Equine herpesviruses in Iceland

Course of infection and immune response against gammaherpesviruses type 2 and 5, and isolation of an alphaherpesvirus, type 3

Lilja Þorsteinsdóttir

Thesis for the degree of Philosophiae Doctor

Supervisor:
Vilhjálmur Svansson

Advisor:
Sigurbjörg Þorsteinsdóttir

Doctoral committee:
Einar G. Torfason, Sigurveig Þ. Sigurðardóttir & Valgerður Andrésdóttir

March 2020

UNIVERSITY OF ICELAND
SCHOOL OF HEALTH SCIENCES

FACULTY OF MEDICINE

Herpesveirur í hestum á Íslandi

Smitferlar og ónæmissvörun gegn gammaherpesveirum af gerð 2 og 5, og einangrun á alfaherpesveiru af gerð 3

Lilja Þorsteinsdóttir

Ritgerð til doktorsgráðu

Umsjónarkennari:
Vilhjálmur Svansson

Leiðbeinandi:
Sigurbjörg Þorsteinsdóttir

Doktorsnefnd:
Einar G. Torfason, Sigurveig Þ. Sigurðardóttir & Valgerður Andrésdóttir

Mars 2020

UNIVERSITY OF ICELAND
SCHOOL OF HEALTH SCIENCES
FACULTY OF MEDICINE

Thesis for a doctoral degree at the University of Iceland. All rights reserved. No part of this publication may be reproduced in any form without the prior permission of the copyright holder.

© Lilja Þorsteinsdóttir 2020

ORCID iD 0000-0003-2410-2960

ISBN 978-9935-9516-4-9

Printing by Háskólaprent

Reykjavík, Iceland 2020

Ágrip

Íslenski hesturinn er eini hestastofninn á Íslandi. Hestar voru fluttir hingað við landnám og hafa verið hreinræktaðir síðan. Vegna landfræðilegrar einangrunar er stofninn berskjaldaður fyrir mörgum sjúkdómsvöldum sem herja á hesta í öðrum löndum.

Fimm gerðir herpesveira eru þekktar hjá hestum (equine herpesvirus, EHV), þrjár alfa (α) EHV 1, 3 og 4 og tvær gamma (γ) EHV 2 og 5. Meirihluti fullorðinna hesta á Íslandi hafa mótefni gegn EHV-4. Sjúkdómseinkenni sem líkjast EHV-3 sýkingu hafa komið fram í hrossum og báðar gammaherpesveirurnar eru algengar í stofninum. Ísland er samkvæmt okkar bestu vitund, eina landið þar sem EHV-1 veiran finnst ekki. Við staðfestum, með einangrun, EHV-3 veiruna í fyrsta skiptið hjá íslenska hestastofninum. Út frá eðli herpesveira má álykta að allar fjórar landlægu herpesveirurnar hafi komið til landsins við landnám.

Við rannsóknir á herpesveirum er mikilvægt að notast við frumur úr sama hýsli og veiran. Með því að innleiða æxlisgen í prímer nýrna- og lungnafrumur bjuggum við til hestafrumulínur. Hægt er að rækta frumulínurnar 40 sinnum, ólíkt upprunalegu frumunum sem einungis er hægt að umrækta 10-12 sinnum. EHV-5 veiran hafði mun hærri títer í nýrnafrumulínunni og innleiðslugeta hennar var um fjórum sinnum betri, borið saman við prímer nýrnafrumurnar. Þessar frumulínur nýtast við rannsóknir á hestaveirum.

Sýkiferill EHV-2 og EHV-5 og ónæmissvar gegn γEHV var skoðað með því að fylgja eftir folöldum frá köstun fram að 22 mánaða aldri. Mæðrum þeirra var fylgt eftir í 6 mánuði. Sértækt γEHV mótefnasvar var mælt, veirumagn var greint með qPCR og veirueinangrun var reynd. EHV-2 var einangruð frá 5 daga gömlu folaldi og EHV-5 á degi 12, fyrr en áður hefur fundist. Sértækt γEHV mótefnasvar var ekki mælanlegt hjá folöldunum áður en þau komust á spena, en hækkaði nokkrum dögum síðar. Mótefnin frá móður lækkuðu síðan fram að 3-4 mánaða aldri og samhliða jókst EHV-2 magn. EHV-5 náði hins vegar ekki hámarki fyrr en folöldin voru eins árs. Folöldin voru flokkuð í tvo hópa, eftir sértæku γEHV heildar IgG mótefnasvari móður í sermi við fæðingu, hátt-svar og lágt-svar. Það var greinileg fylgni milli magns mótefna hjá móður og veirumagns og mótefnaframleiðslu folaldanna. Þetta sást betur hjá EHV-5 þar sem 7 mánuðir voru á milli hópanna tveggja með tilliti til þess hvenær veiran náði hámarki. Fyrir EHV-2 var aðeins

mánuður á milli. Með qPCR fékkst mögnun á erfðaefni γEHV frá hluta af folaldasýnunum sem tekin voru áður en þau komust á spena. Þetta gæti bent til þess að einhver hluti erfðaefnis veiranna sé innlimaður í litninga hestsins. Þessar heildarniðurstöður gefa innsýn í hvernig flutningur á mótefnum frá móður hefur áhrif á γEHV sýkingu og mótefnaframleiðslu afkvæmis. Einnig auka niðurstöðurnar þekkingu okkar á sýkiferli EHV-2 og EHV-5 fyrstu tvö æviárin.

Hönnuð var tjáningarkasetta sem tjáir EGFP prótein samhliða tjáningu EHV-2 glýkópróteins B. Framleidd var endurröðuð EHV-2, rEHV2-gB-egfp, sem gat sýkt bæði hestafrumur og kanínufrumur (RK13). Þessi flúrljómandi veira gæti reynst gagnleg í *in vitro* rannsóknum á EHV-2.

Endurraðaðar baculoveirur með tjáningarkassettu sem hefur stýril sem virkar í spendýrum hafa nýst í tilraunabólusetningum, bæði *in vitro* og *in vivo*. Útbúnar voru sex ólíkar baculoveiruferjur og prófaðar *in vitro*. Þrjár voru síðan prófaðar áfram með bólusetningartilraun í folöldum. Bólusett var í eitla, undir húð og í vöðva. Sértækt mótefnasvar gegn markpróteininu var ekki mælanlegt hjá neinu folaldi.

Niðurstöður verkefnisins veita gleggri innsýn í herpesveirusýkingar í íslenska hrossastofninum og jafnframt voru hönnuð tól sem auðvelda áframhaldandi rannsóknir á sviðinu.

Lykilorð:

Frumulínur, herpesveirur, hestar, ónæmissvar og veiruferjur.

Abstract

The native Icelandic horse is the only horse breed in Iceland. The horses were brought to the country in the 9th and 10th centuries and have been purebred ever since. Due to this geographic isolation the horses are immunologically naïve to various agents known to infect horses in other countries.

Five types of herpesviruses are known in horses, three αEHVs; 1, 3 and 4 and two γEHVs; 2 and 5. In Iceland, the majority of adult horses are antibody positive against EHV-4, clinical symptoms resembling an infection with EHV-3 have been detected and EHV-2 and EHV-5 are endemic and ubiquitous. To our knowledge, Iceland is the only country free of EHV-1. In this study we isolated and confirmed EHV-3 for the first time in our native population. In light of the nature of herpesviruses it is likely the four EHVs endemic in Iceland arrived with the founders of the breed.

Cell lines originating from equines are important when working with EHVs. We established equine cell lines, both kidney and lung, with extended life span by transfecting primary cells with a retroviral vector containing oncogenes. The cell lines can be passaged approximately 40 times, as opposed to the primary cells 10-12 times. EHV-5 grew to a substantially higher titer and the transfection efficiency was four times higher in the kidney cell line than in the primary cells. These cells could have further advantages for equine virus research.

The course of EHV-2 and EHV-5 infections and the immune response against γEHV was studied by following foals from birth to 22 months of age and their dams during the first 6 months postpartum. The γEHV specific antibody responses were evaluated, the viral load was measured with qPCR and virus isolation attempted. Both EHV-2 and EHV-5 were isolated earlier than previously reported on day 5 and 12, respectively. γEHV specific antibodies were not detected before colostrum intake but peaked a few days after birth. The maternal antibodies declined when the foals were 3–4 months of age, followed by a peak in the EHV-2 viral load, whereas the EHV-5 viral load peaked when the foals were one year old. Depending on the mare's γEHV specific total IgG levels in serum at birth, the foals were grouped in two groups, high and low. There was a notable correlation between the level of maternal antibodies on the one hand and viral load and induction of

endogenous antibody production in the foals on the other hand. These effects were more evident for EHV-5 as there were seven months between the viral load peaks for the high and low groups, compared to one month for EHV-2. Amplification of viral DNA was detected in some foals' nasal swab and blood leukocyte samples taken before colostrum intake, indicating possible integration of viral DNA sequences into the host chromosome. Overall, these results provide information on how maternal antibody transfer affects γEHV shedding and antibody production of the offspring and extends our knowledge on the EHV-2 and EHV-5 infection in foals during the first two years of life.

An expression cassette was designed to express EGFP protein as a fusion protein with EHV-2 glycoprotein B. An infectious recombinant EHV-2 virus, rEHV2-gB-egfp, was constructed with homologous recombination. The virus was able to infect both equine and rabbit cells (RK13). The fluorescent virus could be a useful tool in EHV-2 *in vitro* studies.

Recombinant baculoviruses carrying a mammalian cell-active expression cassette have been used for gene delivery *in vitro* and *in vivo.* We constructed and tested 6 different rBac-viral vectors *in vitro* and used three of them in vaccination trials in foals. Regardless of injection site, the foals did not develop measurable antibodies against the target.

Overall, the study provides improved tools for EHV research and deeper understanding of the EHV status in the Icelandic horse population.

Keywords:

Cell lines, Equine, Herpesvirus, Immune response and Viral vector.

Acknowledgements

The study was carried out at the Institute for Experimental Pathology, University of Iceland, Keldur and I would like to thank the administration for allowing me to do my PhD at their facilities.

I thank my supervisors, Vilhjálmur Svansson and Sigurbjörg Þorsteinsdóttir, for the opportunity to work on this project, their guidance, encouragement, support, scientific discussions, friendship and kindness over the years.

I am grateful to the members of my doctoral committee, Einar G. Torfason, Sigurveig Þ. Sigurðardóttir and Valgerður Andrésdóttir for their constructive support and guidance.

I thank Ingólfur Helgason for his help in procuring and taming the mares, Kristinn Guðnason at the Horse Breeder's Association of Iceland for obtaining the stallion, and Sigríður Björnsdóttir and Ólafur Sigurgeirsson for taking care of the horses at Kálfsstaðir. Special thanks to Sigurður H. Helgason and Elvar Ríkharðsson who took care of the foals at Keldur and assisted in the vaccination experiment. I would also like to thank Sævar Ingþórsson and Þórarinn Guðjónsson at the Faculty of Medicine, University of Iceland and Stefanía P. Bjarnason and Helga Kristín Einarsdóttir at the Department of Immunology, Landspítali University Hospital for technical assistance and guidance.

I am thankful to my fellow students and co-workers at Keldur for their companionship and many laughs in and out of the lab. Special thanks to Sigríður Jónsdóttir and Ásbjörg Ósk Snorradóttir for their valuable support and good friendship throughout this project.

The writing of the thesis was performed alongside work at the Bacteriology, Parasitology and Pathology divison at Keldur and I would like to thank Vala Friðriksdóttir, the head of the department and my co-workers, Kristín, Atije, Sandra, Bryndís and Þórunn for their patience and understanding.

I am deeply grateful to my husband and daughters for their endless support, encouragement, understanding and patience. My sincere gratitude goes to my parents for always believing in me; their endless motivation and support taught me to never give up. Special thanks to my mother for all the

babysitting and assistance during the writing of the thesis. My family and friends I wish to thank for their invaluable love, support and understanding.

I dedicate this work to my daughters who light up my life. Just remember, you can do anything you set your mind to.

The work was funded by the Icelandic Research Fund, the University of Iceland Research Fund, the Developmental Fund for Icelandic Horse Breeding, the Icelandic Horse Conservation Fund and the Agricultural Productivity Fund of Iceland.

Contents

Ágrip iii
Abstract v
Acknowledgements vii
Contents ix
List of abbreviations xiii
List of figures xvi
List of tables xviii
List of original papers xix
Declaration of contribution xx
1 Introduction 1
1.1 Viruses – *On the edge of life* 1
1.1.1 Classification 2
1.2 Herpesviruses 5
1.2.1 Classification 6
1.2.2 Structure 7
1.2.2.1 Genetic material 8
1.2.2.2 Capsid 9
1.2.2.3 Tegument 11
1.2.2.4 Envelope 11
1.2.3 Replication cycle 12
1.2.3.1 The entry: Virus and cell fusion 12
1.2.3.2 Transport of the capsid to the nucleus 15
1.2.3.3 Production of viral proteins 15
1.2.3.4 Transport of viruses out of the cell 17
1.2.4 Latent infection 18
1.2.4.1 HSV-1 latent infection 19
1.2.4.2 EBV latent infection 20
1.2.5 Viruses of veterinary concern 21
1.2.6 Viral vectors 22
1.3 Equid herpesviruses 23
1.3.1 Equid alphaherpesviruses (α-EHV) 25
1.3.1.1 Equid alphaherpesvirus 1 (EHV-1) 25
1.3.1.2 Equid alphaherpesvirus 4 (EHV-4) 27
1.3.1.3 Equid alphaherpesvirus 3 (EHV-3) 27

1.3.1.4 Equid alphaherpesviruses in wild equids 28
1.3.2 Equid gammaherpesviruses (γ-EHV) 29
1.3.2.1 Equid gammaherpesvirus 2 (EHV-2) 30
1.3.2.2 Equid herpesvirus 5 (EHV-5) 31
1.3.2.3 Equid gammaherpesviruses in wild equids 33
1.4 The immune system of the horse 33
1.4.1 Immune response against EHVs 35
1.5 The Icelandic horse 35

2 Aims 37

3 Materials and methods 39
3.1 Cells 39
3.1.1 Cells and cell culture conditions (Papers I–IV & unpublished data) 39
3.1.2 Establishment of cell lines with extended life span (Paper I) 39
3.1.3 Isolation of peripheral blood leukocytes (PBL) (Paper II) 40
3.2 Viruses 40
3.2.1 Virus strains (Papers I–III & unpublished data) 40
3.2.2 Virus culture (Papers I–IV) 40
3.2.3 Viral load (Papers I and IV) 41
3.2.4 Cloning of viruses (Paper III) 41
3.3 Microscope figures (Papers I, III and IV) 42
3.4 DNA 42
3.4.1 Isolation of DNA (Papers I–IV) 42
3.4.2 PCR and electrophoresis (Papers I, III, IV & unpublished data) 42
3.4.3 qPCR (Paper II) 42
3.4.4 Purification of amplicons (Papers II–IV & unpublished data) 43
3.4.5 Sequencing 43
3.4.6 Plasmid DNA isolation (Papers III & unpublished data) 43
3.4.7 Transfection (Papers I, III & unpublished data) 43
3.5 rEHV-egfp viruses (Paper III & unpublished data) 43
3.5.1 Construction of EHV2-egfp plasmids 43
3.5.2 Homologous recombination 49
3.6 Construction of rBac-viral vectors and vaccination trials (Unpublished data) 49
3.6.1 Construction of rBac-plasmids 49
3.6.2 Construction of rBac-viral vectors 51

3.6.3 Concentration of rBac-viral vectors 51
3.6.4 Vaccination with rBac-viral vectors 51
3.7 ELISA 52
3.7.1 γEHV ELISA (Paper II) 52
3.7.2 ELISA on serum from rBac-viral vector vaccinated foals (Unpublished data) 52
3.8 Protein methods (Unpublished data) 53
3.8.1 Native purification of glycoproteins 53
3.8.2 Western blot 53
3.9 Polyclonal antibodies (Unpublished data) 53
3.10 Data analysis (Paper II) 54

4 Results 55
4.1 Establishment and characterization of fetal equine kidney and lung cells with extended life span (Paper I) 55
4.1.1 Expression of E6 and E7 55
4.1.2 Cell propagation: Cell lines vs. primary cells 55
4.1.3 Cytopathic effect of EHV-2 and EHV-5 55
4.1.4 Viral load of EHV-2 and EHV-5 in extEqFK vs. prmEqFK 57
4.1.5 Transfection rate and expression of EGFP 57
4.2 The effect of maternal immunity on the equine gammaherpesvirus type 2 and 5 viral load and antibody response (Paper II) 57
4.2.1 γEHV specific antibody levels of the foals and mares 58
4.2.1.1 Set up and standardization of the γEHV ELISA 58
4.2.1.2 γEHV specific antibody levels 60
4.2.1.3 Comparison of γEHV IgG levels between foals of dams with high vs. low maternal antibodies at foaling 62
4.2.2 Isolation of EHV-2 and EHV-5 from nasal swabs and blood samples 65
4.2.3 Viral load of EHV-2 and EHV-5 in nasal swab and buffy coat samples 67
4.2.3.1 Viral load in the foals 67
4.2.3.2 Relation of the viral load to the dam's γEHV specific total IgG antibody status at foaling 69
4.2.3.3 Viral load in the nasal swab of the mares 71
4.2.4 Sequencing of qPCR amplicons (Unpublished data) 72
4.3 Construction and characterization of an infectious EHV-2 virus that expresses enhanced green fluorescent protein (Paper III) 74

4.3.1 Construction of EHV2-egfp insertion plasmids 74
4.3.2 Recombinant EHV2-egfp viruses generated with homologous recombination 75
4.3.3 Cloning of rEHV2-gB-egfp and sorting of EGFP expressing cells 76
4.3.4 Sequencing of the rEHV2-gB-egfp virus 76
4.4 Isolation of equid alpaherpesvirus 3 from a horse in Iceland with equine coital exanthema (Paper IV) 76
4.4.1 Collection of the sample, virus culture and viral load 77
4.4.2 PCR and sequencing 78
4.5 Construction of recombinant baculovirus vectors (Unpublished data) 78
4.5.1 Construction of recombinant pFastBac HT B plasmids and rBac-viral vectors 79
4.5.2 Gene expression under the P_{PH} promoter 80
4.5.3 Expression of the target protein 80
4.5.4 Vaccination of foals with the rBac-viral vector 81
4.5.4.1 Total IgG response following vaccination 82
4.5.4.2 Specific IgG subclass and IgE response in sera following vaccination 82
4.5.5 Glycoprotein specific polyclonal antibodies 84

5 Discussion 85
5.1 The Icelandic horse 85
5.2 Herpesviruses in the Icelandic horse breed 85
5.3 Importance of equine cell lines in virus research 86
5.4 Course of γEHV infections and the immune response against EHV-2 and EHV-5 87
5.5 Possible integration 91
5.6 Recombinant EHV2-EGFP virus 92
5.7 Baculovirus vectors 94

6 Conclusions and future perspectives 97
References 99
Original publications 127
Paper I 129
Paper II 133
Paper III 159
Paper IV 181
Appendix 193

List of abbreviations

aa	Amino acid
AHV	Asinine herpesvirus
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
BC	Buffy coat
BLAST	Basic local alignment search tool
bp	Base pair
CMV	Cytomegalovirus
CPE	Cytopathic effect
Ct	Cycle threshold
Cul n	*Culicoides nubeculosis*
Cul o	*Culicoides obsoletus*
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
ds	Double stranded
EBV	Epstein-Barr virus
ECE	Equine coital exanthema
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EHV	Equine herpesvirus
ELISA	Enzyme-linked sorbent assay
EMPF	Equine multinodular pulmonary fibrosis
extEqFK	Equine kidney cells with extended life span
extEqFL	Equine lung cells with extended life span
F	Foal
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum

FCS	Fetal calf serum
gB	Glycoprotein B
GFP	Green fluorescent protein
gG	Glycoprotein G
gp	Glycoprotein
HCMV	Human cytomegalovirus
HHV	Human herpesvirus
HSV	Herpes simplex virus
IBH	Insect bite hypersensitivity
ICTV	International Committee on Taxonomy of Viruses
IE	Immediate early
IFN	Interferon
Ig	Immunoglobulin
IGR	Intergenic region
IL	Interleukin
i.l.	Intralymphatic
i.m.	Intramuscular
i.p.	Intraperitoneally
i.v.	Intravenous
E	Early
EMPF	Equine multinodular pulmonary fibrosis
kDa	Kilodaltons
L	Late
LAT	Latency-associated transcript
M	Mare
MCF	Malignant catarrhal fever
mRNA	Messenger ribonucleic acid
MT	Microtubules
MTOC	Microtubules organizing center
NCBI	National Center for Biotechnology Information
NS	Nasal swab
OD	Optical density

ORF	Open reading frame
PBL	Peripheral blood leukocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
P_{PH}	Polyhedrin promoter
prmEqFK	Primary equine fetal kidney cells
prmEqFL	Primary equine fetal lung cells
PVDF	Polyvinylidene difluoride
qPCR	Quntitative polymerase chain reaction
RK13	Rabbit kidney cells
RNA	Ribonucleic acid
s.c.	Subcutaneous
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ss	Single stranded
TBS-T	Tris-buffered saline with Tween 20
$TCID_{50}$	Tissue-culture infectious dose, 50% endpoint
TNF	Tumor necrosis factor
VSV-G	Vesicular stomatitis virus protein G
VZV	Varicella zoster virus
W	Week
WB	Western blot

List of figures

Figure 1. Classification schemes for animal viruses. 3
Figure 2. The Baltimore classification. 4
Figure 3. Schematic overview of the ICTV virus classification. 5
Figure 4. Structure of herpesvirus. 7
Figure 5. Simplified illustration of herpesvirus genome structures. 9
Figure 6. Schematic illustration of the icosahedral capsid. 10
Figure 7. Schematic overview of HSV-1 replication cycle. 14
Figure 8. Schematic overview of HSV-1 latent infection. 19
Figure 9. Schematic diagram of the equine herpesvirus genome structure. 22
Figure 10. Schematic diagram illustrating the construction of the EHV2-gB-egfp plasmid, (model A). 45
Figure 11. Schematic diagram illustrating the construction of the EHV2-egfp-NC plasmid, (model B). 46
Figure 12. Schematic diagram illustrating the construction of the EHV2-egfp-IGR plasmid, (model C). 47
Figure 13. Schematic diagram illustrating the construction of the EHV2-egfp-TK plasmid, (model D). 48
Figure 14. Schematic diagram illustrating the construction of rBac-plasmid and rBac-viral vector. 50
Figure 15. Cytopathic effect of EHV-2 and EHV-5 infection and EGFP expression after transfection with pEGFP N3 vector. 56
Figure 16. EHV-2 vs. EHV-5 antibody response of foals and mares. 58
Figure 17. Serial dilutions of serum tested in γEHV specific total IgG ELISA for a representative set of foals. 59
Figure 18. γEHV specific antibody response of foals and mares. 61
Figure 19. γEHV specific total IgG status of the mares at foaling. 62
Figure 20. Comparison of γEHV total IgG response between foals with high vs. low maternal antibodies and between corresponding groups of mares 64

Figure 21. Presence of EHV-2 and EHV-5 in nasal swab and buffy coat samples from the foals. 67
Figure 22. EHV-2 and EHV-5 viral load in nasal swab and buffy coat samples from the foals. 68
Figure 23. Comparison of EHV-2 and EHV-5 viral load between foals with high vs. low maternal antibodies. 70
Figure 24. Comparison of total IgGγEHV specific antibody response and viral load in nasal swab samples from foal. 71
Figure 25. EHV-2 and EHV-5 viral load in nasal swabs from the mares. 72
Figure 26. Schematic diagram illustrating the four different EHV2-egfp plasmids constructed. 75
Figure 27. Expression of gB-egfp fusion protein from rEHV2-gB-egpf virus, seen in RK13 cells. 76
Figure 28. Lesions of equine coital exanthema on the vulva and perineal skin of a mare. 77
Figure 29. Cytopatic effect of EHV-3 in primary equine fetal kidney cells. 77
Figure 30. Schematic diagram illustrating the six different pFastBac plasmids constructed. 79
Figure 31. Proteins expressed by the polyhederin promoter. 80
Figure 32. Expression of target protein after transfection in equine cells. 81
Figure 33. Setup of the vaccination experiment. 81
Figure 34. Specific total IgG response tested for rBac-viral vector 1. 82
Figure 35. Target protein specific antibody response of foals vaccinated with viral vectors and subequently protein boosted intralymhatical. For comparison foals and adult horses vaccinated intralymphatical only with the protein. 83
Figure 36. Specificity of polyclonal EHV2-gB and EHV5-gB mouse antibodies. 84

List of tables

Table 1. Equid herpesviruses 23

Table 2. Isolation of EHV-2 and EHV-5 from NS and PBL samples, from 10 pairs of foals and mares 66

Table 3. Sequencing results on qPCR amplicons from selected buffy coat samples 73

Table 4. Nucleotide sequence of primers, target genes and methods used in the EHV2-egfp virus experiments. 195

Table 5. Primers used in construction of pFastBac plasmids 197

List of original papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. **Establishment and characterization of fetal equine kidney and lung cells with extended lifespan. Susceptibility to equine gammaherpesvirus infection and transfection efficiency.** Thorsteinsdóttir, L., Torsteinsdóttir, S., & Svansson, V., *In Vitro Cell Dev Biol Anim,* 2016; 52(8), 872-877.

II. **The effect of maternal immunity on the equine gammaherpesvirus type 2 and 5 viral load and antibody response.** Thorsteinsdóttir, L., Jónsdóttir, S., Stefánsdóttir, S. B., Andrésdóttir, V., Wagner, B., Marti, E., Torsteinsdóttir, S., & Svansson, V., *PLoS One,* 2019; 14(6):e0218576.

III. **Construction and characterization of a green fluorescent infectious equine gammaherpesvirus.** Thorsteinsdóttir, L., Torsteinsdóttir, S., & Svansson, V., "*Submitted for publication*".

IV. **Isolation of equid alphaherpesvirus 3 from a horse in Iceland with equine coital exanthema.** Thorsteinsdóttir, L., Guðmundsson, G. Ö., Jensson, H., Torsteinsdóttir, S., & Svansson, V., "*Submitted for publication*".

All papers are reprinted by kind permission of the publishers.

Additional related scientific papers published during the Ph.D. period but not included in the thesis:

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

Genetic diversity of equine gammaherpesviruses (γEHV) and isolation of a syncytium forming EHV-2 strain from a horse in Iceland. Thorsteinsdóttir, L., Torfason, E. G., Torsteinsdóttir, S., & Svansson, V., *Res Vet Sci*, 2013, 94(1):170-7.

Declaration of contribution

I took part in the planning, set-up, and execution of the study. I also took part in writing grant applications. I wrote and submitted all four papers and revised papers I and II.

Paper I: I participated in all steps of the experiment including the part that was carried out in the BSL-3 laboratory facility at the Institute for Experimental Pathology at Keldur.

Paper II: I took part in sampling throughout the 2-year sample period, did all of the ELISA experiments, the virus isolation and data analysis.

Paper III: I participated in all steps of the experiment. The FACS was performed at the Department of Immunology, Landspítali University Hospital and the virus culture images acquired from microscopes at the Faculty of Medicine, University of Iceland.

Paper IV: I participated in the virus culture, the cell photography and processing, PCR and sequencing.

Unpublished data: I constructed all of the recombinant plasmids and viral vectors. Also, the *in vitro* experiments and testing of the gene expression. I took part in the vaccination experiment, and the production of polyclonal antibodies.

1 Introduction

1.1 Viruses – *On the edge of life*

The word virus comes from Latin meaning poison (Mahoney, 2019). Viruses are intracellular parasites that infect all life forms, from bacteria to animals. They are completely dependent on living hosts for reproduction and are therefore considered to be organisms on the edge of life (Rybicki, 1990).

Viruses are comprised of nucleic acid, either DNA or RNA, single or double stranded, that is packed into a protein capsid and some viruses have in addition a lipid membrane forming an envelope. Beside these features, there is great diversity among viruses, in the genome size and organization, capsid structure, propagation mechanisms and interaction with host cells (Iyer et al., 2006).

Viruses are found wherever there is life, and they have presumably existed since living cells first evolved. There are theories about the origin of viruses, but the great diversity of viruses suggests that they have probably arisen numerous times in the past by one or more mechanisms (Flint et al., 2004; Iyer et al., 2006, reviewed in Forterre, 2006; Koonin et al., 2015). A common virus ancestor is therefore unlikely to have existed (reviewed in Forterre, 2006). Some of the viruses known today have presumably been with us from the earliest ancestor of mammals and coevolved with their host, while other viruses have entered the population only recently (Flint et al., 2004). The first viruses to become adapted to replication in the earliest human population were likely of low virulence and could therefore establish benign long-term relationship with their hosts, as the modern herpesviruses, papillomaviruses and retroviruses (Flint et al., 2004).

The first virus to be discovered as a contagious filterable agent was the tobacco mosaic virus, first described in 1892 by Dmitri Ivanovsky and then again in 1898 by Martinus Beijernick. The first animal virus was found in 1898, the foot-and-mouth virus, and yellow fever was the first human virus to be discovered in 1901 (Flint et al., 2004).

1.1.1 Classification

In 1962, André Lwoff, Robert W. Horne and Paul Tournier presented a comprehensive scheme for virus classification, based on the Linnaean hierarchical system. Viruses were grouped according to their shared properties (Flint et al., 2004; Lwoff et al., 1962) (Figure 1):

1. The type of nucleic acid, DNA or RNA.
2. Symmetry of the protein capsid (helical, icosahedral or complex).
3. Presence or absence of an envelope.
4. Dimension of the virion and capsid.

Viruses use very diverse methods for encoding information in the nucleic acid, but all viruses must produce mRNA, that is translated by the host ribosomes to produce proteins. In 1971 Baltimore proposed a classification that clusters viruses into seven groups, depending on their type of genome and their method of replication, known today as the Baltimore classification (Flint et al., 2004; ViralZone, 2019, reviewed in Baltimore, 1971) (Figure 2):

I. Double stranded DNA (Herpesviruses, Adenoviruses, Poxviruses).
II. Single stranded + DNA (Parvoviruses).
III. Double stranded RNA (Birnaviruses, Reoviruses).
IV. Single stranded + RNA (Togaviruses, Picornaviruses).
V. Single stranded – RNA (Rhabdoviruses, Orthomyxoviruses).
VI. Single stranded + RNA with a DNA intermediate in lifecycle (Retroviruses).
VII. Double stranded DNA with RNA intermediate (Hepadnaviruses).

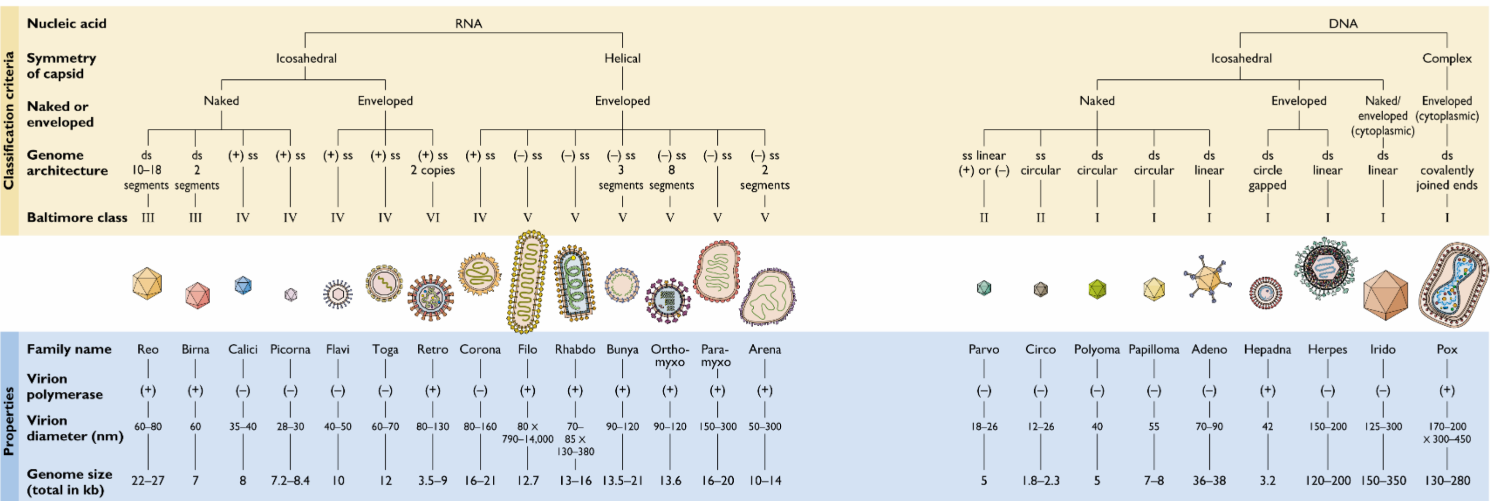

Figure 1. Classification schemes for animal viruses.
Summary of the major characteristics of representative families of vertebrate herpesviruses. ©2004 American Society for Microbiology, adapted from the Academic Press book. Used with permission. No further reproduction or distribution is permitted without the prior written permission of American Society for Microbiology (Flint et al., 2004).

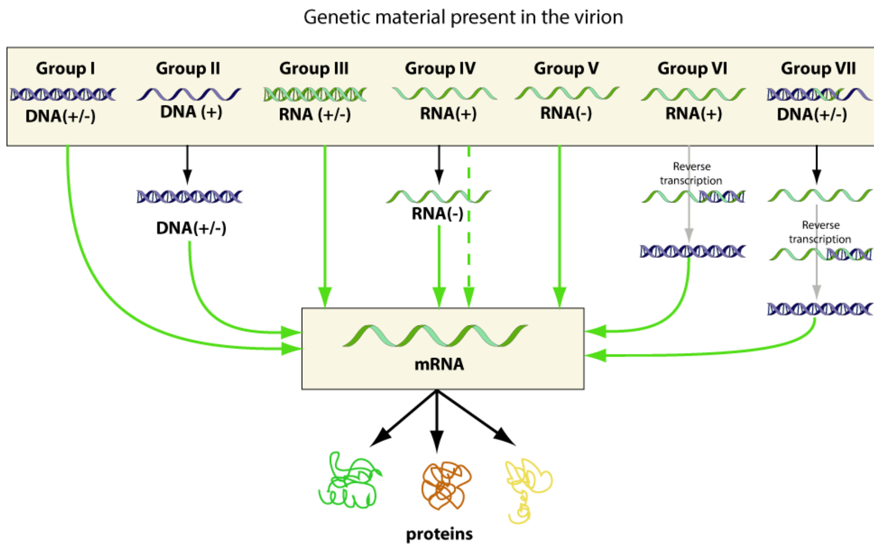

Figure 2. The Baltimore classification.
Image from ViralZone, Copyright © the SIB Swiss Institute of Bioinformation (ViralZone, 2019).

In 1966 the International Committee on Nomenclature of Viruses (ICNV) was established and then renamed in 1977 to the International Committee on Taxonomy of Viruses (ICTV), as the committee regulates both creation and naming of taxa (Adams et al., 2017). The classification is based on various characters that describe and distinguish one virus from another (Lefkowitz et al., 2018). In recent times, sequence comparisons have become the primary set of characters to define and distinguish virus taxa (Lefkowitz et al., 2018). The first report was published in 1971 and, the 10th and the latest one in 2018 (King et al., 2018).

There are over 5500 species of viruses known today, according to the ICTV 2018b release (ICTV, 2019). Figure 3 shows a schematic overview of the ICTV virus classification. *Riboviria* is the only realm, consisting of 1 phylum, 10 orders, 89 families and over 2200 species of RNA viruses and viroids. In addition to the *Riboviria* realm, 4 orders, 46 families and 3 genera have been defined. The order *Caudovirilaes* consists of 1320 species of tailed bacteriophages with dsDNA. There are 122 species in the order *Herpesvirales,* all with dsDNA, animal host, and are all characterized by common morphology. The order *Ligamenvirales* is comprised of 11 species of tailed bacteriophages with dsDNA. There are 225 species in the order *Ortervirales,* both ssRNA and dsDNA viruses that replicate through a DNA and RNA intermediate, respectively.

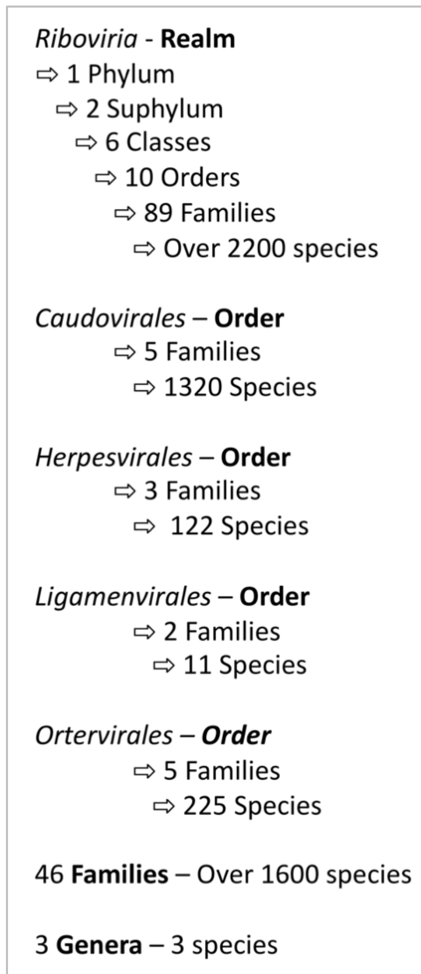

Figure 3. Schematic overview of the ICTV virus classification.
The classification is based on the ICTV 2018b release (ICTV, 2019).

1.2 Herpesviruses

The word *herpes* derives via Latin from Greek, meaning “a creeping”, from *herpein* “to creep” (Dictionary.com, 2019). Today there are over 120 species of herpesviruses known, infecting a wide range of organisms. The infection can vary from being asymptomatic to causing severe diseases or even being lethal.

The first herpesvirus was isolated from a blue wildebeest by the veterinary scientist Walter Plowright (Plowright et al., 1960). The Epstein-Barr virus (EBV), discovered in 1964 was the first human virus to be connected to cancer, Burkitt’s lymphoma (Epstein et al., 1964). Today it is estimated that

EBV contributes to about 1.5% of all human cancer cases worldwide and is associated with many types of lymphomas and carcinomas, e.g. Hodgkin's and Burkitt's lymphoma, and nasopharyngeal and gastric carcinoma (reviewed in Farrell, 2019). Herpesviruses were among the first viruses to be completely sequenced; EBV in 1983, varicella zoster virus (VZV) in 1986, herpes simplex virus 1 (HSV-1) and human cytomegalovirus (HCMV) in 1990 and HSV-2 in 1997 (reviewed in Field et al., 2006).

After primary infection, herpesviruses establish latent infection which is their hallmark and the key to survival. Once a host is infected, a lifelong infection is established. The virus can reactivate at any time, causing another round of lytic infection. Various factors can reactivate the viruses, e.g. physical or emotional stress, fever, UV exposure, and hormonal changes.

Most herpesviruses are species specific and because of the long-term evolutionary relationship with their host, a fine-tuned balance has been reached between the virus and the host. This allows herpesviruses to successfully persist and spread without causing too much damage (Flint et al., 2004). Under certain circumstances, such as in neonates and immunocompromised individuals, they may cause severe diseases such as cancer or lethal infections (reviewed in Adler et al., 2017).

1.2.1 Classification

In the first ICTV report in 1971, the genus *Herpesvirus* was established, consisting of 23 viruses and 4 groups of viruses. In the second report, in 1976, the genus was elevated to family, *Herpetoviridae*. The name was changed to *Herpesviridae* in the third report in 1979 and the family divided into 3 subfamilies, *Alphaherpesvirinae, Betaherpesvirinae* and *Gammaherpesvirinae*. In 2009 the family was elevated to the order *Herpesvirales*, with three families, *Herpesviridae, Alloherpesviridae* and *Malacoherpesviridae (*Davison et al., 2009; Davison, 2010).

Today the order *Herpesvirales* consists of 3 families, 3 subfamilies, 19 genera and 122 species, according to the ICTV 2018b release (ICTV, 2019). The family *Alloherpesviridae* contains 4 genera and 13 species of fish and frog viruses. The *Malacoherpesviridae* family contains 2 genera and 2 species of mollusks viruses. The *Herpesviridae* is the largest family and comprises three subfamilies, *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae,* with 107 species of bird and mammal viruses (ICTV, 2019).

The *Herpesviridae* division into subfamilies is based on host range, replication cycle, cytopathology and latency. The *Alphaherpesvirinae* have a

wide host range and can replicate in various cells of the host, a short reproductive cycle that results in rapid spread of infection and latent infection in ganglia (Cohrs & Gilden, 2001; Pellett et al., 2011; Roizman, 1996). *Beta-herpesvirinae* have a restricted host range but are capable of infecting a variety of cells within their host, a long replication cycle with the infection progressing slowly and latent infection in secretory glands and the lymphoreticular system (Cohrs & Gilden, 2001; Pellett et al., 2011; Roizman, 1996). *Gammaherpesvirinae* have a limited host range and slow replication cycle (Cohrs & Gilden, 2001; Pellett et al., 2011; Roizman, 1996). Cells infected with alpha- and betaherpesviruses support lytic infection while only specific subsets of cells harbor latent virus. Gammaherpesviruses conversely favor the establishment of latency in B lymphocytes, while subsets of cells support lytic infection (Ackermann, 2006, reviewed in Weidner-Glunde et al., 2020).

1.2.2 Structure

Herpesviruses are large viruses that are morphologically distinct from all other viruses. The virion is approximately 150–200 nm in diameter, consists of double stranded DNA core, encased by a 125–130 nm icosahedral nucleocapsid, surrounded by a tegument protein layer which is enclosed within a lipid bilayer envelope (Figure 4), (Liu & Hong Zhou, 2007).

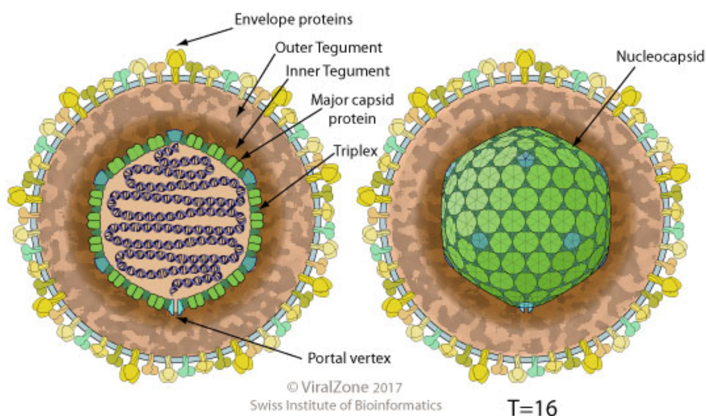

Figure 4. Structure of herpesvirus.
Image from ViralZone, Copyright © the SIB Swiss Institute of Bioinformation (ViralZone, 2019).

1.2.2.1 Genetic material

Herpesviruses have large, double-stranded linear DNA, that encodes for 70–200 genes. The genomes that have been examined in detail have a single unpaired nucleotide extension at the 3'- end and no terminal proteins (Davison, 1984; Mocarski & Roizman, 1982; Tamashiro & Spector, 1986, reviewed in McGeoch et al., 2006). The molecular size and the base composition of the DNA varies between herpesviruses, the size ranging from 125–295 kbp with 32–75 % G + C content (Honess, 1984; Roizman, 1996). The DNA is packed at high density in a liquid crystalline form, presumably like a spool lacking a spindle (Booy et al., 1991; Zhou et al., 1999).

One of the most interesting features of the herpesvirus genome is the sequence arrangement. Direct or inverted repeats, either terminal, internal or both result in a number of different genome structures (Figure 5), (Davison, 2007; Pellett et al., 2011; Roizman, 1996). The explanation for these repeats is probably connected with the mode of DNA replication, thought to occur by circularization, followed by production of concatemers and cleavage of unit-length genomes during packaging into capsids (Davison, 2007, reviewed in Boehmer & Lehman, 1997). These repeats ofter contain no protein-coding regions (Davison, 2007). Figure 5 shows a simplified description of herpesvirus genome structures. Structure 1 has a unique sequence flanked by a direct repeat at both termini, varying in size from 30 bp to 10 kbp. Structure 2 also contains a single unique sequence but is flanked by variable copy numbers of tandemly repeated sequences at each terminus. Many gammaherpesviruses have a structure 2 genome. Different elements are at each terminus in structure 3, also present internally in an inverted orientation, dividing the genome in two unique regions. Structure 4 contains long and short unique regions, flanked by large inverted repeat sequences and contains a short terminal repeat which is present internally in inverse orientation in the junction region. Some herpesviruses, like the human Epstein-Barr virus have none of these structures but have large repetitive elements within the genome that are unrelated to those found at the termini (Pellett et al., 2011).

The number of ORFs within the herpesvirus genomes that potentially encode proteins, ranges from 70 to more than 200 (Pellett et al., 2011). Most of the herpesvirus genome is occupied by protein coding regions with introns in a minority of the genes. Beta- and gammaherpesviruses have more intron-containing genes than alphaherpesviruses (reviewed in McGeoch et al., 2006).

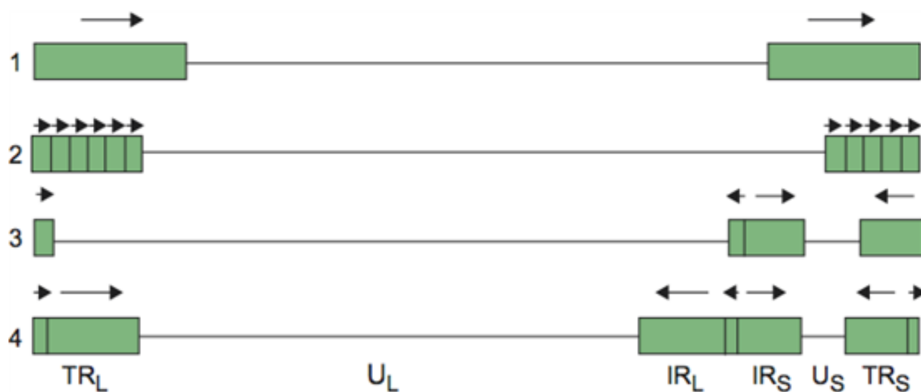

Figure 5. Simplified illustration of herpesvirus genome structures.
Unique and repeated sequences are shown as solid lines and rectangles, respectively. The orientations of repeated sequences are indicated by arrows. The nomenclature used to describe regions of type 3 and 4 genomes is shown: U_L = unique long; U_S = unique short. The repeated sequences flanking the unique regions are named "terminal repeat short" (TR_S) and "internal repeat short" (IR_S), etc. The figure and figure text are reprinted from (Pellett et al., 2011), with permission from ICTV, Copyright © 2019, International Committee on Taxonomy of Viruses (ICTV).

Members of the order *Herpesvirales* are phylogenetically very distant from each other. Only two genes are highly conserved in all herpesviruses, encoding DNA polymerase and the putative ATPase subunit of the terminase, a complex that is responsible for packing virus DNA into the capsids (Davison et al., 2009; Pellett et al., 2011).

About 40 protein-coding genes are conserved among the *Herpesviridae* family (Pellett et al., 2011). These genes encode for example components of the DNA replication and packaging machinery, nucleotide modifying enzymes, capsid proteins, membrane proteins and tegument proteins (Pellett et al., 2011).

1.2.2.2 Capsid

The herpesvirus nucleocapsid has many functions. It protects the genome and transports it from the host cell plasma membrane to the nuclear membrane where it is released to the nucleus and mediates the egress of nascent capsid from the cell nucleus (Ojala et al., 2000; Pasdeloup et al., 2013).

The capsid is a highly organized icosahedral-shaped protein shell, about 125–130 nm in diameter and 16 nm thick (Liu & Hong Zhou, 2007; Pellett et al., 2011). The mature capsid is a T16 = icosahedron consisting of 11 pentons and 150 hexons, a total of 161 capsomers composed primarily of five and six copies, respectively, of the same protein that are joined together by masses, called triplexes (Figure 6) (Liu & Hong Zhou, 2007; Pellett et al., 2011). The triplexes are made of two smaller proteins, present in a 2:1 ratio (Pellett et al., 2011). The twelfth pentameric position is employed by a ring-like structure, composed of 12 copies of capsid portal protein (Pellett et al., 2011).

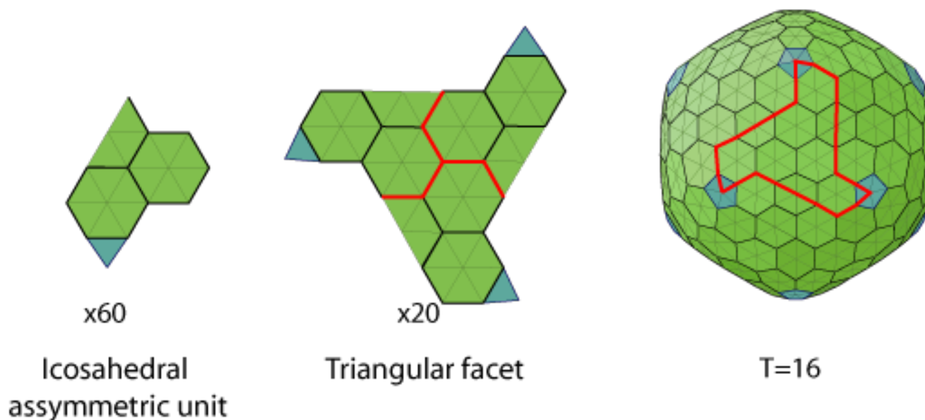

Figure 6. Schematic illustration of the icosahedral capsid.
The capsid consists of 60 asymmetric units, each made of 16 proteins (T = 16). Image from ViralZone, Copyright © the SIB Swiss Institute of Bioinformation (ViralZone, 2019).

Three capsid-like structures, A-, B- and C-capsids have been isolated from herpesvirus infected cells (Gibson & Roizman, 1972; Liu & Hong Zhou, 2007). A-capsids are empty, contain neither viral DNA nor discernible internal structure and result from abortive DNA packing. The B-capsids have an inner array of scaffolding proteins, but it is still debated whether the capsids are abortive forms or assembly intermediates. C-capsids are packed with the viral DNA genome, have no scaffolding proteins and mature into an infectious virion (Liu & Hong Zhou, 2007).

1.2.2.3 Tegument

The tegument of herpesviruses or granular, occupying the space between the capsid and the envelope (Flint et al., 2004; Harrison et al., 1996). The tegument comprises a significant part of the virion space and contains about 40% of the virion protein mass (Liu & Hong Zhou, 2007). There are indications of inner and outer tegument layers, capsid and envelope associated, respectively (Pellett et al., 2011). The structure of the tegument layer is not well defined, but there are evidence of icosahedral symmetry in the inner layer, immediately adjacent to the capsid, while the outer layer appears to be amorphous (Flint et al., 2004; Newcomb & Brown, 2010; Zhou et al., 1999). Tegument proteins have many diverse structural and regulatory functions, including adjustment of the host-cell environment immediately after entry, transport of the capsid to the nucleus during infection, and assembly of virions during egress (reviewed in Kelly et al., 2009; Owen et al., 2015).

The number of proteins in the tegument layer is variable between herpesviruses, and individual proteins can vary markedly in abundance (Pellett et al., 2011). For example, the tegument of HSV-1 contains more than 20 virus-encoded proteins and at least 30 have been found in the HCMV tegument (Liu & Hong Zhou, 2007).

1.2.2.4 Envelope

The envelope, a lipid bilayer, is the outermost element of herpesviruses (Flint et al., 2004). The envelope is sensitive to various chemicals and physical agents, e.g. solvents, desiccation and heat. Viruses have therefore limited tenacity outside the host and are usually transferred directly from host to host. More than 10 different integral viral glycoproteins are embedded in the envelope, in multiple copies, forming a network of closely spaced spikes, of at least three distinct morphologies (Liu & Hong Zhou, 2007; Pellett et al., 2011). They function in attachment and entry of the virus to a host cell at the beginning of infection (Flint et al., 2004). The envelope also contains numerous host proteins or constituents that may participate in the induction of host cellular responses (Liu & Hong Zhou, 2007).

Herpesviruses have a single envelope, but they undergo two rounds of budding. The primary envelope comes by budding from the inner nuclear membrane, followed by a process of de-envelopment by fusion with the outer nuclear membrane. The final envelope is derived from cytoplasmic membranes, such as the Golgi, the trans-Golgi network, and endosomes, see section 1.2.3.4, (reviewed in Johnson & Baines, 2011).

1.2.3 Replication cycle

The members of the order *Herpesvirales* are genetically diverse, and the detail of their replication strategy varies, but all herpesviruses replicate their DNA in the host cell nucleus, where the viral DNA is transcribed to mRNA. Primary herpesvirus infection is lytic, then the virus becomes latent, but can reactivate and start another round of lytic infection. The lytic replication cycle can be categorized in four main parts: entry of the virus with virus and cell fusion, transport of the capsid to the nucleus, production of viral proteins and finally transport of virus out of the host cell. Each part will be discussed in more detail in the following sections, mainly based on HSV-1.

1.2.3.1 The entry: Virus and cell fusion

To enter a target cell, the herpesvirus envelope has to fuse with the host cell membrane. Herpesviruses use glycoproteins embedded in their envelope to bind to receptors on the target cell (Flint et al., 2004). This is a complex process; it involves multiple glycoproteins, each with multiple functions. Each glycoprotein can bind to multiple receptors, interact with other glycoproteins and/or undergo conformational changes to induce membrane fusion (reviewed in Connolly et al., 2011). This process varies between herpesvirus types, the target cells and other factors.

Herpesviruses require at least three glycoproteins for entry and membrane fusion, glycoprotein B (gB) and a gH/gL heterodimeric complex (reviewed in Connolly et al., 2011; Eisenberg et al., 2012; Ibáñez et al., 2018). Glycoprotein B is structurally conserved across all herpesviruses, while gH/gL is more variable (reviewed in Eisenberg et al., 2012). HSV-1 and EBV gB have structural homology to glycoprotein G in the vesicular stomatitis virus (VSV-G), which is the sole VSV fusion protein, and also to glycoprotein 64 (gp64) of baculovirus which is necessary and sufficient for their cell entry (Heldwein et al., 2006; Kadlec et al., 2008; Roche et al., 2006). The gH/gL complex is stable and the glycoproteins are always found together, in the ratio 1:1, where gL is required for correct folding and trafficking of gH (reviewed in Eisenberg et al., 2012).

Some herpesviruses use additional receptor binding proteins in the entry/fusion process, e.g. HCMV which uses UL128, UL130 and UL131, that interact with the gH/gL complex when entering epithelial and endothelial cells (Ryckman et al., 2006; Sinzger, 2008). EBV uses gp42, that binds to human leucocyte antigen (HLA) receptor on B cells as the first step in the fusion process, but gp42 is not used when entering epithelial cells (reviewed in

Connolly et al., 2011; Eisenberg et al., 2012). The gp42 protein is unique to EBV but has functional homologs in other herpesviruses, such as gD in HSV-1 (Kristensen et al., 2010).

For HSV-1 entry, the fusion begins with gD protein binding to target receptors, e.g. nectin-1, nectin-2 or herpesvirus entry mediator (HVEM). The gD then undergoes conformational changes that activate the gH/gL complex, that then promotes the activation of gB, which acts as the viral fusion protein that brings together the cell membrane and virus envelope. The two membranes fuse and the capsid is delivered into the cell (reviewed in Eisenberg et al., 2012; Ibáñez et al., 2018). The alphaherpesvirus VZV does not have gD but uses gE instead as a receptor binding protein (reviewed in Heldwein & Krummenacher, 2008). Glycoprotein E is not required in the HSV-1 entry, but it enhances cell-to-cell spread (Polcicova et al., 2005; Saldanha et al., 2000).

Some glycoproteins tether the virus to the cell but are not essential for entry. This includes gC in HSV-1 and gp350 and BMRF2 in EBV. These glycoproteins serve to accumulate the virus at the cell surface but do not trigger the fusion. Viral entry can still occur in the absence of these glycoproteins, but at lower efficiency (reviewed in Connolly et al., 2011).

Figure 7 shows a schematic overview of the HSV-1 reproductive cycle, which is comparable for all herpesviruses. Steps 1–3 show how the virion fuses with the target cell membrane by binding glycoproteins to cell receptors.

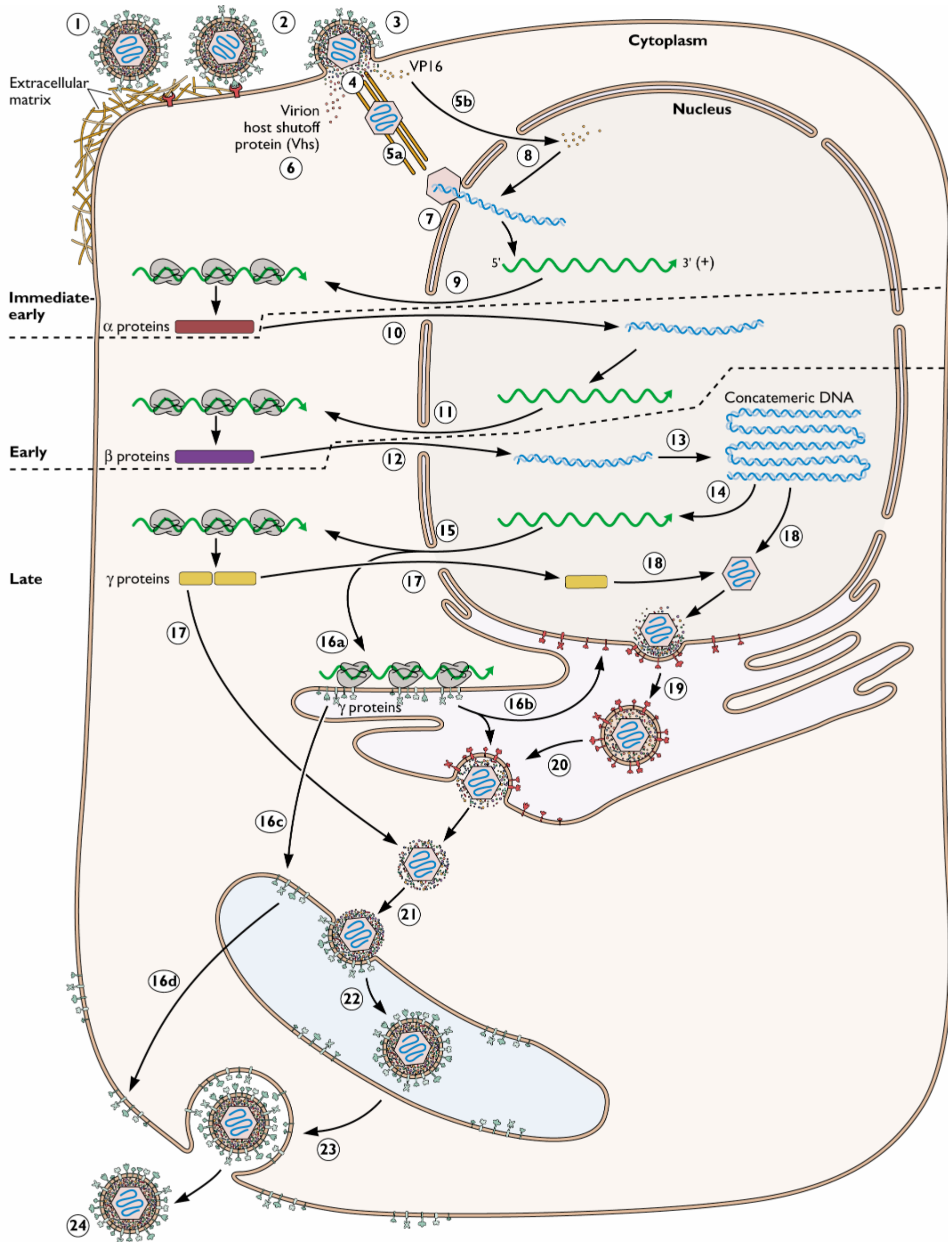

Figure 7. Schematic overview of HSV-1 replication cycle.

Numbers in figure are explained in the main text, sections 1.2.3.1–1.2.3.4. ©2004 American Society for Microbiology. Used with permission. No further reproduction or distribution is permitted without the prior written permission of American Society for Microbiology (Flint et al., 2004).

1.2.3.2 Transport of the capsid to the nucleus

After the fusion, the capsid and some tegument proteins are released into the cytoplasm of the target cell (Flint et al., 2004). A number of tegument proteins associated with the capsid remain and function in the cytoplasm, such as the virion host shutoff (vhs) protein (UL41 gene product) that inhibits host protein synthesis (Pellett et al., 2011). Other proteins, such as the VP16 (UL48 gene product) migrate to the nucleus and contribute to the transcription of viral genes needed for the replication cycle (Pellett et al., 2011; Roizman & Zhou, 2015, reviewed in Ibáñez et al., 2018).

The capsid is transported rapidly and efficiently to the nucleus by microtubules (MT) (Flint et al., 2004; Pellett et al., 2011; Sodeik et al., 1997). MTs are long and hollow cytoskeletal filaments with biochemically distinct ends. The plus end points towards the cell periphery and the minus end is attached to the centrosome, the major microtubule organizing center (MTOC) (reviewed in Garner, 2003; Ibáñez et al., 2018; Nogales, 2000). The viral cytoplasmic dynein transports the capsid along the MTs to the MTOC. Adenosine triphosphate (ATP) is needed for this capsid transport (Dohner et al., 2002; Lee et al., 2006; Wolfstein et al., 2006). It has been suggested that the viral tegument protein VP26 plays a key role in the interaction between the capsids and dynein (Douglas et al., 2004). From the MTOC the capsid is moved further to the nuclear pore, where the viral genome is imported into the nucleoplasm (Dohner et al., 2006; Douglas et al., 2004). Steps 4–7 in figure 7 show the transport of the capsid to the nucleus.

1.2.3.3 Production of viral proteins

During a lytic infection the genes are coordinately transcribed and temporally regulated in an immediate early (IE), early (E), and late (L) fashion. The production of viral proteins can be seen in steps 8–18 in figure 7. (Flint et al., 2004, reviewed in Ibáñez et al., 2018).

IE (α) genes are largely distinct among various herpesvirus families and subfamilies. They protect the virus against innate host immunity and regulate subsequent gene expression by transcriptional and post-transcriptional mechanisms. IE genes can be transcribed in the absence of de novo protein synthesis. In HSV-1 the VP16 protein interacts with the host transcription proteins to stimulate transcription of IE genes using the host cell RNA polymerase II. IE mRNAs are spliced and transported to the cytoplasm where they are translated. The IE proteins are then transported to the nucleus

where they activate transcription of E genes. (Flint et al., 2004; Pellett et al., 2011, reviewed in Ibáñez et al., 2018).

E (β) genes encode the DNA replication complex and a variety of enzymes and proteins involved in modifying host cell metabolism. E gene transcription is dependent on expression of IE proteins. E gene transcripts are rarely spliced but are transported to the cytoplasm for translation. Some E proteins function in the cytoplasm but others are transported to the nucleus. Synthesis of viral DNA occurs from one or more origin of replication, probably by a rolling circle mechanism (Ibáñez et al., 2018). HSV-1 DNA replication requires seven gene products: an origin-binding protein, a ssDNA-binding protein, a DNA polymerase composed of two subunits and a helicase-primase (H/P) complex composed of three gene products. Homologs for all of these proteins except for the origin-binding protein have been identified in all three *Herpesviridae* subfamilies. DNA replication and recombination produce long DNA, through a replication coupled process, with replication fork, which is used as a template for L gene expression. (Flint et al., 2004; Pellett et al., 2011, reviewed in Ibáñez et al., 2018). Two models of the replication fork have been described (reviewed in Weller & Coen, 2012). The first model is simpler, the DNA is first unwound by one molecule of H/P. The polymerase subunit performs leading strand synthesis on the top strand. A second molecule of H/P on the bottom strand lays down an RNA primer and the single stranded DNA is then coated by the ssDNA binding protein (Chen et al., 2011). In the second model the method is the same for the leading strand, but the lagging strand forms a replication loop to align it with the leading strand. A second polymerase subunit on the lagging strand initiates Okazaki fragment synthesis using an RNA primer (Stengel & Kuchta, 2011). The replication forks and associated proteins were recently purified and analysed, showing that several DNA repair complexes and key transcription factors were recruited to, or near the replication fork (Dembowski et al., 2017).

L (γ) genes primarily encode the virus structural proteins and additional proteins needed for virus assembly and particle egress. L gene transcription is dependent on viral DNA synthesis, with the exception of a small number of non-translated RNAs expressed by specific herpesviruses. L mRNAs are rarely spliced but are transported to the cytoplasm where they are translated. Some L proteins are inserted into the membranes of the rough endoplasmic reticulum, many of these membrane proteins are glycosylated. Some precursor viral membrane proteins are thought to be situated on both the inner and outer membrane as well as in the membrane of the endoplasmic

reticulum. L proteins are also transported to the Golgi for further adjustments and processing. Mature glycoproteins are then transported to the cell plasma membrane. Some L proteins are transported to the nucleus for capsid assembly. The newly synthesized DNA is packed from the concatemer into preformed immature capsids within the nucleus, a process that involves several viral proteins. (Flint et al., 2004; Pellett et al., 2011, reviewed in Ibáñez et al., 2018). The scaffolding proteins form the inner shells in the procapsids and B capsids (Wu et al., 2016). These internal scaffolding proteins are not present in the mature virion. Two genes specify these proteins, UL26.5 which encodes the major component of the protein core, and UL26 that specifies the minor constituent (Preston & McDougall, 2002). The number of tegument proteins varies between herpesviruses. They can be major or minor, and some are conserved in all herpesviruses. The tegument proteins have many important roles in the life cycle, such as in the assembly and egress of the virus particles (reviewed in Guo et al., 2010).

1.2.3.4 Transport of viruses out of the cell

The host cell nuclear envelope is composed of an inner and outer membrane, separated by a perinuclear space and connected by nuclear pore complexes. The diameter of the viral capsid is approximately 120 nm but the nuclear pores only support passage of particles that are up to 36 nm in diameter (Panté & Kann, 2002, reviewed in Mettenleiter et al., 2013). The capsid therefore exits the host cell nucleus by traversing the nuclear membranes and the space in between (reviewed in Mettenleiter et al., 2013). The transport and release of the herpesvirus from the host cell can be seen in steps 19–24 in figure 7.

The capsid containing DNA and some tegument proteins bud from the inner nuclear membrane into the perinuclear lumen, forming a primary envelope (Flint et al., 2004, reviewed in Mettenleiter et al., 2013). This process is driven by a heterodimeric complex of two conserved proteins, designated as pUL34 and pUL31 in HSV-1. The pUL34 is a tail-anchored transmembrane protein located in the nuclear envelope and orthologs of pUL31 are soluble proteins (reviewed in Bailer, 2017; Mettenleiter et al., 2013).

To get to the cytoplasm, the virion fuses with the outer nuclear membrane, releasing the viral capsid into the cytosol of the host cell (Flint et al., 2004; Mettenleiter, 2002, reviewed in Mettenleiter et al., 2013). Not much is known about this process, but one line of evidence favors the view that the

enveloped particles in the perinuclear space become “de-enveloped” by fusion with the outer nuclear membrane. Resulting nucleocapsids are then re-enveloped in a Golgi or post-Golgi compartment (Pellett et al., 2011).

Capsid and tegument proteins are transported to a late trans-Golgi compartment or endosomes that contain mature viral membrane proteins. Tegument proteins, which are added in the nucleus remain with the capsid and other tegument proteins, are added in the cytoplasm. The capsid buds into the Golgi, where it acquires an envelope containing mature viral envelope proteins and the complete tegument layer. The enveloped virus then buds into a vesicle, which is transported to the plasma membrane for release with exocytosis (reviewed in Ibáñez et al., 2018).

1.2.4 Latent infection

The ability to enter into latency is an important survival strategy for herpesviruses as they can persist long-term in the host. Latent viruses require hosts that stay alive and interact with others and therefore select long living cell populations to establish latency (reviewed in Brown, 2017; Grinde, 2013; Weidner-Glunde et al., 2020; Wilson & Mohr, 2012). During latent infections there are no symptoms, no infectious viruses are produced and no transmission occurs (reviewed in Brown, 2017; Nicoll et al., 2012). The viral genome is transported to the host cell nucleus as in lytic infection, but when it is released from the capsid is circularized, probably by the host DNA repair machinery. The genome exists as an episomal DNA element packed in histones, probably caused by lack of IE gene expression (Deshmane & Fraser, 1989; Pellett et al., 2011, reviewed in Brown, 2017; Grinde, 2013; Wilson & Mohr, 2012). The latently infected cells are less recognized by the immune system due to restricted DNA replication and limited viral gene expression (Reese, 2016, reviewed in Brown, 2017).

Reactivation is a risky option for the virus, as active replication will induce various host immune system mechanisms that can lead to the death of the infected cell (reviewed in Grinde, 2013). Changes in the transcription factor environment of the latently infected cell due to external stimulation factors, e.g. physical or emotional stress, fever, UV exposure, hormonal changes, or cell differentiation leads to IE gene expression and consequent reactivation of the virus and entry into the productive life cycle (Pellett et al., 2011).

Latent infection can be beneficial for the host. Latency induced protection is not antigen specific but involves prolonged production of the antiviral cytokine IFN-γ and systemic activation of macrophages. This leads to a

heightened state of immune activation against subsequent infection (Barton et al., 2007). It has been shown that mice latently infected with either murine gammaherpesvirus 68 or murine cytomegalovirus which are highly similar to the human herpesviruses EBV and CMV, respectively, are somewhat resistant to *Listeria monocytogenes* and *Yersinia pestis.* The mice were protected from *L. monocytogenes* up to 3 months after infection with murine γHV68, and replication and systemic spread of *Y. pestis* decreased (Barton et al., 2007).

1.2.4.1 HSV-1 latent infection

Lytic HSV-1 infection occurs in epithelial cells while latent infection is in neurons (Flint et al., 2004). The initiation of latent infection occurs in the same manner as lytic infection; HSV-1 binds to receptors on the cell surface, membrane fusion events lead to deposition of the nucleocapsid into the cytoplasm, the capsid traffics to the nucleus where the virus DNA is released to the host cell nucleus. At this point the infection stalls. The viral DNA circularizes, and virus DNA synthesis and production of progeny viruses are blocked completely (Flint et al., 2004, reviewed in Brown, 2017). Figure 8 shows a schematic view of the HSV-1 latent infection.

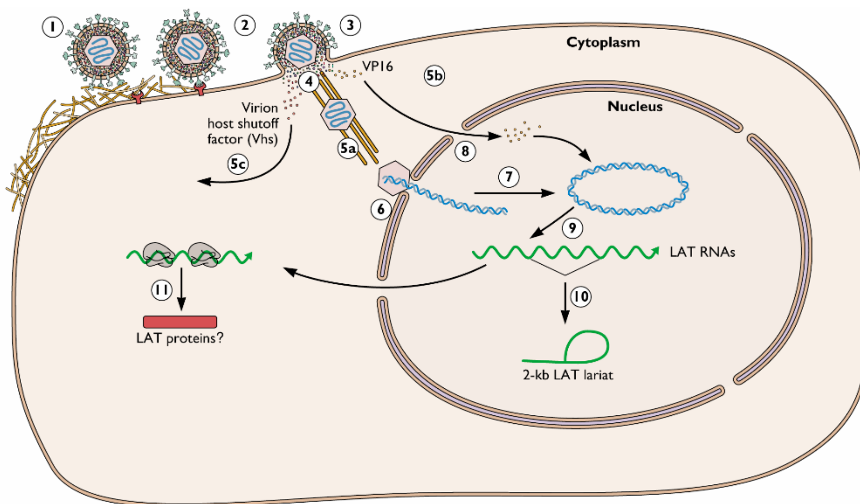

Figure 8. Schematic overview of HSV-1 latent infection.
©2004 American Society for Microbiology. Used with permission. No further reproduction or distribution is permitted without the prior written permission of American Society for Microbiology (Flint et al., 2004).

The transcription is severely restricted and only a single pre-mRNA is produced from the latency-associated transcript (LAT) promoter. Two hypotheses exist about LAT function: one says that LAT blocks apoptosis upon primary infection of neurons (or upon reactivation), and the other one that LAT maintains the latent state through antisense inhibition of ICP0 translation, which is a crucial transcription factor (Flint et al., 2004). Most latent infected neurons contain 10–100 copies of viral genome, but some harbor >1000 copies (Sawtell, 1997), suggesting that a latent state can be established after initiation of the productive cycle (Pellett et al., 2011).

Reactivation is proposed to be the reverse of entry into latency (reviewed in Brown, 2017). Expression of ICP0 activates other IE gene products, that then activate E and L gene expression (Leib et al., 1989). Reactivated viruses migrate back to the site of the initial infection in the oral epithelium, producing lytic infection (reviewed in Brown 2017).

1.2.4.2 EBV latent infection

EBV infects both B lymphocytes and epithelial cells but becomes latent in memory B cells (Flint et al., 2004, reviewed in Kempkes & Robertson, 2015) at a frequency of ~1 in $1x10^5$ to $1x10^6$ cells in a healthy individual (reviewed in Thompson & Kurzrock, 2004). In latent infection the genome is maintained as a circular episome, in 10–50 copies per cell (Flint et al., 2004). The memory B cells are dividing cells and the EBV has therefore to retain the potential for replication of its genome during latency (Roizman, 1996). The circular DNA is replicated in the S phase of the host cell (Flint et al., 2004).

Latent infected cells express at least 11 viral proteins, including 6 nuclear proteins (EBNA1, 2, 3A, 3B, 3C and LP), three viral membrane proteins (LMP1, 2A and 2B) and two small RNA molecules (EBER1 and 2) (reviewed in Kempkes & Robertson, 2015).

Latent EBV exhibits three distinct latent infectious statuses: Latency I, II and III, each with a limited and distinct set of viral proteins and RNAs (reviewed in Grinde, 2013; Kang & Kieff, 2015). Upon infecting a resting B cell, the virus enters Latency III. The set of proteins and RNAs produced induce the B cell to proliferate, subsequently the virus shuts off genes and enters Latency II. A more limited set of proteins and RNAs are produced in Latency II, and this induces the B cell to differentiate into a memory B cell. The gene expression then becomes more restricted, and Latency I is established where only one protein and some non-coding RNAs are expressed (reviewed in Amon & Farrell, 2005; Grinde, 2013).

1.2.5 Viruses of veterinary concern

Herpesviruses infect, in addition to humans and horses, a wide range of animals. Primary infection can vary from being asymptomatic to very severe diseases. Most herpesviruses are well adapted to their host and have coevolved with their specific host species for millions of years. However, some herpesviruses are capable of crossing species barriers.

Alphaherpesvirinae are the most pathogenic of the three subfamilies. Suid herpesvirus 1 (suHV-1), also known as pseudorabies virus (PRV) causes Aujeszky's disease in wild and domestic swine, with clinical symptoms such as hyperexcitability, ataxia, progressive paralysis, coma and abortion. Some animals are asymptomatic but shed the virus with nasal discharge and saliva. Infection in sows can result in 50% abortion and the mortality rate among piglets can reach 100%. With vaccination the virus has been eradicated from domestic swine in many countries, but the virus remains enzootic among free-ranging animals. A number of secondary hosts are also known, such as horses, dogs, sheep, cattle and cats, where infectioun can cause severe neurological symptoms that frequently lead to death (reviewed in Azab et al., 2018; Wozniakowski & Samorek-Salamonowicz, 2015).

Another alphaherpesvirus that can cross the species barrier is Cercopithecine herpesvirus 1 (CeHV-1) or B virus which is common in macaques. The virus can also infect humans, causing paralysis and severe encephalitis, with high mortality. Most of the known cases in humans were traced to inappropriate handling or breeding of the animals (reviewed in Azab et al., 2018; Wozniakowski & Samorek-Salamonowicz, 2015).

Bovine herpesvirus 1 (BoHV-1) causes symptoms such as upper respiratory tract disorder, conjunctivitis and genital disorder in cattle, i.e. infectious rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV) and infectious balanoposthitis (IBP) as well as abortion and neurological signs (reviewed in Graham, 2013). IBR is the most common and costly cattle disease in the US (reviewed in Jones & Chowdhury, 2007). There is also evidence of BoHV-1 in deer species (Fabisiak et al., 2018). BoHV-2 is another severe virus known in cattle, causing mammillitis (Kemp et al., 2008; Petrovski, 2005) and pseudo-lumpy skin disease (Brenner et al., 2009).

Another common animal alphaherpesvirus is gallid herpesvirus 2 (GaHV-2), infecting chickens and causing malignant T cell lymphomas. The disease, also known as Marek's disease (MD) has a major economic impact on the poultry industry (reviewed in Gennart et al., 2015).

The gammaherpesviruses alcelaphine herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OvHV-2) cause malignant catarrhal fever (MCF). Wildebeest is the natural host for AlHV-1 and sheep for OvHV-2. Infection in the natural host is usually asymptomatic. However, symptoms like fever, inappetence, ocular and nasal discharge result in severe or fatal MCF in susceptible species like cattle, deer, bison, water buffalo and pigs (reviewed in Russell et al., 2009).

1.2.6 Viral vectors

Herpesviruses have large genome with non-coding areas which makes gene insertion feasible and the virus advantageous as viral vaccine vectors. Viral vectors have mostly been constructed from alphaherpesviruses, and have been made from all of the three known alpha equine herpesviruses (Akhmedzhanov et al., 2017; Azab et al., 2009; Ibrahim el et al., 2004). An EHV-1 viral vector-based vaccine has shown to be a promising alternative in vaccination against influenza in pigs (Said et al., 2013) and the Rift Valley fever virus (RVFV) in sheep (Said et al., 2017). The genome structure of equine herpesviruses is shown in figure 9, and the general features of the EHVs genomes are described in following sections.

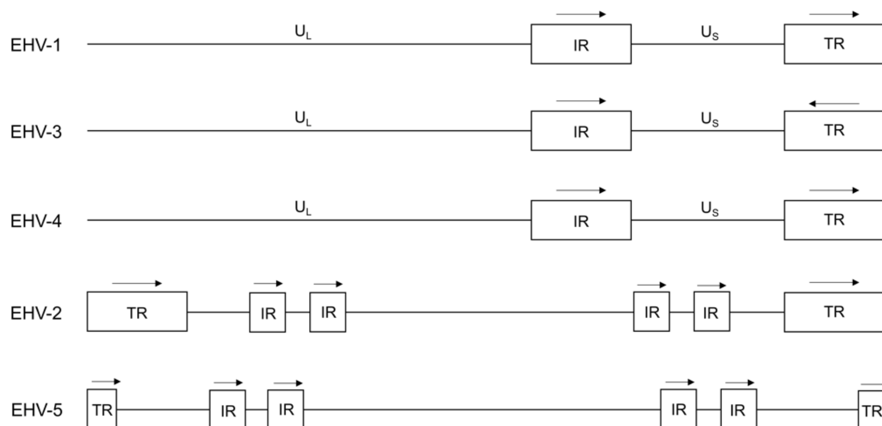

Figure 9. Schematic diagram of the equine herpesvirus genome structure.
U_L: Unique long. U_S: Unique short. IR: Internal repeat. TR: Terminal repeat.

1.3 Equid herpesviruses

Equid herpesviruses (EHV) consist of pathogens that infect all members of the *Equidae* family (Slater, 2014). Five EHVs are known to infect domestic horses, EHV-1 to EHV-5, and three infect wild equids, including donkeys and zebra, EHV-7 to EHV-9 (Table 1). These viruses are ubiquitous, and their long-lasting success as pathogens is probably the result of ancient co-evolution with the *Equidae* family (Slater, 2014).

It is generally accepted that viruses with mild pathology and a narrow host range are likely to be well adapted to their host. Of the five domestic EHVs, EHV-1 has the most severe pathology and widest host range. Iceland is the only country to our knowledge that is free of EHV-1. Horses in Iceland have been isolated from other breeds for over a thousand years and this suggests that EHV-1 may have evolved later than the other EHVs, or was not as common in the 9th and 10th centuries as it is today (Thorsteinsdóttir et al., 2013).

In addition to affecting the health of the horses, EHV infection, especially EHV-1, can cause significant economic losses to the horse industry as symptoms such as respiratory diseases, abortion and paralysis can influence both breeding and competition (Slater, 2014).

Table 1. Equid herpesviruses

	Subfamily	Genus	Natural host	Primary infection	Latency	Disease	Cross-infection known in	Endemic in Iceland
EHV-1	α	Varicellovirus	Horses	Respiratory tract	Trigeminal ganglia	Respiratory diseases, abortion, neurological symptoms and neonatal death	Cattle, fallow deers, antelopes, alpacas, llamas and camels	No
EHV-2	γ	Percavirus	Horses	Respiratory tract	B lymphocytes	Respiratory diseases and poor performance	No records	Yes
EHV-3	α	Varicellovirus	Horses	Veneral transmission	Undefined	Coital exanthema	No records	Yes
EHV-4	α	Varicellovirus	Horses	Respiratory tract	Trigeminal ganglia	Respiratory diseases but can cause abortion and neurological diseases	No records	Yes
EHV-5	γ	Percavirus	Horses	Respiratory tract	B (and T) lymphocytes	Equine multinodular pulmonary fibrosis (EMPF), respiratory diseases and dermatitis	No records	Yes
EHV-7	γ	Not assigned	Donkey	Undefined	Undefined	Undefined. Virus has been found in nasal secretion of donkeys and mules.	Mule	No
EHV-8	α	Varicellovirus	Donkey	Undefined	Undefined	Respiratory disease in donkeys and horses	Horses	No
EHV-9	α	Varicellovirus	Zebra	Undefined	Undefined	Neurological diseases, can cause encephalitis	*Equidea*, *Rhinocerotidae* and *Bovidae*	No

EHV-6 is a related virus that may be member of the Varicello virus.

1.3.1 Equid alphaherpesviruses (α-EHV)

Three α-herpesviruses are known in domestic horses, EHV-1, EHV-3 and EHV-4, all members of the subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. EHV-1 and EHV-4 were the first EHVs to be described (Crabb & Studdert, 1995; Dimock & Edwards, 1933; Sabine et al., 1981; Studdert et al., 1981; Turtinen et al., 1981). EHV-1 was first isolated from abortion material in the 1930s (reviewed in Ata et al., 2018). Before 1981, EHV-1 and EHV-4 were considered to be the same virus or subtypes of the same virus (Crabb & Studdert, 1996). But restriction endonuclease DNA fingerprints showed that it was comprised of two distinct viruses, EHV-1 and EHV-4 (Studdert et al., 1981). These two viruses show high immunological cross reactivity, with 55–84% nucleotide and 55–96% amino acid homology (Telford et al., 1998). However, they differ significantly in host range and pathogenicity.

EHV-8 and EHV-9 infect wild equids; both are members of the subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. EHV-8 was first isolated from donkeys and is also known as asinine herpesvirus 3 (AHV-3) (Browning, 1988). EHV-9 is the newest member of the EHVs, first isolated from a Thomson's gazelle in 1993 (Fukushi et al., 1997). EHV-9 was originally classified as a gazella herpesvirus type 1 (GHV-1), but was later redesigned as the virus showed serological cross-reactivity with EHV-1 in a neutralization test. It has also been shown that the nucleotide sequences of ORFs in EHV-9 are 86-95% identical to those in EHV-1 (Fukushi et al., 2012**)**.

1.3.1.1 Equid alphaherpesvirus 1 (EHV-1)

EHV-1 causes respiratory infections, neurological diseases, abortion, neonatal death, myeloencephalopathy and chorioretinopathy (Anagha et al., 2017; Bryant et al., 2018; Hussey et al., 2013; Nagy et al., 1997; Walter et al., 2016). Direct contact is necessary for virus spread. EHV-1 has a broad host range, and in addition to horses the virus spillover has been isolated from cattle (Crandell et al., 1988), fallow deer (Kinyili & Thorsen, 1979; Thorsen et al., 1977), antelopes (Chowdhury et al., 1988), alpacas and llamas (Rebhun et al., 1988) and from camels (Bildfell et al., 1996).

The genome of EHV-1 was completely sequenced in 1992 (Telford et al., 1992). The EHV-1 genome is about 150 kbp in size, with 57% G + C base composition, 76 distinct genes, four of which are duplicated, resulting in a total of 80 open reading frames (ORF), and both internal and terminal repeats (Figure 9), (Telford et al., 1992).

Primary EHV-1 infection occurs in epithelial cells of the upper respiratory tract, resulting in erosion of the upper respiratory mucosal surface and nasal viral shedding. The virus then spreads to local lymphatic tissues, and infects peripheral blood mononuclear cells (PBMC) that enter the bloodstream, followed by a cell-associated viremia (Allen & J.T., 1986; Gryspeerdt et al., 2010; Lunn et al., 2009; Van de Walle et al., 2008). The viremia is directly responsible for virus transfer and replication at the vascular endothelium of the target organs, including the pregnant uterus and the central nervous system, that can lead to abortion and neurological disorders, respectively (Holz et al., 2019). After primary infection, latency is established in the lymphoreticular system and in the trigeminal ganglia (Slater et al., 1994).

Abortion is one of the most severe symptoms caused by EHV-1 and the virus is known to cause abortion "storms". The abortion usually occurs in the last trimester, but if infected during late gestation the foals can be born alive (Holz et al., 2019; Hussey, 2019, reviewed in Foote et al., 2012). It has been shown that EHV-1 can replicate in the vaginal mucosa (Negussie et al., 2016), and the virus has been detected in semen of infected stallions (Hebia-Fellah et al., 2009; Tearle et al., 1996; Walter et al., 2012). Equine herpesvirus myeloencephalopathy (EHM) is another severe manifestation of EHV-1 infection. The central nervous system is targeted, especially the spinal cord, and this can result in head pressing, ataxia, and paralysis that can progress to recumbency requiring euthanasia (Holz et al., 2019; Hussey, 2019). A single nucleotide polymorphism in the DNA polymerase gene (ORF30) at a highly conserved position is associated with increase in neurovirulence, N752 is replaced by D752. Abortions are thought to be more associated with the N752 genotype (Holz et al., 2019; Nugent et al., 2006). EHV-1 can also induce chorioretinopathy, causing permanent focal or multifocal lesions with "shotgun" morphology. On rare occasions the lesions can diffuse and affect the whole eye, which can have significant impact and cause loss of vision (Holz et al., 2019; Hussey, 2019). There are vaccines available against EHV-1, both inactive and modified-live, none of them are completely effective at eliminating nasal shedding or cell-associated viremia (Goodman et al., 2012).

As mentioned above, Iceland is free of EHV-1 (Thorsteinsdóttir et al., 2013; Torfason et al., 2008). An outbreak in our naïve population would most likely have catastrophic consequences.

1.3.1.2 *Equid alphaherpesvirus 4 (EHV-4)*

EHV-4 has been studied to a much lesser extent than EHV-1. EHV-4, formerly known as equine rhinopneumonitis virus, causes respiratory tract diseases in horses with symptoms such as fever, anorexia, nasal discharge, enlargement of mandibular lymph nodes and mucosal inflammation of the upper respiratory tract (Mizukoshi et al., 2002). Entry and replication remains limited to the upper respiratory tract, and the virus replicates poorly in non-equine cells (Azab & Osterrieder, 2012; Whalley et al., 2007). Leukocyte-associated viremia is typically not established following EHV-4 infection and non-respiratory diseases such as abortion and neurological disorders are therefore rare (Osterrieder & Van de Walle, 2010, reviewed in Patel & Heldens, 2005). Latency is established in the trigeminal ganglia (Borchers et al., 1997).

The genome of EHV-4 has been completely sequenced: 145 kbp with 57% G + C base composition, 76 different genes, three of which are duplicated, and both internal and terminal repeats (Figure 9), (Telford et al., 1998).

EHV-4 is endemic in Iceland, and the majority of adult horses are antibody positive (Nordengrahn et al., 1998; Torfason et al., 2008).

1.3.1.3 *Equid alphaherpesvirus 3 (EHV-3)*

EHV-3 causes equine coital exanthema (ECE) that is an acute, venerally transmitted disease. It is characterized by formation of papules, vesicles, pustules and ulcers on the vaginal and vestibular mucosal and the perineal skin of mares, and on the penis and prepuce of stallions (reviewed in Barrandeguy & Thiry, 2012). The incubation time can be as short as 2 days but is usually around 5–9 days (Studdert, 1996, reviewed in Barrandeguy & Thiry, 2012). Then the lesion appears as small raised and reddened papules, 1–2 mm in diameter. They progress to vesicles, then pustules that rupture and can form ulcers. Healing is usually completed in 10–14 days in uncomplicated cases, but de-pigmented cutaneous scars can persist for many weeks (reviewed in Barrandeguy & Thiry, 2012). General signs of infection, such as fever, anorexia and dullness are occasionally more intense in stallions than mares. Stallions with extensive ECE symptoms can sometimes show diminished libido and refuse to copulate with mares (Studdert, 1996).

Foals get EHV-3 antibodies with colostrum, which persist for 4–6 months. In foals 1–2 years of age, antibodies are infrequently found in the serum. The

number of seropositive cases increases steadily in horses older than 2 years, supporting that the virus is primarily transmitted through coitus (Studdert, 1996). Occasionally lesions can be seen on the skin of the lips and the mucous membrane of the upper respiratory tract after non-coital transmission (reviewed in Barrandeguy & Thiry, 2012). Transmission by contaminated fomites have also been implicated (reviewed in Metcalf, 2001). Despite being highly contagious, the virus is non-invasive, and the disease is relatively benign (Sijmons et al., 2014).

EHV-3 replicates in the stratified epithelium of epidermal tissue present at the mucocutaneous margins or within the skin. Destruction of the epithelium, caused by the lytic virus infection triggers strong, localized inflammatory response, resulting in the characteristic cutaneous lesions of ECE (reviewed in Barrandeguy & Thiry, 2012).

EHV-3 is distinct from other EHVs in regard to antigens, pathogenicity and genetics. The virus is highly host specific and replicates only in equid cells (reviewed in Barrandeguy & Thiry, 2012). The complete EHV-3 genome sequence was published in 2014: 151 kbp in size with 68% G + C base composition, 76 different genes, four of which are duplicated, resulting in a total of 80 genes, and both internal and terminal repeats (Figure 9), (Sijmons et al., 2014).

EHV-3 was first described in the early 1900s and was first isolated in 1986 (reviewed in Barrandeguy & Thiry, 2012). Today the virus has worldwide distribution, including Iceland (**Paper IV**).

1.3.1.4 Equid alphaherpesviruses in wild equids

EHV-8, formerly known as asinine herpesvirus 3 (AHV-3), was first isolated in 1987 from the nasal cavity of a donkey (Browning, 1988). The pathogenesis has not been studied much, but the virus has been associated with respiratory diseases in both donkeys and horses, and, recently, abortion in horses (Garvey et al., 2018). Donkeys have been thought to be the natural host for EHV-8 infections, but recent findings demonstrate that the virus is able to cross to other species. The complete genome sequence of EHV-8 was published recently and showed that the virus is phylogenetically closer to EHV-9 than EHV-1 (Garvey et al., 2018).

EHV-9 is the newest member of the equine herpesviruses and was first described in 1993 (Fukushi et al., 1997). EHV-9 has a broad host range across mammalian families, including members of the *Equidae*, *Rhinocerotidae* and *Bovidea* (Abdelgawad et al., 2015). The virus has been

tested in experimental infections in different animals, including hamsters, dogs, pigs and goats, causing numerous and sometimes lethal diseases (Fukushi et al., 2000; Narita et al., 2000; Taniguchi et al., 2000; Yanai et al., 2003). It has been suggested that the natural host for EHV-9 is the zebra (Schrenzel et al., 2008), but serological prevalence indicate that African rhinoceros species may serve as a reservoir or even the natural host (Abdelgawad et al., 2015). Latent infection is thought to be in the trigeminal ganglion (Borchers et al., 2005). The complete genome sequence of EHV-9 has been published: 148 kbp in size, with 56.1% G + C base composition, and 80 ORF (Fukushi et al., 2012).

EHV-6, formerly known as AHV-1, was thought to be closely related to EHV-3 and caused similar symptoms, venereal lesions, in donkeys (Crabb & Studdert, 1995). In 1998 EHV-6 was abolished as a species and listed as tentative species. ICTV has now removed all tentative species from the taxonomy and requires them to be classified properly. Because little information is available for EHV-6, the virus was listed as “related viruses which may be members of the genus *Varicellovirus,* but have not been approved as species” in the 9th ICTV report (ICTV, 2019).

1.3.2 Equid gammaherpesviruses (γ-EHV)

Two γ-herpesviruses are known in domestic horses, EHV-2 and EHV-5, both members of the subfamily *Gammaherpesvirinae*, genus *Percavirus.* The first equine gammaherpesvirus was isolated in 1962 from a horse with an upper respiratory tract disease (Plummer & Waterson, 1963). A second virus was then described in 1987 (Browning & Studdert, 1987). At first these two viruses were considered betaherpesviruses, but partial nucleotide sequence analysis showed that EHV-2 and EHV-5 were distinctively gammaherpesviruses (Telford et al., 1993).

These two viruses are closely related (Thorsteinsdóttir et al., 2013) with strong serological cross-reactivity, making serological distinction difficult (Agius et al., 1994; Plummer et al., 1973). Both viruses can coexist in the same horse (Bell et al., 2006a; Franchini et al., 1997; Nordengrahn et al., 2002; Reubel et al., 1995; Torfason et al., 2008) and different strains of viruses can infect the same animal (Brault et al., 2011; Fortier et al., 2013). Both viruses are ubiquitous, and they have probably coevolved with their host for millions of years (Thorsteinsdóttir et al., 2013, reviewed in Hartley et al., 2013).

In 1998-1999 an epidemic of infectious pyrexia of unknown viral etiology swept through the entire horse population in Iceland (Svansson, 2000). This disease was highly infectious with morbidity most likely near 100%. The mortality was, however, low, or approximately 0.2%. In search of a possible pathogen, PMBCs were isolated and co-cultured on prmEqFK cells. According to the cytopathic effects observed, the slow growth in cell culture and staining by post-infectious serum in an indirect immunofluorescent assay, there was a suspicion of gammaherpesviruses. Subsequently, we designed a semi-nested type specific PCR and showed that both EHV-2 and EHV-5 are endemic in Iceland (Torfason et al., 2008).

EHV-7, formerly known as ASH-2, is an unassigned species in the *Gammaherpesvirinae* subfamily, a virus known in donkeys.

1.3.2.1 Equid gammaherpesvirus 2 (EHV-2)

Foals get infected with EHV-2 soon after birth, despite high titers of maternal antibodies (Brault et al., 2010; Fu et al., 1986). The majority of foals are infected by the age of 2–4 months, when maternal antibodies decline (reviewed in Hartley et al., 2013; Marenzoni et al., 2015). Foals get infected horizontally from their mothers, probably via the upper respiratory tract (Bell et al., 2006a; Murray et al., 1996). Vertical transmission has not been shown. However, EHV-2 and EHV-5 have been detected in fetuses and in the placenta (Galosi et al., 2005; Léon et al., 2007; Marenzoni et al., 2013). Young foals typically shed strains of EHV-2 identical to those infecting their dams, but more strain variation is observed in older foals, probably due to multiple infections through contact with other horses (Bell et al., 2006a; Brault et al., 2011). EHV-2 is typically present in respiratory secretion and PBMC (Bell et al., 2006a). Foals shed more virus than adult horses (Hue et al., 2014) and it has also been proposed that EHV-2 behaves differently in foals and adults. In foals the virus is probably not strictly cell-bound and therefore easily isolated from the upper respiratory tract and eye swab samples. In adult horses the virus is however thought to be strictly cell-bound in macrophages and possibly lymphocytes (reviewed in Marenzoni et al., 2015). After primary infection the virus is thought to establish latency in B lymphocytes (Drummer et al., 1996).

EHV-2 has been detected from both symptomatic and healthy horses of all ages. This makes it difficult to define the precise clinical impact of EHV-2 and the role in disease causation. The virus has been implicated in keratoconjunctivitis (Borchers et al., 2006; Collinson et al., 1994; Kershaw et

al., 2001), mild respiratory disease (Dunowska et al., 2002a; Dynon et al., 2007; Wang et al., 2007), pneumonia (Dunowska et al., 2011; Nordengrahn et al., 1996), pharyngitis (Blakeslee et al., 1975) and poor performance (Studdert, 1974). The cellular immune response to EHV-2 in foals, i.e. increased number of PBMC and fever, suggests an immunologically mediated disease comparable to infectious mononucleosis in humans, caused by EBV (Brault et al., 2010). It is known that herpesviruses modulate their host immune responses, which can result in secondary bacterial or viral infection (Donofrio et al., 2005; Donofrio et al., 2008; Welchman et al., 2012). The EHV-2 genome encodes proteins that potentially down-regulate the host immune response, IL-10 homologus and three proteins with some similarity to chemokine receptors (Camarda et al., 1999; Sharp et al., 2007). There is evidence for EHV-2 as a predisposing factor for *Rhodococcus equi* infections, resulting in pneumonia (Nordengrahn et al., 1996).

The complete sequence of the EHV-2 genome was published in 1995: 184 kbp in size, with 57.5% G + C base composition, 79 ORFs that are predicted to encode 77 distinct proteins. About a third of the sequence is distributed in several large blocks that appear not to encode proteins, but the genome has both internal and terminal repeats (Figure 9), (Telford et al., 1995).

As mentioned before, EHV-2 is widespread in Iceland. We have investigated the genetic diversity of Icelandic EHV-2 strains and compared them with foreign strains. Four genes, glycoprotein B (gB), gH, DNA polymerase and DNA terminase, were sequenced and nucleotide and amino acid comparisons made. No specific Icelandic genotype was identified, no genetic difference was seen between the Icelandic and foreign strains, and all strains blended in the phylogenetic trees. As Icelandic horses have been isolated for over a thousand years without introduction of new viruses, it is likely that the founder population was infected with gammaherpesviruses (Thorsteinsdóttir et al., 2013).

1.3.2.2 Equid herpesvirus 5 (EHV-5)

EHV-5 infection occurs later than EHV-2 (Dunowska et al., 2002a; Nordengrahn et al., 2002). Transmission is thought to be similar as for EHV-2, i.e. horizontally from mare to foal via the respiratory tract. A recent study showed that EHV-5 was unable to infect the epithelial cells lining the mucosa of the nasal and tracheal as well as primary equine respiratory epithelial cells. The virus however infected lung alveolar cells (Van Cleemput et al., 2019).

Based on these results, the authors suggested a model for EHV-5 pathogenesis, where the virus is propelled by the mucociliary escalator towards the tonsillar crypts, embedded in the nasopharynx. The epithelium of the tonsillar crypts has gaps that reach down to the lymphoid follicles, where the virus can directly infect T and B lymphocytes (Sherman et al., 1977; Van Cleemput et al., 2019).

Our previous findings indicate that EHV-5 could have other target cells for latency than EHV-2. In our studies, fewer horses with infectious pyrexia had EHV-2 compared with healthy horses, while EHV-5 was found in approximately the same frequency in both groups. Decrease in total peripheral blood lymphocyte count was a common finding in pyrexia horses during the outbreak, but whether it is B or T lymphocytes, or both is unclear. Therefore, it can be speculated that the target cells of EHV-2 decrease in the pyrexia horses, but not the EHV-5 target cells (Torfason et al., 2008). Mekuria et al. conclude that B lymphocytes are the major target cells of latency for both EHV-2 and EHV-5 (Mekuria et al., 2017). However, Van Cleemput et al. showed that both B and T lymphocytes support EHV-5 infection, whereas replication was not seen in monocytes (Van Cleemput et al., 2019).

EHV-5 has been detected from both healthy and diseased horses of all ages and the virus is typically present in peripheral blood leukocytes and respiratory secretions (Bell et al., 2006a; Dunowska et al., 1999). The virus has been implicated in dermatitis (Herder et al., 2012), upper respiratory tract disease such as pharyngitis, and keratoconjunctivitis with clinical signs such as nasal and ocular discharge, tachypnea, coughing, fever, enlarged lymph nodes, anorexia, depression and poor body condition (Dunowska et al., 2002a; Franchini et al., 1997; Hart et al., 2008; Rushton et al., 2013; Wong et al., 2008). EHV-5 viral load is higher in younger horses compared to adults (Hue et al., 2014; Marenzoni et al., 2010).

The most severe manifestation of EHV-5 is the development of equine multinodular pulmonary fibrosis (EMPF) (Marenzoni et al., 2011; Poth et al., 2009; Williams et al., 2007; Wong et al., 2008). EMPF was first described and associated with EHV-5 in 2007 (Williams et al., 2007), but the role of EHV-5 in EMPF development has not been determined. EMPF is characterized by multiple fibrotic nodules throughout the lungs. Histologically, interstitial fibrosis with an "alveolar-like" architecture is seen, lined by epithelial cells and thickening of the alveolar walls (Marenzoni et al., 2011; Poth et al., 2009; Williams et al., 2007; Wong et al., 2008).

The complete sequence of the EHV-5 genome was published in the National Center for Biotechnology Information (NCBI) database in 2018 (GenBank accession no. KU315329): 182 kbp in size, with similar G + C % base and structural composition as EHV-2, but with shorter terminal repeats (Figure 9), (Agius et al., 1992).

EHV-5 is ubiquitous, and the virus is also widespread in Iceland. However, isolation and culture of the virus can be difficult as EHV-2 tends to overgrow in cell culture and both viruses are present in most samples. Fewer isolations of EHV-5 have therefore been made. In 2010 we published a paper on isolation and partial sequencing of EHV-5 from a horse in Iceland. The strain, BB5-5 was isolated from co-culture of PBMC cells and prmEqFK followed by plaque purification after alternate passages in prmEqFK and RK13 cells. We completely sequenced three genes of the BB5-5 strain: gB, gH and DNA terminase and in addition sequenced a large part of the DNA polymerase gene. All sequences were published in NCBI GenBank (Thorsteinsdóttir et al., 2010).

1.3.2.3 Equid gammaherpesviruses in wild equids

EHV-7 was first isolated from blood of a healthy donkey (Browning, 1988), and later from nasal secretions of a healthy mule (Bell et al., 2008). Recently the virus was isolated from the placenta of a third trimester abortion in a donkey (LeCuyer et al., 2015). The DNA polymerase gene has been partially sequenced, and comparison shows greatest homology to EHV-2 (Bell et al., 2008; Ficorilli et al., 1995).

AHV-4, AHV-5 and AHV-6 are viruses in donkeys that have been associated with pneumonia, characterized by marked syncytial cell formation (Kleiboeker et al., 2002; Kleiboeker et al., 2004). These viruses have not been studied much, and they have not been classified by ICTV.

1.4 The immune system of the horse

The immune system is a network of cells, organs and molecules that protect the host against pathogens and diseases. The immune response of horses is mediated by more or less the same cells, molecules and mechanisms as in other mammals (Fellipe, 2016). Horses have five major immunoglobulin isotypes, IgA, IgD, IgE, IgG and IgM. The IgA has neutralizing activity and is involved in mucosal immunity (Lewis et al., 2010), IgE binds to both high and low affinity IgE receptors and is increased in parasite infection and type I hypersensitivity (reviewed in Schaffartzik et al., 2012; Wagner, 2009), IgM

activates the complement system, also known as the complement cascade, but the function of IgD is unknown (reviewed in Perkins & Wagner, 2015). IgG neutralizes many horse pathogens and is the major immunoglobulin in horse serum and colostrum (Sheoran et al., 1998; Sheoran et al., 2000). Horses have seven IgG subclasses, IgG1 to IgG7, each distinguished by the gene encoding the constant heavy chain region (Wagner et al., 2004). IgG4/7 and IgG1 are important in protection against intracellular infection (Hussey et al., 2011; Lopez et al., 2002; Nelson et al., 1998) and IgG3/5 against extracellular pathogens, (Dowdall et al., 2002) and these are the most abundant IgG subclasses. IgG2 and IgG6 are found in smaller concentrations and their specific functions are unknown (Fellipe, 2016).

The epitheliochorial placenta in equines prevents transfer of maternal immunoglobulins to the fetus. The neonates have to absorb the immunoglobulin, as well as cytokines and maternal immune cells, from the colostrum as soon after birth as possible (reviewed in Perkins & Wagner, 2015). The transport, from the mammary gland to colostrum through the neonatal gut is mediated by a specific IgG Fc-receptor that is transiently expressed and conserved throughout mammals (reviewed in Cervenak & Kacskovics, 2009; Perkins & Wagner, 2015; Roopenian & Akilesh, 2007). The greatest absorption capacity of the neonatal gut is around 6–8 h after birth, the absorption then declines and ceases 24–36 h after birth (Baird et al., 1987; Jeffcott, 1974; Korosue et al., 2012). Failure of passive transfer (FPT) to the foal increases risk of infection and can lead to death (McGuire et al., 1975; McGuire et al., 1977).

Initially, the IgG compositions of the foal serum after colostrum intake mirror those of the mare's colostrum (reviewed in Perkins & Wagner, 2015). Then the concentration of the maternal immunoglobulins declines steadily. They reach nadir at different time points, depending on the isotype, the antibody status of the mother and colostrum intake. The subsequent increase in IgG isotypes represents endogenous production. The exact time point of when the foal starts to produce IgG is hard to determine, except for IgG1 (reviewed in Perkins & Wagner, 2015). The production of IgG1 and IgM starts before birth and continues after the foal is born (McGuire & Crawford, 1973; Sheoran et al., 2000). Endogenous IgG3/5 is detectable within the first 5–8 weeks of life, Ig4/7 by 16–20 weeks and then increases slowly during the first year (Holznagel et al., 2003), and IgE is not produced until 6–11 months after birth (Marti et al., 2009, reviewed in Wagner, 2006).

1.4.1 Immune response against EHVs

Very few studies have been done on the nature of the immune response to EHV infections. A recent study showed that *in vitro* EHV-1 and EHV-4 infected PBMC induced strong IFN-α, β and γ responses. In addition, EHV-4 induced a moderate inflammatory TNF-α and IL-6 response. When PBMC cells were infected with EHV-2 a strong IFN-γ cytokine response was measured, followed by a moderate IFN-β expression (Hue et al., 2017). Similarly, we found that after *in vitro* stimulation of PBMC with EHV-2, production of IFN-γ was higher than IL-4 (Svansson et al., 2009). We also previously reported the predominance of γ-EHV specific IgG4/7 over IgG1 for both foals and adults (Svansson et al., 2009), and this has also been shown for the response to EHV-1 (Goodman et al., 2012), and EHV-4 (Mizukoshi et al., 2002), suggesting that IgG4/7 is the long-lasting antibody isotype in equine herpesvirus infections.

1.5 The Icelandic horse

The native Icelandic horse is the only horse breed in Iceland. It was brought to the country during the settlement in the 9th and 10th centuries and has been pure bred ever since (Arnórsson, 1997; Beazley, 1999; Torfason et al., 2008). No historical records exist regarding the number of the breed founders, but the population is believed to have been founded by a limited number of horses.

Studies on mitochondrial DNA and Y-chromosomes from contemporary Icelanders have shown ancestry to Scandinavia and the British-Irish Isles (Goodacre et al., 2005; Helgason et al., 2000a; Helgason et al., 2000b) and whole genome sequencing from ancient Icelanders showed combination of Norse, Gaelic and admixed individuals (Ebenesersdóttir et al., 2018).

The settlers probably brought with them their very best riding horses, stallions and breeding mares, as it must have been difficult to ship many animals in open boats across the rough seas (Arnórsson, 1997; Björnsson & Sveinsson, 2006). It is likely that the horses orginated from the same places as the settlers. Old references, antiquities of bones, and comparisons with present breeds are used when the origin of the Icelandic horse is examined (Aðalsteinsson & Þorkelsson, 1991; Aðalsteinsson, 2001). Blood types have also been examined, which indicated that the Icelandic horse is most related to the Shetland pony (Aðalsteinsson & Þorkelsson, 1991; Aðalsteinsson, 2001; Björnsson & Sveinsson, 2006). Approaches, employing molecular technology, have shown genetic relationships between the Icelandic horse

and horses in Norway, the British Isles, and the Mongolian horse (Bjørnstad et al., 2003; Jansen et al., 2002). A study based on the genome-wide SNP data found the Icelandic horse to be clustered with the Shetland pony (Petersen et al., 2013), confirmed with a TreeMix method that groups the Icelandic horse and the Shetland pony together in a phylogenetic tree (Fages et al., 2019). Based on genotyping it has been suggested that the Norse people brought the Icelandic horse from the British Isles to Iceland (Wutke et al., 2016).

Import of horses to Iceland has been prohibited by law since 1882 (Björnsson & Sveinsson, 2006; Torfason et al., 2008). There have been several bottlenecks in the history of the breed, mainly because of harsh weather conditions and volcanic eruptions. One example was in the years 1784–1785, when the population size was reduced to no more than 8000 horses, as a consequence of a volcanic eruption (Bjarnason, 1966). Today the Icelandic horse population is counting approximately 70.000–80.000 horses in Iceland, relatively high for a human population of 350.000 people. The Icelandic horse is also popular outside the country and approximately 1.200–1.400 horses are exported per year. WorldFengur is a database that maintains all information about Icelandic horses, located all over the world, both alive and deceased. It was opened in 2001 and functions as a studbook of origin of the horses. Only pure-bred Icelandic horses are in WorldFengur (Lorange, 2011). There were around 275.000 living Icelandic horses recorded in WorldFengur in 2018, based in 30 different countries. Outside of Iceland most horses are in Europe, but also in the United States and Canada (Horse, 2019).

Due to isolation, Icelandic horses are immunologically naïve to various agents known to infect horses in other countries, e.g. equine influenza and EHV-1. Presently, thirteen out of more than eighty viruses known to infect horses have been detected in the Icelandic horse population. Increased travel between countries, and growing popularity of the Icelandic horse, create new threats to this status (Torfason et al., 2008).

2 Aims

The only horse breed in Iceland is the Icelandic horse, brought to the country during the settlement in the 9th and 10th centuries and has been purebred ever since. Due to this geographic isolation the horses are immunologically naïve to various agents known to infect horses in other countries. This provides a unique opportunity to investigate viruses in an environment where there is no introduction of new virus strains. Two γEHVs are known in horses, EHV-2 and EHV-5. They were confirmed in Iceland in 1998. We have found that both viruses are common in Iceland and that they probably came with the founders. Three αEHVs are known in horses, EHV-1, EHV-3 and EHV-4. EHV-1 is the most pathogenic and not found in Iceland. Antibodies against EHV-4 have been detected, and symptoms resembling EHV-3 infection, but neither virus has been isolated.

The overall aim of this research was to study equine herpesviruses in Iceland.

The specific aims of the project were to:

- Establish equine cell lines to facilitate the viral work.
- Study the course of γEHV infections and the immune response against EHV-2 and EHV-5.
- Construct an infectious rEHV2-egfp virus.
- Isolate and confirm the existence of EHV-3 in Iceland.
- Construct viral vectors for use in vaccination trials against allergy.
- Produce EHV-2 and EHV-5 specific polyclonal antibodies.

3 Materials and methods

This chapter summarizes the main materials and methods used in the project. The experiments not included in the papers are described in more details.

3.1 Cells

3.1.1 Cells and cell culture conditions (Papers I–IV & unpublished data)

Primary kidney and lung cells were derived from a 5-month-old equine fetus, prmEqFK and prmEqFL, respectively. In **paper I** cells with extended lifespan were established from the primary cells, extEqFK and extEqFL (see chapter 3.1.2). These cells were used in later experiments. RK13, COS-7, Vero and 293T cell lines were used (ATCC®) for herpesviruses. Cells were propagated in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific) supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin with 1–10% fetal bovine serum (FBS, Thermo Fisher Scientific) and cultured at 37°C in a humidified atmosphere with 5% CO_2.

The insect cell lines Sf-9 cells (ATCC®) and High Five™ (ATCC®) were used for baculoviruses. The cells were cultured in a complement Sf-900™II Serum Free Medium (SFM, Thermo Fisher Scientific), supplemented with 100 IU/ml penicillin, 100 IU/ml streptomycin, the Sf-9 also with added 2% FBS. The cells were cultured in closed culture at 27°C.

3.1.2 Establishment of cell lines with extended life span (Paper I)

prmEqFK and prmEqFL cells in second passage were transduced with sterile filtered retrovirus containing a supernatant from the PA317 LXSN HPV16E6E7 packaging cell line (ATCC®) in the presence of 8 μg/mL polybrene (Sigma-Aldrich). Transduced cells were selected in the presence of 600 μg/mL G418 (Thermo Fisher Scientific). This concentration of G418 resulted in almost complete cell death after 5 days. In maintenance of transformed extEqFK and extEqFL 800 μg/mL of G418 were used until passage 15 and 19, respectively.

3.1.3 Isolation of peripheral blood leukocytes (PBL) (Paper II)

Blood was collected in EDTA tubes (Vacuette, Greiner Bio-One) and the red blood cells allowed to sediment at RT for 30 min for the mares and 90 min for the foals. The enriched plasma containing the PBLs was used directly for virus isolation.

3.2 Viruses

3.2.1 Virus strains (Papers I–III & unpublished data)

The Icelandic EHV-2 strain EHV2-Bj (Thorsteinsdóttir et al., 2013) and EHV-5 strain BB5-5 (Thorsteinsdóttir et al., 2010) were used in the study. Both strains were isolated from healthy horses, EHV2-Bj from an 8-year-old horse and BB5-5 from a 9-year-old mare. Blood was collected in heparin vacutainer tubes (Vacuette, Greiner) and the PBMC cells separated with Ficoll-Paque, 2–6 x 10^5 cell/mL in PBS were inoculated onto confluent prmEqFK cells (Torfason et al., 2008). When CPE was observed, viruses were plaque purified according to Svansson et al., 2009.

The complete glycoprotein B (gB), gH, DNA polymerase and DNA terminase genes have been sequenced from strain EHV2-Bj, accession numbers HQ247738, HQ247757, HQ247775, and HQ247795, respectively (Thorsteinsdóttir et al., 2013). For BB5-5 the gB, gH and the DNA terminase genes have been completely sequenced, accession numbers GQ325592, GQ325594, and GQ325598, respectively, and the DNA polymerase gene sequenced partially, accession number GQ325596 (Thorsteinsdóttir et al., 2010).

3.2.2 Virus culture (Papers I–IV)

Cells were infected with EHV-2 or EHV-5 either when seeding the cells or when the cell layer was over 90% confluent. The cells were washed with PBS or DMEM, infected with the appropriate virus dilution for 1 h at culture conditions and rocked gently periodically. The supernatand was either aspirated and the cells washed before adding the culture medium or culture medium added directly onto the virus dilution.

For baculovirus propagation confluent Sf-9 cells in T75 flasks (14.5 mL) were infected with 0.5 mL recombinant baculovirus for 3 days at 27°C. For production of baculovirus antigen 10^8 High five cells in 97 mL SFM in an

erlenmeyer flask were infected with 3 mL of baculovirus stock, ~$10^{7.5}$ $TCID_{50}$/mL and incubated in 27ºC on a shaker in closed culture for 4 days.

Virus isolation was attempted from peripheral blood leukocytes (PBL) (**Paper II**), nasal swab samples (**Paper II**) and swab sample from the ECE lesions (**Paper III**). PBL samples: 100 µL of peripheral blood leukocytes (PBL) were added directly to the extEqFK cell culture. Nasal swab samples: the transport medium with the swab-pin was vortexed, spun at 20.817 x g for 1 min and 200 µL of supernatant added to extEqFK cell layer after wash. ECE swab: 2 mL of DMEM with 10% FBS was added to the swab, vortexed and 500 µL added to confluent prmEqFK cells.

3.2.3 Viral load (Papers I and IV)

Viral load was determined with the Reed-Muench method (Reed & Muench, 1938) i.e. the 50% endpoint was determined with titration of the viruses in ten-fold dilutions, 10^{-1} to 10^{-10}, eight wells per dilution, added to 25–50 x 10^{3} cell/well, in 96-well flat bottoms plates (Thermo Fisher Scientific). Plates were examined 7 days post inoculation, infected wells counted, and the viral load calculated.

3.2.4 Cloning of viruses (Paper III)

Different methods were used for virus cloning:

- Limiting dilution: Ten-fold dilutions, 10^{-1} to 10^{-10}, eight wells per dilution, added to 25 x 10^{3} cells/well, in 96 well plates.
- Plaque purification: 80–90% confluent cell layer infected with appropriate virus dilution for 1 h, aspirated and overlay of 1% agarose DMEM with 1% FBS added. Plaques were harvested after approximately 7 days and sub-cultured on fresh confluent cells.
- Cell sorting with FACS: Fluorescence-activated cell sorting (FACS) was used to sort fluorescent EHV2-egfp infected cells from non-infected and cells infected with wild type EHV-2. The cells were trypsinized, then washed in DMEM and centrifuged at 1000 x g for 10 min twice, dissolved in sterile filtered PBS with 4mM EDTA and 2% FCS and stored in tubes coated with FCS to prevent adhesion. The cells were kept on ice before sorting on a Sony LE-SH800 cell sorter with a 100 µM sorting chip. Sorted cells were seeded on fresh RK13 cells.

3.3 Microscope figures (Papers I, III and IV)

Images in **papers I** and **IV** were acquired with an inverted phase contrast microscope (Leica DM IL LED), using the LAS V3.6 program. Images in **paper III** were acquired with Evos FL Auto 2 microscope (Invitrogen by Thermo Fisher Scientific) and Olympus FL1200 Confocal microscope (Olympus).

3.4 DNA

3.4.1 Isolation of DNA (Papers I–IV)

DNA was isolated from prmEqFK, extEqFK, prmEqFL, extEqFL cells and buffy coat (BC) with a Gentra Puregene Blood Kit (Qiagen), according to manufacturer's protocol. DNA was isolated from nasal swab (NS) and supernatant from infected cells with a High Pure Viral Nucleic Acid Kit (Roche), according to manufacturer's protocol. There is an exception from this is in **paper IV** where DNA was isolated from virus supernatant with the Gentra Puregene Blood Kit.

3.4.2 PCR and electrophoresis (Papers I, III, IV & unpublished data)

PCRs were done with Taq DNA Polymerase (New England BioLabs) or Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Fisher Scientific), according to manufacturer's protocol. The PCR products were visualized after electrophoresis on agarose gel with ethidium bromide in InGenius (Syngene). The qPCR amplicons in **paper II** were electrophoresed with 2100 Bioanalyze (Agilent), according to manufacturer's protocol, prior to sequencing.

3.4.3 qPCR (Paper II)

EHV-2 and EHV-5 type-specific quantitative Real-Time assay targeting glycoprotein B were set up according to Rushton et al., 2013. DNA sequences encoding glycoproteins were cloned into pFastbac^{TM}HT B vectors (see chapter 3.6) and used as positive controls, for constructing a standard curve and for calculation of viral copy per 100 ng sample. DNA, 100 ng were tested in duplicate, positive control in triplicate and negative control in duplicate, with AgPath-ID One-Step RT-PCR Kit master mix (Life Technologies), total reaction volume of 25 μL per well, StepOnePlus^{TM} Real-Time PCR System (Applied Biosystem).

3.4.4 Purification of amplicons (Papers II–IV & unpublished data)

Before sequencing, PCR products were purified with gel extraction using the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's protocol. qPCR products were purified with Monarch® PCR & DNA Cleanup Kit (5 μg) (New England Biolabs), according to the manufacturer's protocol.

3.4.5 Sequencing

Sequencing performed at Keldur was done with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem) and sequencing capillary electrophoresis was carried out on an ABI PRIMS 310 Genetic Analyzer (Applied Biosystem), according to protocol. Products were also sequenced with sanger sequencing by GeneWiz Inc. (Leipzig, Germany). Results were analysed in the Sequencher™ program (Gene Codes Corporation).

3.4.6 Plasmid DNA isolation (Papers III & unpublished data)

DNA plasmids were extracted and purified from bacterial cultures with either QIAprep Spin Miniprep Kit or Qiagen® Plasmid Plus Midi Kit, according to the manufacturer's protocol (Qiagen).

3.4.7 Transfection (Papers I, III & unpublished data)

Transfections with plasmids or purified PCR amplicons were done with Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific) and rBacmids with Cellfectin™ II Reagent (Thermo Fisher Scientific), according to the manufacturer's protocols.

3.5 rEHV-egfp viruses (Paper III & unpublished data)

3.5.1 Construction of EHV2-egfp plasmids

Four different EHV2-egfp plasmids were constructed, model A to D (Figures 10–13). The intergenic region (IGR) between glycoprotein B and the DNA polymerase, the non-coding region downstream of the DNA polymerase gene, the posterior part of ORF20, the anterior part of ORF22 and the thymidine kinase gene were sequenced from strain EHV2-Bj prior to construction of the plasmids. Primers used are listed in table 1 in **paper III** and appendix table 4. EHV-2 fragments were amplified from the EHV2-Bj genome (Thorsteinsdóttir et al., 2013) and the EGFP gene and EGFP expression cassette from pEGFP-N3 vector (Clontech) with Phusion Hot

Start II High-Fidelity DNA polymerase (Thermo Fisher Scientific). Before TOPO-TA cloning the inserts were A-tailed with Taq DNA polymerase (New England Biolabs) and then cloned according to manufacturer's protocol (Invitrogen). PCR and sequencing were performed to confirm insert and orientation.

Model A (EHV2-gB-egfp): 3206 bp fragment from the EHV2-Bj genome was amplified and cloned in pCR®4-TOPO® vector. The EGFP gene was amplified omitting both start and stop codons and ligated after *Age*I site digestion at the C-terminal of the gB gene (Figure 10).

Model B (EHV2-egfp-NC): 1195 bp fragment from the EHV2-Bj genome was amplified and cloned in pBAD-TOPO® vector. A 1337 bp of continuing non-coding region was amplified and inserted after *Sda*I and digestions downstream of the previous cloned insert. EGFP expression cassette was inserted at sites *Sda*I og *Spe*I, between the two previous EHV-2 inserts (Figure 11).

Model C (EHV2-egfp-IGR): 1707 bp fragment from the EHV2-Bj genome was amplified and cloned to pBAD-TOPO® vector. A second EHV2-Bj amplification was inserted with after *Pme*I and *Avr*II digestion downstream of the previous cloned insert. EGFP expression cassette was inserted at sites *Avr*II og *Bsi*WI between the two previous EHV-2 inserts (Figure 12).

Model D (EHV2-egfp-TK): 3157 bp fragment from the EHV2-Bj genome was amplified and cloned to pBAD-TOPO® vector. With *Sda*I og *Avr*II digestion 250 bp of the TK gene were removed. The EGFP expression cassette was inserted after corresponding digestion (Figure 13).

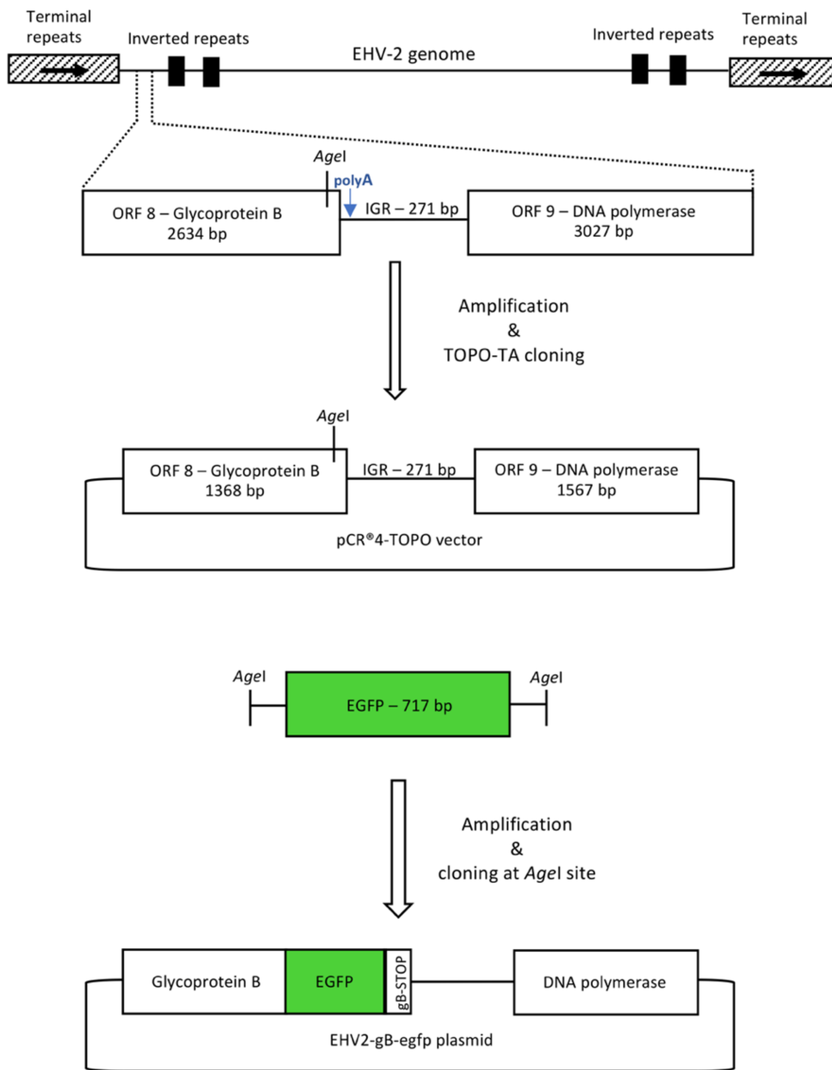

Figure 10. Schematic diagram illustrating the construction of the EHV2-gB-egfp plasmid, (model A).

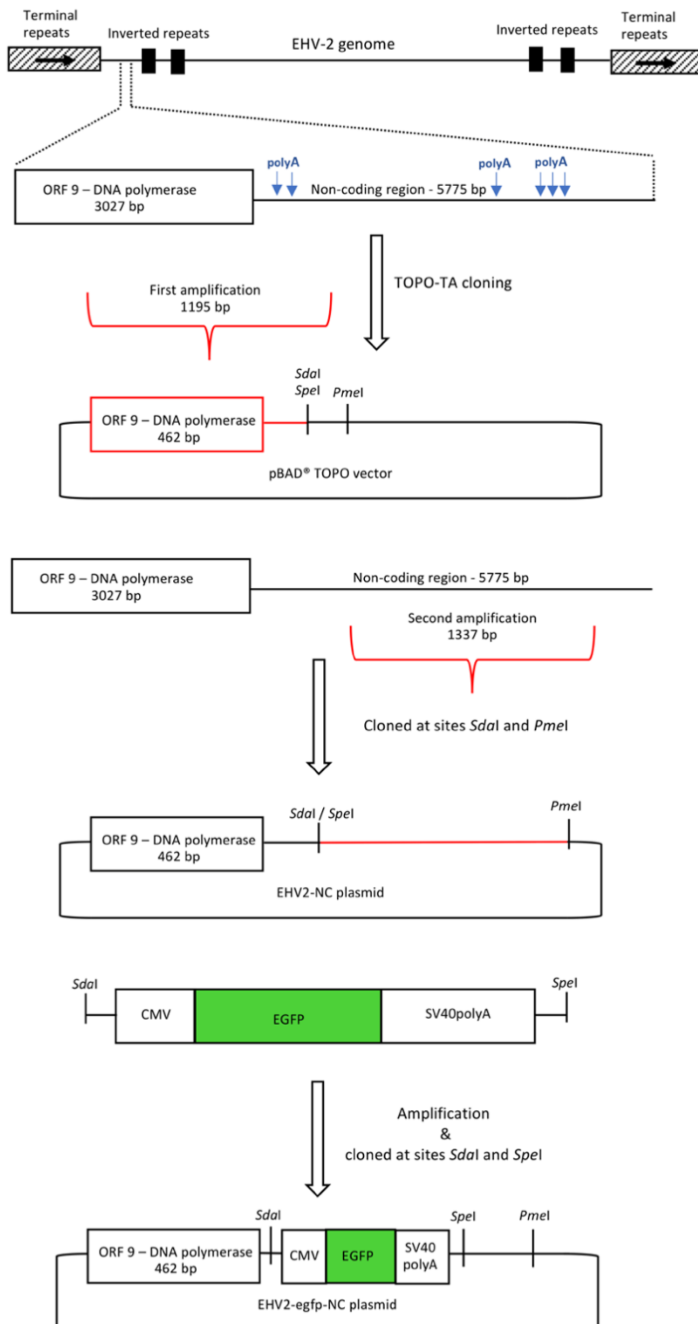

Figure 11. Schematic diagram illustrating the construction of the EHV2-egfp-NC plasmid, (model B).

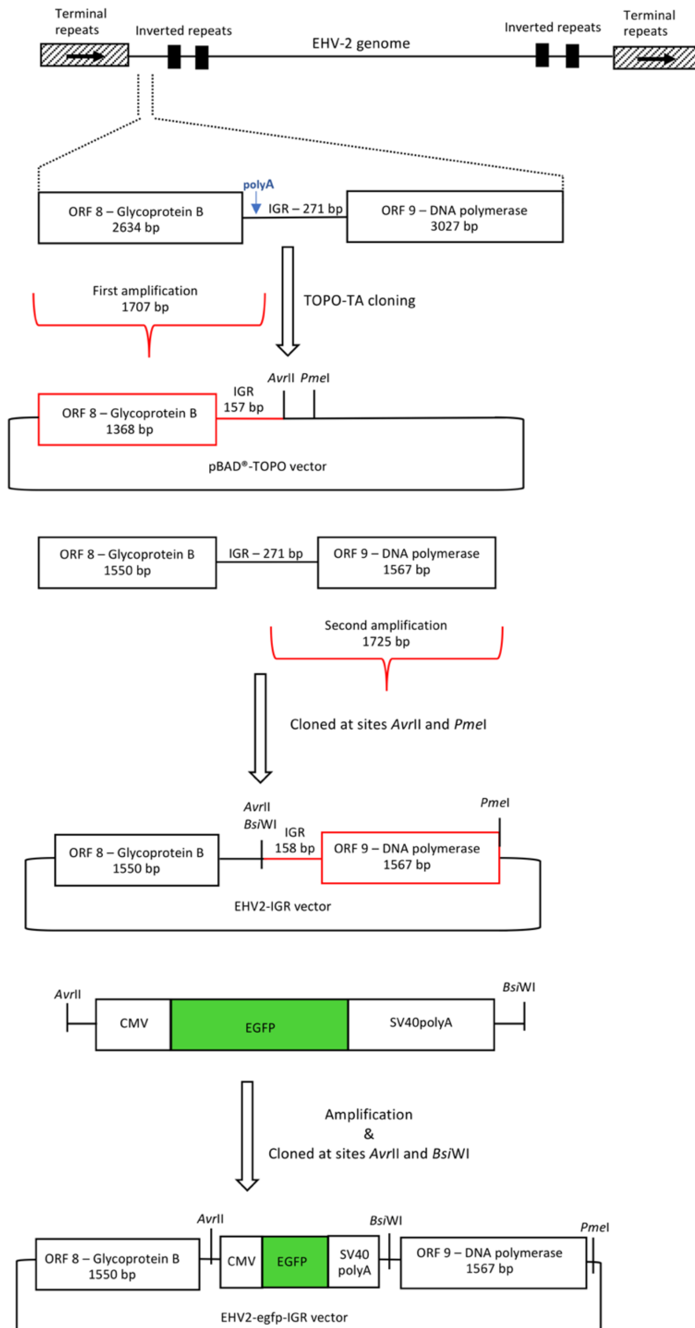

Figure 12. Schematic diagram illustrating the construction of the EHV2-egfp-IGR plasmid, (model C).

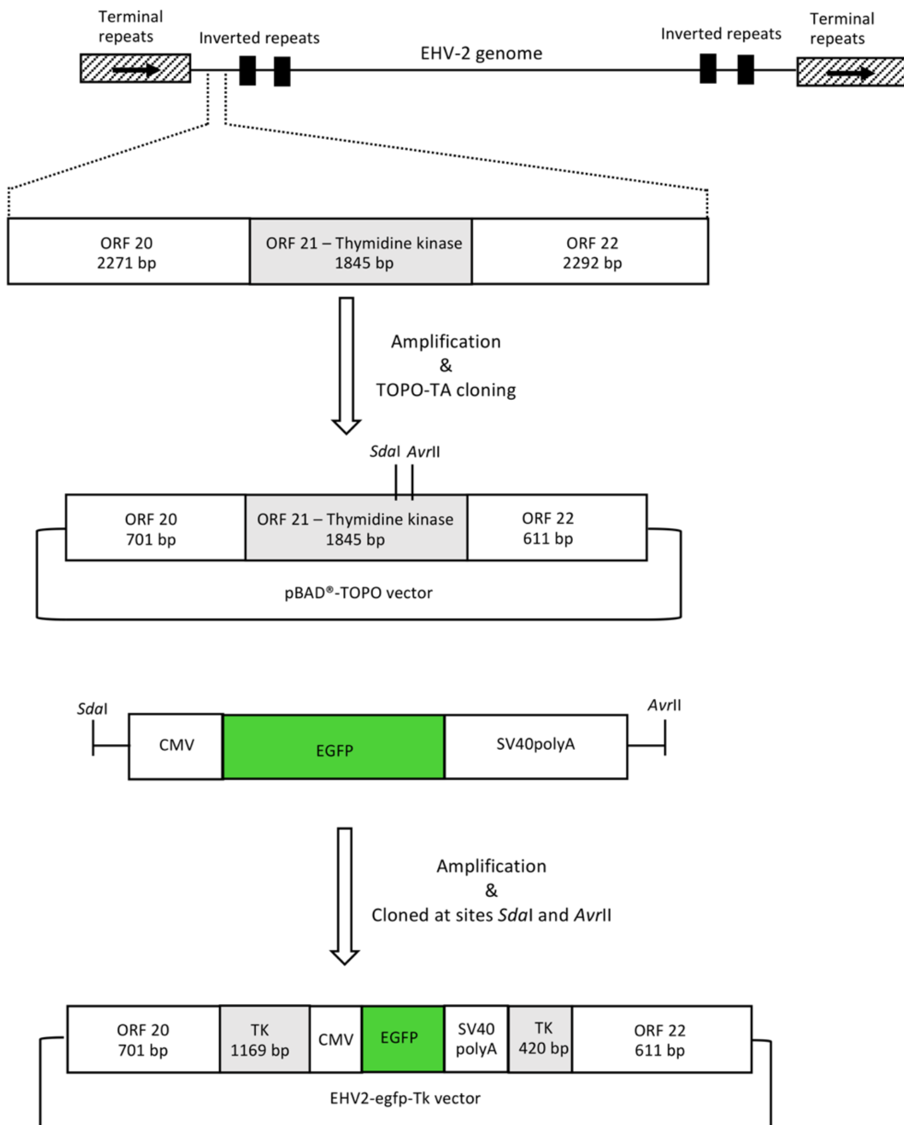

Figure 13. Schematic diagram illustrating the construction of the EHV2-egfp-TK plasmid, (model D).

3.5.2 Homologous recombination

Homologous recombination was carried out to construct recombinant EHV2-egfp viruses, or EHV2-egfp viral vectors. The transfection was done either with linearized plasmids or purified PCR amplicons. Plasmids were linearized by digestion, model A with *Nco*I, models B and D with *Pvu*I and model C with *Eco*RV. The cloned sections were amplified from each plasmid, total of 3680 bp for model A, 4615 bp for B, 3994 bp for C and 4211 bp D, with Phusion Hot Start polymerase and the bands purified from the agarose gel before transfection. Primers used are listed in appendix table 4. Transfection was carried out in prmEqFL and RK13 cells. Cells were either infected with EHV2-Bj 24 h or 48 h prior to transfection or transfected 24 h prior to infection.

3.6 Construction of rBac-viral vectors and vaccination trials (Unpublished data)

3.6.1 Construction of rBac-plasmids

The aim was to construct viral vectors for use in vaccination trials against allergy. Six different pFastBac HT B plasmids were constructed, (Figures 14 & 30) Glycoprotein B from EHV-2 was amplified from strain EHV2-Bj, EHV5 gB from strain BB5-5, glycoprotein G from the Vesicular stomatitis virus (VSV-G) from pMDG plasmid (kindly provided by Dr. Stefán Ragnar Jónsson). The target protein used in the rBac-viral vectors was Cul n 2 (Schaffartzik et al., 2011) a hyaluronidase originated from *Culicoides nubeculosus*, an allergen in insect bite hypersensitivity (IBH) of horses, amplified from a pFastBac-Culn2 plasmid (kindly provided by Dr. Sigríður Jónsdóttir). Primers used are listed in appendix table 5.

The glycoproteins were inserted under the polyhederin promoter P_{PH} in frame with 6xHis tag upstream of the gene, EHV-2 gB at sites *Nco*I and *Spe*I and EHV-5 gB at *Eco*RI and *Not*I. The VSV-G was amplified omitting the Stop codon and inserted at sites *Bam*HI and *Not*I in a modified pFastBac HT B with additional 6xHis Tag and V5 Tag downstream (Sigurðardóttir, 2011). All plasmids were sequenced to confirm the right sequence of the construct and to confirm the reading frame.

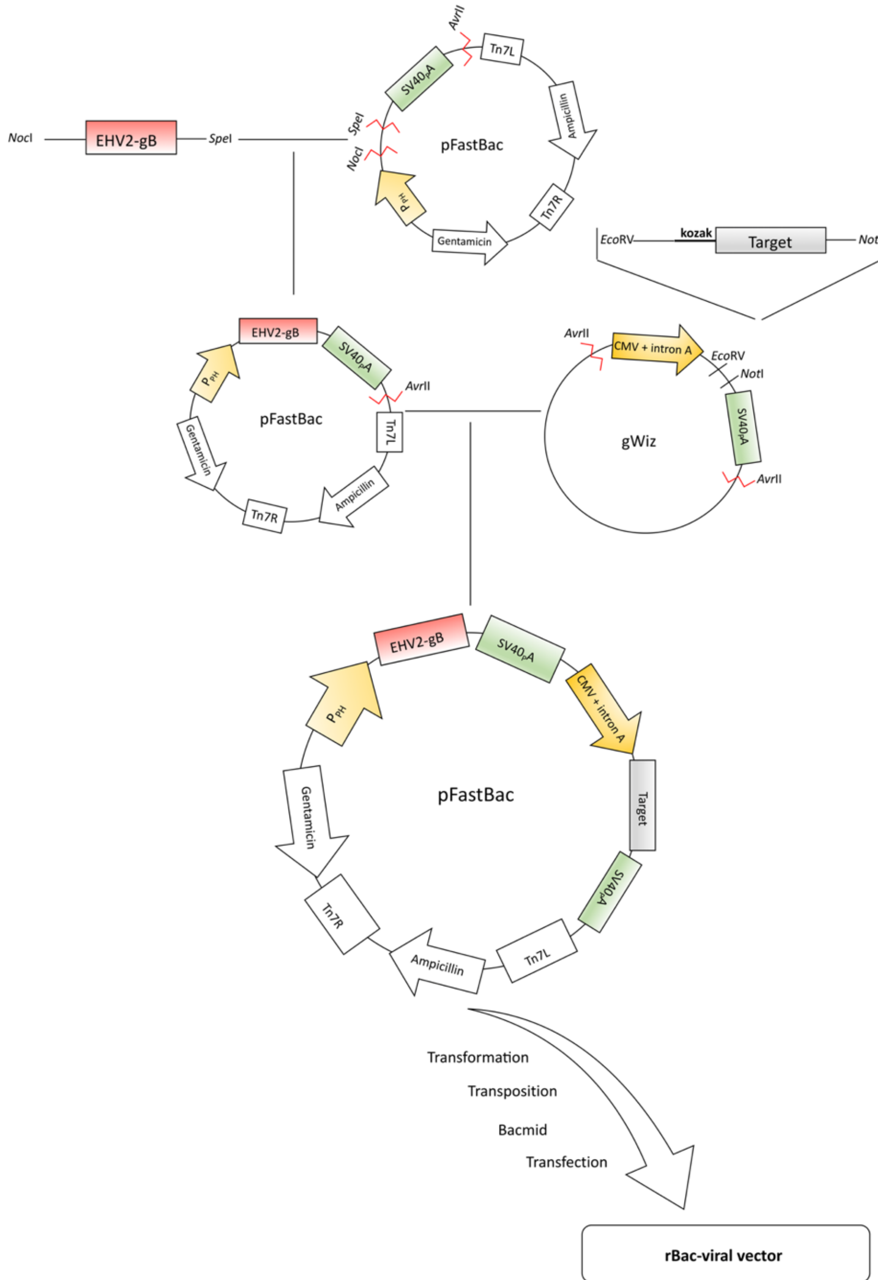

Figure 14. Schematic diagram illustrating the construction of rBac-plasmid and rBac-viral vector.
The diagram is shown for plasmid 1 (rBac-gB2-Target) but is representative for all six rBac-viral vectors constructed.

Construction of the expression cassette was as follows: The target gene was amplified with a Kozak consensus sequence (GCCACCATG) and cloned at restriction sites *Eco*RV and *Not*I. The expression cassette containing the target gene was amplified and inserted to the pFastBac plasmids at the *Avr*II restriction site after shrimp alkaline phosphatase (rSAP, New England BioLabs) treatment of the plasmid, according to the manufacturer's protocol. The pFastBac plasmids were sequenced to confirm the correct orientation of the expression cassette.

3.6.2 Construction of rBac-viral vectors

Recombinant baculoviruses, or rBac-viral vectors were constructed with the Bac-to-Bac Expression System (Thermo Fisher Scientific), according to the manufacturer's protocol, in short: Plasmids were transformed in DH10Bac *E.coli* cells, followed by transposition and selection of recombinant bacmids with antibiotic selection. Sf-9 insect cells were transfected with the bacmids. Supernatant containing the infectious recombinant baculoviruses, hereinafter referred to as rBac-viral vectors was used to infect a fresh confluent layer of Sf-9 cells for virus amplification and cloning the virus with limiting dilution.

3.6.3 Concentration of rBac-viral vectors

rBac-viral vectors were produced in High five insect cells and the culture spun at 11.953 x g at 4°C for 16–18 h in a Sorvall® RC 5C centrifuge. The supernatant was aspirated, and the sediment dried and dissolved in 800 μL PBS, transferred to 1.5 ml eppendorf tubes, vortexed and spun at 20.817 x g for 1 min. This supernatant was used in the 3rd, 4th and 5th vaccination. For the 1st and 2nd vaccination the supernatant was laid carefully on top of 2 mL 40% sucrose and spun at 65.000 x g for 150 min at 4°C in a Beckman TL-100 Ultracentrifuge. The supernatant was aspirated, and the sediment dissolved in 200 μL PBS and used in the 1st and 2nd vaccination. The protein concentration of the rBac-viral vectors was determind using the Bradford assay.

3.6.4 Vaccination with rBac-viral vectors

Twelve healthy foals (5–10 months) were used in the study. They were divided in pairs, colt and filly and kept in isolation stalls during the experiment. One week after each vaccination they were let out, but without close contact with the other pairs. The foals were hay-fed during the experiment. Throughout the study the foals were regularly observed for

health status. Blood was collected by jugular venepuncture into EDTA and serum vacutainer tubes (Vacuetta, Greiner Bio-One). The experiment was with the formal approval from the Icelandic Ethical Committee on Animal Research, 0113-16.

Six foals received intralymphatic (i.l.) vaccination with virus suspension, and six foals subcutaneous and intramuscular (s.c./i.m.), two foals with each rBac-viral vector per inoculation site. The foals were anaesthetised before vaccination with Chanecin, 2.5 mL i.v.. Each foal was vaccinated 5 times, at three-week intervals, the first two times with 20 μg of viral rBac-viral vector and then three times with 200 μg, in 500 mL PBS. Seven weeks after the final vaccination the foals were boosted with the target protein, Cul n 2 produced in *E.coli* in Alum hydroxide gel/Monophosphoryl lipid A (Alum/MPL) i.l.. No clinical signs were seen in the foals during the observation period.

Three foals and two adult horses were vaccinated with the target protein, Cul n 2 produced in *E.coli* in Alum/MPL i.l.. Blood samples were collected before and 2 weeks after the vaccination. The experiment was with the formal approval from the Icelandic Ethical Committee on Animal Research, 2016-01-03.

3.7 ELISA

3.7.1 γEHV ELISA (Paper II)

γEHV specific total IgG, IgG1, IgG4/7, IgG5 and IgE levels were measured in serum from foals and mares at days 0, 5, 12 and at months 1, 2, 3, 4, 5, 6 and for foals at months 9, 12, 15, 20 and 22 (**Paper II**, Supplementary S1 Table). The EHV-2 and EHV-5 antigens were made in prmEqFK and RK13 cells, respectively, according to (Svansson et al., 2009). The IgG ELISAs were done according to (Jonsdottir et al., 2018) and the IgE ELISA according to (Jonsdottir et al., 2015).

3.7.2 ELISA on serum from rBac-viral vector vaccinated foals (Unpublished data)

Cul n 2 specific IgG1, IgG4/7, IgG5 and IgE levels were measured. Four timepoints were tested, before vaccination, 2 weeks after the last vaccination, before protein boost and 2 weeks after the protein boost. The purified Cul n 2 was produced in insect cells. As the rBac-viral vectors were produced in insect cells the ELISA was carried out as well on the target protein from *Culicoides obsoletus,* expressed in yeast, *Pichia*-rCul o 2, (van der Meide et al., 2013). The ELISAs were done according to (Jonsdottir et al., 2015).

3.8 Protein methods (Unpublished data)

3.8.1 Native purification of glycoproteins

Glycoproteins B from EHV-2 and EHV-5, produced in recombinant baculoviruses were purified under native conditions with HIS-Select® HF Nickel Affinity Gel (Sigma Aldrich), in conformity with the manufacturer's protocol.

3.8.2 Western blot

Proteins were separated by SDS-PAGE under denaturing conditions and then transferred to a PVDF membrane (Millipore) by wet transfer (Mini-protean II system, Bio-Rad). Membranes were blocked with TBS-T (Tris buffered saline containing 0.1% Tween 20), with extra 2% Tween for 30 min at RT. After washing they were incubated O/N at 4°C with primary antibody: Mouse-anti-HIS (Biorad) 1:1000, Cul n 2 specific monoclonal (Jonsdottir et al., 2017) 1:10.000, or a pool of serum from 10 horses sampled post pyrexia infection 1:1000. Then after washing incubated for 1 h at RT with the secondary antibody: Goat-anti-mouse-AP (Dako) 1:5000, rabbit-anti-mouse-HRP (Dako) 1:5000 or mouse-anti-horse-AP (Biorad) 1:5000. The membranes were developed with either BCIP/NBT stock solution (Sigma) or Pierce ECLE Plus (Thermo Fisher Scientific), according to the manufacturer's protocols. PageRuler™ Prestained Molecular Mass Marker (Thermo Fisher Scientific) and SuperSignal™ Molecular Weight Protein Ladder (Thermo Fisher Scientific) were used to estimate the size of the proteins.

Concentrated rBac-gB2-Target vector (rBac-viral vector 1) and dissolved baculovirus sediment from rBac-Target-∆ vector (rBac-viral vector 3) were separated by SDS-PAGE and transferred to a PVDF membrane, as described above. The PVDF membranes were then cut into strips and stored in the dark at RT. Strip were incubated with serum from each vaccinated foal, at 4°C O/N, with secondary antibody and development as for the WB.

3.9 Polyclonal antibodies (Unpublished data)

Polyclonal antibodies were produced in mice according to (Overkamp et al., 1988) against EHV-2 and EHV-5 glycoprotein B, expressed in baculoviruses. Female BALB/c mice were injected intraperitoneally (i.p.) with 20 µg of the native purified protein in 200 µL of Freunds complete adjuvant (FCA), three times, day 0, 14 and 21. In the following days the mice were sacrificed and the ascitis collected, aliquoted and stored at -80°C. The antibodies were tested in WB.

3.10 Data analysis (Paper II)

Statistical analysis and graphs were performed with GraphPad Prism 6.0 (GraphPad Software). In **paper II**, each time point was examined separately between group-high and group-low when analysing the statistical difference. This was done for the total IgG, IgG1 and IgG4/7 in foals and mares, and for the EHV-2 and EHV-5 viral load in the NS of the foals. The total IgG and IgG4/7 values were normally distributed according to Shapiro-Wilk ($p \geq 0.05$) and analysed with a two-tailed unpaired t-test ($p<0.05$). The IgG1, EHV-2 and EHV-5 viral load values were not normally distributed and were analysed with the Mann-Whitney test ($p<0.05$). The EHV-2 and EHV-5 viral load from both NS and BC were compared internally, i.e. each time point compared with the others, done with the Kruskal-Wallis multiple comparison test and Dunn’s test was used to correct for multiple comparisons, $p \leq 0.05$ was considered significant.

4 Results

4.1 Establishment and characterization of fetal equine kidney and lung cells with extended life span (Paper I)

Due to the slow growth of gammaherpesviruses, cells that can be propagated long term are crucial in all *in vitro* experiments. We established equine fetal kidney (extEqFK) and lung (extEqFL) cell lines with extended life span from primary cells (prmEqFK and prmEqFL) by transfecting the cells with a retroviral vector containing oncogenes E6 and E7 from the human papilloma virus type 16. These cells are now available at abm® (catalog no. T0084 and T0095, https://www.abmgood.com).

4.1.1 Expression of E6 and E7

Both cell lines, extEqFK and extEqFL expressed the E6 and E7 genes stably, in high and low passage. DNA isolated from primary cells was used as negative control (Figure 1 in **Paper I**).

4.1.2 Cell propagation: Cell lines vs. primary cells

The cell lines, extEqFK and extEqFL needed less serum and grew faster than the primary cells, were split twice a week 1:10 and propagated in medium with 5% and 2% FBS, respectively. Both cell lines grew at a similar rate until passage 35–40 when the growth gradually slowed. The primary cells, prmEqFK and prmEqFL were cultured in medium with 10% FBS and split 1:5 twice a week, for 10–12 passages when their growth started to decline.

4.1.3 Cytopathic effect of EHV-2 and EHV-5

Cytopathic effects (CPE) in the kidney and lung cell lines, the comparable primary cells and RK13 cells were compared (Figure 15 & Figure 2 in **Paper I**). CPE in all four types of horse cells, extEqFK, extEqFL, prmEqFK and prmEqFL, were seen as round cells in defined foci, most distinct in both of the kidney cells, prmEqFK and extEqFK. CPE of both EHV-2 and EHV-5 were more local and isolated in the primary kidney cells (prmEqFK) but scattered throughout the extEqFK cell layer.

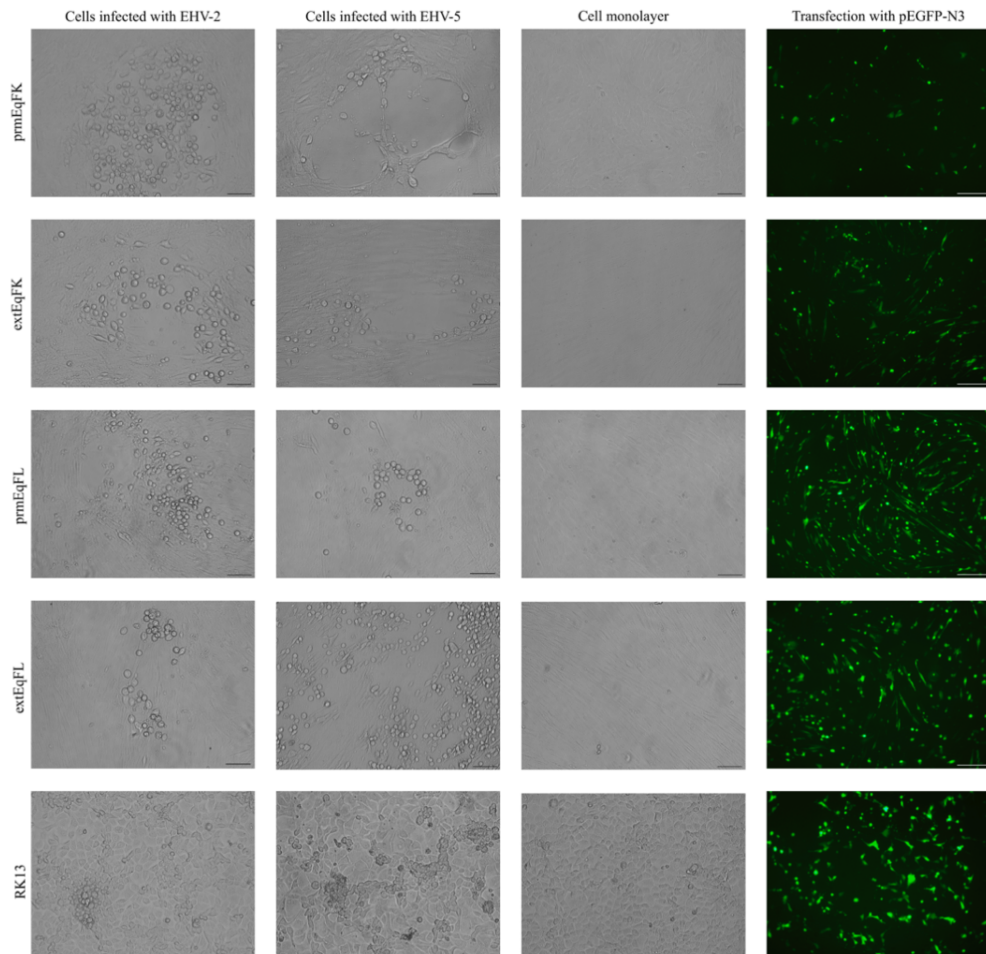

Figure 15. Cytopathic effect of EHV-2 and EHV-5 infection and EGFP expression after transfection with pEGFP N3 vector.
prmEqFK, extEqFK, prmEqFL, extEqFL and RK13 cells infected with EHV-2 and EHV-5 and transfected with pEGFP-N3 vector. Cell monolayer for all cell types showed as control. CPE was observed in a light microscope, scale bar 20 μM and EGFP expression in phase contrast fluorescent microscope, scale bar 50 μM.

The lung cells, both primary and the cell line, grew rapidly and tended to overgrow the CPE of both EHV-2 and EHV-5. CPE of EHV-2 was only observed in the lung cells, both extEqFL and prmEqFL, if the cells were grown in OptiMEM without serum. The optimal method to observe the CPE of EHV-5 was to infect the lung cells, both extEqFL and prmEqFL, simultaneously with seeding of the cells. The infection efficiency and CPE in

both of the horse cell lines (extEqFK and extEqFL) was stable up to passage 35–40, or as long as they kept their proliferation capacity (data not shown).

CPE of both EHV-2 and EHV-5 in RK13 cells was difficult to discriminate from cell death due to necrosis.

4.1.4 Viral load of EHV-2 and EHV-5 in extEqFK vs. prmEqFK

The infectious virus titer of EHV-2 and EHV-5 in extEqFK and prmEqFK was measured with end point dilution assay and compared between the two cell types. There was no difference in the EHV-2 viral load, a titer of 10^5 $TCID_{50}$/mL was obtained in both extEqFK and prmEqFK. EHV-5 yielded a much higher titer in extEqFK cells, $10^{9.5}$ $TCID_{50}$/mL, compared to $10^{5.6}$ $TCID_{50}$/mL in the primary cells. CPE of EHV-5 was observed in extEqFK after 3 days, compared to 6 days in prmEqFK, and after only a few days almost the complete extEqFK cell layer was infected, compared to single CPE scattered throughout the prmEqFK cells.

4.1.5 Transfection rate and expression of EGFP

The extended kidney and lung cell lines (extEqFK and extEqFL), the primary cells (prmEqFK and prmEqFL), RK13 and COS-7 cell lines were transfected with pEGFP-N3 vector and the expression of EGFP examined (Figure 15 & Figure 3 in **Paper I**). Twenty-four hours after the transfection the ratio of fluorescent cells was found by counting EGFP expressing cells vs. total cells.

The extEqFK cell line had about four times higher transfection rate compared to prmEqFK cells, or 13.9% vs 3.5%. No difference was observed between the two types of lung cells, prmEqFL with 10.5% vs. 10.7% in the extEqFL. The transfection efficiency in the kidney and lung cell lines was stable up to passage 20–25. The highest transfection rate was observed in the RK13 and COS-7 cell lines, with 23% and 45% of the cells showed green fluorescence, respectively.

4.2 The effect of maternal immunity on the equine gammaherpesvirus type 2 and 5 viral load and antibody response (Paper II)

To investigate γEHV infection and interaction with specific antibodies, both maternal and endogenous, fifteen foals were followed from birth to 22 months of age and their dams during the first 6 months. Samples were taken regularly for evaluation of antibody responses, virus isolation and viral load.

4.2.1 γEHV specific antibody levels of the foals and mares

4.2.1.1 Set up and standardization of the γEHV ELISA

The EHV-2 and EHV-5 antigens were made from cell associated virus; EHV-2 was cultured in prmEqFK cells (Svansson et al., 2009) and EHV-5 in RK13 cells. γEHV specific total IgG antigen response to EHV-5 was tested with a pilot ELISA experiment on serum from three foal/mare pairs (F/M: 2, 9 and 13), from all time points and the results compared to an analogous EHV-2 test. All serum tested had a lower total IgG response to EHV-5 than EHV-2, except on day 12 where both foals and mares had the same response to both. The EHV-5 curves between time points was parallel to the EHV-2 curves (Figure 16). Based on these results the EHV-2 antigen was used in continuing experiments and the ELISA interpreted as a γEHV ELISA.

Serum from a representative set of foals (F1 and F10) of dams with low and high γEHV specific total IgG at day 0 (pre-suckling) was tested in different dilutions to determine the optimal dilution of the serum for the γEHV ELISA (Figure 17). Based on these results serum was diluted 1:100 in all the γEHV ELISA tests.

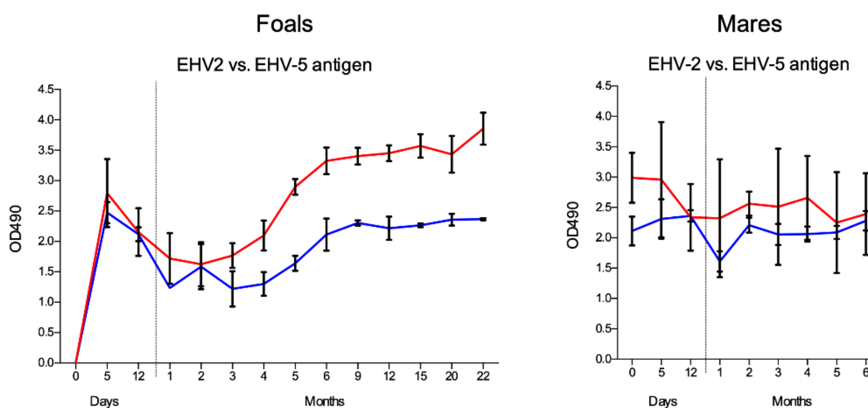

Figure 16. EHV-2 vs. EHV-5 antibody response of foals and mares.
γEHV specific total IgG against EHV-2 (red lines) vs. EHV-5 (blue line) in serum from 3 foals and mares over 22- and 6-month period, respectively. The x-axis shows the age of the foals.

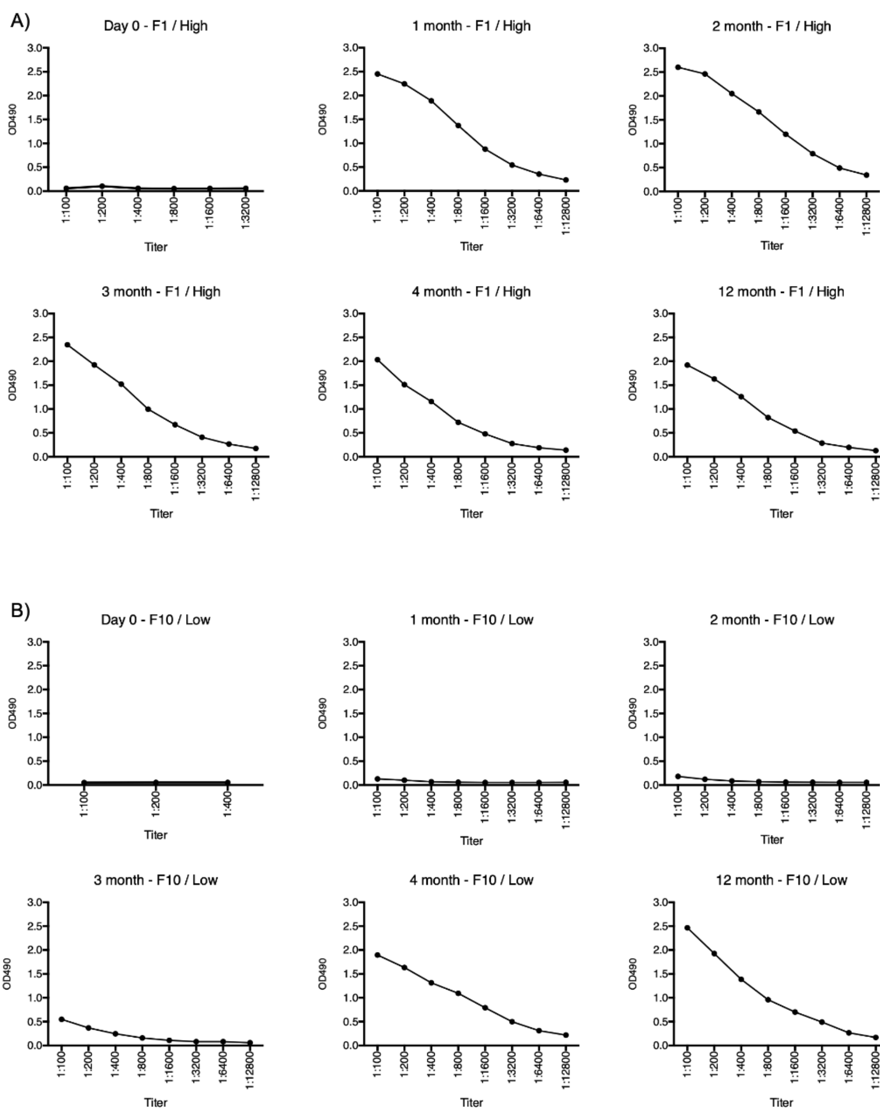

Figure 17. Serial dilutions of serum tested in γEHV specific total IgG ELISA for a representative set of foals.

A) Foal 1, from mare with high γEHV specific total IgG at foaling. B) Foal 10, from a mare with low γEHV specific total IgG at foaling.

4.2.1.2 γEHV specific antibody levels

Serum was collected from all 15 foals and mares throughout the study period (**Paper II**, Supplementary S1 Table) and tested for γEHV specific total IgG, IgG1 and IgG4/7. Serum from the foals pre-suckling were negative for all γEHV antibodies tested.

The specific γEHV total IgG decreased from day 12 in the foals and reached nadir at month 2 (Figure 18 & Figure 1A in **Paper II**). From then on the levels increased throughout the study, with the exception of month 20, where a small decrease was seen. From day 12 on, the IgG1 levels also decreased in the foals and reached nadir at month 2. The levels then increased and peaked at month 5. From then on the levels overall decreased throughout the study with small fluctuations at months 12 and 22 where the levels increased. The IgG4/7 levels in the foals decreased from month 1, reached nadir at month 3, increased and peaked at month 9. From then on the levels were fairly stable throughout the study with minor fluctuations between timepoints. The γEHV specific total IgG, IgG1 and IgG4/7 levels varied between the mares but was relatively stable within each individual (Figure 18 & Figure 1A in **Paper II**).

The γEHV specific IgG5 and IgE levels were tested in serum from three foal/mare pairs. The pairs were chosen with regard to the total IgG levels of the mare at day 0 (pre-suckling): mares with low, medium and high levels (F/M no: 2, 9 and 13). All serum was negative for γEHV specific IgG5 and IgE at all timepoints tested (data not shown).

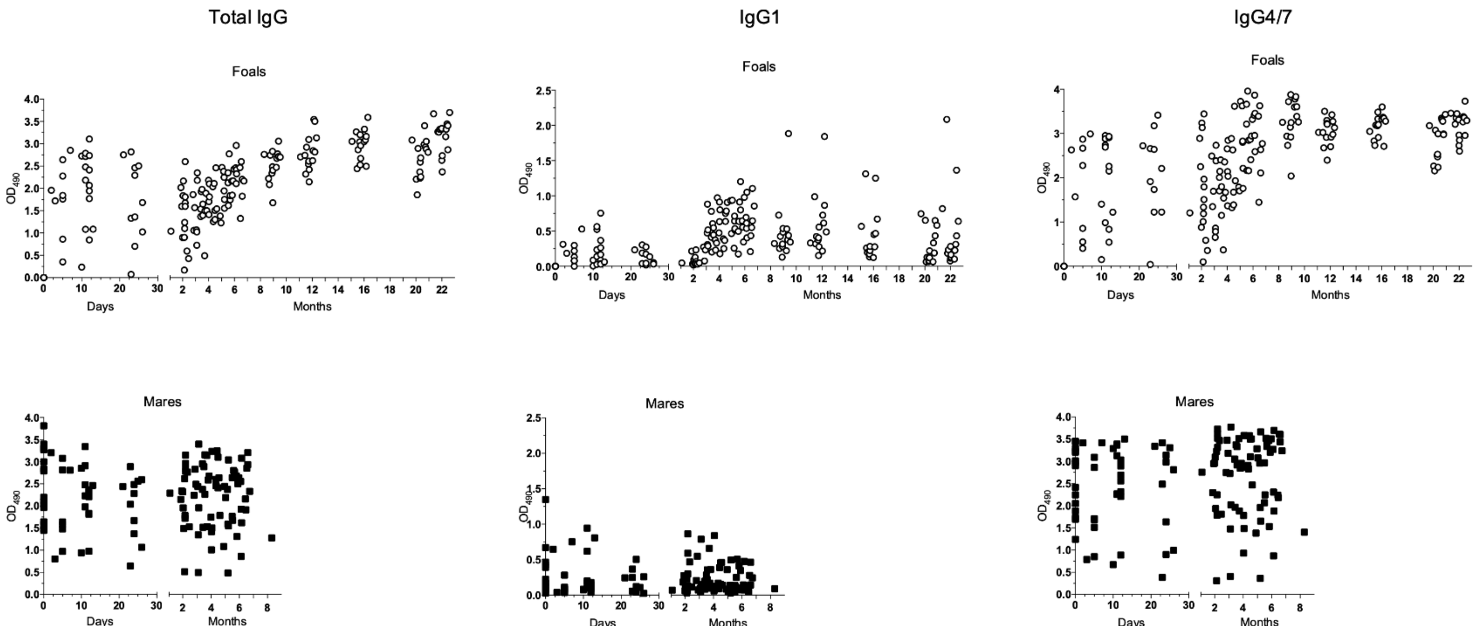

Figure 18. γEHV specific antibody response of foals and mares.
Total IgG, IgG1 and IgG4/7 in serum from 15 foals (upper graphs, ○) and their dams (lower graphs, ■) over 22- and 6-month period, respectively. Each value is represented separately and plotted at the exact time of sampling. The x-axis shows the age of the foals and is in two segments, days and months, with the first month relatively larger.

4.2.1.3 Comparison of γEHV IgG levels between foals of dams with high vs. low maternal antibodies at foaling

At foaling the γEHV specific antibody levels of the mares varied considerably. The mares were grouped in two groups, depending on their γEHV specific total IgG values at day 0 (pre-suckling): low-mares ($OD_{490} < 2.5$, Mares: 2, 5, 6, 10 and 12–15) and high-mares ($OD_{490} > 2.5$, Mares: 1, 3, 4, 6, 8, 9 and 11). The data were normally distributed and the difference between the two groups significant (Figure 19 & S1 Figure in **Paper II**). In addition, at day 0 the differences in IgG1 and IgG4/7 levels between the low-mares and high-mares were also significant (Figure 1B in **Paper II**).

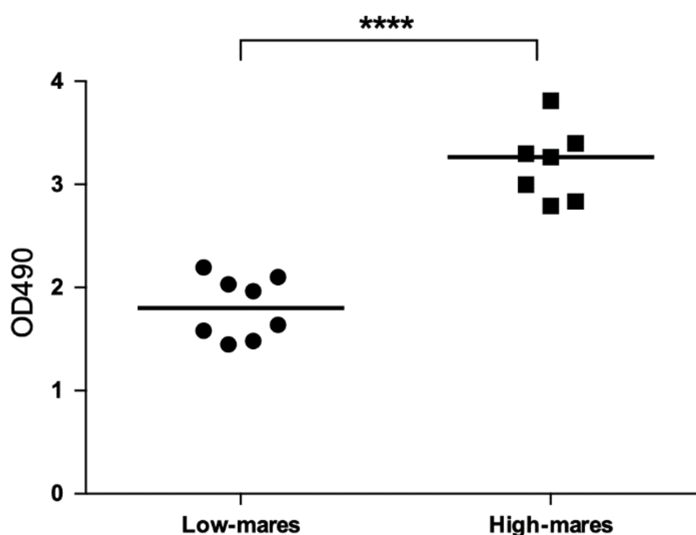

Figure 19. γEHV specific total IgG status of the mares at foaling.
Group low-mares (●) (n = 8) versus group high-mares (■) (n = 7). The data were normally distributed according to Shapiro-Wilk ($p \geq 0.05$) and the results presented as mean, two tailed unpaired t-test ****$p < 0.0001$.

The foals were then grouped in corresponding groups, group-low (foals of dams with low γEHV total IgG at foaling) and group-high (foals of dams with high γEHV total IgG at foaling). Group-high foals had higher γEHV specific total IgG levels compared to group-low for the first 3 months, and this was significant at day 12 and months 2 and 3 (Figure 20 and Figure 1B in **Paper II**). From day 5 the antibody level of group-high decreased and reached nadir at month 4. For group-low decreas was seen from day 12 and nadir at month

2. At months 4 to 6 group-low surpassed group-high and was significantly higher at month 6. No difference was between the two groups from month 9 and onwards.

For the γEHV specific IgG1 group-high foals had higher levels for the first month, this was significant at day 12 (Figure 20 & Figure 1B in **Paper II**). Nadir for both groups was at month 2 and from months 2 to 4 little difference was between the two groups. Group-high had higher levels from month 5 and throughout the study, significant at month 9.

The overall pattern for the γEHV specific IgG4/7 levels was the same as for the total IgG, group-high foals had higher levels than group-low for the first 3 months, significant at month 3. Group-high reached nadir at month 4 and group-low at month 3. Group-low had higher levels at months 4–6, significant at months 6. From month 9 and onwards no differenece was between the groups (Figure 20 & Figure 1B in **Paper II**).

For the mares, day 0 was the only timepoint in the study period where the difference between low-mares and high-mares was significant, seen for the total IgG, IgG1 and IgG4/7. Overall, the antibody levels for the high-mares decreased over the 6-month study period but increased for the low-mares (Figure 20 & Figure 1B in **Paper II**).

.

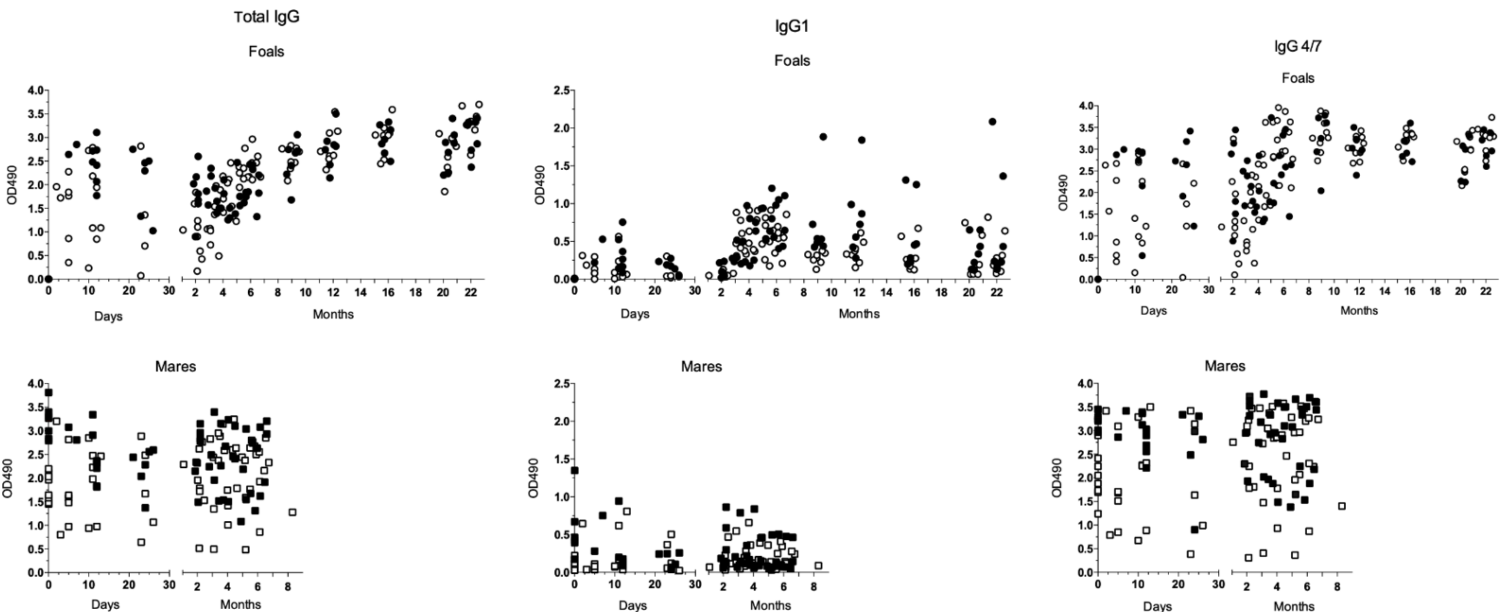

Figure 20. Comparison of γEHV total IgG response between foals with high vs. low maternal antibodies and between corresponding groups of mares

Group-high (●/■) vs. group-low (○/□), for foals (upper graphs) and mares (lower graphs), examined for γEHV specific total IgG, IgG1 and IgG4/7. Each value is represented separately and plotted at the exact time of sampling. The x-axis shows the age of the foals and is in two segments, days and months, with the first month relatively larger.

4.2.2 Isolation of EHV-2 and EHV-5 from nasal swabs and blood samples

Virus isolation was attempted from nasal swabs (NS) and blood samples (PBL) from ten foal/mare pairs (F/M no: 1-4, 6, 10-13 and 15) (**Paper II** Supplementary S1 Table). Exceptions were at day 5 where only three pairs were tested and at month 2 where only NS samples were tested. Virus isolation was done directly after sample collection, except at month 2 where NS samples were stored at -80°C prior to isolation.

A total of 133 NS and 123 PBL samples were tested from the foals, and 83 NS and 73 PBL samples from the mares. If CPE was observed in the culture, DNA was isolated from the supernatant and tested for EHV-2 and EHV-5 with type specific qPCR. A Ct value of 35 was chosen as the cut-off value for sample positivity, based on results of DNA isolated from extEqFK, the cells used for the virus isolation, see chapter 4.2.4, sequencing of qPCR amplicons.

Virus was most frequently isolated from the foals' NS samples, CPE was detected in 27% (36/133) of the cultures (Table 2 & Figure 2 in **Paper II**). EHV-2 was first isolated on day 5 in all three samples tested. On day 12 EHV-2 was isolated from three foals and a mixture of EHV-2 and EHV-5 from one foal. Overall EHV-2 had been isolated from 6 out of the 10 foals on day 12 (Table 2). All nine isolations made in the first two months were EHV-2, with the exception of the one mixture at day 12. At month two EHV-2 had been overall isolated from 7 out of the 10 foals. All NS samples were positive at month three, 50% were EHV-2 and 50% were a mixture of both viruses. Similarly, all foals' PBL samples were positive at month 3, 40% EHV-2 and 60% mixture of both EHV-2 and EHV-5 (Table 2 & Figure 2 in **Paper II**). Month 3 was the only time when virus was isolated from foals' PBL. When the foals were 5–12 months of age, all positive NS cultures were mixture of EHV-2 and EHV-5. No virus was isolated from foals after the first year. Overall, EHV-2 was isolated from all foals within the first three months, and EHV-5 from 8 out of the 10 foals during the first year, always in a mixture with EHV-2.

Virus was less frequently isolated from the mares. Only 4.8% (4/83) of the NS samples and 1.4% (1/73) of the PBL samples were positive (Table 2 & Figure 2 in **Paper II**).

Table 2. Isolation of EHV-2 and EHV-5 from NS and PBL samples, from 10 pairs of foals and mares.
NS: Nasal swab. PBL: Peripheral blood leukocytes. F: Foal. M: Mare.

Day 5	F2	EHV-2	3 month	F1	EHV-2 & EHV-5	Day 12	M1	EHV-2	3 month	M1	EHV-2
	F10	EHV-2		F2	EHV-2		M3	EHV-2 & EHV-5			
	F15	EHV-2		F3	EHV-2 & EHV-5	1 month	M15	EHV-2			
Day 12	F6	EHV-2 & EHV-5		F4	EHV-2 & EHV-5	5 month	M15	EHV-2			
	F10	EHV-2		F6	EHV-2 & EHV-5						
	F11	EHV-2		F10	EHV-2						
	F13	EHV-2		F11	EHV-2 & EHV-5						
2 month	F12	EHV-2		F12	EHV-2 & EHV-5						
	F13	EHV-2		F13	EHV-2						
3 month	F1	EHV-2		F15	EHV-2						
	F2	EHV-2									
	F3	EHV-2 & EHV-5									
	F4	EHV-2 & EHV-5									
	F6	EHV-2 & EHV-5									
	F10	EHV-2									
	F11	EHV-2 & EHV-5									
	F12	EHV-2									
	F13	EHV-2 & EHV-5									
	F15	EHV-2									
4 month	F1	EHV-2 & EHV-5									
	F2	EHV-2									
	F4	EHV-2									
	F6	EHV-2									
	F10	EHV-2									
	F12	EHV-2 & EHV-5									
5 month	F1	EHV-2 & EHV-5									
	F2	EHV-2 & EHV-5									
	F3	EHV-2 & EHV-5									
	F6	EHV-2 & EHV-5									
	F11	EHV-2 & EHV-5									
	F13	EHV-2 & EHV-5									
6 month	F4	EHV-2 & EHV-5									
12 month	F1	EHV-2 & EHV-5									
	F3	EHV-2 & EHV-5									
	F6	EHV-2 & EHV-5									
	F13	EHV-2 & EHV-5									

4.2.3 Viral load of EHV-2 and EHV-5 in nasal swab and buffy coat samples

4.2.3.1 Viral load in the foals

The viral load of EHV-2 and EHV-5 from nasal swab (NS) and buffy coat (BC) samples from the foals was measured with qPCR. A total of 198 NS samples were tested throughout the study period (**Paper II** Supplementary S1 Table). EHV-2 was amplified alone from 35% of the samples, EHV-5 from 2%, 53.5% of the samples were positive for both viruses and 9.5% of the samples were negative (Figure 21). For the 199 BC samples that were tested, EHV-2 was amplified alone from 43% of the samples, EHV-5 from 1%, 54.5% of the samples were positive for both viruses and no amplifications were in 1.5% of the samples, (Figure 21).

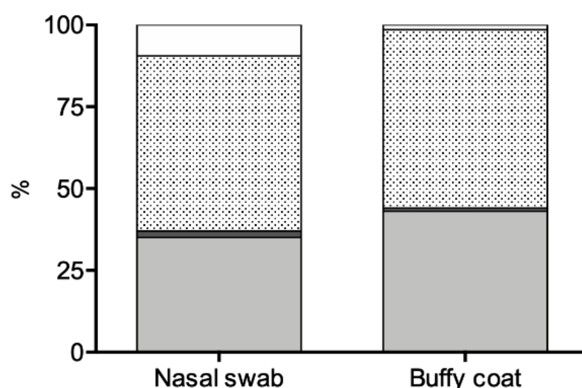

Figure 21. Presence of EHV-2 and EHV-5 in nasal swab and buffy coat samples from the foals.
Total percentage of EHV-2 (light grey), EHV-5 (dark gray), EHV-2 & EHV-5 (pattern), negative samples (white) tested with type specific qPCR.

For the first month the EHV-2 viral load in the foals' NS samples increased only slightly, then increased significantly (**Paper II** Supplementary S4 Table), or almost 5-log and peaked when the foals were 2–4 months old (Figure 22). The viral load then decreased steadily, with small fluctuation between timepoints, over the study period and was at month 22 significantly lower than months 3 to 5. In total 11.5% of the NS samples were negative for EHV-2, mostly from the first month.

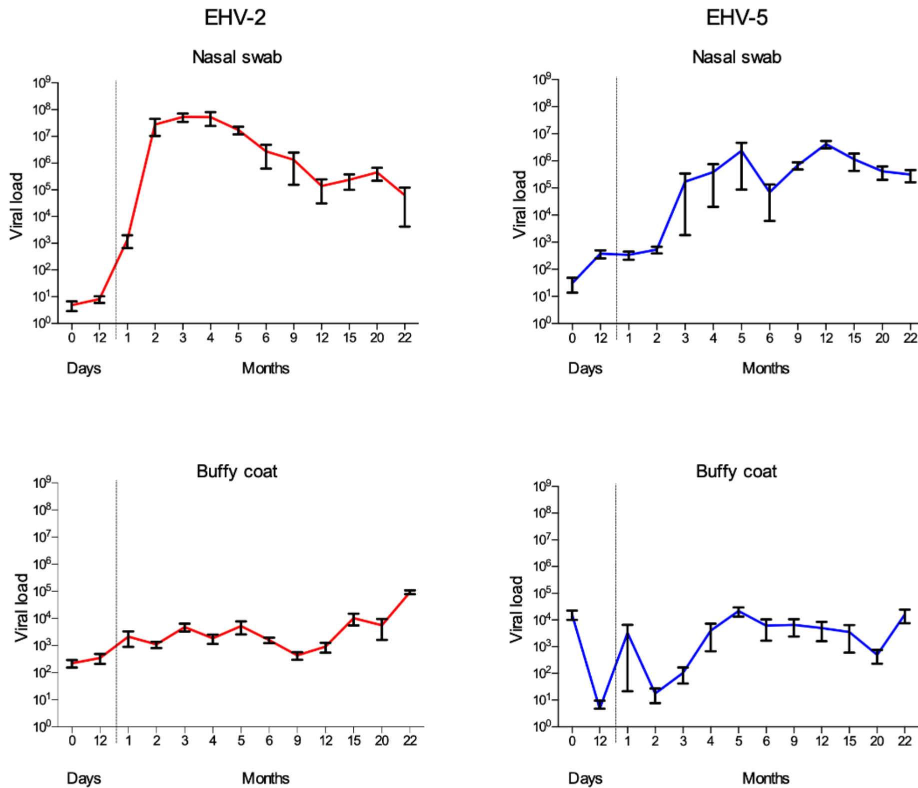

Figure 22. EHV-2 and EHV-5 viral load in nasal swab and buffy coat samples from the foals.
EHV-2 (red lines) and EHV-5 (blue lines) viral load measured with qPCR in nasal swab (upper graphs) and buffy coat (lower graphs). The y-axis is log-scale. Viral load: Copy per 100 ng/DNA. The x-axis shows the age of the foals.

The EHV-5 viral load in the NS samples increased slowly for the first 5 months (Figure 22). High individual variability was observed from month 3 to 6. The viral load peaked twice, at month 5 and at month 12, with the second peak slightly higher and significantly higher than day 0 to month 6, (**Paper II** Supplementary S4 Table). From month 12 and throughout the study the viral load decreased steadily. A total of 16.5% of the NS samples were negative for EHV-5, most were from the first 6 months.

For the first 5 months the EHV-2 viral load in the BC increased steadily, with small fluctuation between timepoints (Figure 22). From then the viral load decreased and was at month 9 significantly lower than months 3 to 5 (**Paper II** Supplementary S4 Table). The viral load rose again and was significantly higher at month 15 than month 9 and 12. At month 20 the viral load decreased slightly and then increased again with the highest viral load measured at month 22. Month 22 was significantly higher than other time points, except for months 3, 5 and 15. Only five BC samples were negative for EHV-2.

Few BC samples were positive for EHV-5 for the first 4 months. The viral load fluctuated for the first 2 months, then increased and was relatively constant from month 5 and throughout the study, with exception of month 20 when the viral load decreased suddenly (Figure 22). A total of 44% of the BC samples were negative for EHV-5, most from the first 6 months.

4.2.3.2 Relation of the viral load to the dam's γEHV specific total IgG antibody status at foaling

The viral load was examined in relation to the dam's γEHV specific IgG level at foaling, group-low (foals of dams with low total γEHV IgG antibody status at foaling) vs. group-high (foals of dams with high total γEHV IgG antibody status at foaling), same groups as in chapter 4.2.1.3.

In the nasal swab, group-low had higher EHV-2 viral load at month 2 and 3 and this was significant at month 3 (Figure 23 & Figure 3B in **Paper II**). For both group-high and group-low the peak in the viral load was consistent with the decrease of maternal antibodies (Figure 24 & Figure 4 in **Paper II**). Little difference was in the viral load between the groups at months 4 to 15. At month 20 group-low had significantly higher viral load and a decrease in antibodies (Figures 23 & 24). At month 22 the group-high surpassed and was significantly higher.

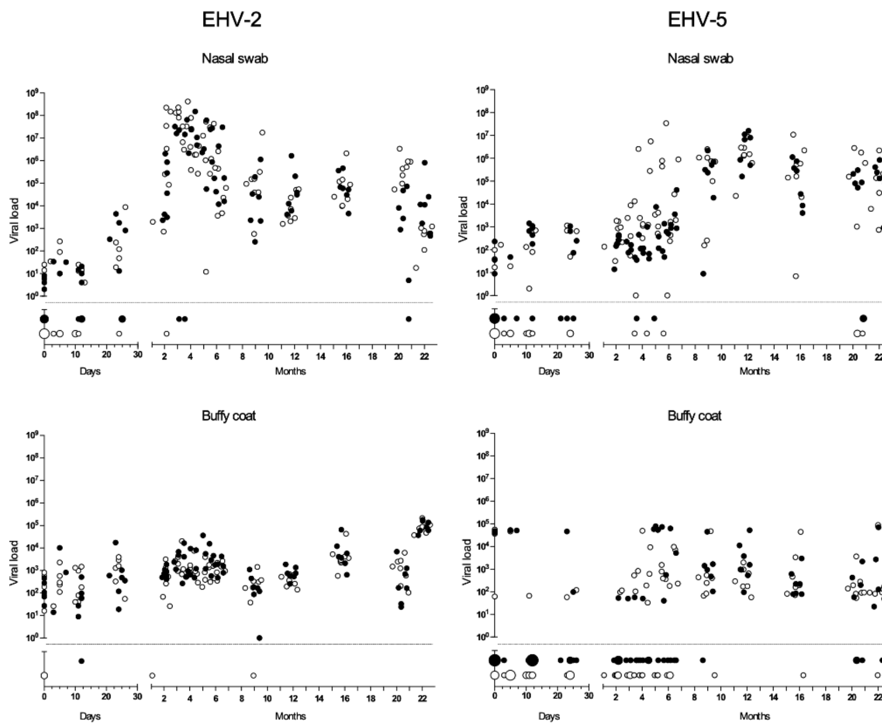

Figure 23. Comparison of EHV-2 and EHV-5 viral load between foals with high vs. low maternal antibodies.

Group-low ○ vs. Group-high ●. Viral load: copy per 100 ng/DNA. The y-axis is log-scale and in two segments, the dots under the line symbolize the value zero. The size of the dots is in proportion to the number of values they symbolize. The x-axis shows the age of the foals in days from birth and is in two segments, days and months, with the first month relatively larger.

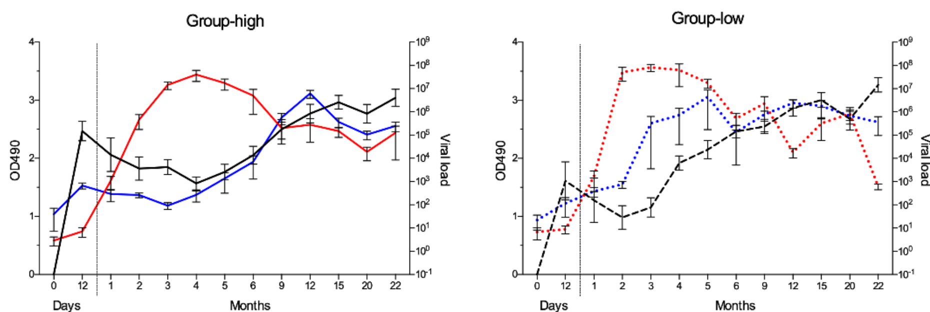

Figure 24. Comparison of total IgGγEHV specific antibody response and viral load in nasal swab samples from foal.
The total IgG antibody level (black lines, lefthand scale) in the foals was compared to the EHV-2 (red lines) and EHV-5 (blue lines) viral load (righthand log-scale), viral load: copy per 100 ng/DNA. Examined separately for group-high (solid lines) and group-low (dotted lines). Results are presented as mean ± SEM. The x-axis shows the age of the foals.

Group-low had higher EHV-5 viral load in the nasal swab at months 2 to 6 and this was significant at month 4 (Figure 23 & Figure 3B in **Paper II**). The γEHV specific total IgG for group-high reached nadir at month 4, and then 8 months later the EHV-5 viral load peaked for group-high, despite of high endogenous antibody production (Figure 24 & Figure 4 in **Paper II**). Group-low reached nadir at month 2, and the viral load peaked three months later, at month 5. From then the viral load remained relatively high throughout the study. In contrast to EHV-2, the EHV-5 viral load appeared to synchronize to the endogenous antibody level over the sample period, for both group-high and group-low.

In the buffy coat samples, there was not much difference between group-high and group-low, both in the EHV-2 and EHV-5 viral load.

4.2.3.3 Viral load in the nasal swab of the mares

The EHV-2 viral load was relatively stable over the 6-month study period, with the exception of day 12 where an approximately 2-log increase was observed. Two peaks were observed in the EHV-5 viral load, at day 12 and month 5. Beside these peaks, the viral load was relatively stable in the mares (Figure 25 & S2 Figure from **Paper II**).

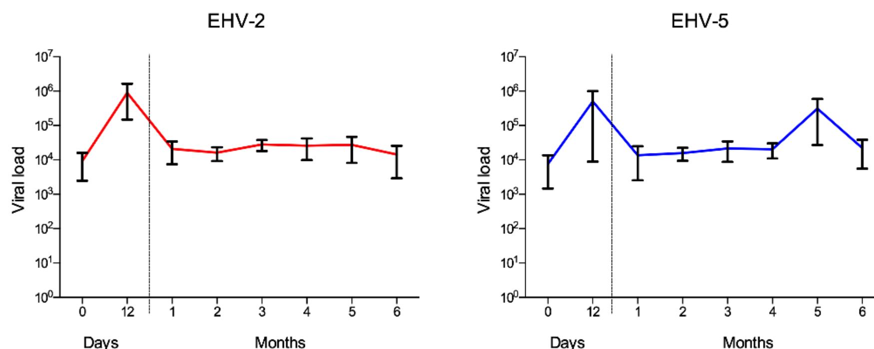

Figure 25. EHV-2 and EHV-5 viral load in nasal swabs from the mares.
EHV-2 (red lines) and EHV-5 (blue lines) viral load measured with qPCR in nasal swabs. Results are presented as mean ± SEM. The y-axis is log-scale. Viral load: copy per 100 ng/DNA. The x-axis shows the age of the foals.

4.2.4 Sequencing of qPCR amplicons (Unpublished data)

Amplification was seen in some of the day 0 (pre-suckling) samples from the foals. For the NS, EHV-2 was amplified from 50% of the samples and EHV-5 from 43%, for the BC this were 86% and 43%, respectively. All of the day 0 samples were tested repeatedly with invariably the same or very similar results. Amplification was very rarely seen in the negative controls and if seen the Ct value was >40. DNA was extracted from extEqFK and extEqFL cells (chapter 4.1) and tested in the EHV-2 and EHV-5 type specific qPCR. Amplification was seen in both cell types, for EHV-2 and EHV-5.

Subsequent qPCR amplicons were chosen from both EHV-2 and EHV-5 reactions for sequencing: extEqFK, extEqFL, standards and negative controls. Two BC amplicons were chosen from EHV-2 reactions (+qPCR), Day 0 – foal no. 13 (F13) and month 5 – F11, and two negative samples (-qPCR), Day 0 – F11 and Day 0 – F 6. A comparable set of BC amplicons was chosen from EHV-5 reaction (+qPCR), Day 0 – F8 and month 22 – F15, and two negative samples (-qPCR), Day 0 – F11 and Day 0 – F6 (Table 3).

Table 3. Sequencing results on qPCR amplicons from selected buffy coat samples

A) EHV-2, amplicon size 76 bp

Sample	qPCR results	Ct value	Copy no	No of bases sequenced	Identity (%)
EHV-2 standard	+	29	1.000	76 bp	93-100
extEqFK cells	+	32	137	26 bp / 31 bp	96-100 / 100
extEqFL cells	+	30	621	—	—
Day 0 - Foal no. 13	+	28	1.879	32 bp / 41 bp	100 / 97-100
5 months - Foal no. 11	+	17	1.687.338	32 bp / 41 bp	100 / 97-100
Day 0 - Foal no. 11	-	Undetermined	0	—	—
Day 0 - Foal no. 6	-	Undetermined	0	—	—
Negative control (ddH_2O)	-	Undetermined	0	—	—

B) EHV-5, amplicon size 82 bp

Sample	qPCR results	Ct value	Copy no	No of bases sequenced	Identity (%)
EHV-5, Standard	+	36	1.000	34 bp / 23 bp	100 / 100
extEqFK cells	+	37	285	—	—
extEqFL cells	+	36	553	82 bp	99-100
Day 0 - Foal no. 8	+	40	48	82 bp	99-100
22 months - Foal no. 15	+	31	27.050	82 bp	99-100
Day 0 - Foal no. 11	-	Undetermined	0	—	—
Day 0 - Foal no. 6	-	Undetermined	0	—	—
Negative control (ddH_2O)	-	Undetermined	0	—	—

—: no sequencing results. Identity to known EHV-2 and EHV-5 strains, BLAST, NCBI.

The complete 76 bp amplicon was sequenced from the EHV-2 standard, with 93–100% identity to known EHV-2 strains (blastn, NCBI), the Ct value was 29 with 1000 copies detected (Table 3A). A total of 57 bp were sequenced from the extEqFK cells, 26 bp at the 5’end and 31 bp at the 3’end, with 96–100% and 100% identity to EHV-2 strains, respectively. The Ct value was 32, but only 137 copies detected. Only the primers were sequenced from the EHV-2 extEqFL amplicon, the Ct value was 30, and 621 copies were detected. A total of 73 bp were sequenced from both of the +qPCR samples, day 0 and month 5, both with 32 bp at the 5’end and 41 bp at the 3’end and 100% and 97-100% identity to known EHV-2 strains, respectively. The Ct value for the month 5 sample was 17 with over millions of copies detected, but for the day 0 the Ct value was 28 with 1879 copies. No sequencing results were obtained from the -qPCR samples or the negative control (Table 3A).

A total of 57 bp were sequenced from the EHV-5 standard amplicon, 34 bp at the 5’end and 23 bp at the 3’end, both parts with 100% identity to known EHV-5 strains. The Ct value in the reaction was 36 with 1000 copies detected (Table 3B). Only part of the primers was sequenced from the EHV-5 extEqFK amplicon. However, the complete 82 bp amplicon was sequenced from the extEqFL cells and the two +qPCR samples, from day 0 and month 22, all with 99-100% identity to EHV-5 strains and Ct values of 36, 40 and 31

respectively. Of these three samples the one from month 22 had the highest copy number, 27.050 copies detected, 553 copies were in the extEqFL sample and only 48 in the day 0 +qPCR sample. No sequencing results were obtained for the -qPCR samples or the negative control (Table 3B).

4.3 Construction and characterization of an infectious EHV-2 virus that expresses enhanced green fluorescent protein (Paper III)

The structure of the EHV-2 genome and large non-coding regions make insertion of foreign genes into the genome feasible. Different approaches were tried to produce EHV-2 expressing enhanced green fluorescent protein (EGFP).

4.3.1 Construction of EHV2-egfp insertion plasmids

Four different EHV2-egfp plasmids were constructed (Figure 26). In model A the EGFP gene was spliced to the C-terminus of the glycoprotein B (gB) gene without its start and stop codons and expressed as an elongation of the gB gene. In models B, C and D an expression cassette where the EGFP gene had its own CMV-promoter was inserted in the genome at different locations, in model B the non-coding region downstream of the DNA polymerase gene, model C in the intergenic region (IGR) between gB and the DNA polymerase gene and in model D in the thymidine kinase (TK) gene, inactivating the gene. See more detailed description and figures of the plasmid construction in chapter 3.5.1 (Figures 10–13).

Correct orientation of inserts was confirmed with sequencing. No variations were in the nucleotide sequence. The model B, C and D plasmid constructs showed fluorescence expression after transfection in RK13 and extEqFK cell.

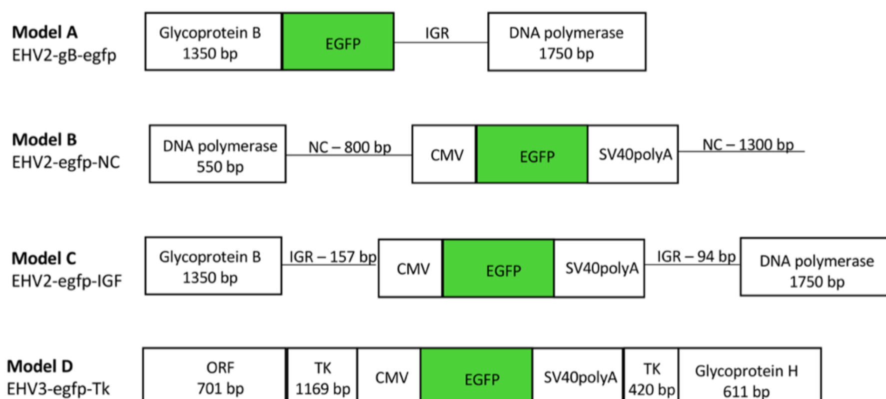

Figure 26. Schematic diagram illustrating the four different EHV2-egfp plasmids constructed.

4.3.2 Recombinant EHV2-egfp viruses generated with homologous recombination

Several protocols for homologous recombination were tested. In the first trials both RK13 and extEqFK cells were infected with EHV2-Bj stock solution, 10^5 $TCID_{50}$/mL (Thorsteinsdóttir et al., 2016), 24 h prior to the transfection. Transfection was either done with linearized plasmids or purified PCR amplicons. This was tested three times, with all four models, and was always unsuccessful.

The approach was tested in reverse, i.e. RK13 and extEqFK cells infected with EHV2-Bj 24 h post transfection. This was tested for models A and B, with both linearized plasmids and purified PCR amplicons. This was successful for model A. After 2 weeks, clusters of green fluorescent cells were observed in the RK13 cells transfected with linearized model A plasmid. The rEHV2-gB-egfp virus was able to infect in addition to RK13 (Figure 27 & Figure 2 in **Paper III**), prmEqFK, extEqFK, prmEqFL and extEqFL cells (data not shown*)*. Other models were not tested further.

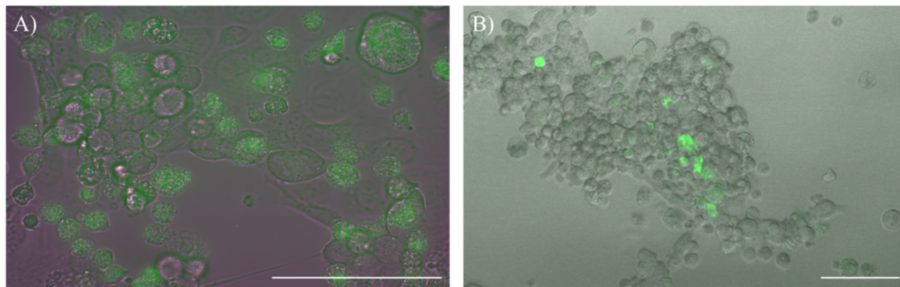

Figure 27. Expression of gB-egfp fusion protein from rEHV2-gB-egpf virus, seen in RK13 cells.
Images acquired with: A) Evos FL Auto 2. B) Olympus FV 1200. Scale bar 100 μM.

4.3.3 Cloning of rEHV2-gB-egfp and sorting of EGFP expressing cells

Cloning of the rEHV2-egfp-gB virus was attempted with limiting dilution and plaque purification. However, the stock was still a mixture with the wild type virus overgrowing. In a further attempt to clone the virus, fluorescent cells were selected with FACS sorting. After two rounds of sorting, brighter fluorescent cells were observed at a higher ratio than seen previously. However, the stock was still a mixture, confirmed with PCR (data not shown).

4.3.4 Sequencing of the rEHV2-gB-egfp virus

The egfp-gB fusion gene of the rEHV2-gB-egfp virus was sequenced. A deletion of aa no. 773–876 in the gB was detected (Figure 3 in **Paper III**). This deletion probably weakened the infectivity capability of the rEHV2-gB-egfp virus.

4.4 Isolation of equid alpaherpesvirus 3 from a horse in Iceland with equine coital exanthema (Paper IV)

EHV-3 causes equine coital exanthema (ECE), a contagious venereal disease. Symptoms resembling ECE have been noted in horses in Iceland. We verified EHV-3 with virological methods for the first time in the native Icelandic horse population.

4.4.1 Collection of the sample, virus culture and viral load

Lesions resembling those of ECE, pustules and ulcers were noted on the vulva and perineum of a mare (Figure 28 & Figure 1 in **Paper IV**). Scrapings from the lesions were collected and inoculated on prmEqFK cells. After four days CPE were observed, characterized by rounded cells and syncytial formation (Figure 29 & Figure 2 in **Paper IV**).

Figure 28. Lesions of equine coital exanthema on the vulva and perineal skin of a mare.

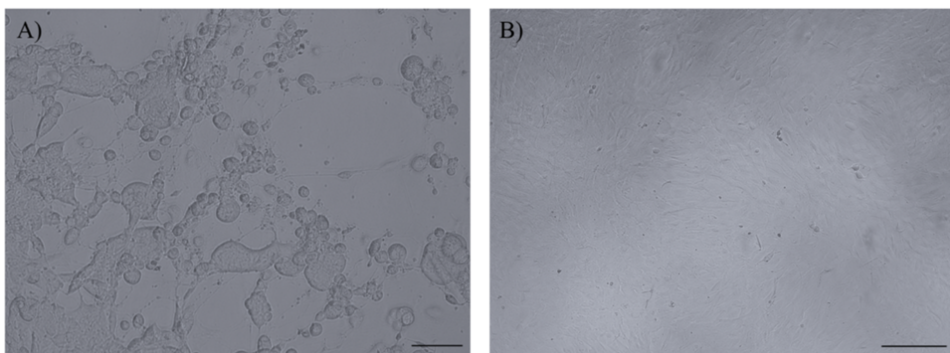

Figure 29. Cytopatic effect of EHV-3 in primary equine fetal kidney cells.
A) EHV-3 infected prmEqFK cells, scale bar 50 μM. B) prmEqFK cell monolayer, scale bar 20 μM.

Growth of the EHV-3 virus was compared in different cells by titration. The titer was similar in the three horse cells tested, prmEqFK, extEqFK and extEqFL, or $10^{5.7}$, $10^{5.11}$ and $10^{5.56}$ $TCID_{50}$/mL, respectively. No CPE was observed in the RK13 and Vero cells.

4.4.2 PCR and sequencing

DNA was isolated from the supernatant of a cell culture with 100% CPE. Primers targeting glycoprotein G (gG) and the DNA polymerase gene were designed. PCR amplification of both gG and DNA polymerase gave single strong bands of the correct size, 616 bp and 321 bp, respectively (Figure 3 in **Paper IV**).

The PCR products were sequenced for further confirmation. The sequences have been submitted to the NCBI database, GenBank accession numbers MN689934 and MN689935 for gG and the DNA polymerase gene, respectively. Glycoprotein G had 99% nucleotide homology and the DNA polymerase gene 100% when compared to the EHV-3 strain AR/2007/C3A (GenBank accession number NC_024771.1) (Table 1 in **Paper IV**). These results confirmed that the mare was infected with EHV-3.

4.5 Construction of recombinant baculovirus vectors (Unpublished data)

The baculovirus expression system is well known for production of recombinant proteins in insect cells. Baculoviruses containing mammalian active promoters have been used to deliver and express genes in mammalian cells, a technology known as BacMam.

The pFastBac plasmids have a polyhederin promoter (P_{PH}) which is designed for protein expression in insect cells. If a surface protein, e.g. a glycoprotein, is inserted under the P_{PH} promoter it is expressed on the viral envelope and can enhance transduction to mammalian cells. For protein expression in mammalian cells an expression cassette with a suitable mammalian promoter is inserted into the plasmid.

Six different recombinant baculoviruses were constructed and tested *in vitro,* three were used in vaccination trials in foals and two were used to produce glycoproteins in insect cells.

4.5.1 Construction of recombinant pFastBac HT B plasmids and rBac-viral vectors

The six plasmids constructed are shown in figure 30. Glycoprotein encoding genes were inserted under the P_{PH}, in four of the plasmids (no. 1, 2, 5 and 6), EHV-2 gB in 1 & 2, gB from EHV-5 in 5 and glycoprotein G from the vesicular stomatitis virus (VSV-G) in 6. The gene coding for the target protein was inserted into plasmid 3. Nothing was inserted under the P_{PH} promoter in plasmid 4. The plasmids were sequenced to confirm the correct phase for protein coding with the 6x-His tag (data not shown). An expression cassette with a CMV promoter and a target gene was inserted in plasmids 1, 4 and 6 (Figure 30). A blank expression cassette, i.e. without the target gene, was inserted in plasmid 2. PCR was done to confirm the right orientation of the expression cassettes (data not shown). ExtEqFL cells were transfected with plasmids 1, 4 and 6 cells and the expression of the target protein confirmed with WB (data not shown). For more detailed description and figure of the construction of the plasmids see chapter 3.6.1 (Figure 14). The Bac-to-Bac expression system was used to generate rBac-viral vectors.

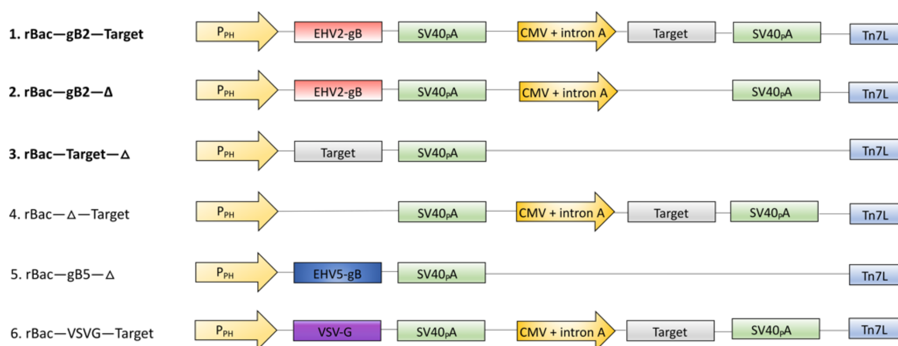

Figure 30. Schematic diagram illustrating the six different pFastBac plasmids constructed.

rBac-viral vectors 1–3 (bold) were used in vaccination trials.

4.5.2 Gene expression under the P_{PH} promoter

Sf-9 cells infected with the rBac-viral vectors were tested in WB for expression of the proteins under the P_{PH} promoter (Figure 31).

The calculated size of EHV-2 glycoprotein B is 98 kDa. In rBac-vector 1 an approximately ~100 kDa band is seen in addition to a ~130 kDa fuzzy band, showing possibly the glycosylated protein (Figure 31). This fuzzy band is detectable in rBac-vector 2. The calculated size of the EHV-5 glycoprotein B is 97 kDa, and in rBac-vector 5 a twofold band is observed ~100 kDa in size and in addition a ~130 kDa fuzzy probably glycosylated band. The calculated size of the target protein is 48 kDa and a clear band of the correct size is seen for rBac-vector 4. The calculated size of VSV-G is 57 kDa but two bands are seen that are both less than 40 kDa. VSV-G is expressed in frame with 6x-His tags both upstream and downstream, suggesting that the protein is expressed in two parts (Figure 31).

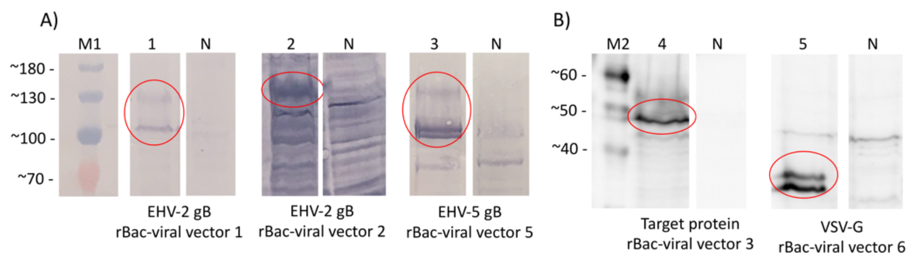

Figure 31. Proteins expressed by the polyhederin promoter.
1: rBac-vector 1, EHV-2 gB. 2: rBac-vector 2, EHV-2 gB. 3: rBac-vector 5, EHV-5 gB. 4: rBac-vector 3, target protein. 5: rBac-vector 6, VSV-G. Primary antibody: 1 and 3: Mouse-anti-his-AP 1:1000, 2: Pool of horse serum 1:1000, 4: Cul n 2 specific monoclonal 1:10.000. 5: Mouse-anti-his-HRP 1:1000. Secondary antibody, 1 and 3: Anti-mouse-AP 1:5000, 2: Mouse-Anti-Horse-AP 1:2000. 4 and 5: Anti-mouse-HRP 1:5000. M1: PageRuler. M2: SuperSignal. N: Negative control.

4.5.3 Expression of the target protein

Both equine cell lines, extEqFL and extEqFK were transfected with the rBac-viral vectors. Expression of the target protein was tested in WB for rBac-viral vectors 1, 4 and 6. The target protein from rBac-vector 1 was expressed in both extEqFL and extEqFK cells (Figure 32).

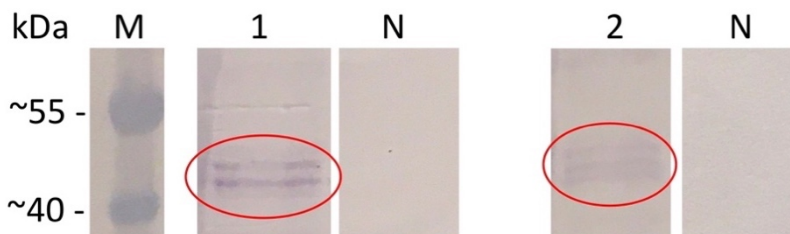

Figure 32. Expression of target protein after transfection in equine cells.
Western blot showing expression of target protein in rBac-vector 1 (rBac-gB2-ExpCas.Target) after transfection in 1: extEqFL and 2: extEqFK cell. Primary antibody: Cul n 2 monoclonal 1:10.000. Secondary antibody: Anti-mouse-AP 1:5000. N: Negative control. M: PageRuler

The target protein could not be detected after transfection with rBac-viral vectors 4 and 6 in the mammalian cells tested; equine cells, COS-7 or 293T. In the rBac-viral vector 4 there was no glycoprotein gene under the P_{PH} promoter, a possible cause of why the virus entry was unsuccessful. In rBac-viral vector 6 the VSV-G glycoprotein was expressed in two parts, which could have prevented the cell entry (data not shown).

4.5.4 Vaccination of foals with the rBac-viral vector

Three rBac-viral vectors were used in the vaccination trial, 1, 2 and 3 (Figure 30). rBac-viral vector 1 (rBac-gB2-Target) was the main vector, with gB from EHV-2 for cell entry and the target protein expressed under the CMV promoter. rBac-vectors 2 (rBac-gB2-Δ) and 3 (rBac-Target-Δ) served as controls.

Twelve foals were vaccinated 5 times, with three-week intervals, six i.l. and six s.c./i.m., 2 foals with each vector per inoculation site (Figure 33).

Figure 33. Setup of the vaccination experiment.
Each foal was vaccinated 5 times, with three-week intervals, the first two times with 20 μg of viral vector and then three times with 200 μg of viral vector. Seven weeks after the final vaccination the foals were boosted i.l. with the target protein produced in *E.coli* in Alum/MPL. The blue dots represent timepoints tested by ELISA.

4.5.4.1 Total IgG response following vaccination

Serum was tested regularly throughout the vaccination trial in WB on the target protein. No difference was observed in specific total IgG response, neither between the rBac-viral vectors nor between week 0 and other timepoints (Figure 34A). When tested on strips containing dissolved baculovirus sediment from rBac-viral vector 3 a difference was observed between week 0 and other timepoints but not between rBac-viral vectors (Figure 34B). Specific total IgG responses were observed after the protein boost, but no difference was observed between rBac-viral vectors (Figure 34C). Overall, the foals responded to the injected material, but no difference was between the rBac-viral vectors.

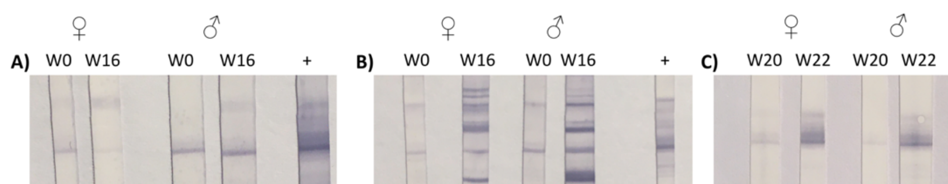

Figure 34. Specific total IgG response tested for rBac-viral vector 1.
Binding of total IgG serum antibodies from foals vaccinated with rBac-viral vector 1, results representative for the three rBac-vectors and both inoculation sites. Reactivity to A) and C) the Cul n 2 target protein and B) baculovirus sediment. Incubated with serum diluted 1:2000. Secondary antibody: Mouse-anti-horse-AP 1:2000. W: Week. +: Positive control. ♀: Female. ♂: Male.

4.5.4.2 Specific IgG subclass and IgE response in sera following vaccination

Serum was tested for the target protein specific IgG1, IgG4/7 antibody response (Figure 35). The results are presented as an ELISA increment where for each foal the OD value before treatment was subtracted from the OD values after vaccination, before and after the boost. Overall, there was no specific response after the five rBac-viral vector vaccinations, but all foals responded strongly to the protein boost, regardless of the rBac-viral vector used for the initial vaccination.

To test if the response following the boost was due to priming, three foals and three adult horses were vaccinated i.l. with the target protein produced in *E.coli* in Alum/MPL (Figure 35). The foals responded strongly as compared to the adult horses.

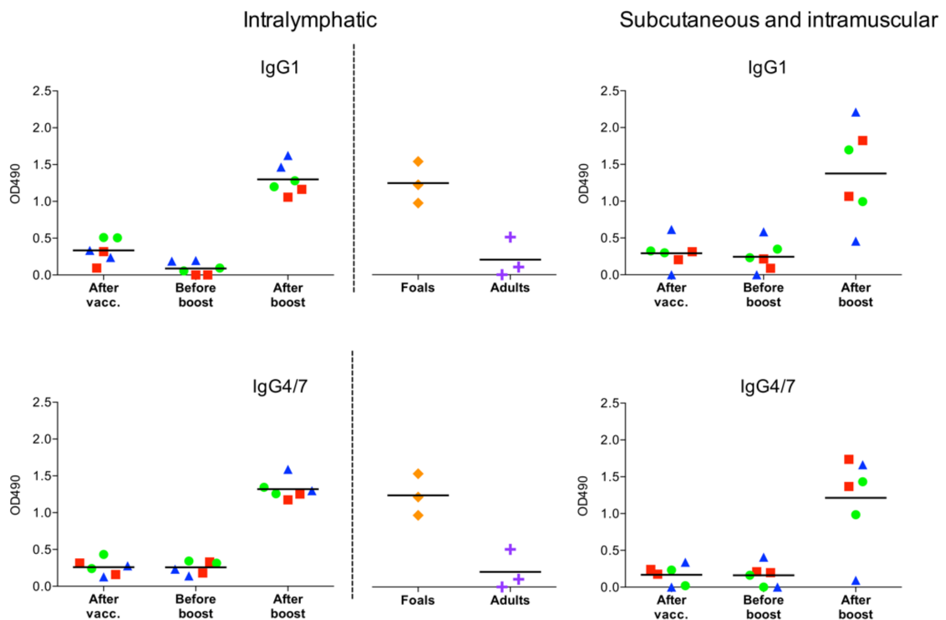

Figure 35. Target protein specific antibody response of foals vaccinated with viral vectors and subequently protein boosted intralymhatical. For comparison foals and adult horses vaccinated intralymphatical only with the protein.
IgG1 and IgG4/7 responses against Cul n 2. For the rBac-viral vaccinated foals tested after five vector vaccinations, before protein boost and 2 weeks after protein boost. rBac-viral vector 1 (rBac-gB2-Target) (red box). rBac-vector 2 (rBac-gB2-Δ) (blue triangle). rBac-vector 3 (rBac-Target-Δ) (green circle). Protein vaccinated foals and adults, tested 2 weeks after the vaccination: Foals (orange diamond) and adults (purple cross). The dotted line separates the two different experiments. Results are shown as ELISA increment (the OD value before treatment has been subtracted from the OD value measured) for each horse, with median for each time point.

The target protein used was hyaluronidase, from *Culicoides nubeculosis,* Cul n 2, an allergen in insect bite hypersensitivity of horses. The ELISA plates were coated with denatured purified baculovirus produced target protein. As the rBac-viral vectors were produced in the same cells, there was a problem with background. Therefore, the ELISA was repeated, and the plates coated with a purified recombinant hyaluronidase from *Culicoides obsoletus,* rCul o 2. When these tests were done we were not able to purify the recombinant hyaluronidase from *Culicoides nubeculosis* (Cul n 2). Therefore, we used purified hyaluronidase from *Culicoides obsoletus* (Cul o 2) that we obtained from collaborators and had been produced in Pichia

(*Pichia*-rCul o 2). No difference was observed between the time points tested (data not shown).

Specific IgG5 and IgE levels were tested, on both Cul n 2 and Pichia-Cul o 2, but all time points were negative (data not shown).

4.5.5 Glycoprotein specific polyclonal antibodies

Glycoprotein B from EHV-2 and EHV-5 was produced with rBac-vectors 2 and 5, respectively in High Five cells. The glycoproteins were purified and used for production of polyclonal antibodies in ascites in mice. The ascites was tested on the native purified gB used for injection (Figure 36). Both Anti-EHV-2 gB and Anti-EHV-5 gB reacted with the proteins used for injection.

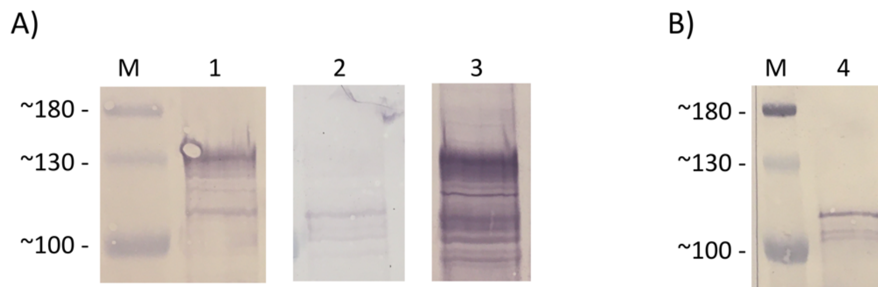

Figure 36. Specificity of polyclonal EHV2-gB and EHV5-gB mouse antibodies. Binding of mouse polyclonal antibodies made against A) EHV2-gB and B) EHV5-gB, produced in baculoviruses. A) Comparison of different primary antibodies, 1: EHV2-gB-polycolonal 1:5000. 2: Mouse-anti-his-AP 1:5000. 3: Pool of horse serum 1:1000. B) 4: EHV5-gB-polyclonal 1:5000. M: PageRuler.

5 Discussion

5.1 The Icelandic horse

The native Icelandic horse is the only horse breed in Iceland. The horses were brought to the country during the settlement in the 9th and 10th centuries and have been purebred ever since. Import of live animals to Iceland has been prohibited by law since 1882. Due to this geographic isolation the horses are immunologically naïve to various agents known to infect horses in other countries. Presently, only thirteen out of more than eighty viruses known to infect horses have been detected in Iceland. Viral infections that have never been found in horses in Iceland are equine influenza, EHV-1 and equine viral arteritis, as well as the bacterial pathogens *Streptococcus equi* subsp. equi (Bjornsdottir et al., 2017) and *Rhodococcus equi* (Robinson et al., 2013). Increased travel between countries, and growing popularity of the Icelandic horse create new threats to this unique infectious status. Therefore, it is important to have an overview and knowledge about the infectious agents present in the population.

5.2 Herpesviruses in the Icelandic horse breed

With semi-nested PCR we verified both EHV-2 and EHV-5 for the first time in Iceland (Torfason et al., 2008). We examined the genetic variation by comparing sequences of four genes between Icelandic and foreign strains and found that the Icelandic strains did not differ phylogenetically from the foreign ones (Thorsteinsdóttir et al., 2010; Thorsteinsdóttir et al., 2013). In light of the nature of herpesviruses in general, i.e. latency and being unstable outside the host, it is likely that these viruses came to the country with the founders.

Clinical symptoms resembling equine coital exanthema, caused by EHV-3 have been noted in horses in Iceland (Svansson, 2000). In this study we isolated EHV-3 for the first time in our native population. The distribution and prevalence have yet to be examined. The virus is thought to be endemic in most horse breeding populations worldwide. However, there is limited information about the prevalence (Barrandeguy & Thiry, 2012). EHV-3 is highly host specific, non-invasive and the coital exanthema relatively benign. As it is generally accepted that viruses with mild pathology and narrow host

range are well adapted to their host, we can speculate that EHV-3 has been in the population since the settlement period, like EHV-2 and EHV-5, although description of coital exanthema cannot be found in older Icelandic writings.

EHV-4 is endemic in Iceland, and a majority of adult horses are antibody positive (Nordengrahn et al., 1998). EHV-4 has also been detected with the qPCR method as described in (Pusterla et al., 2005) from nasal swab DNA. These were from the same samples as used in the virus culture research in **paper II**, from 10 foal/mare pairs over a 20- and 6-month period, respectively. A total of 3.75% of the mare samples and 7.5% of the foal samples were positive for EHV-4 (Unpublished data). The virus has however not been isolated and the course of infection in the native population is not known.

EHV-1 is the most severe equine herpesvirus and has a wide host range. Iceland is to our knowledge the only country that is free of EHV-1. This suggests that the virus evolved later than the other EHVs and/or that EHV-1 infections were not as common in the 9th and 10th centuries as they are today.

5.3 Importance of equine cell lines in virus research

Primary cells are isolated directly from tissues. They have a finite lifespan and limited expansion capacity and are often sensitive and contaminated with a wide range of viruses derived from the same animal species. Their normal morphology and maintenance of important functional can be an advantage over cell lines (Alge et al., 2006; Pan et al., 2009). On the other hand, primary cells are harder to manage considering time and cost as compared to cell lines that are generally highly proliferative, easier to culture and transfect.

Although many viral diseases are prevalent in horses, only a few equine cell lines are available (Andoh et al., 2009; Maeda et al., 2007; Oguma et al., 2013). A rabbit kidney cell line (RK13) and African green monkey kidney cells (Vero) are frequently used, but CPE can be difficult to detect in these cells (Dialli et al., 2008; Dynon et al., 2007). Cell lines originating from equids can be crucial when working with herpesviruses, as many of the EHVs have restricted cell tropism.

EHV-1 has the broadest cell tropism of the EHVs. Although best adapted to equine cells (Kasem et al., 2012), the virus can propagate in cell lines derived from hamster, rabbit, mouse, bovine, pig, monkey and cat (Whalley et al., 2007). EHV-3 and 4 are mainly restricted to equine cells (Whalley et

al., 2007, reviewed in Barrandeguy & Thiry, 2012). The cell tropism of EHV-2 and EHV-5 has not been studied in as much detail but both viruses replicate in RK13 cells in addition to the equine cells (Thorsteinsdóttir et al., 2010; Thorsteinsdóttir et al., 2016).

Virus isolation is considered to be the gold standard for diagnosis of viruses (reviewed in Hematian et al., 2016). It provides clear evidence of the presence of infectious virus in clinical samples, which is then often followed by other techniques like PCR to confirm the identity. For our continuing research we constructed equine cell lines by transfecting primary cells with a retroviral vector containing oncogenes from the human papilloma virus type 16, from both kidney and lung, extEqFK and extEqFL, with extended life span. They can be propagated four times more often than the primary cells, ~40 versus ~10 passages, they grow faster and need less serum. Clear CPE is observed in these cells, for both EHV-2, 5 and 3, which is important when detecting infection as well as in some virological methods, such as plaque purification and limiting dilution. EHV-2 and EHV-5 are usually both present in samples tested on cell culture and EHV-2 tends to overgrow EHV-5, making isolation of EHV-5 strains more difficult (Bell et al., 2006b; Radalj et al., 2018; Thorsteinsdóttir et al., 2010). EHV-5 had a much higher titer in the extEqFK cells compared to the primary cells, $10^{9.5}$ $TCID_{50}$/mL vs. $10^{5.6}$ $TCID_{50}$/mL, indicating that these cells might be a good choice for isolation and growth of EHV-5. In our later experiments with construction of recombinant plasmids and viral vectors, high transfection efficiency was important. The kidney cell line had four times higher transfection efficiency than the primary cells. Whether these cell lines can be made immortal is yet to be seen.

5.4 Course of γEHV infections and the immune response against EHV-2 and EHV-5

In the spring of 2011, 15 pregnant mares from different locations in Iceland, all sired by the same stallion were kept at Keldur. The mares foaled from 17 May to 23 June. As we wanted to take samples from the foals pre-suckling, we took turns watching over them 24/7 until the last foal was born. We followed the mares for 6 months and their foals for 22 months with regular sampling.

EHV-2 and EHV-5 are closely related viruses. They share many common epitopes resulting in strong serological cross reactivity (Agius et al., 1994). Using whole virus antigen in ELISA did not distinguish between these viruses as the response to EHV-2 and EHV-5 were parallel. The EHV-5 antigen

responses were lower and therefore the EHV-2 antigen was used in the ELISAs and the results presented as γEHV specific. With serum dilution of 1:100, γEHV specific total IgG response was not detected in the negative samples but high responses were observed in the positive samples. This was also the optimal dilution in our previous study on the immune response against γEHV (Svansson et al., 2009).

γEHV specific antibodies were not detected in the foals' serum before colostrum intake, supporting previous findings (Murray et al., 1996; Wilks & Studdert, 1974). Two peaks are in the foals' antibody levels, the first being maternal antibodies and the second when the endogenous production has taken over (reviewed in Perkins & Wagner, 2015). The first peak in the γEHV specific total IgG was at day 12, the levels then decreased with the second peak at month 3 and from then on levels rose steadily throughout the study. The IgG4/7 reached the highest level at month 1 and 9, and for IgG1 this was at day 12 and month 5. Overall, the γEHV IgG4/7 antibodies of both foals and mares were comparable to the total IgG throughout the study period. However, the IgG1 levels were low in both foals and mares. These results show the predominance of the IgG4/7 in the mature antibody response, supporting our previous findings (Svansson et al., 2009). This is also the case for EHV-1 and EHV-4 (Goodman et al., 2012; Mizukoshi et al., 2002) and together these results support that IgG4/7 is the long-lasting antibody isotype in EHV infections.

For the first 2–3 months, or when the maternal antibodies were predominant, the γEHV specific total IgG and IgG4/7 varied considerably between the foals. Maternal antibodies are crucial in protecting young animals from diseases. The amount transferred to the offspring can vary significantly between mothers (Coakley et al., 2014). The γEHV specific antibody levels of the mares varied considerably throughout the 6-month study period. Many biological factors can influence the transfer and uptake, e.g. concentration in colostrum, time of intake and the amount transferred through the gut barrier. The foals were grouped in two groups, according to their dam's γEHV specific total IgG levels in serum at birth, group-low and group-high. The immunoglobulin absorption of the neonatal gut is completed after 24 to 36 hours and the endogenous production does not start until later (Baird et al., 1987; Jeffcott, 1974; Korosue et al., 2012, reviewed in Perkins & Wagner, 2015). Therefore, it had been optimal to base the foal grouping on serum from foals on day 3-5. Unfortunately, we did not have samples from all the foals at that age. We did however have samples from all the foals at day 12, but by then most of them were already infected with EHV-2 and EHV-5

and the specific γEHV endogenous production had started. The foals in group-high had higher levels of γEHV specific total IgG and IgG4/7 antibodies for the first 3 months. Group-low then surpassed with higher levels at months 4 to 6, probably because of their higher endogenous antibody production initially. From month 9 no difference was observed between the groups.

We expected that group-low would peak in viral load sooner than group-high as fewer maternal antibodies were protecting the foals from infection. This was seen in the nasal swab (NS) samples, for both EHV-2 and EHV-5 were cell-free viruses are detected. However, this was not found in the blood. EHV-2 group-low peaked with significantly higher viral load in the NS samples at months 2–3, as compared to group-high at month 3. Group-high however peaked in viral load at month 4. For EHV-5 group-low the viral load in the NS was higher at months 2–6, compared to group-high, significant at month 4 and peaked at month 5. The group-high then peaked seven months later, or at month 12. The antibodies from the mother seemed to delay the infection and this was more evident for EHV-5.

A few days before the last sampling, the foals were transported to a new location. At month 22 the viral load of EHV-2 and EHV-5 in the buffy coat (BC) increased significantly, compared to the NS. Stress is one of the triggers that can reactivate herpesviruses from latent infection (Padalino et al., 2018). As γEHVs are thought to be latent in lymphocytes (Mekuria et al., 2017; Torfason et al., 2008; Van Cleemput et al., 2019) it is reasonable that this possible stress effect may have only been noted in the BC. In contrast, a recent study showed an increase in EHV-2 shedding after transport, but the viral load in blood was not measured (Muscat et al., 2018).

The viral load in the foals‘ NS samples was examined with regard to the total IgG response, separately for group-high and group-low. As expected, the EHV-2 viral load increased following reduced antibody levels, seen for both groups. The EHV-5 viral load on the other hand somewhat synchronized to the endogenous antibody level, seen for both groups over the samples period. This could suggest antibody-dependent enhancement of the lytic infection (Guzman & Vazquez, 2010; Willey et al., 2011).

Virus isolation was attempted from from ten foal & mare pairs, both NS and leukocyte enriched plasma (PBL) samples. The EHV-2 virus was isolated from all three NS samples tested from day 5. To our knowledge γEHVs have never been isolated from such young foals. The general understanding is that the majority of foals are EHV-2 positive at 2–3 months of age when the maternal antibodies decline (Bell et al., 2006a, reviewed in Hartley et al.,

2013) and that EHV-5 infection occurs later (Dunowska et al., 2002b; Nordengrahn et al., 2002). We set out to attempt isolation from pre-suckling samples, and then at day 12, month 1 and monthly from then. We expected the first positive virus isolation to be at month 1 or 2. When CPE was observed in the 40% of the NS samples collected on day 12, isolation was attempted from the day 5 samples, of the three last-born foals, with positive virus isolation from all of them. EHV-5 was isolated in a mixture with EHV-2 from NS sample at day 12, also sooner than expected. Considering the high viral load of EHV-2 in NS at month 2, we expected more positive isolations at that time point. However, the samples had been frozen before co-culture, which may have affected the number of virus isolations. Isolation of EHV-5 strains has proved more difficult than EHV-2 strains. As the EHV-5 viral load peaked in the NS at month 12, we hoped for pure EHV-5 cultures at that time, but no pure EHV-5 strains were isolated in the study.

Virus isolation was only successful from the PBL at month 3 in the foals, with all samples positive, and in one sample from a mare. Using enriched plasma is timesaving but yields fewer cells than separating leukocytes. The plasma also contains γEHV neutralizing antibodies. This could have influenced the low isolation efficiency.

No virus isolations were from foals older than one year and only five isolations were from the mares, a lower prevalence than we have previously experienced. Although not measured directly in our γEHV ELISA, this could reflect a higher level and broader spectrum of neutralizing antibodies with age (Craig et al., 2005; Nordengrahn et al., 2001).

Both viruses are more frequently detected in foals, compared to adults (Hue et al., 2014; Marenzoni et al., 2010; Rushton et al., 2013; Stasiak et al., 2018) and our results confirmed this. For the first two months the mares had higher viral load, both EHV-2 and EHV-5, compared to the foals. The viral load of the foals then surpassed and was higher throughout the 6-month sample period of the mares. At month 22 the viral load of both EHV-2 and EHV-5 in the foals NS was still higher than that of the mares at month 6.

Overall in our study the EHV-2 viral load peaked in the nasal swab at months 2 to 4 as a result of decrease in the maternal γEHV IgG levels. In addition, all virus isolations from the foals were positive at month 3 both in PBL and NS. These results confirmed previous findings, that all foals are infected with EHV-2 at 2–3 months of age (Bell et al., 2006a, reviewed in Hartley et al., 2013). We also confirmed that EHV-5 infections occur later in life than EHV-2, as we isolated EHV-5 later than EHV-2 and found that the

EHV-5 viral load peaked 8–10 months later than EHV-2 (Dunowska et al., 2002a; Nordengrahn et al., 2002). This difference between EHV-2 and EHV-5 viral load curves could be due to variation in replication rate and/or maturation of the subpopulation of target cells in the immune system

The exact age of the foals at primary infection is hard to analyze. Both PCR (Stefánsdóttir, 2013) and qPCR methods have detected both viruses in foals soon after birth. EHV-2 has been detected in NS from 2 week old foals (Brault et al., 2010) and in blood samples from 2- to 4-day-old foals (Dunowska et al., 2011). EHV-5 was detected as early as 12–14 h after birth (Bell et al., 2006a). We detected EHV-2 at day 5 and EHV-5 at day 12 with virus isolation. Our viral load results with regard to primary infection are harder to interpret, as amplification of EHV-2 and EHV-5 DNA was observed in many of the day 0 NS and BC samples, but EHV-2 was subsequently detected in a low number in NS at day 2 (35 viral copies/100 ng DNA) and in BC at day 5 (10.000 viral copies/100 ng DNA). EHV-5 was subsequently detected in NS at day 11 (1400 viral copies/100 ng DNA).

5.5 Possible integration

It is generally believed that foals are born free of EHV-2 and EHV-5. All our blood samples taken before colostrum intake were γEHV antibody negative, supporting this. However, both viruses have been detected in the placenta and in aborted fetuses (Galosi et al., 2005; Léon et al., 2007; Marenzoni et al., 2013), but it is possible that the amplified viral DNA was derived from the maternal blood as the placenta is a highly vascular organ. It is also still unclear if EHV-2 and EHV-5 can be transmitted with vaginal secretion or colostrum (Murray et al., 1996; Studdert, 1974).

Integration of γEHV DNA in the horse chromosome has never been reported but an amplification of EHV-2 and EHV-5 DNA was observed in many of the day 0 samples. Amplification was however rarely seen in the negative controls and if seen the Ct value was >40. All of the day 0 samples were tested repeatedly, DNA was isolated from the samples more than once and the qPCR also tested with TaqMan master mix and probes, always with the same or very similar result. Higher viral load was seen in the positive day 0 BC samples than in NS. Overall, amplification was seen in all foals, in at least one day 0 sample, for either virus. For one foal both viruses were amplified from both sample types. To analyze this further, selected qPCR BC amplicons were sequenced, i.e. 76 bp from the EHV-2 gB and 82 bp from the EHV-5 gB. The positive qPCR day 0 sample had a match to both EHV-2 and

EHV-5. No sequencing results were obtained from the negative day 0 samples. Match to EHV-2 was found in the extEqFK and to EHV-5 in extEqFL.

There have been several reports about chromosomally integrated herpesvirus DNA, indicating that under certain conditions herpesviruses can integrate into the host chromosome (reviewed in Morissette & Flamand, 2010). The β-herpesvirus HHV-6 is integrated into the germ lines of approximately 1% of the human population and the Epstein-Barr γ-herpesviruses can be found integrated in the host's chromosome, in B lymphocytes and epithelial cells derived from nasopharyngeal carcinomas at low frequencies (reviewed in Morissette & Flamand, 2010). Chromosomal insertion of DNA segments from EHV-1 and EHV-3 have been reported (Robinson & O'Callaghan, 1983; Sullivan et al., 1986). EHV-2 and EHV-5 are known in horses worldwide and have co-evolved with their host for millions of years (Thorsteinsdóttir et al., 2010, reviewed in Hartley et al., 2013). Our results indicate a possible integration of EHV-2 and EHV-5 DNA into the host chromosome, but this has to be studied further for confirmation.

5.6 Recombinant EHV2-EGFP virus

We have seen that the EHV-2 virus is well adapted to its host, all horses get infected early in life and that the primary infection is mild or asymptomatic. The large double stranded genome of herpesvirues, has large non-coding regions, and many genes that are not essential for growth. The genome can, therefore, potentially accommodate large amounts of foreign DNA. Herpesvirus derived viral vectors have mostly been constructed from alphaherpesviruses, and have been made from all of the three known αEHVs (Akhmedzhanov et al., 2017; Azab et al., 2009; Ibrahim el et al., 2004). Constructing a recombinant EHV-2 virus could give us better understanding of the viral gene function and replication, as well as being a potential viral vector for vaccination.

The enhanced green fluorescent protein (EGPF) was first described in 1996. It has mutations that increase expression in higher eukaryotes and shift its excitation maximum from ultraviolet (395 nm) to blue-green (488 nm) (reviewed in Hogue et al., 2015). EGFP is the most widespread fluorescent protein used today. We constructed four different plasmids using EGFP as our marker (models A to D).

With homologous recombination, we successfully generated an EHV2-EGFP virus (model A), similar to what had been done with EHV-1 (Ibrahim el

et al., 2004). In the EGFP-recombinant virus, named rEHV2-gB-egfp, the EGFP gene was spliced and expressed as a fusion protein of the C-terminal end of gB. Despite numerous different cloning trials, limiting dilution, plaque purification and sorting of green fluorescent cells with FACS we were unable to clone the rEHV2-gB-egfp virus. The FACS has been used with success to enhance HSV-GFP viruses (Kolb & Brandt, 2004) and the same was seen with the rEHV2-gB-egfp. After two rounds of FACS, we saw brighter fluorescence at a higher ratio. Sequencing of the fusion egfp-gB gene showed a 96 aa deletion in the gB. Glycoprotein B is embedded in the virus envelope and plays a role in the herpesvirus entry, spread between cells and is a major target for the immune response (Dunowska et al., 2000; Neubauer et al., 1997). At least three glycoproteins are necessary for herpesvirus entry and fusion, gB and the gH/gL heterodimeric complex (reviewed in Connolly et al., 2011; Eisenberg et al., 2012; Ibáñez et al., 2018). The complex binds to receptors on the host cell and fuses with the cell membrane (Flint et al., 2004). This defect in the gB of the rEHV2-gB-egfp virus was probably the cause of weaker propagation rate compared to the wild type virus. In the previous study on EHV-1, the expression cassette was inserted in a non-coding region, and showed *in vivo* weaker virulence, compared to wildtype virus (Ibrahim el et al., 2004). Perhaps the inserts have these effects, regardless of the place of insertion. Despite the weaker propagation, the rEHV2-gB-egfp virus was able to infect RK13 cells and both primary equine kidney and lung cells and the comparable cells with extended life span. The rEHV2-gB-egfp virus constructed will be useful in future *in vitro* studies.

Despite numerous different homologous recombination trials in equine and RK13 cells, production of recombinant viruses with EGPF-expression cassette was not successful (models B to D). In models B and C, the EGFP-expression cassette was inserted in non-coding regions, as we believed that inserting in these regions would not affect the virus entry or replication. In model B the cassette was inserted in the non-coding region downstream of the DNA polymerase gene and in model C in the intergenic region between gB and the DNA polymerase gene. In model D the EGFP-expression cassette was inserted in the thymidine kinase gene. Research on gammaherpesvirus MHV-68 has shown that a mutant virus with a disrupted TK gene grew normally *in vitro*, but after intranasal inoculation a severe attenuation of replication was observed in lungs (Coleman et al., 2003). These results indicate that the TK is not essential to establish persistent infection but plays a crucial role in the capacity of the virus to replicate efficiently in its natural host. Mutagenesis by homologous recombination in

eukaryotic cells has proved to be very useful in generation of a variety of recombinant viruses. However, this method can be laborious and time consuming and is only possible in cells that are virus susceptible and amenable for introduction of foreign genetic material (reviewed in Adler et al., 2003; Hall et al., 2012). In retrospect it might have been easier to use bacterial artificial chromosomes (BACs) that now is commonly used for construction of mutant viruses. Stable and infectious BACs have been constructed for all the αEHVs (Akhmedzhanov et al., 2017; Azab et al., 2009; Pan et al., 2017).

EHV-1 viral vectors have been tested as potential vaccines where the vectors express target proteins from pathogens (Pan et al., 2017; Said et al., 2013; Said et al., 2017). Likewise, we were interested in constructing an EHV-2 viral vector for this purpose and made four plasmids with a target gene instead of the EGFP (data not show). The target protein was Cul n 2, a hyaluronidase originating form *Culicoides nubeculosus,* an allergen in insect bite hypersensitivity of horses (IBH). However, due to the difficulties with the homologous recombination method this was not continued, but construction of baculovirus vectors tried instead.

5.7 Baculovirus vectors

BacMam viruses, or recombinant baculoviruses carrying mammalian cell active expression cassettes have been used for gene delivery *in vitro* and *in vivo*. The first BacMam viruses were designed by two separate groups in 1995 and 1996 (Boyce & Bucher, 1996; Hofmann et al., 1995) and have since then been tested *in vivo* and showed promise as vaccine candidates (Dai et al., 2018; Lee & Chang, 2017; Wu et al., 2009; Zhou et al., 2017). In addition, baculoviruses are effective inducers, i.e. have strong adjuvant activity (Abe et al., 2003; Abe et al., 2009; Abe & Matsuura, 2010; Gronowski et al., 1999; Hervas-Stubbs et al., 2007). The exact process of BacMam entry into mammalian cells has not been characterized. The transduction can be enhanced by co-expressing surface proteins, like glycoproteins, which are then incorporated in the baculovirus envelope, and take part in the virus entry (reviewed in Mansouri & Berger, 2018). Using BacMam for gene delivery in mammalian cells has many advantages, such as the viruses are easy to generate with large transgene capacity, they can be transduced in many different types of cells, they do not replicate in mammalian cells, little or no CPE is observed in the transduced cells and this method is relatively cost-effective (reviewed in Brun et al., 2008; Mansouri & Berger, 2018).

We constructed six different baculoviruses, three of which were used in vaccination trials in foals. We tried to use glycoprotein B from the EHV2-Bj strain to enhance the transduction efficiency of the rBac-viral vector. The *rBac-gB2-Target* was our main viral vector, having the gB gene expressed under the P_{PH} promoter and the target protein expressed in a CMV-expression cassette. The two other rBac-viral vectors were controls, the *rBac-gB2-Δ* as a control for the transduction in equine cells, *and rBac-Target-Δ* as a control for responses against recombinant baculovirus. Before the vaccination trials these viruses were tested *in vitro* and the protein expression examined with a western blot. All viruses were successfully transduced to insect cells, with the *rBac-gB2-Target* and *rBac-gB2-Δ* viruses expressing the EHV2-gB and the *rBac-Target-Δ* virus expressing the target protein.

Four foals were vaccinated with each rBac-viral vector, two intralymphatic (i.l.) and two subcutaneous and intramuscular (s.c./i.m.) and all foals were boosted i.l. with the target protein produced in *E. coli* in Alum/MPL. No difference was observed in the specific total IgG response between the rBac-viral vectors nor between week 0 and other timepoints. The target protein used was Cul n 2 and indications were of weak specific IgG1, IgG4/7 antibody responses against the denatured purified baculovirus produced Cul n 2. To ascertain whether this was due to background, the response was tested against Cul o 2, protein from *Culicoides obsoletus*, expressed in yeast, Pichia-rCul o 2. The amino acid identity between Cul n 2 and Cul o 2 is 75% (van der Meide et al., 2013). Competitive inhibition ELISA has shown cross-reactivity between Cul n 2 and Cul o 2 (Jonsdottir et al., 2017). No Pichia-rCul o 2 specific IgG1, IgG4/7 antibody responses were detected. However, we saw in western blots that the foals responded to the injected material and strongly to the protein boost regardless of the rBac-viral vector used for the initial vaccination. Apparently, the strong IgG response seen in the foals after the protein boost was not due to priming by the rBac-viral vector vaccination as just one protein vaccination sufficed to induce the same level of antibody response in the non-primed foals.

Recombinant baculoviruses do not diffuse within injected tissue and one study showed that transduced cells were only detected in the proximity of the needle track (Heikura et al., 2012). It has also been shown that uptake of the virus can differ between organs (Borchers et al., 2008; Nishibe et al., 2008). However, many studies on recombinant baculovirus-based vaccines, with intramuscular vaccination have given strong responses (Dai et al., 2018; Lee & Chang, 2017; Wu et al., 2009).

The glycoprotein G of the vesicular stomatitis virus (VSV-G) has been used in many recombinant baculovirus vaccine trials with good results (Facciabene et al., 2004; Wu et al., 2009). The VSV-G protein can bind to receptors on many different cell types, enabling entry. We had high hopes for the VSV-G rBac-viral vector, *rBac-VSVG-Δ*. To our disappointment the VSV-G was always expressed in two parts, possibly because of proteolytic degradation, and the virus unable to enter the mammalian cells tested *in vitro*. We used the glycoprotein B from the EHV-2 virus in the hope of enhancing the transduction. The gB from pseudorabies virus has been used with success in recombinant baculoviruses and intramuscular vaccination resulted in gB-specific antibody titers (Aoki et al., 1999). However, the herpesvirus entry is a complex process involving more glycoproteins and other factors. Therefore, possibly in our study it would have been more successful to use all the glycoproteins, gB, gH and gL.

6 Conclusions and future perspectives

Few equine cell lines are available and in virus research it can be crucial to use cells originating from the same animal as the virus. We constructed fetal equine kidney and lung cells with extended life spans. These cell lines show clear cytopathic effects with equine gammaherpesviruses and can be passaged approximately four times more often than the comparable primary cells. The kidney cell line has a higher transfection rate and supports higher titers of EHV-5 than primary cells. There is a constant threat of new agents infecting our naïve horse population, viruses of both known and unknown etiology. In those situations, these cell lines will be useful tools.

We confirmed the existence of EHV-3 in the native Icelandic horse population. The virus was isolated from a mare with equine coital exanthema symptoms and confirmed by sequencing. The frequency and strain variation in the population is unknown and yet to be examined. In light of the nature of herpesviruses in general it is likely that EHV-3 has been in the population since the settlement period, as we have also speculated for EHV-2 and EHV-5.

EHV-2 and EHV-5 infections were detected with qPCR earlier than expected. EHV-2 two days after birth and EHV-5 at day 11. Virus isolation was also earlier than expected, EHV-2 in 5-day old foals and EHV-5 at day 12. To our knowledge γEHVs have never been isolated from so young foals. EHV-2 peaked in viral load when the foals were 2–3 months of age, but EHV-5 peaked 8–10 months later. The level of maternal antibodies had a notable effect on the viral load and endogenous antibody production. The first samples from the foals taken before colostrum intake were negative for all γEHV antibodies tested. It is still unclear whether EHV-2 and EHV-5 are capable of crossing the placenta or if they can be transmitted with colostrum or vaginal secretion, but it is the general belief that foals are born free of these viruses. We detected amplification of both viruses with our qPCR method from a large part of the pre-suckling foals' samples and also in DNA isolated from the equine cell lines. Integration of γEHVs DNA in the horse chromosomes has never been reported. Our results indicate a possible integration of EHV-2 and EHV-5, but this has to be studied further for confirmation.

The rEHV2-gB-egfp virus expressed the EGFP protein as a fusion protein with the gB. A 96 aa deletion in the gB gene did not have a deleterious effect on the propagation as the virus was infectious *in vitro*. The rEHV2-gB-egfp virus could be a useful tool for *in vitro* studies of EHV-2, for example, to confirm the target cells of the virus.

One of the initial aims was to construct a viral vector for gene vaccination. Using EHV-2 for this purpose was abandoned. Six different baculovirus vectors were constructed, and all tested *in vitro*. One rBac-viral vector with a target gene and two control vectors were tested in vaccination trials in foals. Regardless of injection site the rBac-viral vector vaccinated foals did not develop measurable antibodies against the target, and they were not primed before the protein boost.

We constructed equine cell lines, fluorescent EHV-2, and EHV-2 & EHV-5 specific polyclonal antibodies facilitating further research of equine herpesviruses. The course of EHV-2 and EHV-5 infections was examined in more detail than before, elucidating the connection between antibodies and viral load. The existence of EHV-3 in the Icelandic horse population was confirmed. Overall, the study provides deeper understanding of the EHV status in the Icelandic horse population.

References

Abdelgawad, A., Hermes, R., Damiani, A., Lamglait, B., Czirjak, G. A., East, M., Aschenborn, O., Wenker, C., Kasem, S., Osterrieder, N., & Greenwood, A. D. (2015). Comprehensive Serology Based on a Peptide ELISA to Assess the Prevalence of Closely Related Equine Herpesviruses in Zoo and Wild Animals. *PLoS One, 10*(9), e0138370.

Abe, T., Takahashi, H., Hamazaki, H., Miyano-Kurosaki, N., Matsuura, Y., & Takaku, H. (2003). Baculovirus Induces an Innate Immune Response and Confers Protection from Lethal Influenza Virus Infection in Mice. *J Immunol, 171*(3), 1133-1139.

Abe, T., Kaname, Y., Wen, X., Tani, H., Moriishi, K., Uematsu, S., Takeuchi, O., Ishii, K. J., Kawai, T., Akira, S., & Matsuura, Y. (2009). Baculovirus induces type I interferon production through toll-like receptor-dependent and -independent pathways in a cell-type-specific manner. *J Virol, 83*(15), 7629-7640.

Abe, T., & Matsuura, Y. (2010). Host innate immune responses induced by baculovirus in mammals. *Curr Gene Ther, 10*(3), 226-231.

Ackermann, M. (2006). Pathogenesis of gammaherpesvirus infections. *Vet Microbiol, 113*(3), 211-222.

Aðalsteinsson, S., & Þorkelsson, F. (1991). *Íslenski hesturinn, litaafbrigði*. Reykjavík: Íslandsmyndir.

Aðalsteinsson, S. (2001). *Íslenski hesturinn, litir og erfðir*. Reykjavík: Ormstunga.

Adams, M. J., Lefkowitz, E. J., King, A. M., Harrach, B., Harrison, R. L., Knowles, N. J., Kropinski, A. M., Krupovic, M., Kuhn, J. H., Mushegian, A. R., Nibert, M. L., Sabanadzovic, S., Sanfacon, H., Siddell, S. G., Simmonds, P., Varsani, A., Zerbini, F. M., Orton, R. J., Smith, D. B., et al. (2017). 50 years of the International Committee on Taxonomy of Viruses: progress and prospects. *Arch Virol, 162*(5), 1441-1446.

Adler, B., Sattler, C., & Adler, H. (2017). Herpesviruses and Their Host Cells: A Successful Liaison. *Trends Microbiol, 25*(3), 229-241.

Adler, H., Messerle, M., & Koszinowski, U. H. (2003). Cloning of herpesviral genomes as bacterial artificial chromosomes. *Rev Med Virol, 13*(2), 111-121.

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., 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.

Akhmedzhanov, M., Scrochi, M., Barrandeguy, M., Vissani, A., Osterrieder, N., & Damiani, A. M. (2017). Construction and manipulation of a full-length infectious bacterial artificial chromosome clone of equine herpesvirus type 3 (EHV-3). *Virus Res, 228*, 30-38.

Alge, C. S., Hauck, S. M., Priglinger, S. G., Kampik, A., & Ueffing, M. (2006). Differential protein profiling of primary versus immortalized human RPE cells identifies expression patterns associated with cytoskeletal remodeling and cell survival. *J Proteome Res, 5*(4), 862-878.

Allen, G. P., & J.T., B. (1986). Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus-1 infections. *Prog Vet Microbiol Immunol, 2*, 78-144.

Amon, W., & Farrell, P. J. (2005). Reactivation of Epstein-Barr virus from latency. *Rev Med Virol, 15*(3), 149-156.

Anagha, G., Gulati, B. R., Riyesh, T., & Virmani, N. (2017). Genetic characterization of equine herpesvirus 1 isolates from abortion outbreaks in India. *Arch Virol, 162*(1), 157-163.

Andoh, K., Kai, K., Matsumura, T., & Maeda, K. (2009). Further Development of an Equine Cell Line that can be Propagated over 100 Times. *J Equine Sci, 20*(2), 11-14.

Aoki, H., Sakoda, Y., Jukuroki, K., Takada, A., Kida, H., & Fukusho, A. (1999). Induction of antibodies in mice by a recombinant baculovirus expressing pseudorabies virus glycoprotein B in mammalian cells. *Vet Microbiol, 68*(3-4), 197-207.

Arnórsson, K. (1997). *The Icelandic horse, a breed apart* (S. Ólafsdóttir & G. Wake, Trans.). Reykjavík: Icelandic Review.

Ata, E. B., Zaghawa, A., Ghazy, A. A., Elsify, A., & Shaapan, R. M. (2018). Equine Herpes Virus Type-1 Infection: Etiology, Epidemiology, Pathogenesis, Identification and Recent Diagnosis. *Asian J Epidemiol, 11*(1), 34-45.

Azab, W., Kato, K., Arii, J., Tsujimura, K., Yamane, D., Tohya, Y., Matsumura, T., & Akashi, H. (2009). Cloning of the genome of equine herpesvirus 4 strain TH20p as an infectious bacterial artificial chromosome. *Arch Virol, 154*(5), 833-842.

Azab, W., & Osterrieder, N. (2012). Glycoproteins D of equine herpesvirus type 1 (EHV-1) and EHV-4 determine cellular tropism independently of integrins. *J Virol, 86*(4), 2031-2044.

Azab, W., Dayaram, A., Greenwood, A. D., & Osterrieder, N. (2018). How Host Specific Are Herpesviruses? Lessons from Herpesviruses Infecting Wild and Endangered Mammals. *Annu Rev Virol, 5*(1), 53-68.

Bailer, S. M. (2017). Venture from the Interior-Herpesvirus pUL31 Escorts Capsids from Nucleoplasmic Replication Compartments to Sites of Primary Envelopment at the Inner Nuclear Membrane. *Cells, 6*(4).

Baird, A. N., Pugh, D. G., Rupp, G. P., Shull, J. W., & Field, R. W. (1987). Detection of immunoglobulin G in the neonate. *J Equine Vet Sci, 7*(3), 124-129.

Baltimore, D. (1971). Expression of animal virus genomes. *Bacteriol Rev, 35*(3), 235-241.

Barrandeguy, M., & Thiry, E. (2012). Equine coital exanthema and its potential economic implications for the equine industry. *Vet J, 191*(1), 35-40.

Barton, E. S., White, D. W., Cathelyn, J. S., Brett-McClellan, K. A., Engle, M., Diamond, M. S., Miller, V. L., & Virgin, H. W. (2007). Herpesvirus latency confers symbiotic protection from bacterial infection. *Nature, 447*(7142), 326-329.

Beazley, M. (1999). Horse breeds. In J. Holderness-Roddam (Ed.), *The life of Horses*. London: Octopus Publishing Group Ltd.

Bell, S. A., Balasuriya, U. B., Gardner, I. A., Barry, P. A., Wilson, W. D., Ferraro, G. L., & MacLachlan, N. J. (2006a). Temporal detection of equine herpesvirus infections of a cohort of mares and their foals. *Vet Microbiol, 116*(4), 249-257.

Bell, S. A., Balasuriya, B. R., Nordhausen, U., MacLachlan, R. W., & James, N. (2006b). Isolation of Equine Herpesvirus-5 from Blood Mononuclear Cells of a Gelding. *J Vet Diagn Invest, 18*(5), 472-475.

Bell, S. A., Pusterla, N., Balasuriya, U. B. R., Mapes, S. M., Nyberg, N. L., & MacLachlan, N. J. (2008). Isolation of a gammaherpesvirus similar to asinine herpesvirus-2 (AHV-2) from a mule and a survey of mules and donkeys for AHV-2 infection by real-time PCR. *Vet Microbiol, 130*(1-2), 176-183.

Bildfell, R., Yason, C., Haines, D., & McGowan, M. (1996). Herpesvirus Encephalitis in a Camel (Camelus bactrianus). *J Zoo Wildlife Med, 27*(3), 409-415.

Bjarnason, G. (1966). *Búfjárfræði (Animal Science)*. Akureyri: Bókaforlag Odds.

Bjornsdottir, S., Harris, S. R., Svansson, V., Gunnarsson, E., Sigurdardottir, O. G., Gammeljord, K., Steward, K. F., Newton, J. R., Robinson, C., Charbonneau, A. R. L., Parkhill, J., Holden, M. T. G., & Waller, A. S. (2017). Genomic Dissection of an Icelandic Epidemic of Respiratory Disease in Horses and Associated Zoonotic Cases. *MBio, 8*(4).

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

Bjørnstad, G., Nilsen, N. Ø., & Røed, K. H. (2003). Genetic relationship between Mongolian and Norwegian horses? *Anim Genet, 34*, 55-58.

Blakeslee, J. R., Jr., Olsen, R. G., McAllister, E. S., Fassbender, J., & Dennis, R. (1975). Evidence of respiratory tract infection induced by equine herpesvirus, type 2, in the horse. *Can J Microbiol, 21*(12), 1940-1946.

Boehmer, P. E., & Lehman, I. R. (1997). Herpes simplex virus DNA replication. *Annu Rev Biochem, 66*, 347-384.

Booy, F. P., Newcomb, W. W., Trus, B. L., Brown, J. C., Baker, T. S., & Steven, A. C. (1991). Liquid-crystalline, phage-like packing of encapsidated DNA in herpes simplex virus. *Cell, 64*(5), 1007-1015.

Borchers, K., Wolfinger, U., Lawrenz, B., Schellenbach, A., & Ludwig, H. (1997). Equine herpesvirus 4 DNA in trigeminal ganglia of naturally infected horses detected by direct in situ PCR. *J Gen Virol, 78*(5), 1109-1114.

Borchers, K., Wiik, H., Frolich, K., Ludwig, H., & East, M. L. (2005). Antibodies against equine herpesviruses and equine arteritis virus in burchell's zebras (equus burchelli) from the serengeti ecosystem. *J Wildl Dis, 41*(1), 80-86.

Borchers, K., Ebert, M., Fetsch, A., Hammond, T., & Sterner-Kock, A. (2006). Prevalence of equine herpesvirus type 2 (EHV-2) DNA in ocular swabs and its cell tropism in equine conjunctiva. *Vet Microbiol, 118*(3-4), 260-266.

Borchers, K., Lieckfeldt, D., Ludwig, A., Fukushi, H., Allen, G., Fyumagwa, R., & Hoare, R. (2008). Detection of Equid Herpesvirus 9 DNA in the Trigeminal Ganglia of a Burchell's Zebra from the Serengeti Ecosystem. *J Vet Med Sci, 70*(12), 1377-1381.

Boyce, F. M., & Bucher, N. L. (1996). Baculovirus-mediated gene transfer into mammalian cells. *Pro Nal Acad Sci, 93*(6), 2348-2352.

Brault, S. A., Blanchard, M. T., Gardner, I. A., Stott, J. L., Pusterla, N., Mapes, S. M., Vernau, W., DeJong, K. D., & MacLachlan, N. J.

(2010). The immune response of foals to natural infection with equid herpesvirus-2 and its association with febrile illness. *Vet Immunol Immunopathol, 137*(1-2), 136-141.

Brault, S. A., Bird, B. H., Balasuriya, U. B. R., & MacLachlan, N. J. (2011). Genetic heterogeneity and variation in viral load during equid herpesvirus-2 infection of foals. *Vet Microbiol, 147*(3-4), 253-261.

Brenner, J., Yadin, H., Perl, S., Stram, Y., & Sharir, B. (2009). Herpesvirus type 2 in biopsy of a cow with possible pseudo-lumpy-skin disease. *Vet Rec, 165*(18), 539-540.

Brown, J. C. (2017). Herpes Simplex Virus Latency: The DNA Repair-Centered Pathway. *Adv Virol, 2017*, 7028194.

Browning, G. F., & Studdert, M. J. (1987). Genomic Heterogeneity of Equine Betaherpesviruses. *J Gen Virol, 68*(5), 1441-1447.

Browning, G. F., Ficorilli N., Studdert M. J. (1988). Asinine herpesvirus genomes: comparison with those of the equine herpesviruses. *Arch Virol, 101*(3-4), 183-290.

Brun, A., Albina, E., Barret, T., Chapman, D. A., Czub, M., Dixon, L. K., Keil, G. M., Klonjkowski, B., Le Potier, M. F., Libeau, G., Ortego, J., Richardson, J., & Takamatsu, H. H. (2008). Antigen delivery systems for veterinary vaccine development. Viral-vector based delivery systems. *Vaccine, 26*(51), 6508-6528.

Bryant, N. A., Wilkie, G. S., Russell, C. A., Compston, L., Grafham, D., Clissold, L., McLay, K., Medcalf, L., Newton, R., Davison, A. J., & Elton, D. M. (2018). Genetic diversity of equine herpesvirus 1 isolated from neurological, abortigenic and respiratory disease outbreaks. *Transbound Emerg Dis, 65*(3), 817-832.

Camarda, G., Spinetti, G., Bernardini, G., Mair, C., Davis-Poynter, N., Capogrossi, M. C., & Napolitano, M. (1999). The Equine Herpesvirus 2 E1 Open Reading Frame Encodes a Functional Chemokine Receptor. *J Virol, 73*(12), 9843-9848.

Cervenak, J., & Kacskovics, I. (2009). The neonatal Fc receptor plays a crucial role in the metabolism of IgG in livestock animals. *Vet Immunol Immunopathol, 128*(1-3), 171-177.

Chen, Y., Bai, P., Mackay, S., Korza, G., Carson, J. H., Kuchta, R. D., & Weller, S. K. (2011). Herpes simplex virus type 1 helicase-primase: DNA binding and consequent protein oligomerization and primase activation. *J Virol, 85*(2), 968-978.

Chowdhury, S. I., Ludwig, H., & Buhk, H. J. (1988). Molecular biological characterization of equine herpesvirus type 1 (EHV-1) isolates from ruminant hosts. *Virus Res, 11*(2), 127-139.

Coakley, C. M., Staszewski, V., Herborn, K. A., & Cunningham, E. J. (2014). Factors affecting the levels of protection transferred from mother to offspring following immune challenge. *Front Zool, 11*, 46.

Cohrs, R. J., & Gilden, D. H. (2001). Human herpesvirus latency. *Brain Pathol, 11*(4), 465-474.

Coleman, H. M., de Lima, B., Morton, V., & Stevenson, P. G. (2003). Murine gammaherpesvirus 68 lacking thymidine kinase shows severe attenuation of lytic cycle replication in vivo but still establishes latency. *J Virol, 77*(4), 2410-2417.

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.

Connolly, S. A., Jackson, J. O., Jardetzky, T. S., & Longnecker, R. (2011). Fusing structure and function: a structural view of the herpesvirus entry machinery. *Nat Rev Microbiol, 9*(5), 369-381.

Crabb, B. S., & Studdert, M. J. (1995). Equine herpesviruses 4 (equine rhinopneumonitis virus) and 1 (equine abortion virus). *Adv Virus Res, 45*, 153-190.

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.

Craig, M. I., Barrandeguy, M. E., & Fernández, F. M. (2005). Equine herpesvirus 2 (EHV-2) infection in thoroughbred horses in Argentina. *BMC Vet Res, 1*, 9-13.

Crandell, R. A., Ichimura, H. C., & S., K. (1988). Isolation and comparative restriction endonuclease DNA fingerprinting of equine herpesvirus-1 from cattle. *Am J Vet Res, 49*(11), 1807-1813.

Dai, S., Zhang, T., Zhang, Y., Wang, H., & Deng, F. (2018). Zika Virus Baculovirus-Expressed Virus-Like Particles Induce Neutralizing Antibodies in Mice. *Virol Sin, 33*(3), 213-226.

Davison, A., Eberle, R., Ehlers, B., Hayward, G., McGeoch, D., Minson, A., Pellett, P., Roizman, B., Studdert, M. J., & Thiry, E. (2009). The order Herpesvirales. *Arch Virol, 154*(1), 171-177.

Davison, A. J. (1984). Structure of the genome termini of varicella-zoster virus. *J Gen Virol, 65 (Pt 11)*, 1969-1977.

Davison, A. J. (2007). Comparative analysis of the genome. In A. A, C.-F. G, & M. E (Eds.), *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis*. Cambridge: Cambridge University Press.

Davison, A. J. (2010). Herpesvirus systematics. *Vet Microbiol, 143*(1), 52-69.

Dembowski, J. A., Dremel, S. E., & DeLuca, N. A. (2017). Replication-Coupled Recruitment of Viral and Cellular Factors to Herpes Simplex Virus Type 1 Replication Forks for the Maintenance and Expression of Viral Genomes. *PLoS Pathog, 13*(1), e1006166.

Deshmane, S. L., & Fraser, N. W. (1989). During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. *J Virol, 63*(2), 943-947.

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

Dictionary.com. (2019). Retrieved from https://www.dictionary.com, LLC

Dimock, W. W., & Edwards, P. R. (1933). Is there a filterable virus of abortion in mares. *Agricultural Experiment Station (AES), 426*, 3-20.

Dohner, K., Wolfstein, A., Prank, U., Echeverri, C., Dujardin, D., Vallee, R., & Sodeik, B. (2002). Function of Dynein and Dynactin in Herpes Simplex Virus Capsid Transport. *Mol. Biol. Cell, 13*(8), 2795-2809.

Dohner, K., Radtke, K., Schmidt, S., & Sodeik, B. (2006). Eclipse phase of herpes simplex virus type 1 infection: Efficient dynein-mediated capsid transport without the small capsid protein VP26. *J Virol, 80*(16), 8211-8224.

Donofrio, G., Cavirani, S., van Santen, V., & Flammini, C. F. (2005). Potential Secondary Pathogenic Role for Bovine Herpesvirus 4. *J Clin Microbiol, 43*(7), 3421-3426.

Donofrio, G., Ravanetti, L., Cavirani, S., Herath, S., Capocefalo, A., & Sheldon, I. M. (2008). Bacterial Infection of endometrial stromal cells influences bovine herpersvirus 4 immediate early gene activation: a new insight into bacterial and viral interaction for uterine disease. *Reproduction (Cambridge, England), 136*(3), 361-366.

Douglas, M. W., Diefenbach, R. J., Homa, F. L., Miranda-Saksena, M., Rixon, F. J., Vittone, V., Byth, K., & Cunningham, A. L. (2004). Herpes Simplex Virus Type 1 Capsid Protein VP26 Interacts with Dynein Light Chains RP3 and Tctex1 and Plays a Role in Retrograde Cellular Transport. *J Biol Chem, 279*(27), 28522-28530.

Dowdall, S. M. J., Matthews, J. B., Mair, T., Murphy, D., Love, S., & Proudman, C. J. (2002). Antigen-specific IgG(T) responses in natural and experimental cyathostominae infection in horses. *Vet Parasitol, 106*(3), 225-242.

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., Meers, J., & Wilks, C. R. (1999). Isolation of equine herpesvirus type 5 in New Zealand. *N Z Vet J, 47*(2), 44-46.

Dunowska, M., Holloway, S. A., Wilks, C. R., & Meers, J. (2000). Genomic variability of equine herpesvirus-5. *Arch Virol, 145*(7), 1359-1371.

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

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

Dunowska, M., Howe, L., Hanlon, D., & Stevenson, M. (2011). Kinetics of Equid herpesvirus type 2 infections in a group of Thoroughbred foals. *Vet Microbiol, 152*(1), 176-180.

Dynon, K., Black, W. D., Ficorilli, N., Hartley, C. A., & Studdert, M. J. (2007). Detection of viruses in nasal swab samples from horses with acute, febrile, respiratory disease using virus isolation, polymerase chain reaction and serology. *Aust Vet J, 85*(1-2), 46-50.

Ebenesersdóttir, S. S., Sandoval-Velasco, M., Gunnarsdóttir, E. D., Jagadeesan, A., Guðmundsdóttir, V. B., Thordardóttir, E. L., Einarsdóttir, M. S., Moore, K. H. S., Sigurðsson, Á., Magnúsdóttir, D. N., Jónsson, H., Snorradóttir, S., Hovig, E., Møller, P., Kockum, I., Olsson, T., Alfredsson, L., Hansen, T. F., Werge, T., et al. (2018). Ancient genomes from Iceland reveal the making of a human population. *Science, 360*(6392), 1028-1032.

Eisenberg, R. J., Atanasiu, D., Cairns, T. M., Gallagher, J. R., Krummenacher, C., & Cohen, G. H. (2012). Herpes virus fusion and entry: a story with many characters. *Viruses, 4*(5), 800-832.

Epstein, M. A., Achong, B. G., & Barr, Y. M. (1964). Virus particles in cultured lymphoblasts from burkitt's lymphoma. *Lancet, 1*(7335), 702-703.

Fabisiak, M., Salamaszynska, A., & Stadejek, T. (2018). Detection of seroconversion to bovine herpesvirus 1 related alphaherpesvirus and bovine viral diarrhea virusin Polish free-living deer. *Pol J Vet Sci, 21*(3), 437-440.

Facciabene, A., Aurisicchio, L., & La Monica, N. (2004). Baculovirus vectors elicit antigen-specific immune responses in mice. *J Virol, 78*(16), 8663-8672.

Fages, A., Hanghøj, K., Khan, N., Gaunitz, C., Seguin-Orlando, A., Leonardi, M., McCrory Constantz, C., Gamba, C., Al-Rasheid, K. A. S., Albizuri, S., Alfarhan, A. H., Allentoft, M., Alquraishi, S., Anthony, D., Baimukhanov, N., Barrett, J. H., Bayarsaikhan, J., Benecke, N., Bernáldez-Sánchez, E., et al. (2019). Tracking Five Millennia of

Horse Management with Extensive Ancient Genome Time Series. *Cell, 177*(6), 1419-1435.e1431.

Farrell, P. J. (2019). Epstein-Barr Virus and Cancer. *Annu Rev Pathol, 14*, 29-53.

Fellipe, J. B. (2016). The Immune System. In J. B. Fellipe (Ed.), *Equine Clinical Immunology*. Ithaca, New York, USA: Wiley Blackwell.

Ficorilli, N., Studdert, M. J., & Crabb, B. S. (1995). The nucleotide sequence of asinine herpesvirus 3 glycoprotein G indicates that the donkey virus is closely related to equine herpesvirus 1. *Arch Virol, 140*(9), 1653-1662.

Field, H. J., Biswas, S., & Mohammad, I. T. (2006). Herpesvirus latency and therapy—From a veterinary perspective. *Antivir Res, 71*(2), 127-133.

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.

Foote, A. K., Ricketts, S. W., & Whitwell, K. E. (2012). A racing start in life? The hurdles of equine feto-placental pathology. *Equine Vet J Suppl*(41), 120-129.

Forterre, P. (2006). The origin of viruses and their possible roles in major evolutionary transitions. *Virus Res, 117*(1), 5-16.

Fortier, G., Richard, E., Hue, E., Fortier, C., Pronost, S., Pottier, D., Lemaitre, L., Lekeux, P., Borchers, K., & Thiry, E. (2013). Long-lasting airway inflammation associated with equid herpesvirus-2 in experimentally challenged horses. *Vet J, 197*(2), 492-495.

Franchini, M., Akens, M., Bracher, V., & Fellenberg, R. (1997). Characterisation of Gamma Herpesviruses in the Horse by PCR. *Virology, 238*, 8-13.

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. *New Zeal Vet J, 34*(9), 152-155.

Fukushi, H., Tomita, T., Taniguchi, A., Ochiai, Y., Kirisawa, R., Matsumura, T., Yanai, T., Masegi, T., Yamaguchi, T., & Hirai, K. (1997). Gazelle Herpesvirus 1: A New Neurotropic Herpesvirus Immunologically Related to Equine Herpesvirus 1. *Virology, 227*(1), 34-44.

Fukushi, H., Taniquchi, A., Yasuda, K., Yanai, T., Masegi, T., Yamaquchi, T., & Hirai, K. (2000). A hamster model of equine herpesvirus 9 induced encephalitis. *J Neurovirol, 6*(4), 314-319.

Fukushi, H., Yamaguchi, T., & Yamada, S. (2012). Complete Genome Sequence of Equine Herpesvirus Type 9. *J Virol, 86*(24), 13822-13822.

Galosi, C. M., de la Paz, V. C., Fernandez, L. C., Martinez, J. P., Craig, M. I., Barrandeguy, M., & Etcheverrrigaray, M. E. (2005). Isolation of equine herpesvirus-2 from the lung of an aborted fetus. *J Vet Diagn Invest, 17*(5), 500-502.

Garner, J. A. (2003). Herpes simplex virion entry into and intracellular transport within mammalian cells. *Adv Drug Deliv Rev, 55*(11), 1497-1513.

Garvey, M., Suárez, N. M., Kerr, K., Hector, R., Moloney-Quinn, L., Arkins, S., Davison, A. J., & Cullinane, A. (2018). Equid herpesvirus 8: Complete genome sequence and association with abortion in mares. *PLoS One, 13*(2), e0192301.

Gennart, I., Coupeau, D., Pejakovic, S., Laurent, S., Rasschaert, D., & Muylkens, B. (2015). Marek's disease: Genetic regulation of gallid herpesvirus 2 infection and latency. *Vet J, 205*(3), 339-348.

Gibson, W., & Roizman, B. (1972). Proteins Specified by Herpes Simplex Virus VIII. Characterization and Composition of Multiple Capsid Forms of Subtypes 1 and 2. *J Virol, 10*(5), 1044-1052.

Goodacre, S., Helgason, A., Nicholson, J., Southam, L., Ferguson, L., Hickey, E., Vega, E., Stefansson, K., Ward, R., & Sykes, B. (2005). Genetic evidence for a family-based Scandinavian settlement of Shetland and Orkney during the Viking periods. *Heredity (Edinb), 95*(2), 129-135.

Goodman, L. B., Wimer, C., Dubovi, E. J., Gold, C., & Wagner, B. (2012). Immunological correlates of vaccination and infection for equine herpesvirus 1. *Clin Vaccine Immunol, 19*(2), 235-241.

Graham, D. A. (2013). Bovine herpes virus-1 (BoHV-1) in cattle-a review with emphasis on reproductive impacts and the emergence of infection in Ireland and the United Kingdom. *Ir Vet J, 66*(1), 15.

Grinde, B. (2013). Herpesviruses: latency and reactivation - viral strategies and host response. *J Oral Microbiol, 5*.

Gronowski, A. M., Hilbert, D. M., Sheehan, K. C., Garotta, G., & Schreiber, R. D. (1999). Baculovirus stimulates antiviral effects in mammalian cells. *J Virol, 73*(12), 9944-9951.

Gryspeerdt, A. C., Vandekerckhove, A. P., Garre, B., Barbe, F., Van de Walle, G. R., & Nauwynck, H. J. (2010). Differences in replication kinetics and cell tropism between neurovirulent and non-neurovirulent EHV1 strains during the acute phase of infection in horses. *Vet Microbiol, 142*(3-4), 242-253.

Guo, H., Shen, S., Wang, L., & Deng, H. (2010). Role of tegument proteins in herpesvirus assembly and egress. *Protein Cell, 1*(11), 987-998.

Guzman, M. G., & Vazquez, S. (2010). The Complexity of Antibody-Dependent Enhancement of Dengue Virus Infection. *Viruses, 2*(12), 2649-2662.

Hall, R. N., Meers, J., Fowler, E., & Mahony, T. (2012). Back to BAC: the use of infectious clone technologies for viral mutagenesis. *Viruses, 4*(2), 211-235.

Harrison, S. C., Skehel, J. J., & Wiley, D. C. (1996). Virus structure. 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. 59-100). New York: Lippincott - Raven Publisher.

Hart, K., Barton, M., Williams, K., Felippe, M. J., & Howerth, E. (2008). Multinodular pulmonary fibrosis, pancytopenia and equine herpesvirus-5 infection in a Thoroughbred gelding. *Equine Vet Educ, 20*, 470-476.

Hartley, C. A., Dynon, K. J., Mekuria, Z. H., El-Hage, C. M., Holloway, S. A., & Gilkerson, J. R. (2013). Equine gammaherpesviruses: Perfect parasites? *Vet Microbiol, 167*(1), 86-92.

Hebia-Fellah, I., Leaute, A., Fieni, F., Zientara, S., Imbert-Marcille, B. M., Besse, B., Fortier, G., Pronost, S., Miszczak, F., Ferry, B., Thorin, C., Pellerin, J. L., & Bruyas, J. F. (2009). Evaluation of the presence of equine viral herpesvirus 1 (EHV-1) and equine viral herpesvirus 4 (EHV-4) DNA in stallion semen using polymerase chain reaction (PCR). *Theriogenology, 71*(9), 1381-1389.

Heikura, T., Nieminen, T., Roschier, M. M., Karvinen, H., Kaikkonen, M. U., Mahonen, A. J., Lesch, H. P., Rissanen, T. T., Laitinen, O. H., Airenne, K. J., & Yla-Herttuala, S. (2012). Baculovirus-mediated vascular endothelial growth factor-D(DeltaNDeltaC) gene transfer induces angiogenesis in rabbit skeletal muscle. *J Gene Med, 14*(1), 35-43.

Heldwein, E., & Krummenacher, C. (2008). Entry of herpesviruses into mammalian cells. *Cell Mol Life Sci, 65*(11), 1653-1668.

Heldwein, E. E., Lou, H., Bender, F. C., Cohen, G. H., Eisenberg, R. J., & Harrison, S. C. (2006). Crystal structure of glycoprotein B from herpes simplex virus 1. *Science, 313*(5784), 217-220.

Helgason, A., Sigurdardottir, S., Gulcher, J. R., Ward, R., & Stefansson, K. (2000a). mtDNA and the origin of the Icelanders: deciphering signals of recent population history. *Am J Hum Genet, 66*(3), 999-1016.

Helgason, A., Sigurdardottir, S., Nicholson, J., Sykes, B., Hill, E. W., Bradley, D. G., Bosnes, V., Gulcher, J. R., Ward, R., & Stefansson, K.

(2000b). Estimating Scandinavian and Gaelic ancestry in the male settlers of Iceland. *Am J Hum Genet, 67*(3), 697-717.

Hematian, A., Sadeghifard, N., Mohebi, R., Taherikalani, M., Nasrolahi, A., Amraei, M., & Ghafourian, S. (2016). Traditional and Modern Cell Culture in Virus Diagnosis. *Osong Public Health Res Perspect, 7*(2), 77-82.

Herder, V., Barsnick, R., Walliser, U., Teifke, J. P., Konig, P., Czerwinski, G., Hansmann, F., Baumgartner, W., & Hewicker-Trautwein, M. (2012). Equid herpesvirus 5-associated dermatitis in a horse--Resembling herpes-associated erythema multiforme. *Vet Microbiol, 155*(2-4), 420-424.

Hervas-Stubbs, S., Rueda, P., Lopez, L., & Leclerc, C. (2007). Insect Baculoviruses Strongly Potentiate Adaptive Immune Responses by Inducing Type I IFN. *J Immunol, 178*, 2361-2369.

Hofmann, C., Sandig, V., Jennings, G., Rudolph, M., Schlag, P., & Strauss, M. (1995). Efficient gene transfer into human hepatocytes by baculovirus vectors. *Proc Natl Acad Sci U S A, 92*(22), 10099-10103.

Hogue, I. B., Bosse, J. B., Engel, E. A., Scherer, J., Hu, J. R., Del Rio, T., & Enquist, L. W. (2015). Fluorescent Protein Approaches in Alpha Herpesvirus Research. *Viruses, 7*(11), 5933-5961.

Holz, C. L., Sledge, D. G., Kiupel, M., Nelli, R. K., Goehring, L. S., & Soboll Hussey, G. (2019). Histopathologic Findings Following Experimental Equine Herpesvirus 1 Infection of Horses. *Front Vet Sci, 6*, 59.

Holznagel, D. L., Hussey, S., Mihalyi, J. E., Wilson, W. D., & Lunn, D. P. (2003). Onset of immunoglobulin production in foals. *Equine Vet J, 35*(6), 620-622.

Honess, R. W. (1984). Herpes simplex and 'the herpes complex': diverse observations and a unifying hypothesis. The eighth Fleming lecture. *J Gen Virol, 65 (Pt 12)*, 2077-2107.

Horse, I. M. o. t. (2019). Icelandic horse. Retrieved from https://web.archive.org/web/20150509051746/http://imh.org/exhibits/online/icelandic-horse#

Hue, E. S., Fortier, G. D., Fortier, C. I., Leon, A. M., Richard, E. A., Legrand, L. J., & Pronost, S. L. (2014). Detection and quantitation of equid gammaherpesviruses (EHV-2, EHV-5) in nasal swabs using an accredited standardised quantitative PCR method. *J Virol Methods, 198*(Supplement C), 18-25.

Hue, E. S., Richard, E. A., Fortier, C. I., Fortier, G. D., Paillot, R., Raue, R., & Pronost, S. L. (2017). Equine PBMC Cytokines Profile after In Vitro α- and γ-EHV Infection: Efficacy of a Parapoxvirus Ovis Based-Immunomodulator Treatment. *Vaccines (Basel), 5*(3).

Hussey, G. S., Hussey, S. B., Wagner, B., Horohov, D. W., Van de Walle, G. R., Osterrieder, N., Goehring, L. S., Rao, S., & Lunn, D. P. (2011). Evaluation of immune responses following infection of ponies with an EHV-1 ORF1/2 deletion mutant. *Vet Res, 42*(1), 23.

Hussey, G. S., Goehring, L. S., Lunn, D. P., Hussey, S. B., Huang, T., Osterrieder, N., Powell, C., Hand, J., Holz, C., & Slater, J. (2013). Experimental infection with equine herpesvirus type 1 (EHV-1) induces chorioretinal lesions. *Vet Res, 44*(1), 118.

Hussey, G. S. (2019). Key Determinants in the Pathogenesis of Equine Herpesvirus 1 and 4 Infections. *Vet Pathol, 56*(5), 656-659.

Ibáñez, F. J., Farías, M. A., Gonzalez-Troncoso, M. P., Corrales, N., Duarte, L. F., Retamal-Díaz, A., & González, P. A. (2018). Experimental Dissection of the Lytic Replication Cycles of Herpes Simplex Viruses in vitro. *Front Microbiol, 9*, 2406.

Ibrahim el, S. M., Pagmajav, O., Yamaguchi, T., Matsumura, T., & Fukushi, H. (2004). Growth and virulence alterations of equine herpesvirus 1 by insertion of a green fluorescent protein gene in the intergenic region between ORFs 62 and 63. *Microbiol Immunol, 48*, 831-842.

ICTV. (2019). International Committee on Taxonomy of Viruses (ICTV). Retrieved from https://talk.ictvonline.org

Iyer, L. M., Balaji, S., Koonin, E. V., & Aravind, L. (2006). Evolutionary genomics of nucleo-cytoplasmic large DNA viruses. *Virus Res, 117*(1), 156-184.

Jansen, T., Forster, P., Levine, M. A., Oelke, H., Hurles, M., Renfrew, C., Weber, J., & Olek, K. (2002). Mitochondrial DNA and the origins of the domestic horse. *Proc Natl Acad Sci U S A, 99*(16), 10905-10910.

Jeffcott, L. B. (1974). Some practical aspects of the transfer of passive immunity to newborn foals. *Equine Vet J, 6*(3), 109-115.

Johnson, D. C., & Baines, J. D. (2011). Herpesviruses remodel host membranes for virus egress. *Nat Rev Microbiol, 9*(5), 382-394.

Jones, C., & Chowdhury, S. (2007). A review of the biology of bovine herpesvirus type 1 (BHV-1), its role as a cofactor in the bovine respiratory disease complex and development of improved vaccines. *Anim Health Res Rev, 8*(2), 187-205.

Jonsdottir, S., Hamza, E., Janda, J., Rhyner, C., Meinke, A., Marti, E., Svansson, V., & Torsteinsdottir, S. (2015). Developing a preventive immunization approach against insect bite hypersensitivity using recombinant allergens: A pilot study. *Vet Immunol Immunopathol, 166*(1), 8-21.

Jonsdottir, S., Svansson, V., Stefansdottir, S. B., Mantyla, E., Marti, E., & Torsteinsdottir, S. (2017). Oral administration of transgenic barley expressing a Culicoides allergen induces specific antibody response. *Equine Vet J, 49*(4), 512-518.

Jonsdottir, S., Stefansdottir, S. B., Kristinarson, S. B., Svansson, V., Bjornsson, J. M., Runarsdottir, A., Wagner, B., Marti, E., & Torsteinsdottir, S. (2018). Barley produced Culicoides allergens are suitable for monitoring the immune response of horses immunized with E. coli expressed allergens. *Vet Immunol Immunopathol, 201*, 32-37.

Kadlec, J., Loureiro, S., Abrescia, N. G., Stuart, D. I., & Jones, I. M. (2008). The postfusion structure of baculovirus gp64 supports a unified view of viral fusion machines. *Nat Struct Mol Biol, 15*(10), 1024-1030.

Kang, M. S., & Kieff, E. (2015). Epstein-Barr virus latent genes. *Exp Mol Med, 47*, e131.

Kasem, S., Safaa, A., Warda, & Khodeir, M. (2012). Susceptibility of different cell cultures to equine herpesvirus-1. 2012.

Kelly, B. J., Fraefel, C., Cunningham, A. L., & Diefenbach, R. J. (2009). Functional roles of the tegument proteins of herpes simplex virus type 1. *Virus Res, 145*(2), 173-186.

Kemp, R., Holliman, A., & Nettleton, P. F. (2008). Atypical bovine herpes mammillitis affecting cows and calves. *Vet Rec, 163*(4), 119-120.

Kempkes, B., & Robertson, E. S. (2015). Epstein-Barr virus latency: current and future perspectives. *Curr Opin Virol, 14*, 138-144.

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. *Virus Res, 80*(1-2), 93-99.

King, A. M. Q., Lefkowitz, E. J., Mushegian, A. R., Adams, M. J., Dutilh, B. E., Gorbalenya, A. E., Harrach, B., Harrison, R. L., Junglen, S., Knowles, N. J., Kropinski, A. M., Krupovic, M., Kuhn, J. H., Nibert, M. L., Rubino, L., Sabanadzovic, S., Sanfaçon, H., Siddell, S. G., Simmonds, P., et al. (2018). Changes to taxonomy and the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses (2018). *Arch Virol, 163*(9), 2601-2631.

Kinyili, J., & Thorsen, J. (1979). Antigenic comparisons between herpesviruses isolated from fallow deer in Alberta and the viruses of infectious bovine rhinotracheitis, equine rhinopneumonitis and DN-599, a non-IBR bovine herpesvirus. *J Wildl Dis, 15*(2), 339-341.

Kleiboeker, S. B., Schommer, S. K., Johnson, P. J., Ehlers, B., Turnquist, S. E., Boucher, M., & Kreeger, J. M. (2002). Association of two newly

recognized herpesviruses with interstitial pneumonia in donkeys (Equus asinus). *J Vet Diagn Invest, 14*(4), 273-280.

Kleiboeker, S. B., Turnquist, S. E., Johnson, P. J., & Kreeger, J. M. (2004). Detection and nucleotide sequencing of a DNA-packaging protein gene of equine gammaherpesviruses. *J Vet Diagn Invest, 16*(1), 67-74.

Kolb, A. W., & Brandt, C. R. (2004). Enhanced isolation of low frequency herpes simplex virus recombinants using green-fluorescent protein and FACS. *J Virol Methods, 115*(1), 73-81.

Koonin, E. V., Dolja, V. V., & Krupovic, M. (2015). Origins and evolution of viruses of eukaryotes: The ultimate modularity. *Virology, 479-480*, 2-25.

Korosue, K., Murase, H., Sato, F., Ishimaru, M., Kotoyori, Y., & Nambo, Y. (2012). Correlation of serum IgG concentration in foals and refractometry index of the dam's pre- and post-parturient colostrums: an assessment for failure of passive transfer in foals. *J Vet Med Sci, 74*(11), 1387-1395.

Kristensen, D. M., Mushegian, A. R., Dolja, V. V., & Koonin, E. V. (2010). New dimensions of the virus world discovered through metagenomics. *Trends Microbiol, 18*(1), 11-19.

LeCuyer, T. E., Rink, A., Bradway, D. S., Evermann, J. F., Nicola, A. V., Baszler, T., & Haldorson, G. J. (2015). Abortion in a Mediterranean miniature donkey (Equus asinus) associated with a gammaherpesvirus similar to Equid herpesvirus 7. *J Vet Diagn Invest, 27*(6), 749-753.

Lee, G. E., Murray, J. W., Wolkoff, A. W., & Wilson, D. W. (2006). Reconstitution of Herpes Simplex Virus Microtubule-Dependent Trafficking In Vitro. *J. Virol., 80*(9), 4264-4275.

Lee, J. Y., & Chang, J. (2017). Recombinant baculovirus-based vaccine expressing M2 protein induces protective CD8(+) T-cell immunity against respiratory syncytial virus infection. *J Microbiol, 55*(11), 900-908.

Lefkowitz, E. J., Dempsey, D. M., Hendrickson, R. C., Orton, R. J., Siddell, S. G., & Smith, D. B. (2018). Virus taxonomy: the database of the International Committee on Taxonomy of Viruses (ICTV). *Nucleic Acids Res, 46*(D1), D708-d717.

Leib, D. A., Coen, D. M., Bogard, C. L., Hicks, K. A., Yager, D. R., Knipe, D. M., Tyler, K. L., & Schaffer, P. A. (1989). Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. *J. Virol., 63*(2), 759-768.

Léon, A., Fortier, G., Fortier, C., Freymuth, F., Tapprest, J., Leclercq, R., & Pronost, S. (2007). Detection of equine herpesviruses in aborted foetuses by consensus PCR. *Vet Microbiol, 126*(1-3), 20-29.

Lewis, M. J., Wagner, B., Irvine, R. M., & Woof, J. M. (2010). IgA in the horse: cloning of equine polymeric Ig receptor and J chain and characterization of recombinant forms of equine IgA. *Mucosal Immunol, 3*(6), 610-621.

Liu, F., & Hong Zhou, Z. (2007). Comparative virion structures of human herpesviruses. In A. A, C.-F. G, & M. E (Eds.), *Human Herpesviruses: Biology, Therapy and Immunoprophylaxis*: Cambridge: Cambridge University Press.

Lopez, A. M., Hines, M. T., Palmer, G. H., Alperin, D. C., & Hines, S. A. (2002). Identification of pulmonary T-lymphocyte and serum antibody isotype responses associated with protection against Rhodococcus equi. *Clin Diagn Lab Immunol, 9*(6), 1270-1276.

Lorange, J. B. (2011). WorldFengur - the studbook of origin for the Icelandic horse. *Acta Vet Scand, 53 Suppl 1*, S5.

Lunn, D. P., Davis-Poynter, N., Flaminio, M. J., Horohov, D. W., Osterrieder, K., Pusterla, N., & Townsend, H. G. (2009). Equine herpesvirus-1 consensus statement. *J Vet Intern Med, 23*(3), 450-461.

Lwoff, A., Horne, R., & Tournier, P. (1962). A system of viruses. *Cold Spring Harb Symp Quant Biol, 27*, 51-55.

Maeda, K., Yasumoto, S., Tsuruda, A., Andoh, K., Kai, K., Otoi, T., & Matsumura, T. (2007). Establishment of a novel equine cell line for isolation and propagation of equine herpesviruses. *J Vet Med Sci, 69*(9), 989-991.

Mahoney, K. D. (2019). Latdict: Latin Dictionary & Grammar Resources. Retrieved from https://latin-dictionary.net/search/latin/virus

Mansouri, M., & Berger, P. (2018). Baculovirus for gene delivery to mammalian cells: Past, present and future. *Plasmid, 98*, 1-7.

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

Marenzoni, M. L., Passamonti, F., Lepri, E., Cercone, M., Capomaccio, S., Cappelli, K., Felicetti, M., Coppola, G., Coletti, M., & Thiry, E. (2011). Quantification of Equid herpesvirus 5 DNA in clinical and necropsy specimens collected from a horse with equine multinodular pulmonary fibrosis. *J Vet Diagn Invest, 23*(4), 802-806.

Marenzoni, M. L., Bietta, A., Lepri, E., Casagrande Proietti, P., Cordioli, P., Canelli, E., Stefanetti, V., Coletti, M., Timoney, P. J., & Passamonti, F. (2013). Role of equine herpesviruses as co-infecting agents in cases of abortion, placental disease and neonatal foal mortality. *Vet Res Commun, 37*(4), 311-317.

Marenzoni, M. L., Stefanetti, V., Danzetta, M. L., & Timoney, P. J. (2015). Gammaherpesvirus infections in equids: a review. *Vet Med (Auckl), 6*, 91-101.

Marti, E., Ehrensperger, F., Burger, D., Ousey, J., Day, M. J., & Wilson, A. D. (2009). Maternal transfer of IgE and subsequent development of IgE responses in the horse (Equus callabus). *Vet Immunol Immunopathol, 127*(3-4), 203-211.

McGeoch, D. J., Rixon, F. J., & Davison, A. J. (2006). Topics in herpesvirus genomics and evolution. *Virus Res, 117*(1), 90-104.

McGuire, T. C., & Crawford, T. B. (1973). Passive immunity in the foal: measurement of immunoglobulin classes and specific antibody. *Am J Vet Res, 34*(10), 1299-1303.

McGuire, T. C., Poppie, M. J., & Banks, K. L. (1975). Hypogammaglobulinemia predisposing to infection in foals. *J Am Vet Med Assoc, 166*(1), 71-75.

McGuire, T. C., Crawford, T. B., Hallowell, A. L., & Macomber, L. E. (1977). Failure of colostral immunoglobulin transfer as an explanation for most infections and deaths of neonatal foals. *J Am Vet Med Assoc, 170*(11), 1302-1304.

Mekuria, Z. H., El-Hage, C., Ficorilli, N. P., Washington, E. A., Gilkerson, J. R., & Hartley, C. A. (2017). Mapping B lymphocytes as major reservoirs of naturally occurring latent equine herpesvirus 5 infection. *J Gen Virol, 98*(3), 461-470.

Metcalf, E. S. (2001). The role of international transport of equine semen on disease transmission. *Anim Reprod Sci, 68*(3-4), 229-237.

Mettenleiter, T. C. (2002). Herpesvirus Assembly and Egress. *J. Virol., 76*(4), 1537-1547.

Mettenleiter, T. C., Muller, F., Granzow, H., & Klupp, B. G. (2013). The way out: what we know and do not know about herpesvirus nuclear egress. *Cell Microbiol, 15*(2), 170-178.

Mizukoshi, F., Maeda, K., Hamano, M., Iwata, H., Matsumura, T., Kondo, T., & Sugiura, T. (2002). IgG antibody subclass response against equine herpesvirus type 4 in horses. *Vet Immunol Immunopathol, 88*(1-2), 97-101.

Mocarski, E. S., & Roizman, B. (1982). Structure and role of the herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA. *Cell, 31*(1), 89-97.

Morissette, G., & Flamand, L. (2010). Herpesviruses and Chromosomal Integration. *J Virol, 84*(23), 12100-12109.

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.

Muscat, K. E., Padalino, B., Hartley, C. A., Ficorilli, N., Celi, P., Knight, P., Raidal, S., Gilkerson, J. R., & Muscatello, G. (2018). Equine Transport and Changes in Equid Herpesvirus' Status. *Front Vet Sci, 5*(224).

Nagy, E., Idamakanti, N., & Carman, S. (1997). Restriction endonuclease analysis of equine herpesvirus-1 isolates recovered in Ontario, 1986-1992, from aborted, stillborn, and neonatal foals. *J Vet Diagn Invest, 9*(2), 143-148.

Narita, M., Uchimura, A., Kimura, K., Tanimura, N., Yanai, T., Masegi, T., Fukushi, H., & Hirai, K. (2000). Brain Lesions and Transmission of Experimental Equine Herpesvirus Type 9 in Pigs. *Vet Pathol, 37*(5), 476-479.

Negussie, H., Li, Y., Tessema, T. S., & Nauwynck, H. J. (2016). Replication characteristics of equine herpesvirus 1 and equine herpesvirus 3: comparative analysis using ex vivo tissue cultures. *Vet Res, 47*, 19.

Nelson, K. M., Schram, B. R., McGregor, M. W., Sheoran, A. S., Olsen, C. W., & Lunn, D. P. (1998). Local and systemic isotype-specific antibody responses to equine influenza virus infection versus conventional vaccination. *Vaccine, 16*(13), 1306-1313.

Neubauer, A., Braun, B., Brandmüller, C., Kaaden, O. R., & Osterrieder, N. (1997). Analysis of the Contributions of the Equine Herpesvirus 1 Glycoprotein gB Homolog to Virus Entry and Direct Cell-to-Cell Spread. *Virology, 227*(2), 281-294.

Newcomb, W. W., & Brown, J. C. (2010). Structure and capsid association of the herpesvirus large tegument protein UL36. *J Virol, 84*(18), 9408-9414.

Nicoll, M. P., Proenca, J. T., & Efstathiou, S. (2012). The molecular basis of herpes simplex virus latency. *FEMS Microbiol Rev, 36*(3), 684-705.

Nishibe, Y., Kaneko, H., Suzuki, H., Abe, T., Matsuura, Y., & Takaku, H. (2008). Baculovirus-mediated interferon alleviates dimethylnitrosamine-induced liver cirrhosis symptoms in a murine model. *Gene Ther, 15*(13), 990-997.

Nogales, E. (2000). Structural insights into microtubule function. *Annu Rev Biochem, 69*(1), 277-302.

Nordengrahn, A., Rusvai, M., Merza, M., Ekström, J., Morein, B., & Belák, S. (1996). Equine herpesvirus type 2 (EHV-2) as a predisposing factor for Rhodococcus equi pneumonia in foals: prevention of the bifactorial disease with EHV-2 immunostimulating complexes. *Vet Microbiol, 51*(1), 55-68.

Nordengrahn, A., Merza, M., Svendlund, G., Ronéus, M., Berndtsson, L. T., Lindholm, A., Drummer, H. E., Studdert, M. J., Abusugra, I., Gunnarsson, E., & 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.

Nordengrahn, A., Klingeborn, B., Lindholm, A., & Merza, M. (2001). The use of a neutralizing monoclonal antibody to detect infections of equine herpesvirus type 2 (EHV-2). *J Vet Diagn Invest, 13*(5), 389-393.

Nordengrahn, A., Merza, M., Ros, C., Lindholm, A., Palfi, V. I., 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.

Nugent, J., Birch-Machin, I., Smith, K. C., Mumford, J. A., Swann, Z., Newton, J. R., Bowden, R. J., Allen, G. P., & Davis-Poynter, N. (2006). Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus nonneuropathogenic disease outbreaks. *J Virol, 80*(8), 4047-4060.

Oguma, K., Ishida, M., Maeda, K., & Sentsui, H. (2013). Efficient propagation of equine viruses in a newly established equine cell line, FHK-13.1 cells. *J Vet Med Sci, 75*(9), 1223-1225.

Ojala, P. M., Sodeik, B., Ebersold, M. W., Kutay, U., & Helenius, A. (2000). Herpes simplex virus type 1 entry into host cells: reconstitution of capsid binding and uncoating at the nuclear pore complex in vitro. *Mol Cell Biol, 20*(13), 4922-4931.

Osterrieder, N., & Van de Walle, G. R. (2010). Pathogenic potential of equine alphaherpesviruses: the importance of the mononuclear cell compartment in disease outcome. *Vet Microbiol, 143*(1), 21-28.

Overkamp, D., Mohammed-Ali, S., Cartledge, C., & Landon, J. (1988). Production of Polyclonal Antibodies in Ascitic Fluid of Mice: Technique and Applications. *J Immunoassay, 9*(1), 51-68.

Owen, D. J., Crump, C. M., & Graham, S. C. (2015). Tegument Assembly and Secondary Envelopment of Alphaherpesviruses. *Viruses, 7*(9), 5084-5114.

Padalino, B., Raidal, S. L., Knight, P., Celi, P., Jeffcott, L., & Muscatello, G. (2018). Behaviour during transportation predicts stress response and lower airway contamination in horses. *PLoS One, 13*(3), e0194272.

Pan, C., Kumar, C., Bohl, S., Klingmueller, U., & Mann, M. (2009). Comparative proteomic phenotyping of cell lines and primary cells to assess preservation of cell type-specific functions. *Mol Cell Proteomics, 8*(3), 443-450.

Pan, Z., Liu, J., Ma, J., Jin, Q., Yao, H., & Osterrieder, N. (2017). The recombinant EHV-1 vector producing CDV hemagglutinin as potential vaccine against canine distemper. *Microb Pathog, 111*, 388-394.

Panté, N., & Kann, M. (2002). Nuclear pore complex is able to transport macromolecules with diameters of about 39 nm. *Mol Biol Cell, 13*(2), 425-434.

Pasdeloup, D., McElwee, M., Beilstein, F., Labetoulle, M., & Rixon, F. J. (2013). Herpesvirus tegument protein pUL37 interacts with dystonin/BPAG1 to promote capsid transport on microtubules during egress. *J Virol, 87*(5), 2857-2867.

Patel, J. R., & Heldens, J. (2005). Equine herpesviruses 1 (EHV-1) and 4 (EHV-4)--epidemiology, disease and immunoprophylaxis: a brief review. *Vet J, 170*(1), 14-23.

Pellett, P., Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., Lacoste, V., Minson, A. C., Nicholas, J., Roizman, B., Studdert, M., & Wang, F. (2011). Herpesvirales. *Virus Taxonomy, 9th Report of the International Committee on Taxonomy of Viruses*, 99-107.

Perkins, G. A., & Wagner, B. (2015). The development of equine immunity: Current knowledge on immunology in the young horse. *Equine Vet J, 47*(3), 267-274.

Petersen, J. L., Mickelson, J. R., Cothran, E. G., Andersson, L. S., Axelsson, J., Bailey, E., Bannasch, D., Binns, M. M., Borges, A. S., Brama, P., da Camara Machado, A., Distl, O., Felicetti, M., Fox-Clipsham, L., Graves, K. T., Guerin, G., Haase, B., Hasegawa, T., Hemmann, K., et al. (2013). Genetic diversity in the modern horse illustrated from genome-wide SNP data. *PLoS One, 8*(1), e54997.

Petrovski, K. R. (2005). An acute outbreak of teat lesions affecting 38% of a dairy herd in Northland. *N Z Vet J, 53*(2), 162-163.

Plowright, W., Ferris, R. D., & Scott, G. R. (1960). Blue wildebeest and the aetiological agent of bovine malignant catarrhal fever. *Nature, 188*, 1167-1169.

Plummer, G., & Waterson, A. P. (1963). Equine herpes viruses. *Virology, 19*, 412-416.

Plummer, G., Goodheart, C. R., & Studdert, M. J. (1973). Equine Herpesviruses: Antigenic Relationships and Deoxyribonucleic Acid Densities. *Infect Immun, 8*(4), 621-627.

Polcicova, K., Goldsmith, K., Rainish, B. L., Wisner, T. W., & Johnson, D. C. (2005). The extracellular domain of herpes simplex virus gE is indispensable for efficient cell-to-cell spread: evidence for gE/gI receptors. *J Virol, 79*(18), 11990-12001.

Poth, T., Niedermaier, G., & Hermanns, W. (2009). Equine Multinodular Pulmonary Fibrosis in association with an EHV-5 infection in 5 horses. *Wien Tierarztl Monat, 96*, 203-208.

Preston, V. G., & McDougall, I. M. (2002). Regions of the herpes simplex virus scaffolding protein that are important for intermolecular self-interaction. *J Virol, 76*(2), 673-687.

Pusterla, N., Leutenegger, C. M., Wilson, W. D., Watson, J. L., Ferraro, G. L., & Madigan, J. E. (2005). Equine herpesvirus-4 kinetics in peripheral blood leukocytes and nasopharyngeal secretions in foals using quantitative real-time TaqMan PCR. *J Vet Diagn Invest, 17*(6), 578-581.

Radalj, A., Nisavic, J., Krnjaic, D., Valcic, M., Jovanovic, T., Veljovic, L., & Milic, N. (2018). Detection and molecular characterization of equine herpesviruses 1, 2, and 5 in horses in the Republic of Serbia. *Acta Vet Brno, 87*(1), 27-34.

Rebhun, W. C., Jenkins, D. H., Riis, R. C., Dill, S. G., Dubovi, E. J., & Torres, A. (1988). An epizootic of blindness and encephalitis associated with a herpesvirus indistinguishable from equine herpesvirus I in a herd of alpacas and llamas. *J Am Vet Med Assoc, 192*(7), 953-956.

Reed, L. J., & Muench, H. (1938). A simple method of estimating fifty per cent endpoints *American Journal of Epidemiology, 27*(3), 493-497.

Reese, T. A. (2016). Coinfections: Another Variable in the Herpesvirus Latency-Reactivation Dynamic. *J Virol, 90*(12), 5534-5537.

Reubel, G. H., Crabb, B. S., & Studdert, M. J. (1995). Diagnosis of equine gammaherpesvirus 2 and 5 infections by polymerase chain reaction. *Arch Virol, 140*(6), 1049-1060.

Robinson, C., Steward, K. F., Potts, N., Barker, C., Hammond, T. A., Pierce, K., Gunnarsson, E., Svansson, V., Slater, J., Newton, J. R., & Waller, A. S. (2013). Combining two serological assays optimises sensitivity and specificity for the identification of Streptococcus equi subsp. equi exposure. *Vet J, 197*(2), 188-191.

Robinson, R. A., & O'Callaghan, D. J. (1983). A specific viral DNA sequence is stably integrated in herpesvirus oncogenically transformed cells. *Cell, 32*(2), 569-578.

Roche, S., Bressanelli, S., Rey, F. A., & Gaudin, Y. (2006). Crystal structure of the low-pH form of the vesicular stomatitis virus glycoprotein G. *Science, 313*(5784), 187-191.

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.

Roizman, B., & Zhou, G. (2015). The 3 facets of regulation of herpes simplex virus gene expression: A critical inquiry. *Virology, 479-480*, 562-567.

Roopenian, D. C., & Akilesh, S. (2007). FcRn: the neonatal Fc receptor comes of age. *Nat Rev Immunol, 7*(9), 715-725.

Rushton, J. O., Kolodziejek, J., Tichy, A., Nell, B., & Nowotny, N. (2013). Detection of equid herpesviruses 2 and 5 in a herd of 266 Lipizzaners in association with ocular findings. *Vet Microbiol, 164*(1), 139-144.

Russell, G. C., Stewart, J. P., & Haig, D. M. (2009). Malignant catarrhal fever: A review. *The Veterinary Journal, 179*(3), 324-335.

Rybicki, E. (1990). The classification of organisms at the edge of life or problems with virus systematics. *S Afr J Sci, 86*(4), 182.

Ryckman, B. J., Jarvis, M. A., Drummond, D. D., Nelson, J. A., & Johnson, D. C. (2006). Human cytomegalovirus entry into epithelial and endothelial cells depends on genes UL128 to UL150 and occurs by endocytosis and low-pH fusion. *J Virol, 80*(2), 710-722.

Sabine, M., Robertson, G. R., & Whalley, J. M. (1981). Differentiation of sub-types of equine herpesvirus I by restriction endonuclease analysis. *Aust Vet J, 57*(3), 148-149.

Said, A., Lange, E., Beer, M., Damiani, A., & Osterrieder, N. (2013). Recombinant equine herpesvirus 1 (EHV-1) vaccine protects pigs against challenge with influenza A(H1N1)pmd09. *Virus Res, 173*(2), 371-376.

Said, A., Elmanzalawy, M., Ma, G., Damiani, A. M., & Osterrieder, N. (2017). An equine herpesvirus type 1 (EHV-1) vector expressing Rift Valley fever virus (RVFV) Gn and Gc induces neutralizing antibodies in sheep. *Virol J, 14*(1), 154.

Saldanha, C. E., Lubinski, J., Martin, C., Nagashunmugam, T., Wang, L., van der Keyl, H., Tal-Singer, R., & Friedman, H. M. (2000). Herpes Simplex Virus Type 1 Glycoprotein E Domains Involved in Virus Spread and Disease. *Journal of Virology, 74*(15), 6712-6719.

Sawtell, N. M. (1997). Comprehensive quantification of herpes simplex virus latency at the single-cell level. *J. Virol., 71*(7), 5423-5431.

Schaffartzik, A., Marti, E., Torsteinsdottir, S., Mellor, P. S., Crameri, R., & Rhyner, C. (2011). Selective cloning, characterization, and production of the *Culicoides nubeculosus* salivary gland allergen repertoire. *Vet Immunol Immunopathol, 139*(2-4), 200-209.

Schaffartzik, A., Hamza, E., Janda, J., Crameri, R., Marti, E., & Rhyner, C. (2012). Equine insect bite hypersensitivity: What do we know? *Vet Immunol Immunopathol, 147*(3), 113-126.

Schrenzel, M. D., Tucker, T. A., Donovan, T. A., Busch, M. D., Wise, A. G., Maes, R. K., & Kiupel, M. (2008). New hosts for equine herpesvirus 9. *Emerg Infect Dis, 14*(10), 1616-1619.

Sharp, E. L., Farrell, H. E., Borchers, K., Holmes, E. C., & Davis-Poynter, N. J. (2007). Sequence analysis of the equid herpesvirus 2 chemokine receptor homologues E1, ORF74 and E6 demonstrates high sequence divergence between field isolates. *J Gen Virol, 88*(9), 2450-2462.

Sheoran, A. S., Lunn, D. P., & Holmes, M. A. (1998). Monoclonal antibodies to subclass-specific antigenic determinants on equine immunoglobulin gamma chains and their characterization. *Vet Immunol Immunopathol, 62*(2), 153-165.

Sheoran, A. S., Timoney, J. F., Holmes, M. A., Karzenski, S. S., & Crisman, M. V. (2000). Immunoglobulin isotypes in sera and nasal mucosal secretions and their neonatal transfer and distribution in horses. *Am J Vet Res, 61*(9), 1099-1105.

Sherman, J., Thorsen, J., Barnum, D. A., Mitchell, W. R., & Ingram, D. G. (1977). Infectious causes of equine respiratory disease on Ontario standardbred racetracks. *J Clin Microbiol, 5*(3), 285-289.

Sigurðardóttir, H. (2011). *Isolation and expression of allergens from midges (Culicoides spp) causing insect bite hypersensitivity in horses.* (M.Sc.). University of Iceland, Reykjavík, Iceland. Retrieved from http://hdl.handle.net/1946/7838

Sijmons, S., Vissani, A., Tordoya, M. S., Muylkens, B., Thiry, E., Maes, P., Matthijnssens, J., Barrandeguy, M., & Van Ranst, M. (2014). Complete genome sequence of equid herpesvirus 3. *Genome Announc, 2*(5).

Sinzger, C. (2008). Entry route of HCMV into endothelial cells. *J Clin Virol, 41*(3), 174-179.

Slater, J. (2014). Chapter 14 - Equine Herpesviruses. In D. C. Sellon & M. T. Long (Eds.), *Equine Infectious Diseases (Second Edition)* (pp. 151-168.e158). St. Louis: W.B. Saunders.

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.

Sodeik, B., Ebersold, M. W., & Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J Cell Biol, 136*(5), 1007-1021.

Stasiak, K., Dunowska, M., & Rola, J. (2018). Prevalence and sequence analysis of equid herpesviruses from the respiratory tract of Polish horses. *Virol J, 15*(1), 106.

Stefánsdóttir, S. B. (2013). *Prevalence of gammaherpesvirus infections in foals and their dams the first year after birth.* (M.Sc.). University of Iceland, Reykjavík, Iceland. Retrieved from http://hdl.handle.net/1946/14270

Stengel, G., & Kuchta, R. D. (2011). Coordinated leading and lagging strand DNA synthesis by using the herpes simplex virus 1 replication complex and minicircle DNA templates. *J Virol, 85*(2), 957-967.

Studdert, M. J. (1974). Comparative aspects of equine herpesviruses. *Cornell Vet, 64*(1), 94-122.

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.

Studdert, M. J. (1996). Equine coital exanthema (equiene herpesvirus 3). In M. J. Studdert (Ed.), *Virus Infection of Vertebrate, Virus Infections of Equines* (Vol. 6, pp. 39-46). Amsterdam: Elsevier.

Sullivan, D. C., Atherton, S. S., Caughman, G. B., Staczek, J., & O'Callaghan, D. J. (1986). Oncogenic transformation of primary hamster embryo cells by equine herpesvirus type 3. *Virus Res, 5*(2), 201-212.

Svansson, V. (2000). Hvað er vitað um herpessýkingar í íslenskum hrossum? *Rit Dýralæknafélag Íslands, 11*(1), 10-13.

Svansson, V., Roelse, M., Ólafsdóttir, G., Thorsteinsdóttir, L., Torfason, E. G., & Torsteinsdóttir, S. (2009). Immune response against equine gammaherpesvirus in Icelandic horses. *Vet Microbiol, 137*(3-4), 363-368.

Tamashiro, J. C., & Spector, D. H. (1986). Terminal structure and heterogeneity in human cytomegalovirus strain AD169. *J Virol, 59*(3), 591-604.

Taniguchi, A., Fukushi, H., Yanai, T., Masegi, T., Yamaguchi, T., & Hirai, K. (2000). Equine herpesvirus 9 induced lethal encephalomyelitis in experimentally infected goats. *Arch Virol, 145*(12), 2619-2627.

Tearle, J. P., Smith, K. C., Boyle, M. S., Binns, M. M., Livesay, G. J., & Mumford, J. A. (1996). Replication of equid herpesvirus-1 (EHV-1) in the testes and epididymides of ponies and venereal shedding of infectious virus. *J Comp Pathol, 115*(4), 385-397.

Telford, E. A., Watson, M. S., McBride, K., & Davison, A. J. (1992). The DNA sequence of equine herpesvirus-1. *Virology, 189*(1), 304-316.

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

Telford, E. A., Watson, M. S., Aird, H. C., Perry, J., & Davion, A. J. (1995). The DNA sequence of equine herpesvirus 2. *J Mol Biol, 249*(3), 520-528.

Telford, E. A., Watson, M. S., Perry, J., Cullinane, A. A., & Davison, A. J. (1998). The DNA sequence of equine herpesvirus-4. *J Gen Virol, 79*(5), 1197-1203.

Thompson, M. P., & Kurzrock, R. (2004). Epstein-Barr Virus and Cancer. *Clin Cancer Res, 10*(3), 803-821.

Thorsen, J., Karstad, L., Barrett, M., & Chalmers, G. (1977). Viruses isolated from captive and free-ranging wild ruminants in Alberta. *J Wildl Dis, 13*(1), 74-79.

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.

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

Thorsteinsdóttir, L., Torsteinsdóttir, S., & Svansson, V. (2016). Establishment and characterization of fetal equine kidney and lung cells with extended lifespan. Susceptibility to equine gammaherpesvirus infection and transfection efficiency. *In Vitro Cell Dev Biol Anim, 52*(8), 872-877.

Torfason, E. G., Thorsteinsdóttir, L., Torsteinsdóttir, 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.

Turtinen, L. W., Allen, G. P., Darlington, R. W., & Bryans, J. T. (1981). Serologic and molecular comparisons of several equine herpesvirus type 1 strains. *Am J Vet Res, 42*(12), 2099-2104.

Van Cleemput, J., Poelaert, K. C. K., Laval, K., & Nauwynck, H. J. (2019). Unravelling the first key steps in equine herpesvirus type 5 (EHV5) pathogenesis using ex vivo and in vitro equine models. *Vet Res, 50*(1), 13.

Van de Walle, G. R., Peters, S. T., VanderVen, B. C., O'Callaghan, D. J., & Osterrieder, N. (2008). Equine herpesvirus 1 entry via endocytosis is facilitated by alphaV integrins and an RSD motif in glycoprotein D. *J Virol, 82*(23), 11859-11868.

van der Meide, N. M., Roders, N., Sloet van Oldruitenborgh-Oosterbaan, M. M., Schaap, P. J., van Oers, M. M., Leibold, W., Savelkoul, H. F., & Tijhaar, E. (2013). Cloning and expression of candidate allergens from Culicoides obsoletus for diagnosis of insect bite hypersensitivity in horses. *Vet Immunol Immunopathol, 153*(3-4), 227-239.

ViralZone. (2019). Retrieved from https://viralzone.expasy.org, SIB Swiss Institute of Bioinfermatics

Wagner, B., Miller, D. C., Lear, T. L., & Antczak, D. F. (2004). The Complete Map of the Ig Heavy Chain Constant Gene Region Reveals Evidence for Seven IgG Isotypes and for IgD in the Horse. *J Immunol, 173*(5), 3230-3242.

Wagner, B. (2006). Immunoglobulins and immunoglobulin genes of the horse. *Dev Comp Immunol, 30*(1-2), 155-164.

Wagner, B. (2009). IgE in horses: Occurrence in health and disease. *Vet Immunol Immunopathol, 132*(1), 21-30.

Walter, J., Balzer, H. J., Seeh, C., Fey, K., Bleul, U., & Osterrieder, N. (2012). Venereal shedding of equid herpesvirus-1 (EHV-1) in naturally infected stallions. *J Vet Intern Med, 26*(6), 1500-1504.

Walter, J., Seeh, C., Fey, K., Bleul, U., & Osterrieder, N. (2016). Prevention of equine herpesvirus myeloencephalopathy - Is heparin a novel option? A case report. *Tierarztl Prax Ausg G Grosstiere Nutztiere, 44*(5), 313-317.

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. *Vet Microbiol, 121*(1-2), 18-28.

Weidner-Glunde, M., Kruminis-Kaszkiel, E., & Savanagouder, M. (2020). Herpesviral Latency-Common Themes. *Pathogens, 9*(2).

Welchman, D. d. B., Verkuijl, A. M., Pepper, W. J., Ibata, G., King, S. A., Davidson, H. M., Mawhinney, I. C., & Banks, M. (2012). Association of gammaherpesviruses and bacteria with clinical metritis in a dairy herd. *Vet Rec, 170*(8), 207-207.

Weller, S. K., & Coen, D. M. (2012). Herpes simplex viruses: mechanisms of DNA replication. *Cold Spring Harb Perspect Biol, 4*(9), a013011.

Whalley, J. M., Ruitenberg, K. M., Sullivan, K., Seshadri, L., Hansen, K., Birch, D., Gilkerson, J. R., & Wellington, J. E. (2007). Host cell tropism of equine herpesviruses: glycoprotein D of EHV-1 enables EHV-4 to infect a non-permissive cell line. *Arch Virol, 152*(4), 717-725.

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

Willey, S., Aasa-Chapman, M. M., O'Farrell, S., Pellegrino, P., Williams, I., Weiss, R. A., & Neil, S. J. (2011). Extensive complement-dependent enhancement of HIV-1 by autologous non-neutralising antibodies at early stages of infection. *Retrovirology, 8*, 16.

Williams, K. J., Maes, R., Piero, F. D., Lim, A., Wise, A., Bolin, D. C., Caswell, J., Jackson, C., Robinsons, N. E., Derksen, F., Scott, M. A., Uhal, B. D., Li, X., Youssef, S. A., & Bolin, S. R. (2007). Equine multinodual pulmonari fibrosis: a newly recognized herpesvirus-associated fibrotic lung disease. *Vet Pathol, 44*(6), 849-862.

Wilson, A. C., & Mohr, I. (2012). A cultured affair: HSV latency and reactivation in neurons. *Trends Microbiol, 20*(12), 604-611.

Wolfstein, A., Nagel, C. H., Radtke, K., Döhner, K., Allan, V. J., & Sodeik, B. (2006). The Inner Tegument Promotes Herpes Simplex Virus Capsid Motility Along Microtubules *in vitro*. *Traffic, 7*(2), 227-237.

Wong, D. M., Belgrave, R. L., Williams, K. J., Del Piero, F., Alcott, C. J., Bolin, S. R., Marr, C. M., Nolen-Walston, R., Myers, R. K., & Wilkins, P. A. (2008). Multinodular pulmonary fibrosis in five horses. *J Am Vet Med Assoc, 232*(6), 898-905.

Wozniakowski, G., & Samorek-Salamonowicz, E. (2015). Animal herpesviruses and their zoonotic potential for cross-species infection. *Ann Agric Environ Med, 22*(2), 191-194.

Wu, Q., Fang, L., Wu, X., Li, B., Luo, R., Yu, Z., Jin, M., Chen, H., & Xiao, S. (2009). A pseudotype baculovirus-mediated vaccine confers protective immunity against lethal challenge with H5N1 avian influenza virus in mice and chickens. *Mol Immunol, 46*(11-12), 2210-2217.

Wu, W., Newcomb, W. W., Cheng, N., Aksyuk, A., Winkler, D. C., & Steven, A. C. (2016). Internal Proteins of the Procapsid and Mature Capsids of Herpes Simplex Virus 1 Mapped by Bubblegram Imaging. *J Virol, 90*(10), 5176-5186.

Wutke, S., Andersson, L., Benecke, N., Sandoval-Castellanos, E., Gonzalez, J., Hallsson, J. H., Lõugas, L., Magnell, O., Morales-Muniz, A.,

Orlando, L., Pálsdóttir, A. H., Reissmann, M., Muñoz-Rodríguez, M. B., Ruttkay, M., Trinks, A., Hofreiter, M., & Ludwig, A. (2016). The origin of ambling horses. *Curr Biol, 26*(15), R697-R699.

Yanai, T., Fujishima, N., Fukushi, H., Hirata, A., Sakai, H., & Masegi, T. (2003). Experimental Infection of Equine Herpesvirus 9 in Dogs. *Vet Pathol, 40*(3), 263-267.

Zhou, X., Zhang, X., Han, Y., Jia, Q., & Gao, H. (2017). Vaccination with Recombinant Baculovirus Expressing Ranavirus Major Capsid Protein Induces Protective Immunity in Chinese Giant Salamander, Andrias davidianus. *Viruses, 9*(8).

Zhou, Z. H., Chen, D. H., Jakana, J., Rixon, F. J., & Chiu, W. (1999). Visualization of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions. *J Virol, 73*(4), 3210-3218.

Original publications

Paper I

Paper II

Paper III

Paper IV

Appendix

Table 4. Nucleotide sequence of primers, target genes and methods used in the EHV2-egf

Gene	Location	Direction	Primers sequence 5'-3'	Method
DNA polymerase	2896-2915	Forward	GCCGTGGACCTGTACTTTGA	Sequencing
non-coding	599-581	Reverse	GGGTGGTGAAAGCGTACAGA	
non-coding	342-361	Forward	CTTAAACTGTCGCCGCTGTG	Sequencing
non-coding	1056-1039	Reverse	ACGCGTGGGCCTCTTTAT	
non-coding	876-894	Forward	GTGTGCGAGATACCGTTCG	Sequencing
non-coding	1587-1569	Reverse	GGGTCCCTGTCATCCATCA	
non-coding	1410-1429	Forward	CCGCTGTACTTTGCAGAACC	Sequencing
non-coding	2120-2101	Reverse	ATCGCAAGTCCTCCTCTCCT	
DNA polymerase	2566-2584	Forward	GGTGCCATGGTATATGAGGGTGGAGGAGGTGT	PCR
non-coding	733-714	Reverse	GGTG**ACTAGT**CC**CCTGCAGG**GCAGTTTTGACAGTCGGTGT	
non-coding	736-755	Forward	GGTG**CCTGCAGG**TT**ACTAGT**ATCTCGCCAGAGCAGCAGAG	PCR
non-coding	2072-2053	Reverse	GGTGGTTTAAACGTGTGTGACCTCTCCACCAG	
pEGFP N3 vector	1-28	Forward	GGTGT**CCTGCAGG**ATAGTTATTAATAGTAATCAATTACGGGG	PCR
pEGFP N3 vector	1645-1627	Reverse	GGTGT**ACTAGT**TACGCCTTAAGATACATTG	
DNA polymerase	2608-2627	Forward	TTCCTCAAGGTGGTGGACAT	PCR
non-coding	1909-1890	Reverse	GGAGTTTACCCGGGAGCTTA	
Glycoprotein B	1516-1535	Forward	ATCTATGGCAGGCCAGTGTC	PCR
DNA polymerase	1567-1548	Reverse	TGGGGATCTTGGCTATCTTG	
Glycoprotein B	1267-1286	Forward	GGTGTACTAGTATGTCCAGTGGTTTGAGGAGGAG	PCR
Intergenic region	157-139	Reverse	GGTGT**CCTAGG**GAGGCGCGAAAACCACAAT	
Intergenic region	158-176	Forward	GGTGT**CCTAGG**T**CGTACG**AGAGTTGTTAACCACATTC	PCR
DNA polymerase	1571-1548	Reverse	GGTAT**GTTTAAAC**CGACTGGGGATCTTGGCTATCTTG	
pEGFP N3 vector	1-28	Forward	GGTGT**CCTAGG**ATAGTTATTAATAGTAATCAATTACGGGG	PCR
pEGFP N3 vector	1645-1627	Reverse	GGTGT**CGTACG**TACGCCTTAAGATACATTG	
ORF20	1551-1570	Forward	CGCGTTTCTCCTGAGACTTG	PCR
ORF22	628-608	Reverse	AGGCATCATAGTCCCTGGTAG	
pEGFP N3 vector	1-28	Forward	GGTGT**CCTGCAGG**ATAGTTATTAATAGTAATCAATTACGGGG	PCR
pEGFP N3 vector	1645-1627	Reverse	GGTGT**CCTGCAGG**TACGCCTTAAGATACATTG	
ORF20	1924-1944	Forward	CTTTGGCAGAGCCTTGTTTC	PCR
ORF22	626-606	Reverse	GGCATCATAGTCCCTGGTAGTG	

Bold: restriction site

Table 5. Primers used in construction of pFastBac plasmids

Gene	Direction	Primers sequence 5'-3'
EHV2-gB	Forward	GGTGT**CCATGG**TTATGGGGGTCGGGGGCGGG
	Reverse	GGTGT**ACTAGT**TTACACACCGGTATCCGCCG
Target gene	Forward	GGTG**GTCGAC**GCGGCC*GCCACCATG* TGGTTGAACGTGG
	Reverse	GGTGT**GCGGCCGC**TGCGTTATGACAAATTTGGGGTAAG
Exp.Cas	Forward	GT**CCTAGG**GCGGCCGGCTATTGGCCATTGC
	Reverse	GGTGT**CCTAGG**GGCGCACACAGATCTCTAG
VSV-G	Forward	GGTGT**GGATCC**CCTATGAAGTGCCTTTTG
	Reverse	CTAC**GCGGCCGC**GCAGTCACTTTCCAAGTCGG
EHV5-gB	Forward	GGTGT**GAATTC**TCATGGTTGCCTGGTTTGGCC
	Reverse	GGTGT**GCGGCCGC**TTACACGCCCGTCTCTGCC

Bold: restriction site. *Italic* : Kozak sequence.