EHV-4

The alphaherpesvirus EHV-4, also known as equine rhinopneumonitis virus, are like EHV-1 found in most equine populations worldwide (Crabb & Studdert, 1996; Harless & Pusterla, 2006; Reed & Toribio, 2004). EHV-4 is endemic in Iceland, and majority of adult horses in Iceland are antibody positive (Nordengrahn *et al*., 1997). EHV-4 is a major cause of acute respiratory disease and most horses become infected during the first two years of life. The symptoms seen in EHV-4 infection are fever, anorexia, nasal discharge, enlargement of mandibular lymph node and mucosal inflammation of upper respiratory tract, figure 8 (Crabb

& Studdert, 1996). Lesions occur on the mucus membranes of the upper, and sometimes lower, respiratory tract and may also involve the lungs. Secondary bacterial infection can induce pneumonia and a mucopurulent nasal discharge, especially with streptococci of Lancefield group C which is most damaging (Crabb & Studdert, 1995; Crabb & Studdert, 1996). In older horses it is assumed that some episodes of respiratory disease represent reactivation of either EHV-4 or EHV-1 (Crabb & Studdert, 1995). Infections with EHV-4 occur throughout the year, whereas EHV-1 infections occur predominantly in the winter season (Patel & Heldens, 2005).

Figure 8: Nasal discharge in equine respiratory disease caused by EHV-4 or -1 Allen *et al*., 2004

It is assumed that reactivation of EHV-4 from latency causes recurrent disease and is accompanied by virus shedding and transmission either directly or indirectly to other horses. Thereby there is usually each year in the annual foals born on a farm, a round of EHV-4 respiratory disease (Crabb & Studdert, 1995).

The complete genome for EHV-4 has been sequenced, 145 kbp with 50.5% G+C base composition. The genomic structure of EHV-4 and EHV-1 is the same, with internal and terminal repeats, group 3 in figure 4 (Allen et *al*., 2004).

3.1.3 Epidemiology

For both EHV-1 and EHV-4 the initial portal of viral entry into the horse is the upper respiratory tract. Primary infection occurs via direct horse to horse contact and inhalation of aerosolized virus. Shared water sources and other fomites contaminated by respiratory secretions may also play role in transmission. However the virus is short lived in the environment (Allen *et al*., 2004; Harless & Pusterla, 2006; Reed & Toribo, 2004). EHV-1 may also be transmitted via the semen of naturally infected stallions at mating or by artificial insemination (Hebia-Fellah *et al*., 2009).

Both viruses are highly contagious, with infection rates approaching 100 percent. Though are several influencing factors for infection such as: age, season, geographic distribution, compromised immune status and high horse stocking concentration (Allen *et al*., 2004).

The epidemiological life styles of both EHV-1 and EHV-4 are characterized by: widespread infection of young, susceptible horses with overall low clinical morbidity; high prevalence of latently infected carrier horses and the frequent shedding of infectious virus from such carrier

that allows efficient and uninterrupted transmission of virus to new generations of horses (Allen *et al*., 2004). Most infections with these viruses are sporadic, but outbreaks can occur annually in concentrated horse populations, especially in horses under two years of age. Naive horses exposed for the first time to EHV-1 and EHV-4 may release infectious virus into the respiratory mucus for as long as 15 consecutive days following infection. In such primary infections, the magnitude of virus present in nasal mucus may be as great as 10^6 pfu/swab for both EHV-1 and EHV-4. Shedding of virus from the respiratory tract of horses with previous virus exposure or after reactivation from latency is more transient (two to four days) and reduced in magnitude (10^2 to 10^5 pfu/swab) (Allen *et al*., 2004).

Prophylactic horses can be routine vaccinated and vaccines that are available are modified live or an inactivated whole virus equine herpesvirus 1 and 4 vaccine (Foote *et al*., 2006). The vaccination appears to reduce the severity of clinical signs, viremia and to decrease the likelihood of abortion (Quinn *et al*., 2009). It is not considered fully protective and therefore frequent boosters are recommended (Harless & Pusterla, 2006).

Foals are born with negligible quantities of serum immunoglobulins and relay on passive transfer of maternally derived antibodies for humoral immunity to common pathogens. Ingestion of colostrum during the first 12 to 24 hours of a foal´s life results in the passive transfer of high levels of maternal antibodies to the foal. Maternal antibodies may be present in the foal for 3-4 months in most cases and up to 6 months or more in some foals. Maternal antibodies appear to block the serologic response of most foals to inactivated EHV-1/4 vaccines until they are at least five months old (Harless & Pusterla, 2006).

Most serological tests can not differentiate between EHV-1 and EHV-4 antibodies due to the extensive antigenic cross reactivity between these viruses. Furthermore seroepidemiological studies can be complicated in horse population in which vaccination is practiced. Crabb and Studdert were the first to use a type-specific serological test to demonstrate the different, in a random sample of thoroughbred horses, where all were positive for EHV-4, but only 9% was positive for EHV-1. The serum used in their study was collected in 1967-1974 in Australia, prior to the first confirmed case of abortion there in 1977 (Crabb & Studdert, 1993). In a more recent survey, the seroprevalence of EHV-1 specific antibodies had increased to 30%, whereas the seroprevalence of EHV-4 specific antibodies was again 100% (van Maanen, 2002).

Gilkerson *et al*. (1999a) studied prevalence of EHV-4 and EHV-1 infections in mares and foals by using type specific ELISA on serum samples in Australia. They attempted to find if the dams are the source of EHV-1 infection for unweaned foals. They found EHV-1

antibodies in 26.2% of the mares but 11.4% in the foals, whereas over 99% of mares and foals tested were EHV-4 antibody positive.

An Australian study reports that EHV-1 infection of suckling foals can occur as early as 30 days of age (Patel & Heldens, 2005), but the average age in Gilkerson´s study of 229 foals was 123,9 days or 4 months (Gilkerson et al., 1999a). In another study, where the aim was to investigate whether EHV-1 and EHV-4 could be detected in young foals from vaccinated mares, the youngest EHV-1 PCR positive foal was 22 days of age, while the youngest EHV-4 PCR positive foal was only 11 days of age (Foote *et al*. 2004). In a longitudinal cohort designed study of 40 vaccinated mares and their unweaned, unvaccinated foals in the first five weeks of life, serological evidence was described in blood samples, indicating infection by both EHV-1 and EHV-4 (Foote *et al*., 2006). Pusterla *et al*. (2012) detected 33 of 130 (25%) horses that were under one year of age positive in qPCR.

In Gilkerson´s study seropositive foals for EHV-1 were 24% only 12 hours after colostrums ingestion, whereas 100% of foals had maternally derived EHV-4 antibodies at the same time. In that same study identification of new EHV-1 cases peaked at 90 days of age when over 80% of the foals were seropositive for EHV-1, indicating significant exposure and subclinical infection during the preweaning period. Unvaccinated mares from that same study, were 2.8 times more likely to be EHV-1 antibody positive than foals (Gilkerson et al., 1999a; Gilkerson et al., 1999b).

The studies described above show that EHV-1 and EHV-4 infections start early in life, were the foals become infected from their dams or from other lactating mares in the group, with subsequent foal to foal spread of infection prior to weaning (Gilkerson *et al*., 1999a). Reactivated latent virus in the mares is likely to be the source of such transmission (Patel & Heldens, 2005; Allen *et al*., 2004). The infection circulates in vaccinated populations of mares and their unweaned, unvaccinated foals and infection of young foals ensures the survival of EHV-1 and EHV-4 in the population (Foote *et al*., 2006). The results of these studies support the long standing management practice of separating pregnant mares from other group of horses to reduce the incidence of EHV-1 abortion (Gilkerson et al., 1999b).

3.1.4 Diagnosis

Differential diagnosis to EHV-1 and EHV-4 are infections with equine influenza virus, *Streptococcus equi* spp. *equi* and *Streptococcus equi* spp. *zooepidemicus*, equine arteritis virus, equine rhinovirus and equine adenovirus (Harless & Pusterla, 2006). Diagnosis of EHV-1 and EHV-4 can be based on rapid spread of the disease and the presence of the

clinical symptoms. A classical test such as virus isolation has been the gold standard for diagnosing EHV-1 and EHV-4 infections. The sample of choice is a nasopharyngeal swab which should be taken during the early stage in the febrile phase of respiratory infections and dispatched in sterile, cold viral transport medium. Virus isolation from an aborted fetus can also be performed. Virus isolation requires the maintenance of specific cell culture lines, making the process relatively expensive and time-consuming (Harless & Pusterla, 2006; Quinn et al., 2009).

The PCR offers an alternative to virus isolation and has proven to be a sensitive method of detecting EHV-1 and EHV-4 in nasopharyngeal swab, peripheral blood lymphocytes and other tissues. PCR is a nucleic acid amplification method and its advantage is it does not interfere with antibodies. Conventional single or nested PCR assays have inherent risks of carry over contamination due to postamplification steps required to detect PCR products. The real-time PCR have reduced the risk of contamination. PCR assays used in routine diagnostics are based on the detection of viral genomic DNA and are therefore unable to distinguish between different viral states, e.g. if the virus is lytic, non-replicating or latent. Real-time TaqMan PCR techniques have been developed that target several genes which allows discrimination between the different states (e.g. glycoprotein, latency-associated transcripts). Using this approach genomic DNA transcriptional activity of the target genes together with viral load can be measured (Harless & Pusterla, 2006).

Due to the high seroprevalence of the EHV-4 and the cross reactivity of antibodies between the two viruses, most serological tests do not distinguish infection with EHV-1 from EHV-4. However acute and convalescent serum samples have been useful in retrospective serodiagnosis but are only possible to use if showing significant increase (fourfold rise) in antibody titer in paired serum samples. Thereby they can be used for confirmation of a recent outbreak (Harless & Pusterla, 2006; Quinn et al., 2009; Crabb & Studdert, 1995). Virus neutralization tests, complement fixation and ELISAs are the most commonly used serological tests. Developments of type specific ELISA has allowed specific serodiagnosis of EHV-1 and EHV-4 infections and is capable of differentiating between the antibodies (Hartley *et al*., 2005; van Maanen, 2002).

3.1.5 Treatment

There is no specific therapy for EHV-1 and EHV-4 induced respiratory disease. Horses with respiratory signs and fever should get supportive care, minimized stress (as transport, competition and crowding) and stabled in well ventilated and low dust environment. Affected

horses should be rested four to six weeks after the symptoms are last noticed. Supportive veterinary treatment is giving intravenous fluid if dehydrated because of fever and anorexia. Non-steroidal anti-inflammatory drugs (NSAIDs) may decrease the degree of febrile episodes, improve appetite, decrease complications and hasten recovery (Harless & Pusterla, 2006). The use of NSAIDs has to be careful because many of the horses may be unable to drink, making dehydration a serious complication. Administration of corticosteroid, such as dexamethasone and prednisolone is also helpful (Reed & Toribio, 2004). Antibiotics are used to control secondary bacterial infections (Quinn *et al*., 2009). The use of acyclovir has been restricted to congenital EHV-3 and neurologic EHV-1 infections (Harless & Pusterla, 2006, Reed & Toribio, 2004).

In outbreaks the aim should be to reduce spread by infectious aerosols, direct contact and fomites, isolate affected horses and reduce stress that can reactivate latent infection (Harless & Pusterla, 2006). EHV-1 and EHV-4 are environmentally labile and infectivity is quickly destroyed by lipid solvents, detergents, heat and the common disinfectants available for veterinary use (Allen *et al*., 2004)

3.1.6 Latency

During lytic phase of infection, EHV-1 and EHV-4 initially replicate within respiratory epithelial cells and are subsequently drained into local lymphoid tissue, the submandibular, retropharyngeal and bronchial lymph nodes. Local lymphadenopathy occurs when the respiratory tract epithelial cells and lymphoid tissue are destroyed secondary to viral replication and budding. Whereas EHV-4 infections are generally limited to the respiratory tract, EHV-1 infections may spread systemically through the hematogenous dissemination of infected lymphocytes (cell-associated viremia). Local vasculitis and thrombosis may lead to abortion, neonatal mortality and EHM (Harless & Pusterla, 2006).

As all alphaherpesviruses, EHV-1 and EHV-4 appear to establish life-long latent infection. This capacity to persist in the body of the horse in a dormant, but potentially reactivatable, state after recovery from primary infection provides a biological reservoir of the two viruses (Allen *et al*., 2004). Reactivation and shedding of the viruses creates the opportunity for transmission to other horses which is considered important in the epidemiology and might explain why these diseases can occur in closed populations (van Maanen, 2002). EHV-4 typically results in the establishment of latent infection within the first weeks or months after birth (Pusterla *et al*., 2012).

There is evidence of EHV-4 latency sites in the neurons of the trigeminal ganglia, lymphoid

tissues and pulmonary epithelial cells of naturally infected horses, figure 9 (Borchers *et al*., 1997; Borchers *et al*., 1999; Pusterla *et al*., 2012). Latency sites for EHV-1 have been identified in neurons of the trigeminal ganglia, peripheral blood lymphocytes and in lymphoid tissues from horses with experimental induced recrudescence of disease (Borchers *et al*., 1997; Borchers *et al*., 1999).

In a study, where the prevalence of latent alphaherpesviruses in Thoroughbred racing horses was examined, the distribution of latent EHV-1 and -4 infections varied in different samples. The trigeminal ganglia were found to be most commonly infected. These results are in agreement with those of previous studies which found that the trigeminal ganglia, followed by lymphoid tissues (submandibular and bronchial lymph nodes), are common sites of latent infection for alphaherpesviruses. Though, in that same study, non- neuropathogenic EHV-1 strains were more frequently detected than neuropathogenic strains and supports that non-neuropathogenic EHV-1 strains are more prevalent in the horse population. That is consistent with the uncommen occurrence of EHM (Pusterla *et al*., 2012).

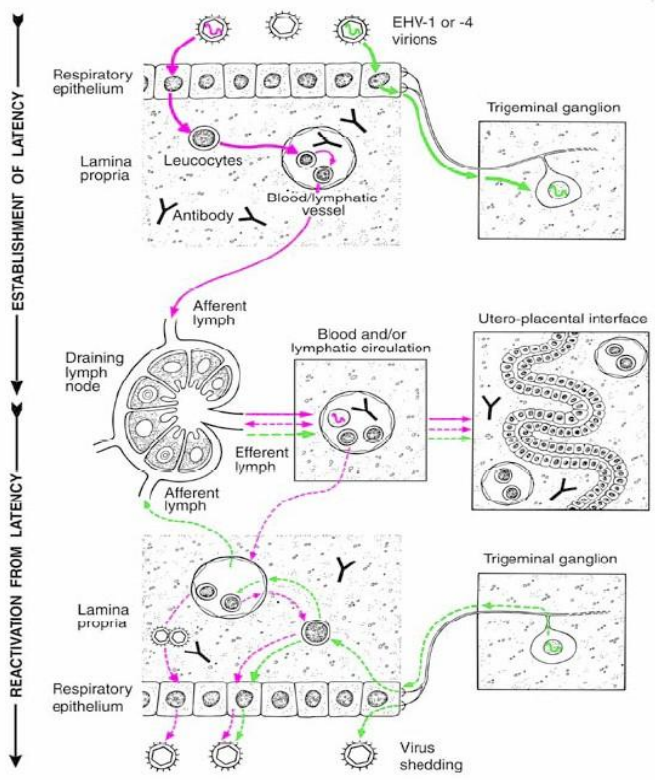

Figure 9: Diagrammatic model illustrating a) the route(s) proposed for establishment (*solid lines*) of EHV-1 and EHV-4 latency in either the trigeminal ganglion (*green lines*) or lymphocytes (*lavender lines*), and b) potential routes and consequences following reactivation (*broken lines*) of latent virus from either of these anatomic sites
Allen et al., 2004

3.2 Other herpesviruses in horses

3.2.1 EHV-3

The alphaherpesvirus EHV-3 is also known as equine coital exanthema virus (Crabb & Studdert, 1995; Studdert, 1996). EHV-3 causes equine coital exanthema (ECE) that is an acute, mild to severe disease characterized by the formation of papules, pustules, ulcers and scabs on the vaginal and vestibular mucosa, the modified skin of the penis and prepuce and on the skin of the perineal region, especially of the mare. Occasionally lesions can be seen on the skin of the lips, nares and mucous membrane of the upper respiratory tract. Secondary bacterial infection is common. ECE transmits venerally (Studdert, 1996; Seki *et al*., 2004). Infection can affect the fertility of stallions as they may refuse to serve mares when penile lesions are severe (Quinn *et al*., 2009).

Figure 10: Clinical symptoms resembling those after EHV-3 infection in a mare in Iceland
Picture from veterinarian Gunnar Örn Guðmundsson

EHV-3 has been isolated in many countries around the world (Seki *et al*., 2004) and clinical symptoms resembling those after a EHV-3 infection have been seen in horses in Iceland, figure 10 (Torfason *et al*., 2008). At the Institute for Experimental Pathology at Keldur, typical herpesvirus-like cytopathy were seen in equine primary kidney cells (EqFKC) when inoculated with sample from clinical case resembling coital exanthema and EHV-3 aetilogy of the infection was verified with PCR and sequencing. The distribution and prevalence of EHV-3 infections in horses in Iceland is not known (Personal communication with Vilhjálmur Svansson, 2013).

3.2.2 Gammaherpesviruses: EHV-2 and EHV-5

EHV-2 and EHV-5 were first described as cytomegalo- or betaherpesviruses, but from nucleotide sequence analysis of their genomic DNA they are known to be members of the genus *Percavirus* of the *Gammaherpesvirinae* subfamily (Allen & Murray, 2004). EHV-2 is endemic in all horse populations that have been tested and more than 90% of horses are seropositive. EHV-5 is also widespread but less common. Both viruses are common in the Icelandic horse population (Torfason et al., 2008). The two viruses have a strong serological cross reactivity and EHV-2 is in latent form in circulating B lymphocytes (Allen & Murray, 2004). EHV-2 and EHV-5 were first isolated from horses in Iceland, when peripheral blood mononuclear cells (PBMCs) from horses with infectious pyrexia were co-cultured on primary

equine fetal kidney cells, in search for the cause of the pyrexia which occurred in horses in Iceland in 1998-99 (Torfason *et al*., 2008).

EHV-2 and EHV-5 are slowly cytopathic. Clinical signs of EHV-2 are usually mild but can be associated with kerato-conjunctivitis, both upper and lower respiratory tract disease, pharyngitis, pneumonia, enlarged lymphnodes, fever and anorexia (Browning & Agius, 1996; Allen & Murray, 2004). Associations have been made with pharyngeal ulceration and loss of racing performance (Browning & Agius, 1996). The clinical symptoms are frequently seen in foals that become infected before sixth months of age, despite maternal antibodies. The upper respiratory tract and epithelia of the conjunctiva and cornea are thought to be the natural portals of EHV-2 entry (Allen & Murray, 2004).

Infection with EHV-5 occurs later than with EHV-2. EHV-5 has been detected in nasal swabs and peripheral blood leukocytes in young and adult horses with or without clinical signs of respiratory disease (Nordengrahn *et al*., 2002).

4. The Icelandic horse

4.1 The origin

The Icelandic horse is the only horse breed in Iceland. For more than 1000 years the Icelandic horse population has been pure bred (Torfason *et*. *al*., 2008). The first horses arrived to Iceland with the Viking settlers who mostly came from Norway in the ninth century. It is likely that the Icelandic horse originated from there, since the Icelandic and the Nordwegian Fjords breed and the Nordland/Lyngen horse, share a similar size and conformation (Aðalsteinsson, 2001; Arnórsson, 1997; Björnsson & Sveinsson, 2006; Sveinsson, 2010). The origin of the Icelandic horse have been examined using old references, antiquities of bones in comparisons with bones from present breeds, blood types examination and analysis of mitochondria, microsatellite and single-nucleotide polymorphism. These examinations have indicated that the Icelandic horse has diverse origin but is most related to the Shetland pony and some of the Scandinavian horse breeds (Aðalsteinsson, 2001; Sveinsson 2010; Bjørnstad *et al*., 2003; Vilá *et al*., 2001; Petersen *et al*., 2013). That can be explain by that Norwegians conquered the Shetland Islands around the year 800 and the Shetland pony was mixed with the Norwegian horse breeds (Aðalsteinsson, 2001).

There are also theories about relationship between the Icelandic horse and the Mongolian horse, because they are similar in size and have similar endurance (Aðalsteinsson, 2001; Sveinsson 2010). This relationship can exist through the Scandinavian horse breeds. New

approaches have revealed genetic association between northern European horse breeds, as the Icelandic horse, Nordwegian Fjords breed, the Nordland/Lyngen horse and horse breeds in the British Islets, and the Mongolian horse (Bjørnstad *et al*., 2003; Petersen *et al*., 2013).

4.2 Diseases

Since the Viking settlement there are no records of import of horses to Iceland until 1867 when an Icelandic gelding was re-imported from Denmark. Importation of horses has been prohibited by law since 1882 (Björnsson & Sveinsson, 2006; Torfason *et*. *al*., 2008). Due to the geographic isolation, the horses in Iceland are immunologically naive to various agents known to infect horses in other countries, such as equine influenza, EHV-1 and strangles (Svansson, 2004). This provides an exceptional opportunity to investigate old and new pathogens in this isolated population that only has few infectious diseases (Torfason *et al*., 2008).

Before the year of 1700 no records exist regarding the number of horses in Iceland. Today the Icelandic horse population is approximately 77.500 horses (Hagtölur landbúnaðarins). There have been several bottlenecks in the breeding history, e.g. in the years 1783-1785, when the horse population was reduced to fewer than 8.000 horses due to the volcanic eruption (Móðuharðindin) in Iceland (Sveinsson, 2010). Volcanic activity and harsh weather conditions have been the main scourges in the breeding history, rather than infectious diseases. Although these environmental factors may primarily be responsible for genetic selection in the past, infectious agents, that were able to persist in a close population, and came with the settlers, cannot be excluded. In modern times increased travel between countries and growing popularity of the Icelandic horse has brought new threats to the unique infectious status of the Icelandic horse population (Torfason *et al*., 2008).

Over 40 viral infections have been documented in horses but only few of these have been reported in horses in Iceland. Symptoms of equine papiloma virus infections are commonly seen in yearlings in Iceland. Berne virus was found to be endemic in the Icelandic horse population in a serological survey conducted in connection to an epidemic of infectious pyrexia in 1998. In the same epidemic, picornavirus-like particles were found in faeces samples by electron microscopy examination. The picornavirus-like virus was thought to be the primary cause of the outbreak (Vilhjálmur Svansson, 2013 personal communication). Presence of several respiratory viruses has been demonstrated in horses in Iceland. In 2004 equine adenovirus was identified for the first time when an outbreak of kerato-conjunctivitis occurred. Serological investigations have shown that equine rhinitis A and B viruses are

endemic in the horse population in Iceland. These equine rhinoviruses were probably introduced to the country in the early nineties. Antibodies to reovirus type 1 were detected for the first time during the epidemic of respiratory disease caused by ST-209 strain of *Streptococcus equi* spp. *zooepidemicus* in Iceland in 2010. As previously mentioned infections with equine gammaherpesvirus type 2 and 5 are common in the Icelandic horse population (Vilhjálmur Svansson, 2013 personal communication).

Clinical symptoms resembling infections with EHV-3 are known to occur and have been verified with PCR and sequencing. Only one report has been published about EHV-1 and EHV-4 in horses in Iceland. In that study antibodies to both EHV-1 and EHV-4 were found with type-specific ELISA. In the study serum samples from 207 horses in Iceland were chosen from stock at the Institute of Experimental Pathology, University of Iceland, Keldur. The horses were sampled in 1993 for export testing of antibodies to equine infectious anemia virus and the horses were no younger than one year of age. The prevalence rate of EHV-4 antibodies in the serum samples was 99.5% and of EHV-1 antibodies 8%. The authors assumed that the low prevalence of EHV-1 in the Icelandic horse population represents a closed population and with indications of low spread between different herds within Iceland (Nordengrahn *et al*., 1998).

Vaccination of horses is not practiced in Iceland. There have been no clinical signs of EHV-1 infections, as abort storms and neurological symptoms that have indicated that EHV-1 is present in the Icelandic horse population. Therefore it is generally accepted that Iceland is free of EHV-1 infections.

On several occasions, since 1990, samples from horses expressing neurological or respiratory signs have been tested for presence of EHV-1. Most of these testing have been done by serology with few exceptions, have virus isolation or PCR been employed. In some of the serum samples, low EHV-1 antibody titer was found by virus neutralization test or complement fixation test.

From 2008 an ongoing surveillance program has been established were sera from 50-60 selected stallions that have been in contact with numerous horses are tested in EHV-1/EHV-4 specific ELISA test (Svanova DK). In this program the sera are also screened for antibodies to equine influenza virus and equine infectious anaemia virus. With one exception, EHV-1 antibodies have not been detected in these samples (Vilhjálmur Svansson, 2013 personal communication).

III. MATERIALS AND METHODS

1. Study population and samples

1.1 The horse population

Nasal swabs from 10 healthy mares and their foals were used in the study. The mares were from diverse places in Iceland born in the years from 1999 to 2007. From February to June 2011 the mares were kept at the Institute for Experimental Pathology, University of Iceland at Keldur in limited contact to other horses. May-June 2011 they foaled. After foaling they were transported to the farm Kálfsstaðir in Skagafjörður in North-Iceland where they were with a stallion until September. After that they were in contact with 20-30 other horses of all ages on the farm. The horses at Kálfsstaðir are in frequent contact with other horses in the Skagafjörður region. Furthermore there is a close connection between Kálfsstaðir and Hólar University College for equine studies (http://www.holar.is) where there are stabled 200 to 300 horses from all over Iceland. In January 2012 the foals were weaned and in February the mares were exported to Cornell University, Ithaca, New York, USA (http://www.cornell.edu).

1.2 The samples

Nasal swabs (Coban®) were collected from the 10 mares and their foals, figure 9. Pre-colostrum samples was taken immediately after foaling and before colostrum intake, called Day 0. Further samples were taken from all the pairs 12 days after foaling and thereafter every month until the foals became six months old. After that the samples were taking every third month and the last samples were taken when the foals became 20 months old. Weaning of the foals was at seven months age and sampling from the mares stopped six months after foaling. Clinical symptoms at the sampling days were not recorded systematically. The samples were stored at -80°C at the Institute for Experimental Pathology, University of Iceland at Keldur.

Figure 11: Author taking a nasal swab sample from one of the young horses at month fifteen

1.3 The positive controls

Positive DNA controls for EHv-1 and EHV-4 DNA were used. The positive controls were a gift from the National Veterinary Institute of Technical University of Denmark (Veterinærinstituttet, Danmarks Tekniske Universitet, DTU). The EHV-1 DNA was extracted from a horse serum. Prior to extraction the serum was verified EHV-1 positive by virus isolation in rabbit kidney cells (RK13) and equine embryonic lung cells (EEL). The EHV-4 DNA control was a cell culture suspension (one passage EEL cells) infected with EHV-4. The control DNA was isolated with QIAmp DNA Mini Kit (Qiagen) after manufacturer´s instruction.

2. DNA

2.1 Isolation of DNA

DNA was isolated from the nasal swabs with High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer´s protocol. A cell free supernatant from the nasal swab samples was used. Nasal swab, 200 µl, was mixed with 200 µl binding buffer supplemented with poly(A) and 50 µl Proteinase K and incubated for 10 min at 72°C. Then mixed with 100 µl binding buffer and centrifuged for 1 min at 8.000 x g. The flowthrough was discarded and 500 µl of Inhibitor removal buffer added and centrifuged for 1 min at 8.000 x g. The flowthrough discarded, 450 µl of Wash buffer added and centrifuged again for 1 min at 8.000 x g. This step was repeated and then centrifuged again for 10 sec at maximum speed (13.000 x g). The flowthrough discarded and 50 µl Elution buffer added and centrifuged for 1 min at 8.000 x g. The purified viral DNA/nucleic acids were kept at -80°C until used.

2.2 DNA quantification

Quantification and quality assessment of the DNA was carried out in NanoDrop® ND-1000 Spectrophometer (NanoDrop Technologies, Inc.) according to manufacturer´s user manual.

2.3 Primers and probes

Primers used for polymerase chain reaction were designed with the Primer 3 program (http://primer3.wi.mit.edu/) within the glycoprotein B (gB) gene.

The forward primer for EHV-1 is located in the gene from base 1 to 20 and the reverse primer from base 857 to 876. The forward primer designed for EHV-4 is located in the gene from base 1 to 20 and the reverse primer from base 809 to 828.

Primers and probes for the gB gene used in the real-time polymerase chain reaction were from

a known EHV-1 and EHV-4 sequences and have been described in a published article (EHV-1: GenBank accession number: NC001491; EHV-4: GenBank accession number: NC001844) (Pusterla *et al*., 2011). All primers and the probe for EHV-1 were from TAG Copenhagen. The probe for EHV-4 was from Applied Biosystems. All primers and probes are listed in tables 3 and X.

2.4 Polymerase Chain Reaction (PCR)

The PCR is a nucleic acid amplification method. The method is based on the use of different temperature in three steps of the reaction. These steps are denaturation where bonds are broken in the double stranded DNA, creating single strands of DNA that are susceptible to copying; annealing where the primers (forward and reverse primer) binds to the DNA; and extension where the Taq polymerase enzyme adds DNA nucleotides and makes two double stranded DNA molecules from each one double stranded DNA molecule that was denatured. These steps are usually repeated 25-40 times to get enough amplification of the gene fragment that lies between two specific designed primers.

The PCR reactions were performed in 20 µl reaction volume with Taq DNA polymerase and 10x Taq buffer (New England BioLabs) in PTC-200 DNA Engine Thermal Cycler (MJ Research). The PCR primers are listed in table 3.

Table 3: Sequences of PCR primers

Virus	Primer name	Primer sequences (5´→3´)	Location in gB	Ampicon size (bp)	Tm	GC %
EHV-1	Forward	ATGTCCTCTGGTTGCCGTTC	1-20	876	59,4°C	55
EHV-1	Reverse	CGTTGACGTGTAGTGCCTCC	857-876	-	61,4°C	60
EHV-4	Forward	ATGTCCACTTGTTGCCGTGC	1-20	828	59,4°C	55
EHV-4	Reverse	AGACGTGGTGTCGTTAGTGG	809-828	-	59,4°C	52,6

Table 4: PCR reaction solution

Reaction Component	Volume per sample
DNA	1 µl
10 x Taq Buffer	2 µl
10 x dNTP mix (2 mM)	2 µl
Forward primer (20 µM)	1 µl
Reverse primer (20 µM)	1 µl
Taq polymerase	0,1 µl
ddH_2O	12,9 µl
Total	20 µl

Table 5: PCR program
Steps 2-4 were repeated 40 times

Step 1 (denaturing)	94°C	4 min
Step 2 (denaturing)	94°C	10 sec
Step 3 (annealing)	45-60°C	30 sec
Step 4 (elongation)	72°C	30 sec-1 min
Step 5 (elongation)	72°C	7 min

The temperature in step 3 depended on the primer´s melting point. In step 4 the time depended on the size of the PCR fragment being amplified (30 seconds for each 500 base amplified).

2.5 Electrophoresis on agarose gel

PCR products were run on a 1% agarose gel, smaller DNA fragments (>200 bp) on a 2% agarose gel. Gels were made with "Agarose Basic" (AppliChen GmbH). Agarose was dissolved in 0.5x TBE buffer (Tris borate-EDTA; 0.045 M Tris borate and 0.001 M EDTA, with pH 8.0). The agarose was melted in the TBE buffer and fragments dissolved, and then cooled down to 50-60°C and 1-3 drops of ethidium bromide (10 mg/ml) (Sigma) added. One tenth volume of restriction buffer, 10x (RSB) (50% glycerol, 15 mM EDTA, 0.25 bromophenol blue), was added to the PCR product before loading onto the gel. Electrophoresis was carried out at 60-75 V for 40-60 min, depending on the fragment size, with 2-log DNA ladder (New England BioLabs) and with TBE buffer, 0.5x was used as a running buffer. The PCR products were visualized under UV light in a White/UV Transilluminator (UVP, UK), photographed using IN Genius, Syngene Bio Imaging and viewed in GeneSnap (Syngene).

2.6 DNA extraction

DNA extraction from agarose gel was done with a QIAquick Gel Extraction kit (QIAGEN) according to the manufacturer´s protocol. DNA bands were visualized under UV light and cut from the gel.

2.7 TOPO cloning

Cloning was performed with TOPO TA Cloning® Kit for sequencing with pCR®4-TOPO®Vector (Invitrogen). Partial sequence (876 and 828 bp) of the gB gene from the EHV-1 and EHV-4 DNA from DTU were cloned into the vector and used as positive controls for the real-time PCR assay. The parts had been amplified with the primers listed in table 3. The TOPO cloning reaction; 3 µl of PCR product, 1 µl of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$), 0.5 µl TOPO vector and 1.5 µl ddH_2O, were mixed gently and incubated at room temperature for 5-30 min depending on the PCR products size. One Shot® TOP10 competent cells were used for transformation. The cells, kept in from -80°C, were put directly on ice, the TOPO cloning reaction mixture added to the cells and incubated on ice for 30 min. Heat-shock transformation was done at 42°C for 30 sec, cooled on ice for 2 min, 250 µl of SOC

medium (Super Optimal Broth medium with added glucose) was added and pre-cultured at 37°C for one hour. The transformation mixture was then spread on LB agar plates containing 100 µg/ml ampicillin, and cultered at 37°C overnight. Colonies were picked and cultured in 3 ml of LB amp medium (100 µg/ml ampicillin) overnight shaking at 37°C and tested in PCR for successful inserts.

2.8 Isolation of plasmids

Plasmids were isolated with miniprep or midiprep kits from Qiagen (QIAprep® Spin Miniprep Kit and Quiagen® Plasmid Midi Kit). Minipreps colonies were cultered in 3 ml of LB amp (100 µg/ml ampicillin) medium overnight, shaking at 37°C. Midipreps colonies were cultured in 2 ml of LB amp for 8 hours, shaking at 37°C, then the culture was added to 50 ml of LB amp and cultered overnight, shaking at 37°C. Mini- and midiprep were made according to the manufacturer´s instructions. Plasmid DNA was collected in 30 µl (miniprep) or 100 µl (midiprep) elution buffer (EB) and stored at -20°C.

2.9 Restriction enzyme digestion

Restriction enzyme digestion was performed to cut out the cloned fragments from the vector. From the sequence of the pCR®4-TOPO® vector the restriction sites were evaluated. The restriction enzyme EcoRI was found to be optimal for both the EHV-1 and EHV-4 at both sites of the sequences. That enzyme did only cut at the restriction sites of the vector but neither the EHV-1 nor EHV-4 sequence.

Table 6: The solution for the restriction enzyme digestion

Component	Volume
DNA	*1-2 µl
EcoRI buffer x10	2 µl
EcoRI retriction enzyme	1 µl
ddH_2O	15-16 µl
Total	20 µl

*The volume depends on the DNA quantification

The reaction was incubated at 37°C overnight and inactivated at 65°C for 20 min. The reaction was electrophoreses on agarose gel to see if the restriction digestion worked. Extraction of the DNA fragment from the agarose gel was done as described before. Tenfold dilutions were made of the extracted DNA in ddH_2O and were used as standard curves for real-time PCR.

2.10 Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction assay, also called quantitative PCR or qPCR, was performed with TaqMan assay quantification using StepOnePlus™ Real-Time PCR System (Applied Biosystems) to determine the amount of a gene target during each amplification cycle of PCR.

Every TaqMan assay consists of two standard PCR target specific primers that define the region to amplify. Between the primers is a Taqman probe. The probe is labeled with two dyes, a reporter dye (FAM) at the 5´end and a quencher dye at the 3´end. The quencher dye at the 3´-end quenches the reporter dye. The TaqMan assay is based on the 5´-3´ exonuclease activity of the Taq DNA polymerase that extends primer and displaces and digests the TaqMan probe during PCR amplification. When the TaqMan probe is degraded the two dyes are separated and the reporter dye is no longer quenched and can therefore be detected. Increasing amounts of the reporter dye can be detected after each amplification cycle (Pusterla *et al*., 2006).

The EHV-1 and EHV-4 assays used in present study were according to Pusterla *et al*. 2006 with minor modifications. First the assay was performed for EHV-4 with AgPath Mix (AgPath-ID™ One-Step RT-PCR kit, Applied Biosystems) and probes labeled with black hole quencher (BHQ) dye at the 3´end, purchased from TAG Copenhagen. This combination gave unacceptable cycle threshold (CT) value for the ddH_2O negative controls. The value supposed to be undetected, showed all always some values from 31-39, while CT-values in the standard curve were at ca. 19-29. The results were therefore not reliable.

To exclude possible contamination of the reaction components, the work process was changed. The ddH_2O and pipettes were replaced. After preparation of the reaction mix the DNA was added to the qPCR tubes in a separate laboratory and the positive control was added last. New primers from TAG Copenhagen were bought and the AgPath mix was replaced for TaqMan® Universal PCR Master Mix (Applied Biosystems). At least new TaqMan probe, for EHV-4, with Minor Groove Binder (MGB), which is nonfluorescent quencher (NFQ), at the 3´end (Applied Biosystems), were bought. The CT-values in the EHV-4 assay for the ddH_2O controls was first acceptable when the new TaqMan probes with MGB quencher came in use. Anyhow some ddH_2O controls still showed some values from 35 to 40.

The DNA was in duplicate on the qPCR plate and the ddH_2O negative controls were in triplecate. Standard curves were made with tenfold dilution (10^{-5}-10^{-8}) both for EHV-1 and EHV-4 of positive controls and used to calculate the amplification efficiency, table 13.

Positive samples were tested twice. The results used in the study were from reactions with TaqMan® Universal PCR Master Mix (Applied Biosystems), BHQ-probe and TaqMan probe with MGB respectively for EHV-1 and EHV-4. The real-time primers and probes are listed in table 7.

Table 7: Sequences of qPCR primers and probes

Virus	Primer name	Primers sequences (5´→3´)	Location in gB	Amplicon size (bp)	Tm	GC %
EHV-1	Forward	TATACTCGCTGAGGATGGAGACTTT	387-411	90	61,3°C	44
EHV-1	Reverse	TTGGGGCAAGTTCTAGGTGGTT	455-476	-	60,3°C	50
EHV-4	Forward	CGCAGAGGATGGAGACTTTTACA	384-406	78	60,6°C	47,8
EHV-4	Reverse	CATGACCGTGGGGGTTCAA	443-461	-	58,8°C	57,9
	Probe name					
	EHV-1	5´-Fam-ACACCTGCCCACCGCCTACCG-BHQ-1-3´			67,6°C	71,4
	EHV-4	5´-Fam-CTGCCCGCCGCCTACTGGATC-BHQ-1-3´			67,6°C	71,4
	EHV-4	5'-Fam-CTGCCCGCCGCCTACTGGATC-MGBNFQ-3´				71,4

Table 8: Real-time PCR reaction solution with the TaqMan Universal PCR Master Mix

Reaction Component	Volume per sample
DNA	2 µl
TaqMan Universal PCR Master Mix (2x)	12,5 µl
Forward primer (20 µM)	0,2 µl
Reverse primer (20 µM)	0,2 µl
Probe (20 µM)	0,04 µl
ddH_2O	5,06 µl
Total	20 µl

Table 9: Real-time PCR reaction solution with the AgPath Mix

Reaction Component	Volume per sample
DNA	2 µl
25x RT-PCR Enzyme Mix	1 µl
2x RT-PCR Buffer	10,0 µl
Forward primer (20 µM)	0,2 µl
Reverse primer (20 µM)	0,2 µl
Probe (20 µM)	0,04 µl
ddH_2O	6,56 µl
Total	20 µl

Table 10: Temperature cycling

Step 1 (holding stage)	50°C	2 min
Step 1 (polymerase activation)	95°C	10 min
Step 2 (denaturing)	95°C	15 sec
Step 3 (annealing and elongation)	60°C	1 min

Cycle steps 2-3 were repeated 40 times

2.11 Sequencing

DNA sequencing was performed using the BigTye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and sequencing capillary electrophoresis was carried out on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

The PCR products were purified with gel extraction before sequencing. For the sequencing 1-3 ng/µl were needed for 100-200 bp products.

Table 11: Reaction solution

Reaction Component	**Volume per sample**
DNA	1 µl
BigDye	1 µl
Buffer x5	1,5 µl
Primer (2 µM)	1,6 µl
ddH_2O	Up to 10 µl final volume

Table 12: PCR program

Step 1 (denaturing)	95°C	5 min
Step 2 (denaturing)	95°C	20 sec
Step 3 (annealing)	50°C	15 sec
Step 4 (elongation)	60°C	4 min

Cycle steps 2-4 were repeated 30 times. Step 3 depends on primer melting point

Prior to sequencing was 40 µl of 75% isopropanol added to the PCR product, mixed briefly, incubated at RT for 15 min and centrifuged at 20.800 x g for 30 min. The supernatant was discharged immediately and 100 µl of 75% isopropanol, centrifuged at 20.800 x g for five min and the supernatant discarded. The pellet was dried at 90°C for 1 min, dissolved in 15 µl Hi-Di™ Formamide (Applied Biosystems), mixed briefly and heated at 95°C for two min. Then mixed briefly again and centrifuged at 20.800 x g for few seconds. Results from sequencing were analyzed in Sequencher™ 4.8 program from Gene Codes Corporation.

IV. RESULTS

1. Generation of the standard curve

By sequencing the positive controls were confirmed to be true EHV-1 and EHV-4. Partial sequences of the gB gene from the EHV-1 and EHV-4 positive controls were cloned into a vector and then excised by restriction enzyme digestion. Electrophoreses on agarose gel was done to see if the restriction digestion worked, figure 12. Extraction of the DNA fragment from the gel was done and the standard curve for the qRCR assay was then generated on tenfold dilutions of the extracted DNA. The standard curve was tested in qPCR before the samples were examined, and electrophoreses on agarose gel was done for confirmation of the standard curve, figure 13.

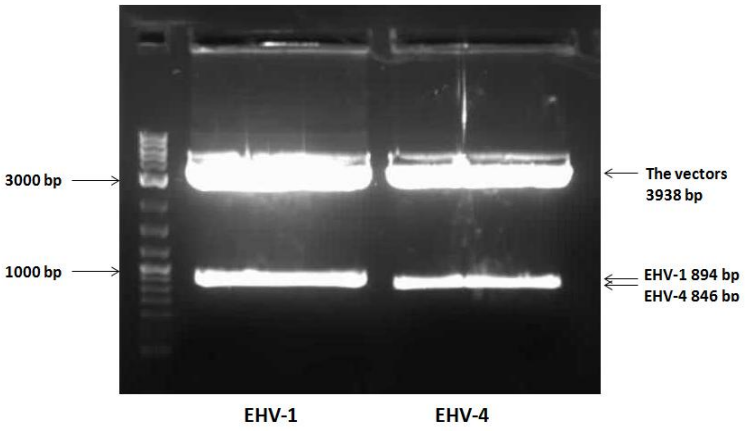

Figure 12: Electrophoreses of EHV-1 and EHV-4 positive DNA controls

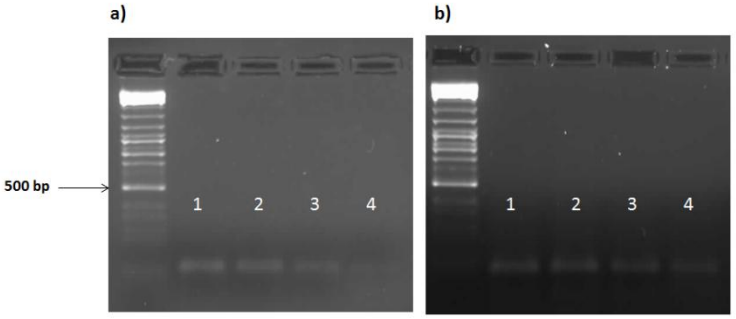

Figure 13: Electrophoreses of the dilutions used for the standard curve
a) EHV-1: 1. 10^{-4}, 2. 10^{-5}, 3. 10^{-6}, 4. 10^{-7}. b) EHV-4: 1. 10^{-5}, 2. 10^{-6}, 3. 10^{-7}, 4. 10^{-8}

A working solution of 20 ng/µl was made from the gel extractions of the EHV-1 and EHV-4 gB fragments. The number of viral DNA copies was found for the EHV-1 and EHV-4 gB gene fragment, used for the standard curve, with a special calculator (http://www.endmemo.com/bio/dnacopynum.php). Two µl of the working solution included approximately 4 billion viral DNA copies. Based on that it was known how many copies of DNA were in each dilution, table 13.

Table 13: Number of viral copies in 2µl of each dilute together with estimates of the mean CT value

Dilutions	Number of viral copies	CT value
1	$4*10^{10}$	-
10^{-1}	$4*10^{9}$	7,6
10^{-2}	$4*10^{8}$	9,6
10^{-3}	$4*10^{7}$	13,4
10^{-4}	$4*10^{6}$	16,7
10^{-5}	$4*10^{5}$	19,8
10^{-6}	$4*10^{4}$	23,4
10^{-7}	4.000	26,2
10^{-8}	400	29,9
10^{-9}	40	32,3
10^{-10}	4	34,5

The amplification efficiency of the gB gene assay was calculated from the slope of the standard curve. The amplification efficiency varied from 95 to 115 % between reactions and the regression coefficient (R^2) was around 1 for both EHV-1 and EHV-4. The standard curve slope, made from tenfold dilution, for EHV-4 is shown in figure 14.

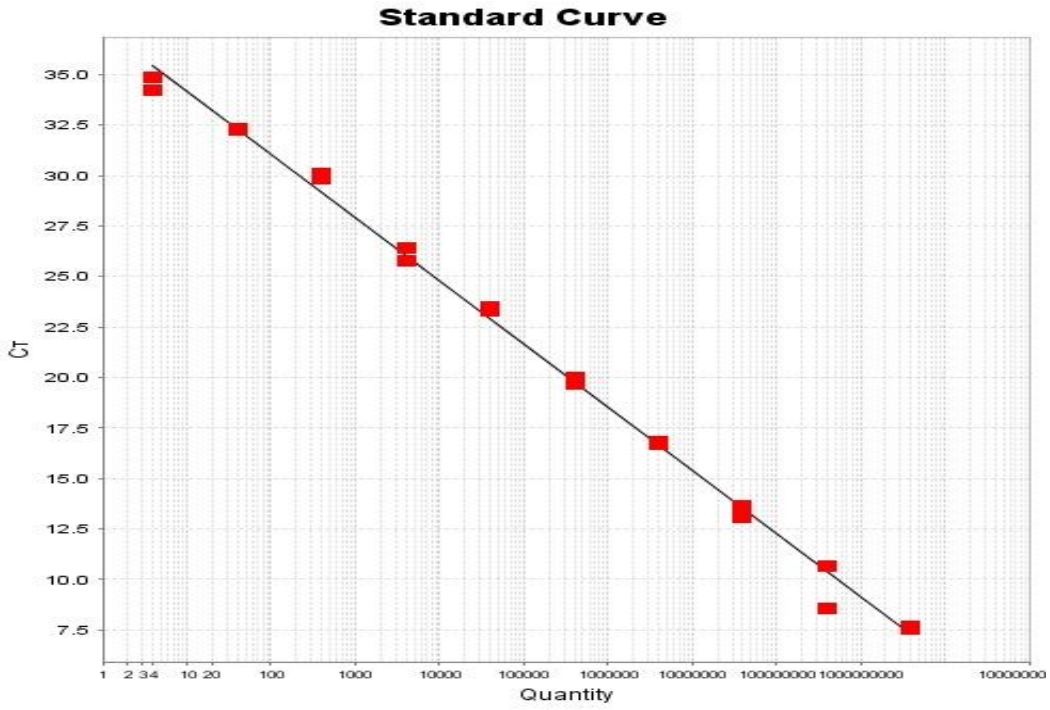

Figure 14: Standard curve for EHV-4
Tenfold dilution (10^{-1}-10^{-10}). R^2 = 0.995. Efficiency%= 108.9. y =0.995x + 37.296.

2. Detection of EHV-1 and EHV-4 positive samples in qPCR

To exclude possible cross reactivity of the primers, PCR was performed with the published primers from a known EHV-1 and EHV-4 sequences, used for qPCR, and the positive controls of EHV-1 and EHV-4. The primers for EHV-1 were used on EHV-4 DNA and vice versa. No cross reactivity was detected.

In the qPCR assay a positive reaction is detected by a fluorescent signal. The CT (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). CT value is a relative measure of the concentration of the target gene in the qPCR reaction. CT-values are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the CT-value the greater the amount of the target nucleic acid in the sample). In this study the qPCR amplification was repeated 40 times.

Table 14: CT-values for qPCR

CT-values ≤29	Are strong positive reactions indicative of abundant target nucleic acid in the sample
CT-values of 30-35	Are positive reactions indicative of moderate amounts of target nucleic acid
CT-values of 36-40	Are weak reactions indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination

CT values of the ddH_2O controls were almost always undetected, as they should be, when the qPCR assay for EHV-1 was performed. But it was not always the case when the assay was performed for EHV-4. The CT-values varied from 35-40 in ca. 33% (ca. one of each triplicate on each qPCR plate) of the ddH_2O controls. Therefore the DNA samples that showed some reactions in 35-40 were not reliable. It was decided to have a cut-off value at CT-value 35, every reaction in 35 and above was assumed as negative.

3. EHV-1

Nasal swabs samples from the ten foals and their dams were tested for presence of EHV-1 DNA. For the foals the nasal swab samples were 120, 12 from each foal. The samples were collected at day 0, day 12 and months 1, 2, 3, 4, 5, 6, 9, 12, 15 and 20 after birth. All the samples from the foals were negative for EHV-1 in qPCR.

For the mares 80 samples were collected, eight from each mare. The samples were collected at day 0, day 12 and months 1, 2, 3, 4, 5 and 6 after foaling. All the samples from the mares were negative for EHV-1 in qPCR.

4. EHV-4

The same samples were tested for presence of EHV-4 DNA. Of the 80 samples from the mares, over the sampling period of six months, three mares (3.75%) were tested positive for EHV-4, all in month two (mares number 2, 3 and 15). The positive mares had CT values from 32.46 to 34.63, table 15, or $10^{3.80}$- $10^{4.20}$ viral DNA copies/swab.

Of the 120 samples from the foals, over the sampling period of 20 months, nine samples (7.5%) were positive for EHV-4 in qPCR or six (F1, F2, F4, F6, F10 and F13) of the ten foals at least once. Foal number 1 was tested positive once in month fifteen. Foals number 2 and 10 were positive twice in months one and two. Foal number 4 was also tested positive twice on day twelve and in month fifteen. Foals number 6 and 13 were positive once in month twenty and nine respectively. The positive foals had CT values from 25.60 to 34.90, table 15, or $10^{4.00}$ - $10^{6.95}$ viral DNA copies/swab. Few foal samples, or four, had CT values over 35, and were not considered valid.

Once, both the mare and her foal were found positive at the same time (M2 and F2 at month 2). In the same month most samples were also found positive for EHV-4 (41.67% of positive samples). Figure 15 shows the number of samples from the foals detected positive and negative in qPCR at each month.

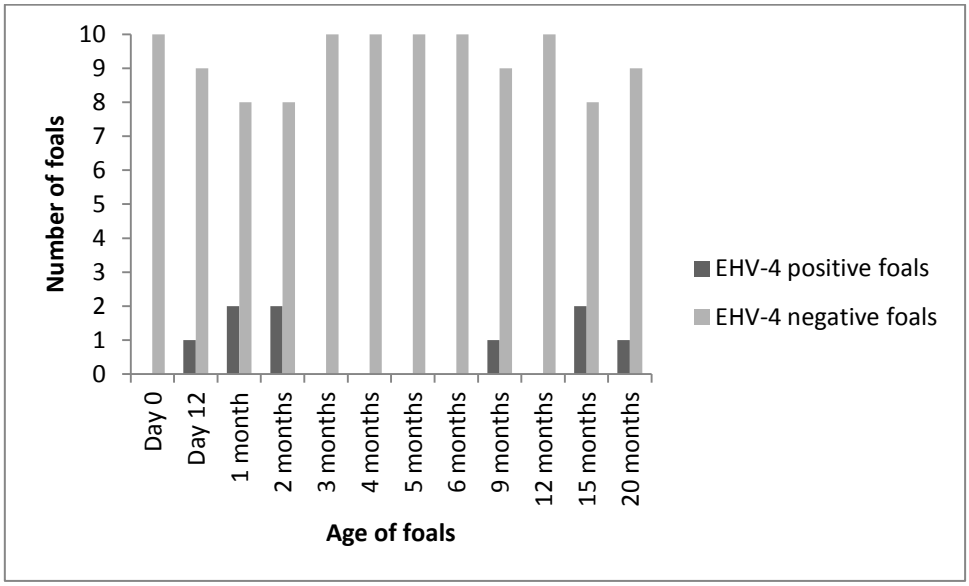

Figure 15: Number of EHV-4 positive and negative foal samples in each sampling time point

The average age of first EHV-4 detection was 209.5 days (ca. 7 month old). The youngest foal sampled that was found positive was twelve days old (one foal), while the oldest was twenty months (one foal).

The total prevalence of the samples from both the mares and the foals found positive for

EHV-4 in the qPCR was 6%. Positive samples were tested twice for confirmation. When all the positive samples were tested together the ddH_2O controls were negative.

Table 15 displays the outcomes from all the 200 nasal swab samples when they were tested for EHV-4 in qPCR. The positive results are shown with the CT values from the assay were all the positive samples were tested together. Values in red color are above the cut off value at 35.

Table 15: EHV-4 detection by qPCR in nasal swabs

Mare nr.	Born	d 0	d 12	1 m	2 m	3 m	4 m	5 m	6 m	9 m	12 m	15 m	20 m
M1	2006	-	-	-	-	-	-	-	-				
M2	2005	-	-	-	34.62*	-	-	-	-				
M3	2007	-	-	-	34.31	-	-	-	-				
M4	2006	-	-	-	-	-	-	-	-				
M6	2003	-	-	-	-	-	-	-	-				
M10	2004	-	-	-	-	-	-	-	-				
M11	2004	-	-	-	-	-	-	-	-				
M12	2006	-	-	-	-	-	-	-	-				
M13	2003	-	-	-	-	-	-	-	-				
M15	1999	-	-	-	32.46	-	-	-	-				
Foal nr.	**												
F1	June	-	-	-	-	-	-	-	-	-	-	33.50	-
F2	June	-	-	34.28	33.90	-	-	-	-	-	-	35.30	35.89
F3	June	-	-	-	-	-	-	-	-	-	-	37.35	-
F4	May	-	33.68	-	-	-	-	-	-	-	-	25.60	-
F6	May	-	-	-	-	-	-	-	-	-	-	-	34.40
F10	June	-	-	34.90	34.05	-	-	-	-	-	-	-	-
F11	May	-	-	-	-	-	-	-	-	-	-	-	-
F12	May	-	-	-	-	-	-	-	-	-	-	-	-
F13	May	-	-	-	-	-	-	-	-	34.83	-	-	-
F15	June	-	-	-	36.39	-	-	-	-	-	-	-	-

*The positive results are registered with CT value in black and cut off value was set at CT 35. Samples with values >35 are marked in red

**All the foals were born in 2011

M=mare, F=foal, d= day, m= month, - =negative

V. DISCUSSION

The Icelandic horse population has been isolated from other horse breeds for over 1000 years. They were brought to the country during the Viking settlement in the 9th and 10th century and have been purebred ever since. The population is presently around 77.500 horses. Due to the geographic isolation many known pathogens endemic in other horse populations are not found in horses in Iceland and vaccination of horses is not practiced.

1. qPCR

Until this project the EHV-1 and EHV-4 have not been systematically looked for in Iceland with modern molecular techniques. The qPCR method was chosen instead of PCR because qPCR can both detect the viral DNA and give an estimation of the viral load.

When testing for EHV-4 using AgPath Mix and a probe from TAG Copenhagen it gave some problem with the ddH_2O negative controls. The ddH_2O controls had CT values from 31 to 39. It was attempted to exclude possible contamination. When the probe for EHV-4 was replaced with TaqMan probe (Applied Biosystems), the CT values for the ddH_2O controls were much better. However some of them had CT values from 35 to 40 and therefore cut of value was set at 35. I assume it was more likely due to the probes for the EHV-4, or something else in the assay, rather than contamination in the system.

The CT value increases with decreasing amount of the template. Artifacts from the reaction mix or instruments can change the fluorescence measurements associated with the CT calculation and will result in template independent changes to the CT value. Optimally one reaction solution should have been prepared for each virus type and used for all the qPCR reactions to ensure homogeneity and comparability.

PCR efficiency is dependent on the assay, the reaction mix performance and the sample quality. The amplification efficiency in the reactions was from 95% to115% and efficiency between 90% and 110% is considered acceptable. Efficiency is also the key factor in determining the sensitivity of a reaction. It is calculated using the slope of the regression line in the standard curve. A slope closed to -3.32 indicates optimal, 100% qPCR amplification efficiency. The slopes in the reactions were close to that value.

Sometimes the CT values were not the same for the duplicates, though they were similar. Different CT value between duplicates could be explained by different amount of the DNA template in the samples, assuming all other factors such as instruments, reagents and assays are equal.

2. The nasal swab samples

The initial portal of entry of EHV-1 and EHV-4 is the upper respiratory tract (Allen *et al*., 2004; Harless & Pusterla, 2006; Reed & Toribo, 2004). During lytic phase of infection EHV-1 and EHV-4 initially replicate within respiratory epithelial cells (Harless & Pusterla, 2006). Naive horses exposed for the first time to the viruses may release infectious progeny virus into the respiratory mucus for as long as 15 consecutive days following infection. In such primary infections, the magnitude of virus present in nasal mucus may be as great as 10^6 pfu/swab for both EHV-1 and EHV-4. Shedding of virus from the respiratory tract of horses with previous virus exposure or after reactivation from latency is more transient (two to four days) and reduced in magnitude (10^2 to 10^5 pfu/swab) (Allen *et al*., 2004).

Because the upper respiratory tract is the initial portal and respiratory epithelial cells the site of replication for the viruses, nasal swab samples were used. As cell free supernatant from the swabs was used for extraction of DNA, it is assumed that nasal shedding of free virus from the replicating (lytic phase) cycle was mainly detected in present study. The viral load of detected EHV-4 was $10^{3.80}$- $10^{6.95}$ viral DNA copies/swab and includes all EHV-4 viruses, both infectious and non-infectious. Plague forming unit (pfu) detect only infectious viral particles.

3. EHV-1

It has been assumed that Iceland is free of EHV-1 because no clinical signs of the virus, as abort storms and neurological symptoms, have been detected. However, in the only publication on EHV-1 and EHV-4 in horses in Iceland, antibodies to both viruses were found with a type-specific ELISA. In the study 207 horses in Iceland were chosen from serum sample stock at the Institute of Experimental Pathology, University of Iceland, Keldur. The horses were sampled prior to export in 1993 and tested for antibodies to equine infectious anemia virus. The horses were no younger than one year of age (Nordengrahn *et al*., 1997). Nordengrahn *et al*., 1997 found antibodies to EHV-1 in 8% of the samples and concluded that the low prevalence of EHV-1 in the Icelandic horse population represented a closed population and the spread between different herds within Iceland was low. This conclusion is in contrast to the experience made in recent outbreaks of infectious agens in horses in Iceland where rapid spread has been observed. The prevalence of EHV-1 in the Icelandic samples was similar to the prevalence in sero-arceological study done on serum samples from horses in Australia prior to the first cases of EHV-1 abort in 1977 (Crabb & Studdert, 1993).

Low antibody titers to EHV-1 have also been detected in virus neutralization test or

complement fixation test in sera from horses in Iceland. In a surveillance program of 50-60 stallions yearly, that have been ongoing since 2008, only one positive sample have been detected with the type specific EHV-1/EHV-4 ELISA assay. Low antibody titers against EHV-1 could be explained by non-specific reactions or antigenic cross reactivity with e.g. EHV-4. Cross reactivity with an unknown EHV strains is also a possibility. EHV-1 has never been detected in the few samples, tested in the period 1990-2012, neither in PCR or by virus isolation attempt.

Based on high prevalence in Nordengrahn´s *et al*. (1997) study EHV-4 is endemic in the horse population in Iceland. Most serological tests, as serum neutralizations test, broad spectrum ELISA and complement fixation, do not distinguish infection with EHV-1 from EHV-4 due to the extensive antigen cross reactivity between the two viruses. It is therefore most likely that the low prevalence of EHV-1 can be explained by cross reactivity with EHV-4 antibodies. In every case where EHV-1 was detected in low titer EHV-4 was found as well.

In a newly published study about genetic diversity of equine gammaherpesviruses in horses in Iceland it was discussed that viruses with mild pathology and narrow host range are well adapted to their host. EHV-1 has the most severe pathology of the equine herpesviruses and a wide host range. The moderate adaption to its host could suggest that it evolved later than other EHV. Iceland is the only country, as far as it is known, to be free of EHV-1. It could be that EHV-1 infections were not as common in the 9th and 10th century as they are today and the Icelandic horse founder population was free of EHV-1 infections. In contrast, the gammaherpesviruses are considered very well adapted and have most likely co-evolved with the equid genus for millions of years (Thorsteinsdóttir *et al*. 2013).

One the other hand it has been demonstrated that distinct strains of EHV-1 circulate in the field and a certain strain groups are geographically restricted (Goodman *et al*., 2007). It is possible that these different strains are different regarding pathogenicity. It cannot be excluded that low antibody titers against EHV-1 in horses in Iceland could be against a low pathogenic virus strain.

In my study qPCR was used to look for EHV-1 and EHV-4 in nasal swab samples. No evidence of EHV-1 was found in 200 samples from 20 horses, among them foals at the most sensitive age. This reinforces the assumption that Iceland is free of a pathogenic EHV-1 strain and the low EHV-1 prevalence in the Nordengrahn *et al*. (1997) study, is possible cross reactivity to EHV-4 antibodies.

4. EHV-4

EHV-4 is endemic in Iceland. Nordengrahn´s *et al*. (1997) found EHV-4 specific antibodies in 99.5% of tested samples. The prevalence of EHV-4 was similar as in studies done in Australia (Crabb & Studdert, 1993; Gilkerson *et al*. 1999a). In these studies the detection was performed with antibody measurements. Antibodies will always been found if the horse has once become infected with the virus, explaining the high (99-100%) detection in studies using serological tests. When the virus DNA is examined with qPCR using the gB gene as the target, the viral DNA has to be present in the sample at that time point. As the sample material in present study was cell free nasal swabs it is assumed that mainly free virus from lytic phase of herpes virus replication was detected.

Three mares of ten were found positive, all at month two. Nine of 120 samples (7.5%) from the foals were positive for EHV-4 or six of the ten foals. Pusterla *et al*. (2012) detected 33 of 130 (25%) horses that were under one year of age positive in qPCR, but they only collected samples from horses with symptoms. All the samples, in my study, found positive, except one ($10^{6.95}$), included low viral load ($10^{3.80}$-$10^{4.18}$ viral DNA copies/swab).

In my study three foals were found EHV-4 positive before and at one month of age like I formulated in my hypothesis and is supported with published results that EHV-4 infection starts early in life, e.g. the youngest foal was only 11 days of age, when EHV-4 was detected in nasal swab sample by PCR (Foote *et al*. 2004). The youngest EHV-4 qPCR positive foal in my study was 12 days old. The reason why some of the foals were detected positive twice could be because of reinfection with the same or different EHV-4 strains, as has been demonstrated for EHV-1. In reinfection the magnitude of the virus in nasal mucus is reduced and could explain only low viral load are found in some of the samples at that time.

The average age of first EHV-4 detection in the foals was 209.5 days or ca. seven month of age. EHV-4 was detected in the foals from day 12 to month 2 and from month 9 to month 20 (except at month 12). The samples that had some reaction, but were above the CT cut off value, were also seen at these time periods. One would maybe have expected to see a kind of outbreak where most of the foals would have been infected at the same time. This could maybe have been seen with more frequent sampling.

5. Maternal antibodies

Foals relay on passive transfer of maternally derived antibodies through colostrum for humoral immunity to common pathogens. Ingestion of colostrum during the first 12 to 24 hours of a foal´s life results in the passive transfer of high levels of maternal antibodies to the

foal. Uptake of maternal antibodies through nursing is important for immune protection of the foal against infectious diseases during the first few months of life until the foal can produce its own antibodies. Maternal antibodies may be present in the foal for 3-4 months in most cases and up to 6 months or more in some foals. The maternal antibodies in the blood become diluted as the foal grows and the active development of acquired immunity is a slow and gradual process. Consequently foals are more susceptible to infections when maternal antibody level has decline than immediately after birth when maternal antibody are at high level.

The positive detections of EHV-4 in the foals at their first months of life could have been when they met the virus for the first time. After that time the foals are protected by maternal antibodies that could explain only low viral load are found in the samples. The foals were weaned when they were 7-8 months old but samples were taken when the foals were 6 and 9 months old, but not between. The maternal antibodies can be present in the foals to ca. month 6 and more likely to detect the virus after that. It could be possible that EHV-4 infection of some foals have occurred around weaning at 7-8 months of age. Then the maternal antibodies do not protect them anymore, the weaning can be stressful and they become more susceptible for infection. Too long time between the sampling at month six to nine can explain why EHV-4 was not detected as an outbreak where most of the foal became infected at the same time.

VI. CONCLUSION

EHV-1 was not detected in the horses in the study and in light of this and previous investigations on EHV-1 in Iceland I think it can be concluded that EHV-1 does not exists in the horse population in Iceland.

EHV-4 was found in the samples with low prevalence (6%) in nasal swabs and with low viral load ($10^{3.80}$- $10^{4.18}$ viral DNA copies/swab), with one exception ($10^{6.95}$ viral DNA copies/swab). The qPCR should be sensitive enough to find the virus both after primary infection and reinfection. There could be several reasons why EHV-4 is not detected more frequently and in more quantity: The virus has to be in lytic phase; the foals were protected by maternal antibodies when they first met the virus; in reinfections, with the same or different strains, the magnitude of the virus in nasal mucus is reduced; too long time was between the sampling at the time when the foals are most sensitive for infection.

EHV-4 was found in three foals before and at one month of age and nine of 120 samples from 6 foals were found positive during the sampling period of 20 months. EHV-4 positive foals were seen from day 12 to month 2 after birth, and from month 9 to month 20 when maternal antibodies do not protect them anymore.

These results confirm that EHV-4 infections starts early in life and the virus circulates in latent infected horse in Iceland.

VII. PERSPECTIVES

In the study I showed that EHV-1 does not exists in horses in Iceland. EHV-4 was only found in low frequency, but further investigation of horses all over the country is needed to get more information of the distribution of the virus. Testing the nasal swab sediment, which is rich of cells, could be an option for further examination of this material.

Increased travel between countries and growing popularity of the Icelandic horse bring new threats to the infectious status of the Icelandic horse population. It is therefore of the utter importance to know the status of EHV-4 and EHV-1 in the country and have the methodology available if EHV-1 is introduced to the horse population in Iceland.

Hopefully Icelanders will manage to prevent EHV-1 to be brought to the country. However if not, it is vital to be prepared and have some contingency plan to fight the threat.

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