Prevalence of gammaherpesvirus infections in foals and their dams the first year after birth

Þróun sýkinga með gammaherpesveirum í folöldum og mæðrum þeirra fyrsta árið eftir köstun

Sara Björk Stefánsdóttir

Raunvísindadeild
Háskóli Íslands
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16 eininga ritgerð sem er hluti af
Baccalaureus Scientiarum gráðu í lífefnafræði

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Verkfræði- og náttúruvísindasvið
Háskóli Íslands
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Reykjavík, apríl 2013

I hereby declare that this thesis is based on my own observations, is written by me and has neither in part nor as whole been submitted for a higher degree.

Hér með lýsi ég því yfir að ritgerð þessi er samin af mér og hún hefur hvorki að hluta til né í heild verið lögð fram áður til hærri prófgráðu.

Reykjavík, apríl 2013

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Sara Björk Stefánsdóttir
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Útdráttur

Í hestum eru tvær tegundir af gamma-herpesveirum þekktar. Nánast allir hestar eru sýktir með EHV-2 og flestir þeirra einnig með EHV-5 og endursýkingar eru algengar. Folöld smitast á unga aldri frá móður og smitleiðin er um efri öndunarfæri. Veirurnar valda yfirleitt vægum eða engum sjúkdómseinkennum. EHV-2 er bendluð við öndunarfærasjúkdóma og augnslímhúðarbólgu. Markmið verkefnisins var að kanna tíðni gammaherpesveira í folöldum og mæðrum þeirra á Íslandi fyrsta árið eftir köstun og athuga hvenær EHV-2 og EHV-5 sýkinga verður fyrst vart í folöldum. Blóð var tekið úr 15 folöldum yfir 12 mánaða tímabil, jafnframt var blóði safnað úr mæðrum þeirra fyrstu 6-7 mánuðina eftir köstun. Keðjufjölföldun eða PCR var notað til þess að greina veiru DNA í blóði meranna og folaldanna. Vísa pör sem magna hluta úr glýkóprótein B geni og pólýmerasa geni veiranna voru fyrst prófuð en reyndust ekki henta fyrir þennan efnivið. Næmt semi-nested PCR týpu-greiningarpróf var þá prófað. Týpu-greiningarprófið fyrir EHV-2 var erfitt í aflestri og vart nothæft fyrir þennan efnivið. Á hinn bóginn reyndist EHV-5 týpugreiningarprófið vel og greindi EHV-5 hjá öllum folöldum á einhverjum tímapunkti nema hjá einu folaldi. Tíðni EHV-5 sýkingarinnar var mest á tólfta mánuði þar sem sýking greindist hjá 12 folöldum af 15. Fyrstu merki um EHV-5 sýkingar meðal folaldana komu fram í öðrum mánuði. Sýkingar varð vart hjá 6 merum af 15 á tímabilinu. EHV-5 greindist í blóði 93% folalda á fyrsta aldursári með semi-nested PCR týpugreiningarprófi og er það umtalsvert hærri tíðni en áður er þekkt.

Abstract

There are two gammaherpesviruses known to infect horses, EHV-2 and EHV-5. Almost all horses are infected with EHV-2 and most with EHV-5. Foals become infected at young age from their mother and the natural route of infection is through the upper respiratory track. EHV-2 and EHV-5 can co-exist within the same horse and re-infections are common. The viruses usually cause mild or no symptoms. EHV-2 has been related to keratoconjunctivitis and upper respiratory tract diseases. The aim of the study was to investigate prevalence of gammaherpesviruses in foals and their dams the first year after birth and the age at which infection can first be detected. Blood was collected from 15 foals over 12 month period and the dams the first 6-7 month after birth of the foals. Polymerase chain reaction or PCR was used to detect viral DNA in the blood of the foals and the mares. Primer pairs which amplify an area in the glycoprotein B gene and the polymerase gene were first tested but turned out not to work in this material. Sensitive semi-nested type-specific PCR was then tested. The EHV-2 type-specific assay was hard to discriminate and did not proof accessible for this material. On the other hand, the EHV-5 type-specific assay proofed to work fine and detected infection in all foals but one foal during the 12 month period. The highest prevalence was at month 12 were the infection was detected in 12 foals of 15. The EHV-5 infection was first detected among the foals at month 2. Infection was detected among 6 mares of 15 in the observation period. EHV-5 was detected in the blood of 93% of the foals the first year after birth which is higher prevalence than previously has been published.

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Abbreviations

bp	Base pair
d	Day
ddH_2O	Distilled deionised water
DNA	Deoxyribonucleic acid
EHV	Equine herpesvirus
F	Foal
HSV-1	Herpes simplex virus 1
M	Mare
m	Month
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature

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1 Introduction

1.1 Viruses

A virus is a small infectious agent that can only replicate and synthesize virion components inside the living cells of an organism. Progeny virions are formed inside the host cell and they transmit the viral genome to the next host cell. Viruses are of variable size, shape and compositions and they can infect all types of organisms, from animals and plants to bacteria and arcahaea. They are classified into orders, families, genera and species by four features; the nature of the nucleic acid (DNA or RNA), symmetry of the capsid (helical or icosahedral), presence or absence of an envelope, and the size of the virion and the capsid (Flint et al., 2004).

1.2 Herpesviruses

Herpesviruses are widespread in the nature and every vertebrate species is infected with at least one species of herpesvirus and some with several (MacLachlan & Dubovi, 2011; Roizman, 1996). Most herpesviruses are species specific, that is, the virus is restricted to infect one host species, but herpesviruses with a wide host range are also known (Flint et al., 2004).

1.2.1 Classification

The classification of herpesviruses is complex due to their genomic diversity. Recently, herpesviruses were assigned to the new order *Herpesvirales*, with three distinct families: the *Herpesviridae,* the *Alloherpesviridae*, and the *Malacoherpesviridae (*Davison *et al.*, 2009*).* The family *Herpesviridae* includes the herpesviruses of birds, mammals and reptiles and is divided into three subfamilies, *Alphaherpesvirinae, Betaherpesviriane* and *Gammaherpesvirinae.* This classification is based on common genetic and biological properties of the viruses within each subfamily (MacLachlan & Dubovi, 2011). Most alphaherpesviruses grow rapidly, lyse infected cells and have a wide host range; they establish latent infections primarily in sensory ganglia. Betaherpesviruses have narrow and highly restricted host range, long replication cycle and delayed cell lysis, the virus can remain latent in secretory glands, lymphoreticular cells, kidneys and other tissues. Gammaherpesviruses have narrow host range and a slow replication cycle, they are lymphotropic and establish latent infection in B and/or T lymphocytes (MacLachlan & Dubovi, 2011; Roizman, 1996).

1.2.2 Virion properties

Herpesviruses have a genome that consists of linear double stranded DNA, but after the release from the capsid into the nuclei of infected cell it takes a circular form (Roizman, 1996). The genome is very variable in composition, size and organization (MacLachlan & Dubovi, 2011). The genomic size of herpesvirus can vary from 125 to 290 kbp with a base composition of G+T that varies from 31% to 75%. One of the most interesting features of herpesviruses is the diversity in the organization of the genome were many herpesviruses have repeated sequences, either terminal, internal or both. (MacLachlan & Dubovi, 2011; Roizman, 1996). The genes of herpesviruses can be classified into three groups: those

encoding proteins with regulatory functions and virus replication, those encoding structural proteins and "optional" genes, which are not found in all herpesviruses and are not necessary for replication (MacLachlan & Dubovi, 2011).

The size of herpesviruses can vary from 120 to 250 nm in diameter; they are enveloped and consist of a core, capsid, and tegument. The core contains the viral DNA which is wrapped around a fibrous spool-like core in the form of torus. The core is wrapped in protein capsid which is approximately 125 nm in diameter and is composed of 162 capsomers. A layer made of virion proteins, known as the tegument, surrounds the capsid and is enclosed in a lipoprotein envelope with many viral glycoprotein spikes (MacLachlan & Dubovi, 2011). The structure of herpesvirus is shown in figure 1.

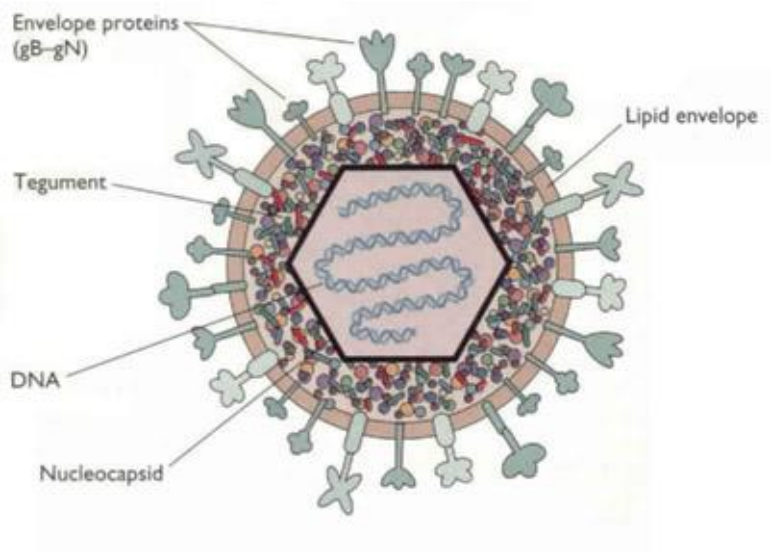

Figure 1. The structure of herpesvirus (Flint et al., 2004).

1.2.3 Replication cycle

The replication cycle of the human herpesviruses, herpes simplex virus 1 (HSV-1), has been most extensively studied but because of the great genomic diversity of the herpesfamily there is probably some variety in the replication cycle of individual herpesviruses (MacLachlan & Dubovi, 2011). The replication cycle starts by virion binding to the extracellular matrix of host-cell through specific interactions of virion glycoprotein spikes. That initiates a fusion of the viral envelope with the plasma membrane were the nucleocapsid enters the cytoplasm along with vhs (virion host shutoff) tegument proteins which inhibit protein synthesis of host-cell. Viral DNA is released into the nucleus and circulates along with the VP16 tegument protein. VP16 is a transcription factor that activates transcription of three classes of mRNA, α, β, and γ by the cellular RNA polymerase II. α RNAs are processed in the nucleus to become mRNAs, which are translated to α proteins in the cytoplasm. The α proteins initiate transcription of β mRNAs, which translate to β proteins and suppress further transcription of α mRNAs. The replication of viral DNA initiates applying some of the viral α and β proteins in addition to host-cell proteins. γ mRNAs are transcribed and translated into γ proteins which are primarily virion structional proteins. The newly synthesized DNA is packaged into immature capsids. The capsids along with some tegument proteins bud out through the

nuclear membrane and envelopment completes. The mature virion is released by exocytosis or cytolysis (Flint et al., 2004; MacLachlan & Dubovi, 2011). The replication cycle is shown in figure 2.

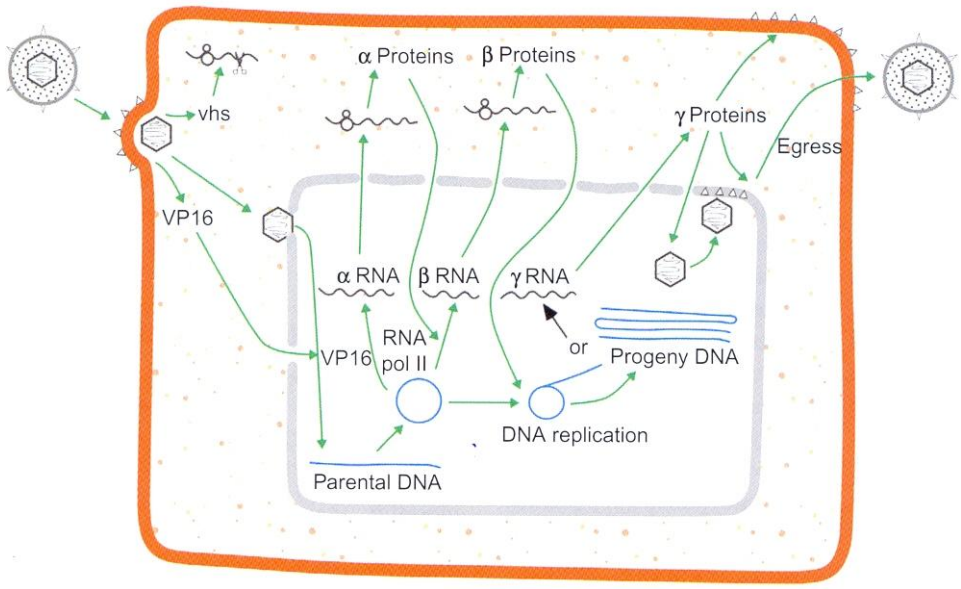

Figure 2. The replication cycle of a typical herpesvirus (MacLachlan & Dubovi, 2011).

1.2.4 Latent infection

The ability to establish a latent infection is a feature that all herpesviruses share. In a latent infection the virus stops reproducing and lies dormant until reactivated. Once infected with herpesvirus you are infected throughout your life (M. J. Studdert, 1996). For latency to act as survival strategy, the latent virus must be able to reactivate so it can spread to other hosts. Reactivation can either occur spontaneous or by stimulation such as trauma, stress, hormonal variance, immunosuppression, UV light or other conditions that makes the host a poor place to continue the latent infection (Ahmed et al., 1996; Flint et al., 2004). In the case of HSV-1, a reinfection can occur following reactivation so the virus can establish latent infection in more neurons (Flint et al., 2004).

Genome of latent viruses is kept in a circular form and can be maintained as a non-replicating chromosome in a non-dividing cell such as neuron (HSV-1) or as autonomous, self-replicating chromosome in a dividing cell (Epstein-Barr virus). Usually there is only restricted set of viral genes transcribed during a latent infection, and the proteins required for productive replication may not be transcribed at all. In the case of HSV only one gene is transcribed, producing latency – associated transcript (LAT), Epstein-Barr virus on the other hand needs several proteins to maintain the latent infection. A latent genome has to be stable, and therefore a balance among the regulators of viral and cellular gene expression is necessary (Flint et al., 2004).

1.3 Equine herpesviruses (EHV)

There are five herpesviruses known to infect horses. Three alphaherpesviruses, EHV-1, 3 and 4, and two gammaherpesviruses, EHV-2 and 5 (Crabb & Studdert, 1996).

1.3.1 Alphaherpesviruses

The alphaherpesvirus EHV-1, also known as equine abortion virus, causes abortion, respiratory diseases and encephalomyelitis in horses. EHV-1 is the most common viral cause of abortion in mares and also known to cause extensive outbreaks of abortion, abortion storms (Crabb & Studdert, 1996; MacLachlan & Dubovi, 2011). EHV-1 establishes latency in trigeminal ganglion (Baxi et al., 1995; Slater et al., 1994) and probably in lymphoid tissue and peripheral blood leukocytes (Welch et al., 1992). EHV-1 is known to infect horses worldwide (Crabb & Studdert, 1996), except for Iceland (Torfason et al., 2008).

The alphaherpesvirus EHV-4, also known as equine rhinopneumonitis virus, causes acute respiratory diseases in horses, it occurs mainly in foals, weanlings and yearlings. Symptoms are fever, anorexia, and profuse serous nasal discharge that later become mucopurulent (Crabb & Studdert, 1996; MacLachlan & Dubovi, 2011). EHV-4 establishes latent infection in trigeminal ganglion (Borchers et al., 1999; G. F. Browning et al., 1988) and is also found latent in lymphoid tissue and peripheral blood leukocytes (Welch et al., 1992). In Iceland, antibodies to EHV-4 have been found in the majority of adult horses (A. Nordengrahn et al., 1998).

EHV-1 and EHV-4 are close related alphaherpesviruses. Until 1981 they were considered one single virus type known as EHV-1 (Crabb & Studdert, 1996) when it was shown that they have different restriction endonuclease DNA fingerprint (M. J. Studdert et al., 1981).

The alphaherpesvirus EHV-3, also known as equine coital exanthema virus, causes venereal disease of horses called equine coital exanthema (ECE). ECE is an acute but usually mild venereal disease. ECE is characterized by the formation of pustular and ulcerative lesions on the vaginal and vestibular mucosae and adjacent perineal skin of affected mares and on the penis and prepuce of affected stallions. Occasionally lesions can be seen on the teats, lips and respiratory muscosa (MacLachlan & Dubovi, 2011). Clinical symptoms resembling infections with EHV-3 have been seen in Icelandic horses (Torfason et al., 2008).

1.3.2 Gammaherpesviruses

The gammaherpesvirus EHV-2 is among the most common viral infections in horses, but its clinical impact remains unclear (F. Browning & Agius, 1996; G. F. Browning & Studdert, 1987b). It has been related to keratoconjunctivitis, upper respiratory tract disease, pneumonia and pharyngitis, fever, enlarged lymph nodes and inappetence/anorexia, general malaise and poor performance (Agius & Studdert, 1994; Collinson et al., 1994; Dunowska et al., 2002b; Kershaw et al., 2001). The upper respiratory tract seems to be the natural route of infection with EHV-2 (Agius & Studdert, 1994) and the transmission of EHV-2 is horizontal, both from mare to foal and from foal to foal (G. F. Browning & Studdert, 1987a). Most foals become infected within 2 months of age (Bell et al., 2006; Dunowska et al., 2011; Fu et al., 1986; Murray et al., 1996; A. Nordengrahn et al., 2002). Horses infected with EHV-2 can be re-infected with other variants of the virus (G. F. Browning & Studdert, 1987a). After primary infection the virus establishes latent infection most likely in the B-lymphocytes (Drummer et al., 1996). The complete EHV-2 genome has been sequenced and is 184 kbp with 57,5% G+C base composition and both internal

and terminal repeats (F. Browning & Agius, 1996; G. F. Browning & Studdert, 1989; E. A. Telford et al., 1995)

The gammaherpesvirus EHV-5 is thought to be widespread but less common than EHV-2 (A. Nordengrahn et al., 2002). The clinical impact of EHV-5 is unclear but it has been associated with the occurrence of equine multinodular pulmonary fibrosis (EMPF) (Williams et al., 2007). Infection with EHV-5 occurs later than with EHV-2 (A. Nordengrahn et al., 2002). The host cell for EHV-5 latency is not known, but could have other target cells than EHV-2 (Torfason et al., 2008). The genome of EHV-5 is smaller than of EHV-2, 179 kbp but has similar G+C % base composition (Agius et al., 1992; F. Browning & Agius, 1996). The EHV-5 genome has not been completely sequenced. Five genes have been completely sequenced, that is DNA polymerase (Genbank accession number: JX125459), DNA terminase gene (Thorsteinsdóttir et al., 2010), glycoprotein H (Thorsteinsdóttir et al., 2010) and glycoprotein B (Holloway et al., 1999; Thorsteinsdóttir et al., 2010).

EHV-2 and EHV-5 were first described as betaherpesviruses, but partial nucleotide sequence analysis demonstrated that they are distinct gammaherpesviruses (E. A. R. Telford et al., 1993). The viruses have close antigenic similarities and can coexist within the same horse (G. F. Browning & Studdert, 1987b). Comparative studies of proteins showed that both viruses had six functionally homologous glycoproteins of similar size but of different abundance (Agius et al., 1994).

EHV-2 and EHV-5 were first isolated from peripheral blood mononuclear cells (PBMC) from Icelandic horses in the search of the cause of infectious pyrexia which occurred in the Icelandic horse population in 1998-99 (Torfason et al., 2008). The summer eczema group at Keldur, has in collaborations with Einar G. Torfason at the Virological department at the University hospital shown that both EHV-2 and EHV-5 are common in horses in Iceland (Torfason et al., 2008).

1.4 The Icelandic horse

The native Icelandic horse is the only horse breed in Iceland. In the early Viking settlement in the 9th and 10th century the native Icelandic horse was brought to Iceland and has been pure bred since then. There are no records of import of horses to Iceland after the Saga-age and from 1882. Import of horses to Iceland became prohibited by law (Björnsson & Sveinsson, 2006).

The horses in Iceland are immunologically naive to various agents known to infect horses in other countries, such as equine influenza, herpes abortion virus EHV-1 and strangles due to the geographic isolation of Iceland. Environmental factors like volcanic activity and harsh weather have been the main scourges in the history of the breed rather than infectious diseases. Although infectious agents that came with the founders cannot be excluded.

Foals are protected from infections by passive transmission of maternal antibodies through colostrum. During the first 12-24 hours after birth, high level of maternal antibodies is transferred passively to the foal with the colostrum. The maternal immunity is vital for the foal the first months of life until it starts to produce its own antibodies. As the foal gets older the maternal antibodies become diluted and the rate of the decline varies both for

individuals and different infectious agent. At 2-3 months of age, the concentration of most of the maternal antibodies has fallen to nonprotective level but some may be present until the foal becomes 6 months of age (Lenn et al., 1998).

Lilja Þorsteinsdóttir has looked at genetic diversity of equine gammaherpesviruses in Iceland. She sequenced and compared four genes (glycoprotein B, glycoprotein H, DNA terminase, and DNA polymerase) from 12 Icelandic and 7 foreign gammaherpesvirus strains. The results showed there was no phylogenetical difference between the Icelandic and foreign virus strains. According to these results and that herpesviruses establish latent infection and are unstable outside of the host, the founders were probably infected with gammaherpesviruses (Thorsteinsdottir et al., 2013; Thorsteinsdóttir, 2009; Thorsteinsdóttir et al., 2010).

The basis for the present project is the gammaherpesvirus work done at Keldur, isolation of Icelandic EHV-2 and EHV-5 strains and sequencing of the EHV-5 glycoprotein B, glycoprotein H, DNA terminase and polymerase genes (Thorsteinsdottir et al., 2013; Thorsteinsdóttir et al., 2010; Torfason et al., 2008).

1.5 Aims of the study

The purpose of this study was to determine the prevalence of EHV-2 and EHV-5 infections in foals and their dams in Iceland the first year after birth and the age at which infection can first be detected.

2 Materials and methods

2.1 The experimental setup

In the spring and summer of 2011 a total of 15 mares (M) sired by the same stallion were kept at Keldur. The mares were kept under observation until they foaled, then nasal swabs and blood were collected from both the mares and their newborn foals (F) before they lactated. In July after 1 month of blood collection, the pairs were moved to Kálfsstaðir in Hjaltadalur and kept with the stallion and other mares. The mare and foal pairs were kept together until the foals were approximately 6 months of age, then the foals were separated from the mares and kept with other horses at Kálfsstaðir.

2.2 Mares and foals

A total of 30 healthy Icelandic horses were used for the study, 15 mares with the mean age 7.1 years (range 4-12 years) and their foals (Table 1).

Table 1. Mares and foals.

Mares	Name	Birth year	Foals	Name	Birth year
M1	Brá	2006	F1	Embla	2011
M2	Galdranótt	2005	F2	Fjörgyn	2011
M3	Valdís	2007	F3	Gandálfur	2011
M4	Silvía	2006	F4	Eiríkur rauði	2011
M5	Hreyfing	2001	F5	Gefjun	2011
M6	Snör	2003	F6	Dúfa	2011
M7	Sandra	2001	F7	Byrjun	2011
M8	Lögg	2006	F8	Káinn	2011
M9	Alba	2005	F9	Jór	2011
M10	Skör	2004	F10	Illingur	2011
M11	Vænting	2004	F11	Birtingur	2011
M12	Perla	2006	F12	Djarfur	2011
M13	Ófeig	2003	F13	Fáfnir	2011
M14	Sigga	2002	F14	Leifur heppni	2011
M15	Gjálp	1999	F15	Huginn	2011

2.3 Samples

Blood was collected by jugular vein puncture into vacutainer tubes (Vacuette, Greiner) containing K_3EDTA. Nine mL of whole blood was centrifuged for 20 min at 760 x g and the buffy coat layer aspired from the red blood cells, 120 µL of the buffy coat was transferred to 400 µL of RBC lysis solution for DNA isolation and the rest of the buffy coat was frozen. Blood collection is shown in Table 2.

DNA was isolated from nasal swab samples by Kristín Þórhallsdóttir, Keldur with High Pure Viral Nucleic Acid kit (Roche) according to manufacturer's protocol.

Table 2. Nasal swab and blood collection.

Mare and foal pair	Foal age (days or months)											
	d0	**d2**	**d5**	**d12**	**1m**	**2m**	**3m**	**4m**	**5m**	**6m**	**9m**	**12m**
M1	x	-	-	x	x	x	x	x	x	x	-	-
F1	x	-	-	x	x	x	x	x	x	x	x	x
M2	x	-	x	x	x	x	x	x	x	x	-	-
F2	x	-	x	x	x	x	x	x	x	x	x	x
M3	x	-	-	x	x	x	x	x	x	x	-	-
F3	x	-	-	x	x	x	x	x	x	x	x	x
M4	x	-	-	x	x	x	x	x	x	x	-	-
F4	x	-	-	x	x	x	x	x	x	x	x	x
M5	x	-	x	x	x	x	x	x	x	x	-	-
F5	x	-	x	x	x	x	x	x	x	x	x	x
M6	x	-	-	x	x	x	x	x	x	x	-	-
F6	x	-	-	x	x	x	x	x	x	x	x	x
M7	-	-	-	-	-	x	x	x	x	x	-	-
F7	-	-	-	-	-	x	x	x	x	x	x	x
M8	x	x	x	x	x	x	x	x	x	x	-	-
F8	x	x	x	x	x	x	x	x	x	x	x	x
M9	x	-	x	x	x	x	x	x	x	x	-	-
F9	x	-	x	x	x	x	x	x	x	x	x	x
M10	x	-	x	x	x	x	x	x	x	x	-	-
F10	x	-	x	x	x	x	x	x	x	x	x	x
M11	x	-	-	x	x	x	x	x	x	x	-	-
F11	x	-	-	x	x	x	x	x	x	x	x	x
M12	x	-	-	x	x	x	x	x	x	x	-	-
F12	x	-	-	x	x	x	x	x	x	x	x	x
M13	x	-	-	x	x	x	x	x	x	x	-	-
F13	x	-	-	x	x	x	x	x	x	x	x	x
M14	x	x	x	x	x	x	x	x	x	-	-	-
F14	x	x	x	x	x	x	x	x	x	-	x	x
M15	x	-	x	x	x	x	x	x	x	x	-	-
F15	x	-	x	x	x	x	x	x	x	x	x	x

x: Nasal swab and blood collected
-: Nasal swab and blood not collected

In the project, viruses from nasal swab and enriched plasma (PBMC) were cultured from 10 mare and foal pairs at each time point. The cultures were observed for cytopathic effect, results are shown in table 7 (appendix I).

2.4 Virus controls

Fourteen virus controls were obtained through the courtesy of Lilja Þorsteinsdóttir, Keldur. Of the viral isolates, twelve were EHV-2 and two were EHV-5.

2.5 DNA

2.5.1 Isolation of DNA

DNA was isolated from buffy coat with a Gentra Puregene Blood Core Kit (QIAGEN) according to the manufacturer‘s protocol. In short, the sample was mixed with RBC lysis solution and centrifuged, the supernatant discarded, the pellet and the residual liquid mixed with Cell lysis solution and incubated at 55°C overnight. Protein Precipitation Solution was added to the sample, mixed and centrifuged, the supernatant collected, mixed with 100% isopropanol in a clean tube and centrifuged. The supernatant was discarded, the DNA pellet washed with 70% ethanol, dried, DNA Hydration Solution added to the pellet, and incubated at 65°C for 30-40 min. Then the samples were incubated at RT overnight with gentle shaking.

2.5.2 Primer design

Primers for the polymerase gene were designed from known EHV-2 and EHV-5 sequences (appendix II) by using the Primer3 program (http://frodo.wi.mit.edu/). Other primers used in this study have been published (Thorsteinsdottir et al., 2013; Thorsteinsdóttir et al., 2010; Torfason et al., 2008). Primers were purchased from TAG Copenhagen and are listed in appendix III.

2.5.3 Polymerase chain reaction (PCR)

PCR was performed in DNA Engine® Peltier Thermal Cycler (PTC-200) from MJ Research with *Taq* DNA polymerase from New England BioLabs.

PCR reaction solution	
DNA	1 µL
10x Thermo Buffer	2 µL
2 mM dNTP	2 µL
20 µM Forward primer	1 µL
20 µM Reverse primer	1 µL
Taq Polymerase	0.1 µL
ddH_2O	12.9 µL
Total Volume	20 µL

PCR reaction		
1. Denaturing	94°C	4 min
2. Denaturing	94°C	10 sec
3. Annealing	50 or 55°C	15 sec
4. Elongation	72°C	30 - 50 sec
5. Elongation	72°C	7 min

Steps 2 to 4 were repeated 29-34 times. The annealing temperature depended on the melting point of the primers and the elongation time depended on the size of the gene amplified (1 min for each 1000 base amplified).

Type-specific semi-nested PCR

Sensitive semi-nested PCR was used to type analyze the samples as EHV-2 or EHV-5 (Torfason et al., 2008). The 1st PCR targets, an 113 bp area in glycoprotein B which is almost identical for both virus types. The 2nd PCR targets, an 76 bp sequence within the target of the 1st PCR, which is different in these two viruses. The same reverse primer is used in all PCR.

PCR reaction solution	
DNA	x µL
10x Thermo Buffer	2 µL
2 mM dNTP	2 µL
20 µM Forward primer	0.5 µL
20 µM Reverse primer	0.5 µL
Taq Polymerase	0.1 µL
ddH_2O	x µL
Total Volume	20 µL

PCR reaction		
1. Denaturing	94°C	12 min
2. Denaturing	94°C	45 sec
3. Annealing	48 or 50 °C	60 sec
4. Elongation	72°C	30 sec
5. Elongation	72°C	5 min

Steps 2 to 4 were repeated 34 times. The annealing temperature depended on the melting point of the primers. The annealing temperature in the 1st PCR is 48°C and in the 2nd 50°C. For the reaction in the 1st PCR, 1 µL of DNA sample was used and 13,9 µL ddH_2O added. In the 2nd PCR, 2 µL of product from the 1st PCR was used and 12,9 µL of ddH_2O. ddH_2O was used as negative control. Virus isolation from nasal swab, foal F4 at 3 month of age, was used as positive control as it was both EHV-2 and EHV-5 positive.

2.5.4 Electrophoresis

The PCR products were run on 1-4 % agarose gel, depending on the size of the gene fragment. For fragments smaller than 100 bp, gels were made with MetaPhor Agarose (Lonza). The agarose powder was added to cold 1.0x TBE (Tris borate – EDTA, appendix IV) and melted. For fragments larger than 100 bp, gels were made with Agarose Basic (AppliChem) melted in 0.5x TBE. Ethidium bromide was added to the melted agar before solidification. Before loading the PCR product, a 10x RSB (restriction buffer, appendix IV) was added to each sample. As a running buffer a 0.5x TBE buffer was used. Electrophoresis was carried out at 65V for 75 min for fragments smaller than 100 bp but at 75V for 45 min for larger fragments. DNA ladder, 2-log (New England Biolabs), was used. PCR products were visualized under UV light in InGenius (SynGene) and photographed using the GeneSnap program (SynGene).

2.5.5 Extraction of DNA from agarose gel

The extraction procedure was performed with a QIAquick Gel Extraction Kit (QIAGEN) according to manufacturer's protocol. The DNA was visualized under UV light and excised from the gel.

2.5.6 DNA quantification

The concentration of nucleic acid in the DNA samples was measured in NanoDrop®ND-1000 Spectrophotometer (NanoDrop Technologies Inc.) according to manufacturer‘s user manual.

2.5.7 TOPO cloning

TOPO cloning was performed with TOPO TA Cloning® Kit for Sequencing (Invitrogen). The TOPO cloning reaction: 4 µl of PCR product, 1 µl of salt solution, 0.7 µl of ddH_2O and 0.5 µl of TOPO vector (pCR®4-TOPO®). The reaction was mixed gently, incubated for 30 minutes at RT, and put on ice. The One Shot® TOP10 chemically competent cells, kept in -80°C, were put directly on ice and 3 µL of the TOPO cloning reaction added, kept on ice for 30 min, heat-shocked at 42°C for 30 sec and cooled on ice for 2 min. After that, 250 µL of SOC medium (Super Optimal Broth medium with added glucose) was added and pre-cultured, shaken at 37°C for 1 hr. The transformation mix was spread on preheated LB agar (appendix IV) plates containing 100 µg/mL ampicillin and cultured overnight at 37°C.

2.5.8 Isolation of plasmids

After transformation of chemically competent cells, colonies were picked and cultured in 2.5 mL of LB medium (appendix IV) with ampicillin (100 µg/mL) overnight, shaking at 37°C. A QIAprep®spin Miniprep kit (QIAGEN) was then used to isolate plasmids according to manufacturer’s protocol.

2.5.9 Sequencing

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

Isolated plasmids were sequenced and for each reaction 200-500 ng/µl of sample was used. The vector primers used for sequencing were from Invitrogen (appendix III).

PCR Sequencing Reaction Solution	
DNA	1 µL
BigDye (v3.1)	1 µL
5x Buffer	1.5 µL
Primer (2 µM)	1.6 µL
ddH_2O	4.9 µL
Total volume	10 µL

PCR sequencing reaction		
1. Denaturing	95°C	5 min
2. Denaturing	95°C	20 sec
3. Annealing	50°C	15 sec
4. Elongation	60°C	4 min
Steps 2 to 4 were repeated 29 times.		

After the PCR 40 µL of 75% isopropanol was added to each PCR product, mixed briefly, incubated at RT for 15 min and centrifuged at 20800 x g for 30 min. The supernatant was discarded, 100 µL of isopropanol added to each sample, mixed briefly, centrifuged for 5 min at 20800 x g, and the supernatant discarded. The pellet was dried at 90°C for 1 min, dissolved in 15 µL of Hi-Di^{TM} Formamide (Applied Biosystems), mixed briefly, heated at

95°C for 2 min, mixed briefly again and centrifuged at 20800 x g for few sec. Then the samples were ready for sequencing.

The data was analyzed in Sequencher^{TM} 4.8 program from Gene Codes Corporation.

3. Results

3.1 PCR with primers for the glycoprotein B gene

3.1.1 Specificity and sensitivity of the primers

Specificity of eight primer pairs was tested in PCR with viral isolates of EHV-2 and EHV-5 viral DNA. Four of the primer pairs were designed to detect EHV-2 and four to detect EHV-5 (Table 3).

Table 3. Specificity of primer pairs for glycoprotein B gene.

Primer pair	Amplicon size (bp)	PCR results	
		EHV-2	EHV-5
EHV-2			
1. Eq2B -140 F Eq2B 458 R	598	(+)	(+)
2. Eq2B 341 F Eq2B 1011 R	670	+	-
3. Eq2B 880 F Eq2B 1545 R	665	+	(+)
4. Eq2B 1837 F Eq2B 2532 R	695	-	-
EHV-5			
5. Eq5B 1399 F Eq5B 2091 R	752	-	+
6. Eq glyB 7 F Eq glyB 730 R	723	-	+
7. Eq glyB 669 F Eq glyB 1581 R	912	-	+
8. Eq glyB 2092 F Eq glyB 2844 R	752	-	+

+: Positive
(+): Weak positive or smear
-: Negative

All EHV-2 primer pairs were tested on both the EHV-2 and EHV-5 DNA (Figure 3). Two EHV-2 primer pairs gave positive results for EHV-2 DNA, one pair gave weak band and one was negative. When the EHV-2 primer pairs were tested on the EHV-5 DNA, two were negative, one gave weak band with many extra bands and one gave smear (Figure 3).

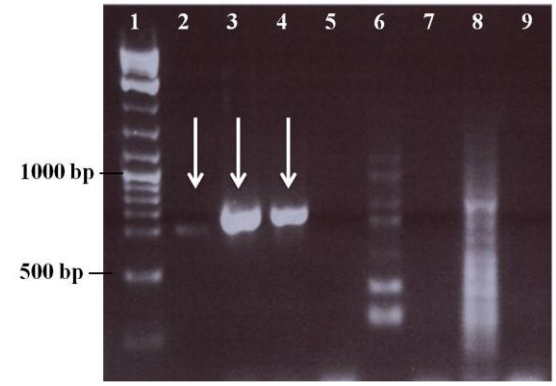

Figure 3. Electrophoresis with the EHV-2 primers on EHV-2 and EHV-5 DNA. Lane 1: 2-log ladder, lanes 2-5: EHV-2 DNA with primer pairs 1-4, respectively, lanes 6-9: EHV-5 DNA with primer pairs 1-4, respectively (Table 3). The arrows represent positive samples.

Primer pair 1 (Table 3) was tested further on isolates from 11 different EHV-2 virus strains (Figure 4). All were positive except one.

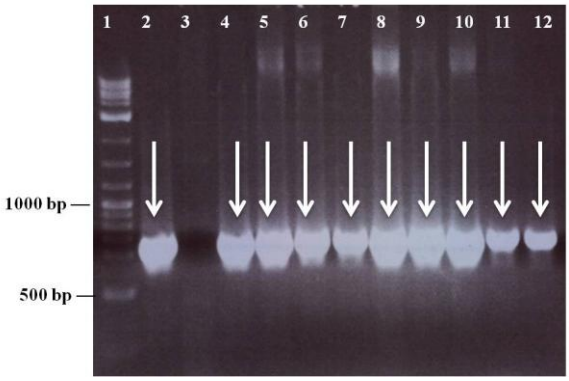

Figure 4. Electrophoresis primer pair number 1 (Table 3) on 11 different EHV-2 virus strains. Lane 1: 2-log ladder, lanes 1-12: isolates of different EHV-2 virus strains. The arrows represent positive samples.

The four EHV-5 primer pairs number 5-8 (Table 3), were tested on both the EHV-5 and EHV-2 DNA (Figure 5). All pairs were positive for the EHV-5 DNA and negative for the EHV-2 DNA (Figure 5).

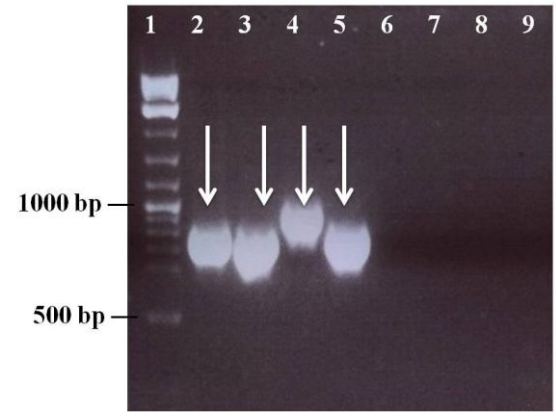

Figure 5. Electrophoresis with the EHV-5 primers on EHV-5 and EHV-2 DNA. Lane 1: 2-log ladder, lanes 1-4: EHV-5 DNA with primer pairs 5-8, respectively, lanes 5-9: EHV-2 DNA with primer pairs 5-8, respectively, (Table 3).The arrows represent positive samples.

The sensitivity of primer pairs 1 and 6 was tested with PCR on serial 10-fold dilutions of purified EHV-2 and EHV-5 DNA, respectively. According to calculations, the detection level of the EHV-2 primer pair 1 (Table 3) was very good, few femtograms (Figure 6). The detection level of the EHV-5 primer pair 6 (Table 3) was few hundred femtograms (Figure 7).

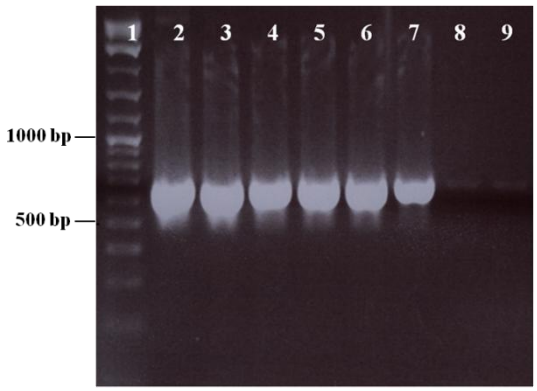

Figure 6. Electrophoresis of PCR with EHV-2 primer pair 1 (Table 3) on glycoprotein B gene on 10-fold diluted EHV-2 DNA. Lane 1: 2-log ladder, lane 2: undiluted EHV-2 DNA (220 ng/µL), lane 3: 10^{-1} dilution, lane 4: 10^{-2} dilution, lane 5: 10^{-3} dilution, lane 6: 10^{-4} dilution, lane 7: 10^{-5} dilution, lane 8: 10^{-6} dilution, lane 9: 10^{-7} dilution.

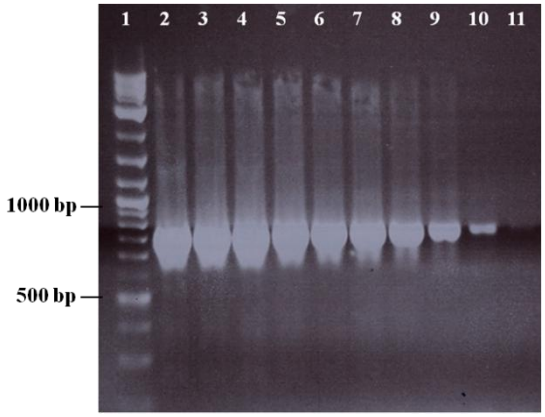

Figure 7. Electrophoresis of PCR with EHV-5 primer pair 6 (Table 3) on glycoprotein B gene on 10-fold diluted EHV-5 DNA. Lane 1: 2-log ladder, lane 2: undiluted EHV-5 DNA (170 ng/µL), lane 3: 10^{-1} dilution, lane 4: 10^{-2} dilution, lane 5: 10^{-3} dilution, lane 6: 10^{-4} dilution, lane 7: 10^{-5} dilution, lane 8: 10^{-6} dilution, lane 9: 10^{-7} dilution, lane 10: 10^{-8} dilution, lane 11: 10^{9}dilution.

3.1.2 PCR on buffy coat

According to virus isolation from blood and nasal swabs all the foals should have been positive at three months of age (Table 7). However, in PCR with the glycoprotein B specific primers all the buffy coat samples from foals at months 2 and 3 were negative both for EHV-2 and EHV-5 (data not shown). Different PCR protocols, with varied anneling temperature, elongation time and repeat of steps, were tried to optimize the amplification of the product with no success.

3.2 PCR with primers for the polymerase gene

3.2.1 Specificity and sensitivity of the primers

Six primer pairs, three designed to detect EHV-2 and three to detect EHV-5 were tested on isolates of EHV-2 and EHV-5 for specificity (Table 4).

Table 4. Specificity of primer pairs for polymerase gene.

Primer pair	Amplicon size (bp)	PCR results	
		EHV-2	EHV-5
EHV-2			
1. EHV2_poly_533F EHV2_poly_1169R	637	+	-
2. EHV2_poly_947F EHV2_poly_1534R	588	+	-
3. EHV2_poly_1895F EHV2_poly_2386R	492	+	-
EHV-5			
4. EHV5_poly_1500F EHV5_poly_2168R	669	-	+
5. EHV5_poly_1658F EHV5_poly_2168R	511	-	+
6. EHV5_poly_1658F EHV5_poly_2361R	704	-	+

+: Positive
-: Negative

All of the primers designed for EHV-2 were positive for EHV-2 and negative for EHV-5 and all of the primers designed for EHV-5 were positive for EHV-5 and negative for EHV-2 (Figure 8, Table 4). One primer pair designed for EHV-2, pair 1, was tested on one other EHV-2 strain, but was negative. The polymerase specific primers gave to much background on foal samples from day 0 and were therefore not tested further.

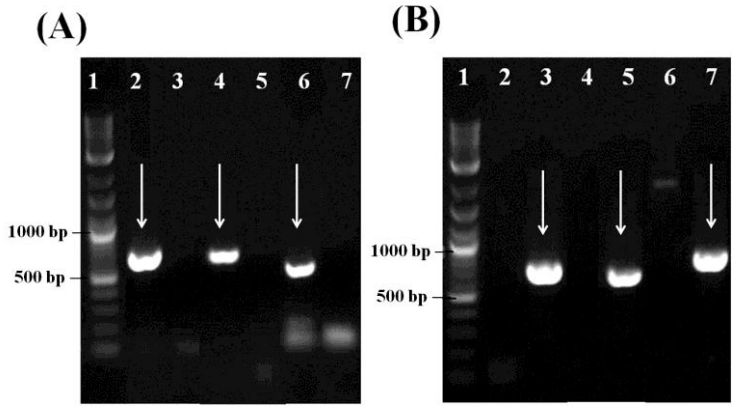

Figure 8. Electrophoresis of PCR with primers on polymerase gene. (A) PCR with primer pairs 1-3. Lanes 2-3: primer pair 1, lanes 4-5: pair 2, lanes 6-7: pair 3. (B) PCR with primer pairs 4-6. Lane 2-3: primer pair 4, lanes 4-5: pair 5, lanes 6-7: pair 6. In both (A) and (B) lane 1: 2-log ladder, lanes 2,4 and 6: EHV-2 DNA, lanes 3,5 and 7: EHV-5 DNA. The arrows represent positive samples.

3.3 EHV-2 and EHV-5 type-specific semi nested PCR on glycoprotein B

3.3.1 PCR on virus isolation from positive nasal swab samples

The primers used in the type-specific PCR, Torfason et al. 2008 were tested on paired nasal swab and buffy coat samples, taken at day 0 and month 3. This was done on foals F2, F3 and F4. The results are shown in figure 9.

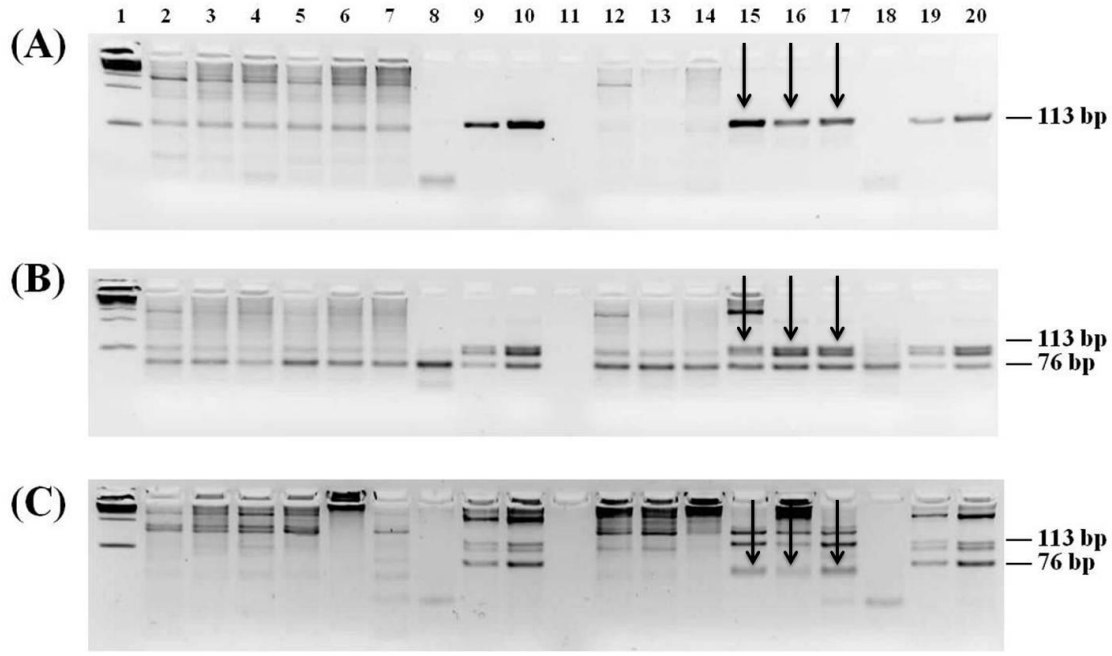

Figure 9. Electrophoresis of type-specific PCR on selected nasal swab and buffy coat samples from foals F2, F3 and F4 at day 0 and month 3. (A) 1st PCR, (B) 2nd PCR for EHV-2, (C) 2nd PCR for EHV-5. Lane 1: 2-log ladder, lanes 2-7: buffy coat samples, lanes 12-17: nasal swab samples, lanes 2 and 12: F2 at day 0, lanes 3 and 13: F3 at day 0, lanes 4 and 14: F4 at day 0, lanes 5 and 15: F2 at month 3, lanes 6 and 16: F3 at month 3, lanes 7 and 17: F4 at month 3, lanes 8 and 18: negative control (ddH_2O), lanes 9, 10, 19, 20: positive control, lane 11: empty. The arrows represent positive nasal swab samples.

The 1st PCR is shown in figure 9(A). The specific band is 113 bp. The day 0 samples, both for nasal swabs and buffy coat, are negative (lanes 2-4 and 12-14). The buffy coat samples from month 3 (lanes 5-7) are also negative whereas the nasal swabs samples (lanes 15-17) are positive.

The 2nd PCR for EHV-2 (Figure 9 (B)) is very hard to interpret, the EHV-2 specific band should be 76 bp. However the band seen at 76 bp is not specific as it is in the negative controls. On the other hand, a band was seen in the positive controls as the upper band of a double band at around size 100 bp. This band was also observed in the month 3 nasal swab samples but not in the negative controls, the day 0 samples or the buffy coat samples. Because of this it was decided that this was a specific EHV-2 band. An attempt was made to sequence this upper band, however it was difficult to discriminate between the double band. The double band was therefore extracted from gel and TOPO cloned. Sequencing from several colonies showed in one case EHV-2 but also Equus caballus protein tyrosine phosphatase, mRNA (data not shown) for more than one colony.

The 2nd PCR for EHV-5 is shown in figure 9(C). The specific band, 76 bp was seen in the nasal swab samples from month 3 (lanes 15-17). The day 0 samples, both for nasal swabs and buffy coat, are negative (lanes 2-7 and 12-14). The buffy coat samples from month 3 (lanes 5-7) are also negative.

3.3.2 Type-specific PCR on buffy coat samples

All buffy coat samples from the 15 mare/foal pairs from all time points (Table 2), total of 280 samples were tested with type-specific PCR. The 1st PCR was carried out for all the samples and then the product was used for the 2nd PCR to test for both EHV-2 and EHV-5. A total of 840 PCRs was done.

EHV-2 type-specific PCR

Example of the electrophoresis of EHV-2 buffy coat samples from the mares at day 0 is shown in figure 10.

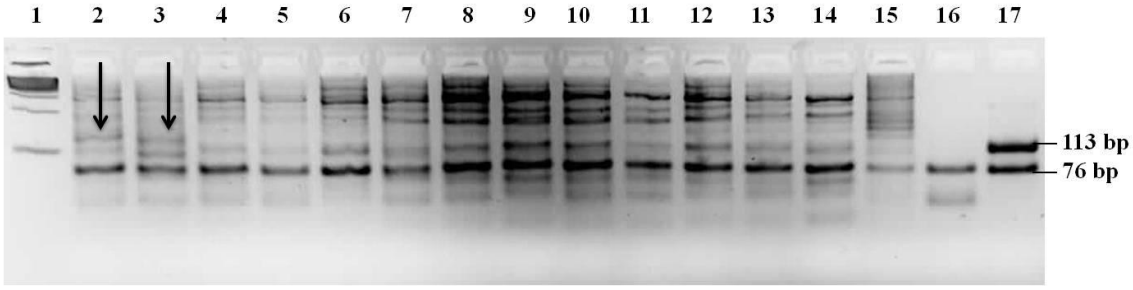

Figure 10. Electrophoresis of EHV-2 type-specific PCR on buffy coat samples from mares at day 0. Lane 1: 2-log ladder, lanes 2-15: mares M1-M6 and M8-M15, lane 16: negative control (ddH_2O), lane 17: positive control. The arrows represent weak positve buffy coat samples.

Twelve mares were negative at day 0 (Figure 10) and two mares showed weak specific EHV-2 band, M1 (lane 2 in Figure 10) and M2 (lane 3 in Figure 10). M7 was not tested (Table 2).

Example of the electrophoresis from mares at day 12 is shown in Figure 11.

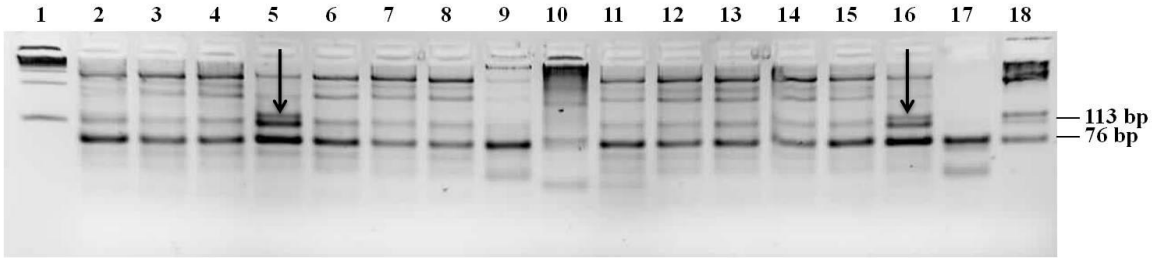

Figure 11. Electrophoresis of EHV-2 type-specific PCR on buffy coat samples from mares at day 12. Lane 1: 2-log ladder, lanes 2-16: mares M1-M15, lane 17: negative control (ddH_2O), lane 18: positive control. The arrows represent positive buffy coat samples.

Mares M4 and M15 showed strong specific EHV-2 band at day 12 (Figure 11), all the others were negative.

Results from the EHV-2 type-specific PCR from all the mares and their foals are shown in table 5.

Table 5. Results from EHV-2 type-specific PCR on buffy coat samples.

Mare and foal pair	Foal age (days or months)											
	d0	**d2**	**d5**	**d12**	**1m**	**2m**	**3m**	**4m**	**5m**	**6m**	**9m**	**12m**
M1	(+)	NT	NT	-	-	-	-	-	-	-	NT	NT
F1	-	NT	NT	-	-	-	-	-	-	-	-	-
M2	(+)	NT	-	-	-	-	-	-	-	-	NT	NT
F2	-	NT	-	-	-	-	-	-	-	-	-	-
M3	-	NT	NT	-	-	-	-	-	-	-	NT	NT
F3	-	NT	NT	-	-	-	-	-	-	-	-	-
M4	-	NT	NT	+	-	-	-	-	-	-	NT	NT
F4	-	NT	NT	-	-	-	-	-	-	-	-	-
M5	-	NT	-	-	-	-	-	-	-	-	NT	NT
F5	-	NT	-	-	-	-	-	-	-	-	-	-
M6	-	NT	NT	-	-	-	-	-	-	-	NT	NT
F6	-	NT	NT	-	-	-	-	-	-	-	-	-
M7	NT	NT	NT	NT	NT	-	-	-	-	-	NT	NT
F7	NT	NT	NT	NT	NT	(+)	-	-	-	-	-	-
M8	-	-	-	-	-	-	-	(+)	-	-	NT	NT
F8	-	-	-	-	?	-	-	-	-	-	-	-
M9	-	NT	-	-	-	-	-	-	-	-	NT	NT
F9	-	NT	-	-	-	-	-	-	-	-	-	-
M10	-	NT	-	-	-	-	-	-	-	-	NT	NT
F10	-	NT	-	-	-	-	-	-	-	-	-	-
M11	-	NT	NT	-	-	-	-	-	-	-	NT	NT
F11	-	NT	NT	-	-	-	-	-	-	-	-	-
M12	-	NT	NT	-	-	-	-	-	-	-	NT	NT
F12	-	NT	NT	-	-	-	-	-	-	-	-	-
M13	-	NT	NT	-	-	-	-	-	-	-	NT	NT
F13	-	NT	NT	-	-	-	-	-	-	-	-	-
M14	-	-	-	-	-	-	-	(+)	(+)	NT	NT	NT
F14	-	-	-	-	-	-	-	(+)	-	NT	-	-
M15	-	NT	-	+	-	-	-	-	-	-	NT	NT
F15	-	NT	-	-	-	-	-	-	-	-	-	-

+: positive
(+): weak positive
?: Only the upper band of the double band present
NT: Not tested
- :Negative

At most time point's mare and foal samples tested negative for EHV-2. Mares 4 and 15 showed strong positive band at day 12. In day 0 M1 and M2 tested weak positive. Weak positive band was also seen in electrophoresis of F7 at month 2, in M8 and M14 and F14 at month 4, and M14 at month 5. Only the upper band of double band at 113 bp was seen in electrophoresis for F8 at month 1 (? in Table 5).

EHV-5 type-specific PCR

Example of the electrophoresis of EHV-5 buffy coat samples from the mares at month 12 is shown in figure 12.

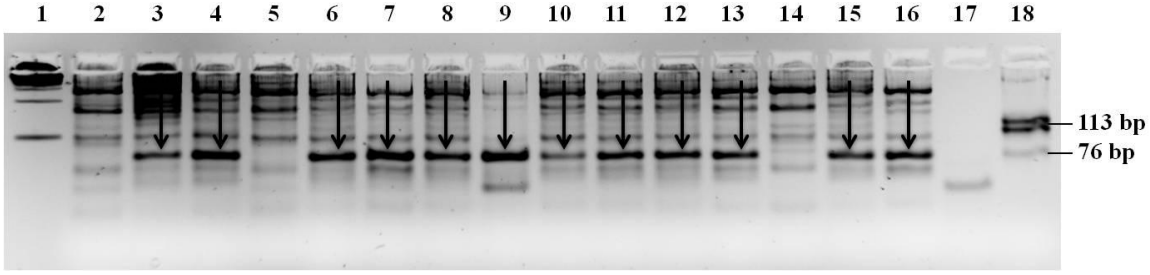

Figure 12. Electrophoresis EHV-5 type-specific PCR of buffy coat samples from the foals at month 12. Lane 1: 2-log ladder, lanes 2-16: foals F1-F15, lane 17: negative control (ddH_2O), lane 18: positive control. The arrows represent positive buffy coat samples.

A total of 12 foals showed strong positive band at age 12 month (Figure 12), three foals were negative.

Results from the EHV-5 type-specific PCR on buffy coat samples from all the mares and foals are shown in table 6.

Table 6. Results from EHV-5 type-specific PCR on buffy coat samples.

Mare and foal pair	Foal age (days or months)											
	d0	**d2**	**d5**	**d12**	**1m**	**2m**	**3m**	**4m**	**5m**	**6m**	**9m**	**12m**
M1	-	NT	NT	-	-	-	-	-	+	-	NT	NT
F1	-	NT	NT	-	-	-	-	-	-	-	-	-
M2	-	NT	-	-	-	-	-	-	-	+	NT	NT
F2	-	NT	-	-	-	-	-	-	-	-	-	+
M3	-	NT	NT	-	-	-	-	-	-	-	NT	NT
F3	-	NT	NT	-	-	-	-	-	-	-	+	+
M4	-	NT	NT	-	-	-	-	-	-	-	NT	NT
F4	-	NT	NT	-	-	(+)	-	-	-	-	+	-
M5	-	NT	-	-	-	-	-	-	-	-	NT	NT
F5	-	NT	-	-	-	-	-	-	-	-	-	+
M6	-	NT	NT	-	-	-	-	-	-	-	NT	NT
F6	-	NT	NT	-	-	-	-	-	-	+	-	+
M7	NT	NT	NT	NT	NT	-	-	-	-	-	NT	NT
F7	NT	NT	NT	NT	NT	-	+	+	-	-	-	+
M8	-	-	-	-	-	-	-	-	-	+	NT	NT
F8	-	-	-	-	-	-	-	-	-	-	-	+
M9	+	NT	+	+	+	+	+	+	+	+	NT	NT
F9	-	NT	-	-	-	-	-	-	-	-	+	+
M10	-	NT	(+)	-	-	-	-	-	-	-	NT	NT
F10	-	NT	-	-	-	-	-	-	-	-	-	+
M11	-	NT	NT	-	-	-	-	-	-	-	NT	NT
F11	-	NT	NT	-	-	-	-	-	-	-	-	+
M12	-	NT	NT	-	-	-	-	-	-	-	NT	NT
F12	-	NT	NT	-	-	-	-	+	-	+	+	+
M13	-	NT	NT	-	-	-	-	+	+	-	NT	NT
F13	-	NT	NT	-	-	-	-	-	+	+	-	-
M14	-	-	-	-	-	-	-	-	-	NT	NT	NT
F14	-	-	-	-	-	-	+	-	+	NT	-	+
M15	-	NT	-	-	-	-	-	-	-	-	NT	NT
F15	-	NT	-	-	-	-	-	-	-	-	-	+

+: Positive
(+): Weak positive
NT: Not tested
-: Negative

Mare 9 was positive at all time points. Five other mares also tested EHV-5 positive, M13 at month 4, M1 and M13 at month 5 and M2 and M8 at month 6. M10 was weak positive at day 5. All the foals except foal 1 tested positive at some point (Table 6). F4 was weak positive at month 2. At month 3 F7 and F14 were positive, F7 and F12 at month 4 and F13 and F14 at month 5. At month 6 three foals were positive, F6, F12 and F13 and at month 9, four foals were positive, F3, F4, F9 and F12. At month 12 there were only 3 foals negative, F1, F4 and F13.

Comparison of number of EHV-5 positive mares and foals is shown in figure 13.

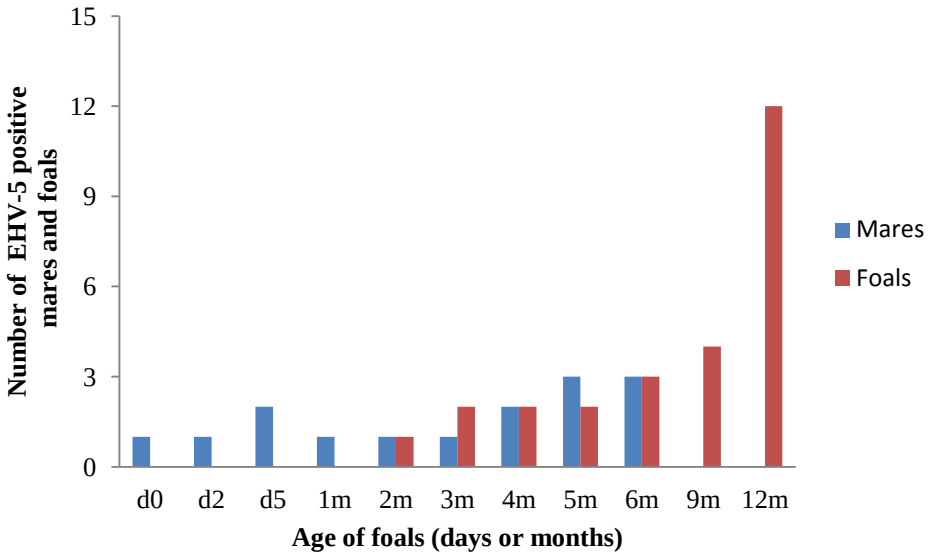

Figure 13. Detection of EHV-5 in buffy coat samples of 15 mares and foals with type-sepecific PCR assay. Blue columns represent positive mares and red columns represent positive foals.

EHV-5 was present in the buffy coat of 1/15 mares at day 5 and at month 4, 2/15 mares at month 5 and month 6 (Figure 13).

EHV-5 was present in the buffy-coat of 1/15 foals at month 2 and in 2/15 at month 3, 4 and 5. At 6 month of age 3/15 of foals were positive and 4/15 at 9 month of age. At the final sampling when the foals were 12 month of age, 12/15 was positive (Figure 13).

4. Discussions

In the spring and summer of 2011 fifteen mares were kept at Keldur under observation until they foaled. Blood and nasal swabs were collected from the mares and their foals before colostrum intake and regularly after that. This gave a great opportunity to investigate gammaherpesvirus infection in the foals, i.e. to see when the infections could first be detected and how it developed. Equine gammaherpesviruses have been investigated at Keldur for several years and it has been shown that both EHV-2 and EHV-5 are common in Iceland (Torfason et al., 2008). The present project is the direct continuation of this work that also involved the isolation of several EHV-2 and EHV-5 strains and sequencing of four different genes (Thorsteinsdottir et al., 2013; Thorsteinsdóttir, 2009; Thorsteinsdóttir et al., 2010).

Foals relay on passive transmission of maternal antibodies through the colostrum for immunity against infections. Even in the presence of maternal antibodies, EHV-2 is transmitted from mares to foals soon after birth (Wilks & Studdert, 1974). The natural portal of entry is through the upper respiratory tract where it infects and replicates first in the respiratory mucosal epithelium (G. F. Browning & Studdert, 1987a; Murray et al., 1996). A secondary infection occurs with entry of infected leukocytes into the blood-vascular circulation and the virus establishes latency in the B lymphocytes (Drummer et al., 1996). The site of latency for EHV-5 is not known.

Virus culture from the enriched plasma and the nasal swabs samples was done. Cytopathic effect typical of herpesvirus was monitored but not tested for virus type-specificity. All the samples taken from the foals at month 3 were positive in virus isolation, demonstrating that they were all virus infected, probably with either EHV-2 or 5 or both at that time point or possibly other herpesviruses endemic in Iceland e.g. EHV-4.

The purpose of my project was to look for EHV-2 and EHV-5 in the peripheral blood leucocytes from the foals and their dams with PCR at different time-points over a year after birth. First I tried primers which target a 600-900 bp area in the glycoprotein B gene (Thorsteinsdottir et al., 2013; Thorsteinsdóttir, 2009). These primer pairs gave good amplification on EHV-2 and 5 isolates. However, they gave negative results both for EHV-2 and EHV-5 for all the samples from month 2 and 3. The protocol for the PCR was varied with regard to annealing temperature, number of cycles and elongation time but without positive results. As virus cultures from nasal swab and enriched plasma from the foals had shown to be positive at month 3, the primers were judged not sensitive enough to detect viral DNA of EHV-2 and EHV-5 in blood leucocytes. It was decided to design primers that target another gene, the polymerase gen, which is more preserved than glycoprotein B. The polymerase gene of EHV-2 and EHV-5 has a very G+C rich area so it was hard to design proper primers for the PCR assay. Three primer pairs were designed for each virus and they worked well on both EHV-2 and EHV-5 virus isolates. On the blood samples they either showed too much smear in electrophoresis or did not amplify the targeted fragment at month 3. Therefore the polymerase gene primers were not sensitive enough for detecting the viruses in blood.

Finally the highly sensitive semi-nested type-specific PCR used by Torfason et al., 2008 was tried. Isolated DNA from nasal swab and DNA isolate from the leucocytes from three foals at 3 month of age was compared. These nasal swab samples had shown herpesvirus

like cytopathic effects in cell culture. The EHV-5 type-specific assay worked well on this material. On the other hand we had problem with the EHV-2 type-specific assay. The expected specific product could not be differentiated in the gel used from another product at a similar size. These two PCR products were TOPO cloned together in reaction and then different colonies were cultured and sequenced. This confirmed the presence of EHV-2 DNA but also equine DNA. It was therefore concluded that this type-specific PCR assay in this project was not applicable to detect EHV-2 in blood leucocytes.

EHV-2 infections are very common and most foals become infected within 2 months of age (Bell et al., 2006; Dunowska et al., 2011; Fu et al., 1986; Murray et al., 1996; A. Nordengrahn et al., 2002). In addition, Dunowska et al 2011 even showed positive tests from foals down to 2-4 days of age. Many studies have been carried out using PCR to look at the prevalence of EHV-2 and EHV-5, most find EHV-2 in higher frequency than EHV-5 (Bell et al., 2006; Dunowska et al., 2002a; A. Nordengrahn et al., 2002; Torfason et al., 2008). There are few exceptions were EHV-5 is found in higher frequency than EHV-2 (Diallo et al., 2008; Wang et al., 2007) as reported by Wang et al., where EHV-5 was detected in PBMC from 89% of foals EHV-2 was detected in 30% of foal. As the EHV-2 test did not work properly we were not able compare the prevalence of EHV-2 and EHV-5

In similar studies, it appears that initial infection of EHV-5 in foals usually occurs later in life than infection with EHV-2. The initial infection of EHV-5 has been found in foals at 2 month of age (Bell et al., 2006; Dunowska et al., 2002a; A. Nordengrahn et al., 2002). The results in this study are consistent with this since EHV-5 was first detected in one foal at that time. The first weeks of life the foals are protected against infections with maternal antibodies. As they get older the maternal antibodies declines and the foals have to rely on their own immune system. The concentration of the maternal antibodies has most likely fallen to no protective level in foals at the age of 2 months when the EHV-5 infections are first detected.

All the foals but one became infected with EHV-5 on at least one occasion over the 12 month period of the study and EHV-5 was also detected in six of the mares. The infection was most frequent at 12 months, where EHV-5 was identified in 12 of the 15 foals. Not many studies have looked at the prevalence of EHV-5 infections in foals and none has shown this high prevalence of the virus (Bell et al., 2006; Dunowska et al., 2002a; A. Nordengrahn et al., 2002). Study on age-dependent prevalence of EHV-5 infections has shown that it is higher in younger group of horses than in older (Marenzoni et al., 2010).

Some of the foals were EHV-5 positive at two or more occasion. That could be because of re-infection with the same or different EHV-5 virus strain. A reactivation of the same EHV-5 virus strain might be caused by increased stress, hormonal variance, immunosuppression or other conditions.

For further investigation on this material a discriminable EHV-2 type-specific assay has to be designed. Our project was aimed at detecting equine gammaherpesvirues in peripheral blood leukocytes. However, to get better picture of the pathogeneses of the EHV-2 and EHV-5 infections, further testing of nasal swab samples collected and the herpesvirus-like virus isolated, along with serological testing, is needed.

References

Agius, C. T., Crabb, B. S., Telford, E. A. R., Davison, A. J., & Studdert, M. J. (1994). Comparative studies of the structural proteins and glycoproteins of equine herpesviruses 2 and 5. *J Gen Virol, 75*(10), 2707-2717. doi: 10.1099/0022-1317-75-10-2707

Agius, C. T., Nagesha, H. S., & Studdert, M. J. (1992). Equine herpesvirus 5: comparisons with EHV2 (equine cytomegalovirus), cloning, and mapping of a new equine herpesvirus with a novel genome structure. *Virology, 191*(1), 176-186.

Agius, C. T., & Studdert, M. J. (1994). Equine herpesviruses 2 and 5: comparisons with other mebmers of the gammaherpesvirinae. *Adv Virus Res, 44*, 357-379.

Ahmed, R., Morrison, L. A., & Knipe, D. M. (1996). Presistence of Viruses. In B. M. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman & S. E. Straus (Eds.), *Fields Virology* (Vol. 1, pp. 219-250). New York: Lippincott-Raven Publisher.

Baxi, M. K., Efstathiou, S., Lawrence, G., Whalley, J. M., Slater, J. D., & Field, H. J. (1995). The detection of latency-associated transcripts of equine herpesvirus 1 in ganglionic neurons. *J Gen Virol, 76*(12), 3113-3118. doi: 10.1099/0022-1317-76-12-3113

Bell, S. A., Balasuriya, U. B., Gardner, I. A., Barry, P. A., Wilson, W. D., Ferraro, G. L., & MacLachlan, N. J. (2006). Temporal detection of equine herpesvirus infections of a cohort of mares and their foals. [Research Support, Non-U S Gov't]. *Vet Microbiol, 116*(4), 249-257.

Björnsson, G. B., & Sveinsson, H. J. (2006) *The Icelandic Horse*. Reykjavík: Mál og Menning.

Borchers, K., Wolfinger, U., & Ludwig, H. (1999). Latency-associated transcripts of equine herpesvirus type 4 in trigeminal ganglia of naturally infected horses. [Research Support, Non-U S Gov't]. *J Gen Virol, 80*(Pt 8), 2165-2171.

Browning, F., & Agius, C. T. (1996). Equine herpesviruses 2 and 5 (equine gammaherpesviruses) and asinine herpesvirus 2 infections. In M. J. Studdert (Ed.), *Virus Infections of Vertebrates, Virus Infections of Equines* (Vol. 6, pp. 47-60). Amsterdam: Elsevier.

Browning, G. F., Bulach, D. M., Ficorilli, N., Roy, E. A., Thorp, B. H., & Studdert, M. J. (1988). Latency of equine herpesvirus 4 (equine rhinopneumonitis virus). *Vet Rec, 123*(20), 518-519.

Browning, G. F., & Studdert, M. J. (1987a). Epidemiology of equine herpesvirus 2 (equine cytomegalovirus). *J Clin Microbiol, 25*(1), 13-16.

Browning, G. F., & Studdert, M. J. (1987b). Genomic Heterogeneity of Equine Betaherpesviruses. *Journal of General Virology, 68*(5), 1441-1447. doi: 10.1099/0022-1317-68-5-1441

Browning, G. F., & Studdert, M. J. (1989). Physical mapping of a genome of equine herpesvirus 2 (equine cytomegalovirus). *Arch Virol, 104*(1-2), 77-86.

Collinson, P. N., O´Rielly, J. L., Ficorilli, N., & Studdert, M. J. (1994). Isolation of equine herpesvirus type 2 (equine gammaherpesvirus 2) from foals with keratoconjunctivitis. *J Am Vet Med Assoc, 205*(2), 329-331.

Crabb, B. S., & Studdert, M. J. (1996). Equine rhinopneumonitis (equine herpesvirus 4) and equine abortion (equine herpesvirus 1). In M. J. Studder (Ed.), *Virus Infections*

of Vertebrates, Virus Infection of Equines (Vol. 6, pp. 11-37). Amsterdam: Elsevier.

Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., McGeoch, D. J., Minson, A. C., . . . Thiry, E. (2009). The order Herpesvirales. *Arch Virol, 154*(1), 171-177.

Diallo, I. S., Hewitson, G. R., de Jong, A., Kelly, M. A., Wright, D. J., Corney, B. G., & Rodwell, B. J. (2008). Equine herpesvirus infections in yearlings in South-East Queensland. *Arch Virol, 153*(9), 1643-1649.

Drummer, H. E., Reubel, G. H., & Studdert, H. J. (1996). Equine gammaherpesvirus 2 (EHV2) is latent in B lymphocytes. *Arch Virol, 141*(3-4), 495-504.

Dunowska, M., Howe, L., Hanlon, D., & Stevenson, M. (2011). Kinetics of Equid herpesvirus type 2 infections in a group of Thoroughbred foals. [Research Support, Non-U S Gov't]. *Vet Microbiol, 152*(1-2), 176-180.

Dunowska, M., Wilks, C. R., Studdert, M. J., & Meers, J. (2002a). Equine respiratory viruses in foals in New Zealand. *N Z Vet J, 50*(4), 140-147.

Dunowska, M., Wilks, C. R., Studdert, M. J., & Meers, J. (2002b). Viruses associated with outbreaks of equine respiratory disease in New Zealand. *N Z Vet J, 50*(4), 132-139.

Flint, S. J., Enquist, L. W., Racaniellio, V. R., & Skalka, A. M. (2004). *Principles of Virology; Molecular Biology, Pathogenesis, and Control of Animal Viruses* (Second ed.). Washington: American Society for Microbiology, ASM PRess.

Fu, Z. F., Robinson, A. J., Horner, G. W., Dickinson, L. G., Grimmett, J. B., & Marshall, R. B. (1986). Respiratory disease in foals and the epizootiology of equine herpesvirus type 2 infection. *N Z Vet J, 34*(9), 152-155.

Holloway, S. A., Lindquester, G. J., Studdert, M. J., & Drummer, H. E. (1999). Identification, sequence analysis and characterisation of equine herpesvirus 5 glycoprotein B. [Research Support, Non-U S Gov't]. *Arch Virol, 144*(2), 287-307.

Kershaw, O., von Oppen, T., Glitz, F., Deegen, E., Ludwig, H., & Borchers, K. (2001). Detection of equine herpesvirus type 2 (EHV-2) in horses with keratoconjunctivitis. [Research Support, Non-U S Gov't]. *Virus Res, 80*(1-2), 93-99.

Lenn, D. P., Hannant, D., & Horohov, D. W. (1998). Immunology of horses and donkeys. In P. P. Pastoret, P. Griebel, H. Bazin & A. Govaerts (Eds.), *Handbook of Vertebrate Immunology* (Vol. 1, pp. 343-361). London: Academic Press Limited.

MacLachlan, N. J., & Dubovi, E. J. (Eds.). (2011). *Fenner's Veterinary Virology* (Fourth ed.). London: Elsevier.

Marenzoni, M. L., Coppola, G., Maranesi, M., Passamonti, F., Cappelli, K., Capomaccio, S., . . . Coletti, M. (2010). Age-dependent prevalence of equid herpesvirus 5 infection. *Vet Res Commun, 34*(8), 703-708.

Murray, M. J., Eichorn, E. S., Dubovi, E. J., Ley, W. B., & Cavey, D. M. (1996). Equine herpesvirus type 2: prevalence and seroepidemiology in foals. *Equine Vet J, 28*(6), 432-436.

Nordengrahn, A., Merza, M., Ros, C., Lindholmc, A., Palfl, V., Hannant, D., & Belak, S. (2002). Prevalence of equine herpesvirus types 2 and 5 in horse populations by using type-specific PCR assays. *Vet Res, 33*(3), 251-259.

Nordengrahn, A., Merza, M., Svendlund, G., Ronéus, M., Berndtsson, L. T., Lindholm, A., . . . Klingeborn, B. (1998). A field study of the application of a type-specific test distinguishing antibodies to equine herpesvirus-4 and -1. *Equine Infection Diseases VIII*, 125.

Roizman, B. (1996). Herpesviridae. In B. M. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman & S. E. Straus (Eds.), *Fields Virology* (Vol. 2, pp. 2221-2230). New York: Lippincott - Raven Publisher.

Slater, J. D., Borchers, K., Thackray, A. M., & Field, H. J. (1994). The trigeminal ganglion is a location for equine herpesvirus 1 latency and reactivation in the horse. *J Gen Virol, 75*(8), 2007-2016. doi: 10.1099/0022-1317-75-8-2007

Studdert, M. J. (1996). Herpesviridae. In M. J. Studdert (Ed.), *Virus Infections of Vertebrates, Virus Infections of Equines* (Vol. 6, pp. 9-10). Amsterdam: Elsevier.

Studdert, M. J., Simpson, T., & Roizman, B. (1981). Differentiation of respiratory and abortigenic isolates of equine herpesvirus 1 by restriction endonucleases. *Science, 214*(4520), 562-564.

Telford, E. A., Watson, M. S., Aird, H. C., Perry, J., & Davison, A. J. (1995). The DNA sequence of equine herpesvirus 2. [Comparative StudyResearch Support, Non-U S Gov't]. *J Mol Biol, 249*(3), 520-528.

Telford, E. A. R., Studdert, M. J., Agius, C. T., Watson, M. S., Aird, H. C., & Davison, A. J. (1993). Equine Herpesviruses 2 and 5 Are gammaherpesviruses. *Virology, 195*(2), 492-499.

Thorsteinsdottir, L., Torfason, E. G., Torsteinsdottir, S., & Svansson, V. (2013). Genetic diversity of equine gammaherpesviruses (gamma-EHV) and isolation of a syncytium forming EHV-2 strain from a horse in Iceland. *Res Vet Sci, 94*(1), 170-177.

Thorsteinsdóttir, L. (2009). *Genetic diversity of equine gammaherpesviruses in Iceland.* M.Sc., University of Iceland, Reykjavík.

Thorsteinsdóttir, L., Torfason, E. G., Torsteinsdóttir, S., & Svansson, V. (2010). Isolation and partial sequencing of equine gammaherpesvirus 5 (EHV-5) from a horse in Iceland. *J Vet Diagn Invest, 22*(3), 420-423.

Torfason, E. G., Thorsteinsdottir, L., Torsteinsdottir, S., & Svansson, V. (2008). Study of equid herpesviruses 2 and 5 in Iceland with a type-specific polymerase chain reaction. *Res Vet Sci, 85*(3), 605-611.

Wang, L., Raidal, S. L., Pizzirani, A., & Wilcox, G. E. (2007). Detection of respiratory herpesviruses in foals and adult horses determined by nested multiplex PCR. [Comparative StudyResearch Support, Non-U S Gov't]. *Vet Microbiol, 121*(1-2), 18-28.

Welch, H. M., Bridges, C. G., Lyon, A. M., Griffiths, L., & Edington, N. (1992). Latent equid herpesviruses 1 and 4: detection and distinction using the polymerase chain reaction and co-cultivation from lymphoid tissues. [Research Support, Non-U S Gov't]. *J Gen Virol, 73*(Pt 2), 261-268.

Wilks, C. R., & Studdert, M. J. (1974). Equine herpesviruses. 5. Epizootiology of slowly cytopathic viruses in foals. *Australian Veterinary Journal, 50*(10), 438-442.

Williams, K. J., Maes, R., Del Piero, F., Lim, A., Wise, A., Bolin, D. C., . . . Bolin, S. R. (2007). Equine multinodular pulmonary fibrosis: a newly recognized herpesvirus-associated fibrotic lung disease. *Vet Pathol, 44*(6), 849-862.

Appendix I

Table 7. Cytopathic effect in virus cultures of nasal swab (NS) and enriched plasma (PBMC) of 10 mare and foal pairs.

Mare and foal pairs	d0		d5		d12		1m		2m		3m		4m		5m		6m		9m		12m	
	NS	PBMC	NS	PBMC	NS	PBMC	NS	PBMC	NS	PBMC	NS	PBMC	NS	PBMC	NS	PBMC	NS	PBMC	NS	PBMC	NS	PBMC
M1	-	-	NT	NT	-	+	-	-	-	-	-	(+)	-	-	-	-	-	-	NT	NT	NT	NT
F1	-	-	NT	NT	-	-	-	-	-	+	+	+	+	-	+	-	-	-	-	-	+	-
M2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT
F2	-	-	+	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-
M3	-	-	NT	NT	(+)	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT
F3	-	-	NT	NT	-	-	-	-	+	+	+	+	-	+	+	-	-	-	-	-	+	-
Nt	-	-	Nt	NT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT
F4	-	-	NT	NT	-	-	-	-	+	+	+	+	+	+	-	-	(+)	-	-	-	-	-
M4	-	-	NT	NT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT
F6	-	-	NT	NT	+	+	-	-	-	-	+	+	(+)	-	+	-	-	-	-	-	+	-
M6	-	-	NT	NT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT
F10	-	-	+	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-
M10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT
F11	-	-	NT	NT	+	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-
M11	-	-	NT	NT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT
F12	-	-	NT	NT	-	-	-	-	+	+	+	+	(+)	-	-	-	-	-	-	-	-	-
M12	-	-	NT	NT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT
F13	-	-	NT	NT	+	-	-	-	+	+	+	+	-	-	+	+	-	-	-	-	+	-
M13	-	-	NT	NT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT
M15	-	-	-	-	-	-	(+)	-	-	-	-	-	-	-	+	-	-	-	NT	NT	NT	NT
F15	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-

+: Positive

(+): Weak positive

-: Negative

Appendix II

GenBank accession numbers for EHV-2 sequences:

EF515176.1

HQ247775.1

HQ247776.1

HQ247777.1

HQ247778.1

HQ247779.1

HQ247780.1

HQ247781.1

HQ247782.1

HQ247783.1

HQ247784.1

HQ247785.1

HQ247786.1

HQ247787.1

HQ247788.1

HQ247789.1

HQ247790.1

HQ247791.1

HQ247792.1

HQ247793.1

HQ247794.1

NC_001650.1

U20824.1

U63461.1

GenBank accession numbers for EHV-5 sequences:

AF141886.1

EF515178.1

GQ325596.1

GQ325597.1

JX125459.1

Appendix III

Primers for glycoprotein B gene

Primers for type-specific PCR

EHVY+	5’- TCAGAAAGAGCATCAACAGG -3’
EHVY-	5’- GACGCTGGTGGGGTTKATCT -3’
EHV2+	5’- CAGGGCATGGTGCAGGGAA -3’
EHV5+	5’- TAGGGTCTGGTGTCAGAAC -3’

K= G or T

Primers for EHV-2

Eq2B -140 F	5’- ACCTGACCTACGAGGGGACT -3’
Eq2B 458 R	5’- ACGGCGCTAAAGCACTGATA -3’
Eq2B 341 F	5’- CCACCATCTACAAGGGTTGG -3’
Eq2B 1011 R	5’- GAAGGAGGCTGTTATATCATTGG -3’
Eq2B 880 F	5’- CTGTCTTGGAAAGCCACCAC -3’
Eq2B 1545 R	5’- CCCTATCAACTTGGCAGACAC -3’
Eq2B 1837 F	5’- GACATACCCACCCTACACACC -3’
Eq2B 2532 R	5’- GCTCCCGTCTCGCTGTAG -3’

Primers for EHV-5

Eq5B 1399 F	5’- TTGGCAACCTCCCAAGTTCAG -3’
Eq5B 2091 R	5’- CCGCTGGCCACGTTCACTATC -3’
Eq glyB 7 F	5’- GGTCTTTGACTCTAAGGGGTACG -3’
Eq glyB 730 R	5’- TGACCGTCGTTCTAGTGGTG -3’
Eq glyB 669 F	5’- TCAGGCGCTATCACAGTCAG -3’
Eq glyB 1581 R	5’- TGGTGGGGTTTATCTTGCTC -3’
Eq glyB 2092 F	5’- CCAAAATCTAAAGGGGCTGA -3’
Eq glyB 2844 R	5’- CGCGTTCCATGTACCATTTT -3’

Primers for DNA polymerase gene

Primers for EHV-2

EHV2_poly_533F	5‘- GCCGGTACCGATATGAGAAA -3‘
EHV2_poly_1169R	5‘- GCCCTGTCTATCACGTAGGG -3‘
EHV2_poly_947F	5‘- AGATCTCCTGCGTRATCTGG -3‘
EHV2_poly_1534R	5‘- TCTCCACGTGCGTCATAAAG -3‘
EHV2_poly_1895F	5‘- AGAAGCACAAGGCGGTCTC -3‘
EHV2_poly_2386R	5‘- GGCACTTGAAGGTCTTTTCG -3‘

R = A or G

Primers for EHV-5

EHV5_poly_1500F	5‘- CGGCMCTGGTGTTAGACTTA -3‘
EHV5_poly_1658F	5‘- ATCCTTCCATCGGGCAAC -3‘
EHV5_poly_2361R	5‘- ACAAACAGGGTGTGGGTCAT -3‘
EHV5_poly_2168R	5‘- TGTAGCGCTTGGACCTCTCT -3‘

M = A or C

Vector primers

M13 F	5‘- GTAAAACGACGGCCAG -3‘
M13 R	5’- CAGGAAACAGCTATGAC -3‘

Appendix IV

5x Tris borate - EDTA (TBE) buffer

0.045 M Tris borate, 0.0001 M EDTA

10x Restriction buffer (RSB)

50% glycerol, 15mM EDTA, 0.25% bromophenol blue

LB medium

1% Tryptone, 0.1% Yeast extract, 1% NaCl

LB agar

1% Tryptone, 0.1% Yeast extract, 1% NaCl, 1.5% Bacto agar