Is Latent Equine Herpesvirus Type 1 (EHV-1) Reactivated by Triggering Activation of the Unfolded Protein Response in Equine Peripheral Blood Leukocytes?

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i

ABSTRACT

Equine Herpesvirus type 1 (EHV-1) is a worldwide threat to the health of horses. It can cause mild respiratory disease, abortions and deaths of newborn foals as well as a potentially fatal neurologic disorder known as Equine Herpesvirus Myeloencephalopathy (EHM). The virus is maintained in populations by stress-induced periodic reactivation of virus in long-term latently infected horses and transmission of the reactivated virus to susceptible individuals. In horses, peripheral blood leukocytes (PBLs) are thought to be an important site for EHV-1 latent genomes. Since the Unfolded Protein Response (UPR) is a cellular response to a variety of stressors that has been linked to reactivation of herpes simplex virus in humans, a virus closely related to EHV-1, I tested the hypothesis that latent EHV-1 relies on the UPR as a pluripotent stress sensor and uses it to reactivate lytic gene expression.

Since little work has been done in defining the UPR in horses, I first successfully developed a quantitative real-time polymerase chain reaction (RT-qPCR) assay to detect and quantitate transcripts for selected UPR genes in equine dermal (E.Derm) cells and PBLs. Activation of the UPR was achieved in both cell types using thapsigargin and a difference in gene expression after activation of the UPR in two equine cell types was found. A nested PCR assay to detect and distinguish latent EHV-1 and EHV-4 was evaluated and the sensitivity of the technique to detect EHV-1 was determined. I discovered that the nested PCR technique was not sensitive enough to detect the estimated one latent viral genome in 50,000 PBLs.

Lytic EHV-1 infection was characterized by single step growth curve in E.Derm cells and consistent detection of temporal EHV-1 gene expression by RT-qPCR was achieved. The relationship between EHV-1 gene expression and UPR gene expression during lytic infection

was investigated. While EHV-1 infection had no effect on UPR gene expression, activation of the UPR appeared to decrease the expression of EHV-1 genes temporarily and reversibly during the first 4 h after infection. Finally, detection of EHV-1 in PBLs from horses presumed to be latently infected by co-cultivation with E. Derm cells permissive to EHV-1 infection was attempted. To detect viral DNA, PBLs were stimulated with thapsigargin or interleukin 2 (IL-2) which was previously reported to induce reactivation of latent EHV-1. I was not able to reproduce previously published experiments of reactivation *in vitro* of latent EHV-1 by stimulation with IL-2, and virus reactivation did not occur after stimulation of PBLs with thapsigargin.

In summary, a RT-qPCR assay to measure the expression of equine UPR genes was developed and activation of the UPR by treatment of E.Derm cells and PBLs with thapsigargin was successfully achieved. A difference in gene expression after activation of the UPR in two equine cell types was found. In contrast to what has been reported for other alphaherpesviruses, there appears to be no, or only little, interaction between the UPR and EHV-1 during viral infection. Detection of latent EHV-1 genomes in PBLs was not achieved by using a nested PCR, as this technique was not sensitive enough to detect the estimated one latent viral genome in 50,000 PBLs. Finally, latent EHV-1 was not detected in presumed latently infected PBLs or reactivated by triggering the UPR in equine PBLs.

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DEDICATION

I dedicate this thesis to my parents, Lili and Pablo. Thanks for teaching me that everything can

be done and for never letting me give up. I love you.

I dedicate it to my grandmother Nelly, because she is my person and the personification of love.

Finally, I dedicate it to Alvaro, my husband, my partner, who conceived the idea of this whole

adventure that took us across the world to show us how much we can accomplish together.

Le dedico esta tesis a mis padres, Lili y Pablo. Gracias por enseñarme que todo se puede y por no

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Se lo dedico a mi abuela Nelly, porque es mi ejemplo de persona y porque es la personificacion

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Finalmente, se la dedico a Alvaro mi esposo, mi compañero, el ideologo de toda esta aventura

que nos llevo al otro lado del mundo para demostrarnos cuanto podemos hacer juntos.

Table of contents

List of figures

xiii

List of Tables

List of Abbreviations

°C	Degree Celsius
2-DG	2-deoxy-D-glucose
Ab4	EHV-1 neuropathogenic strain
ATF	Activating transcription factor
BoHV-1	Bovine herpes virus type 1
BoHV-5	Bovine herpes virus type 5
BiP	Immunoglobulin heavy chain binding protein
BLAST	Basic local alignment search tool
bZIP	Basic leucine zipper domain
CD$^-$	Cluster of differentiation negative
CD$^+$	Cluster of differentiation positive
cDNA	Complementary DNA
CHOP	DNA damage inducible transcript 3protein-like
CaHV-1	Canine herpesvirus 1
cm^2	Centimeter squared
CNS	Central nervous system
CO$_2$	Carbone dioxide
CPE	Cytopathic effect
Cq	Quantification cycle
CREB	cAMP response element binding
CREB3L	CREB3 like protein
CVB3	Coxsackievirus B3
D	Aspartic acid amino acid
d	Dilution factor
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
E	Early
E.Derm	Equine dermal cells
EBV	Epstein-Barr virus (HHV-4)
eCG	Equine chorionic gonadotrophin
EDEM	ER degradation enhancing α mannosidase protein like
EEK	Embryonic equine kidney cells
EHM	Equine herpesvirus myeloencephalopathy
EHV	Equine herpesvirus
EICP	Equine infected cell protein
eIF2α	Eukaryotic translation initiation factor 2α
ElHV-1	Endotheliotropic elephant herpesvirus type 1
ER	Endoplasmic reticulum
Es	Essential for viral replication *in vitro*
E-VP16/ETIF	Equine α-*trans*-inducing factor homologue
FBS	Fetal Bovine Serum
fg	Femtogram
FeHV-1	Feline herpesvirus type 1 (feline rhinotracheitis virus)
G+C	guanine plus cytosine
GADD34	Growth arrest and DNA damage-inducible protein
GaHV-1	Gallid herpesvirus 1 (Infectious laryngo-tracheitisvirus)
GaHV-2	Gallid herpesvirus 2 (Marek's disease herpes virus)
gB	Glycoprotein B
gDNA	Genomic DNA
GRP78	Glucose regulated protein 78KDa
GRP94	Glucose regulated protein 94KDa
HBV	Hepatitis B virus

HBx	Hepatitis B virus X protein
HCF	Host cell factor
HCMV	Human cytomegalovirus (HHV-5)
HCV	Hepatitis C virus
HERP	Homocystein-inducible ER stress inducible ubiquitin like domain member
HHV	Human herpesvirus
hpi	Hours post infection
HSV-1	Herpes simplex virus type 1 (HHV-1)
ICP	Infected cell protein
IE	Immediate early
IIF	Indirect immunofluorescence
IL-2	Interleukin 2
IRE-1α	Inositol–requiring kinase 1 α
IR$_L$	Internal repeated sequence unique long region
IR$_S$	Internal repeated sequence unique short region
IU	International units
JNK	c-Jun-N terminal kinase
kb	Kilobase
KDa	Kilo daltons
Kg	Kilogram
KSHV	Kaposi sarcoma associated herpesvirus (HHV-8)
L	Late
LAT	Latency associated transcripts
MuCMV	Mouse cytomegalovirus (MuHV-1)
mg	Milligram
MHC	Major histocompatibility complex
min	Minutes

ml	Millilitre
MLN	Mandibular lymph node
moi	Multiplicity of infection
mRNA	Messenger ribonucleic acid
N	Asparagine amino acid
NEs	Not essential for viral replication *in vitro*
NF-kB	Nuclear factor kappaB
ng	Nanogram
nm	Nanometer
nM	Nanomolar
NPS	Nasopharyngeal secretions
OASIS	Old astrocyte specifically induced substance(CREB3L1)
Oct-1	Octamer-binding protein 1
ORF	Open reading frames
p58IPK	Protein kinase inhibitor of 58 kDa
PBLs	Peripheral blood leukocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PERK	Pancreatic ER kinase (PKR)-like ER kinase
pfu	Plaque forming unit
pg	Picogram
PHA	Phytohaemagglutinin
PI	Post infection
PRV	Pseudorabies virus (SuHV-1)
PWM	Pokeweed mitogen

qPCR	Quantitative real time PCR
RT-qPCR	Reverse transcriptase qPCR
RIDR	Regulated IRE1 dependant decay
RK-13	Rabbit kidney epithelial cells
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute Medium
SCG	Superior cervical ganglion
SFV	Semliki Forest virus
SMLN	Submandibular lymph nodes
SuHV-1	Suid herpesvirus type 1
TNFα	Tumor necrosis factor α
TR$_L$	Terminal repeated sequence unique long region
TR$_S$	Terminal repeated sequence unique short region
U$_L$	Unique long region
UPR	Unfolded protein response
U$_S$	Unique short region
V	Volume
V592	EHV-1 non-neuropathogenic strain
Vero	African green monkey kidney cells
VP16/α-TIF	α trans-inducing factor
VZV	Varicella-zoster virus (HHV-3)
WNV	West Nile virus
XBP1	X box binding protein 1
XBP1s	XBP1spliced form
XBP1u	XBP1unspliced form
$\gamma_1$34.5	Neurovirulence factor ICP34.5
μM	Micromolar

1 LITERATURE REVIEW, HYPOTHESIS AND OBJECTIVES

1.1 General introduction

Equine herpesvirus type 1 (EHV-1) is present in herds of horses around the world (Matumoto, Ishizaki et al. 1965) and can induce respiratory disease, abortions, mortality of newborn foals and a neurological disease (Charltons, Mitchell et al. 1976) that can be fatal, known as equine herpes virus myeloencephalopathy or EHM (Allen 2004). Dimock and Edwards (1936) described outbreaks of abortion in mares as epizootic or virus abortion. The lesions they described in aborted fetuses were consistent with EHV-1 induced lesions (Dimock and Edwards 1936). The virus was first identified as the cause of abortion in mares and it was thought to be a manifestation of equine influenza. Later, it was determined to be a different agent than the influenza A virus (Doll and Kintner 1954) and several names were assigned to it until equine herpesvirus type 1 was officially proposed by Plummer and Waterson in 1963 (Plummer and Waterson 1963).

There are some characteristics of EHV-1 that allow it to persist and circulate within the equine population. Excretion and entry of the virus through the respiratory tract facilitates transmission between animals. One particular aspect of herpesviral infection is the establishment of latency, a process by which the virus infects a particular type of cells and remains in a non-replicative state, hiding from the immune system of the host for an undetermined period of time. Under appropriate conditions, latent virus can revert to an infective state in a process called reactivation from latency (Roizman and Baines 1991). The ability of the herpesviruses to establish latency and to reactivate from this latent state makes it a challenge to control transmission because, usually, clinical signs are not easily detected during the reactivation process. Latent virus carrier animals reactivating virus can be the source of infection to other

animals, including re-infection of latently infected horses. Diagnostic techniques that are able to detect latently infected animals are needed and research in this area has increased in recent years (Allen 2006). The economic impact of EHV-1 infection in the equine industry is apparent by the restriction on the transport of horses when outbreaks are detected in an area. In addition, the potentially high number of abortions during EHV-1 outbreaks (abortion storms) and the delay in training of young horses affected with respiratory disease pose another threat to the success of the equine industry. The incidence of EHM during outbreaks can range from 10% to more than 30% (Goehring, van Winden et al. 2006). Mortality rates of around 40% raise concerns in the equine industry (Allen 2004; Lunn, Davis-Poynter et al. 2009).

1.2 Herpesviridae family

Viruses within the family *Herpesviridae* were originally classified based on structures identified by electron microscopy. Now that nucleotide sequencing is more accessible, new taxonomic classifications have been proposed to reorganize this diverse group of over 200 viruses that have been identified in mammals, birds and reptiles. The classic identification of herpesviridae has been divided into subfamilies (alpha, beta, and gamma) and each subfamily is subdivided into different genera (Flint, Enquist et al. 2004; Roizman 2007). Table 1.1 shows examples of herpesviruses representing each genus. The following are biological characteristics shared among the family members:

1. **Variety of enzymes:** Even though there is variation between different herpesviruses, all possess a considerable array of enzymes involved in protein processing, nucleic acid metabolism and synthesis.

2. **Nuclear replication**: While the final processing of the mature virion occurs in the cytoplasm, the synthesis of the viral genome and the assembly of the nucleocapsid take place in the nucleus.

3. **Cellular damage:** Damage of the infected cell is inevitable for the egress of the infectious virions from the cell.

4. **Latency:** The viruses have the ability to remain latent in the host, where no detectable infective progeny is produced until reactivation events occur. Also, the genome adopts a circular form in the latently infected cells and only selected latency associated viral genes are expressed.

While members of the family *Herpesviridae* share a considerable number of characteristics, the biological properties that differ among its members include the cell type

where the virus establishes latency, the ability to infect one or more hosts, the rate to infect and destroy the infected cells, and the clinical manifestations of infection (Roizman, Desrosiers et al. 1992; Alder, Shen et al. 2005; Roizman 2007).

1.2.1 Alphaherpesvirinae subfamily

EHV-1 is a member of the *Alphaherpesvirinae* subfamily of the *Herpesviridae* family. EHV-1 is included in this subfamily as a member of the genus *Varicellovirus*. Within this genus there are several viruses that can cause disease to animals and humans: Varicella-zoster virus (HHV-3 or VZV), Bovine herpesvirus 1 and 5 (BoHV-1 and BoHV-5), Canine herpesvirus 1 (CaHV-1), Equine herpesvirus 3, 4, 8 and 9 (EHV-3, 4, 8 and 9), Felid herpesvirus 1 (FeHV-1) and Suid herpesvirus 1 or pseudorabies virus (SuHV-1 or PRV) are some examples. The alphaherpesviruses are characterized by a variable host range, the ability to multiply quickly, to spread rapidly in culture, to produce lysis in infected cells (Roizman and Baines 1991) and to establish latency in long lived differentiated cells, primarily in sensory ganglia (Roizman 2007). In addition to establishing latency in sensory ganglia, BoHV-1, EHV-1 and PRV have been proposed to establish latency in lymphoid tissues, although the evidence for this is inconclusive. EHV-1 has further been described to establish latency in peripheral blood leukocytes (Gleeson and Coggins 1980; Chesters, Allsop et al. 1997) while latent genomes of neither BoHV-1 nor PRV have been detected in PBLs (Balasch, Pujols et al. 1998; Winkler, Doster et al. 2000).

Table 1.1 *Herpesviridae* subfamilies and genus examples with name and abbreviations.

Main characteristics of each subfamily are detailed.

Subfamily	Genus	Common names (abbreviations)	Characteristics of subfamily
Alphaherpesvirinae	*Simplexvirus*	Herpes simplex virus (HSV-1)	Variable host range, rapid spread in culture, short reproductive cycle, destruction of infected cells and establishment of latency primarily (but not exclusively) in sensory ganglia.
	Varicellovirus	Varicella-zoster virus (VZV or HHV-3)	
		Equine herpes virus 1 (EHV-1)	
		Bovine herpes virus 1 (BoHV-1)	
		Pseudorabies virus (PRV or SuHV-1)	
	Iltovirus (avian)	Infectious laryngo-tracheitisvirus (GaHV-1)	
	Mardivirus (avian)	Marek disease herpes virus (GaHV-2)	
Betaherpesvirinae	*Cytomegalovirus*	Human cytomegalovirus (HCMV)	Restricted host range, long reproductive cycle, slow progression in culture
	Muramegalovirus	Murine cytomegalovirus (MuCMV)	
	Roseolovirus	Human herpesvirus 6 (HHV-6)	
	Proboscivirus	Endotheliotropic elephant herpesvirus 1 (ElHV-1)	
Gammaherpesvirus	*Lymphacryptovirus*	Epstein-Barr virus (EBV or HHV-4)	Infection specific to either T or B lymphocytes.
	Rhadinovirus	Kaposi sarcoma associated herpesvirus (KSAH or HHV-8)	

1.3   Equine Herpesvirus type 1

1.3.1   EHV-1 virion properties and genome structure

The EHV-1 genome is a 150,000 bp linear double stranded DNA packed in torus shape forming the core, which is surrounded by an icosahedral capsid. The capsid consists of 162 capsomeres of which 150 are hexons and 12 are pentons and it has an approximate diameter of 100 nm. Core and capsid form the nucleocapsid, which is enclosed by an amorphous and asymmetrical protein-containing layer, known as the tegument. The envelope is the outer lipoprotein layer composed of cellular host membrane and embedded viral glycoproteins (Roizman and Baines 1991; Maclachlan and Dubovi 2010).
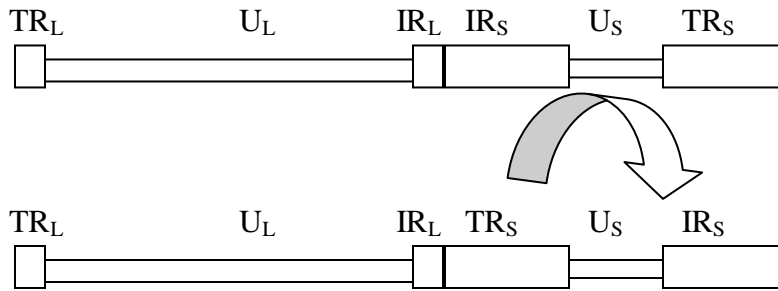
Figure 1.1 EHV-1 genome. The linear disposition of the viral genome consisting of a unique long region (U_L, 112,870 bp) bordered by 32 bp of internal and terminal repeated sequences (IR_L and TR_L, respectively) and a unique short region (U_S, 11,861 bp) bordered by 12,777 bp of internal and terminal repeated sequences (IR_S and TR_S, respectively). The U_S can be present in two directions as a result of inversion in the orientation of the TR_S and the IR_S with respect to the fixed orientation of the U_L, resulting in two different isomers of the viral genome during virus replication.

The viral genome (Figure 1.1) has a base composition of 56.7 % G+C (Telford, Watson et al. 1992) and consists of a long unique region (U_L) of 112,870 bp covalently linked to a short unique region (U_S) of 11,861 bp. Both, the U_L and the U_S regions are bordered by internal and terminal inverted repeated sequences: a small inverted repeated sequence of 32 bp (TR_L; IR_L) bordering the U_L, and a large inverted repeated sequence of 12,777 bp (TR_S; IR_S) bordering the U_S (Yalamanchili and O'Callaghan 1990; Mahy and Van Regenmortel 2008; O'Callaghan and Osterrieder 2008). The U_S can be present in two directions as a result of inversion in the orientation of the TR_S and the IR_S with respect to the fixed orientation of the U_L, resulting in two different isomers of the viral genome during virus replication (Allen 2004). The viral genome comprises 80 open reading frames (ORFs) including four duplicated ORFs present in the TR_S and the IR_S. Within the U_L region, 63 ORFs are organized identically to other alphaherpesviruses (EHV-4, HSV and VZV); however, a number of genes within the U_S region and its IR_S are organized differently. Unique to EHV-1 and EHV-4 is a group of five genes not found in any other alphaherpesviruses. These five distinctive genes of unknown function are presumed to be the genes involved in host specificity (Allen 2004; O'Callaghan and Osterrieder 2008).

1.3.2   EHV-1 proteins

The virus encodes 76 genes that can be differentiated into the immediate early (IE) or α, early (E) or β and late (L) or γ categories based on the order requirements for their expression. EHV-1 possesses only one IE gene and several transcripts have been recognized for the E and the L categories: approximately 45 E transcripts and about 29 L (O'Callaghan and Osterrieder 2008). Efficient expression of the IE gene (ORF64, IR_S) requires the virion-associated transactivator, E-VP16 (ORF12) (Elliott 1994; Purewal, Allsopp et al. 1994), and the IE product trans-activates other viral genes and is also able to regulate itself (Kim, Dai et al. 2012). Four E

gene products act as regulatory proteins: EICP22 (ORF67) is a trans-activator synergistic with the IE protein (Kim, Holden et al. 1997); EICP27 (ORF5) stimulates transcription of E and L genes (Albrecht, Kim et al. 2004); EICP0 has an antagonist effect on the IE protein while it is a potent transactivator of the other two classes of temporal genes; and IR2 (ORF64, TR_S) negatively regulates gene expression. Viral genes encoding proteins involved in virus replication, such as the DNA polymerase gene (ORF30), the thymidine kinase gene (ORF38) and the ribonucleotide reductase gene (ORFs 20, 21) are also found within the early category (Telford, Watson et al. 1992; Allen 2004; O'Callaghan and Osterrieder 2008; Dubovi and Maclachlan 2011). The late genes encode structural proteins. Six genes code for proteins that form part of the capsid (ORFs 22, 25, 35, 35.5, 42 and 43), twelve genes code for proteins associated with the tegument (ORFs 11, 12, 13, 14, 15, 23, 24, 40, 46, 49, 51 and 76) and twelve genes code for glycoproteins present in the envelope (Table 1.2). These glycoproteins can be classified further as essential for virus replication *in vitro* or not essential for virus replication *in vitro* (Table 1.2). The main functions of these glycoproteins are related to viral binding, penetration, assembly, egress and spreading from cell to cell. The late gene 12 (ORF 12) codes for the tegument protein E-VP16/ETIF, a homologue to the HSV-1 VP16/α-TIF (α trans-inducing factor). Although the main regulatory function of EHV-1 E-VP16 is the transactivation of the IE promoter, it is not essential for viral gene expression and viral DNA replication (Von Einem, Schumacher et al. 2006). E-VP16 also plays a key role in secondary viral envelopment and it contributes to virion structure by its presence on the viral tegument (Lewis, Thompson et al. 1993; Von Einem, Schumacher et al. 2006; Sellon and Long 2007; O'Callaghan and Osterrieder 2008).

Table 1.2 EHV-1 glycoproteins: name, ORF, function and classification

Name	ORF	Function	Classification[1]
gK	6	Viral egress and spread from cell to cell	Es
gN	10	Virus assembly; necessary for maturation of gM	NEs
gC	16	Viral egress and viral attachment to cells	NEs
gB	33	Viral binding, penetration and spread from cell to cell	Es
gH	39	Crucial for plaque formation *in vitro*/undetermined *in vivo*[2]	Es
gM	52	Viral binding, penetration and spread from cell to cell	NEs
gL	62	Undetermined	Es
gG	70	Immunomodulatory [3]	NEs
gp2	71	Viral binding, penetration and spread from cell to cell	NEs
gD	72	Viral binding, penetration and spread from cell to cell	Es
gI	73	Viral spread from cell to cell	NEs
gE	74	Viral spread from cell to cell	NEs

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[1] Es: essential for viral replication *in vitro*; NEs: not essential for viral replication *in vitro*
[2] Azab, Zajic et al. 2012
[3] Thormann, Van de Walle et al. 2012

1.3.3 EHV-1 gene expression during lytic infection

As with most herpesviruses, EHV-1 gene expression during lytic infection is arranged in a rigorously regulated temporal mode. Upon entry of the viral nucleocapsid into the infected cell, tegument proteins are released in the cytoplasm and the viral DNA, accompanied by E-VP16, reaches the cellular nucleus. (Figure 1.2) The IE gene expression is strongly activated by the E-VP16 protein and the IE mRNA is transcribed by the cellular RNA polymerase II; the E genes require IE protein activation to be transcribed and translated into E proteins. The IE protein and the E proteins suppress the translation of more IE mRNA while inducing replication of viral DNA and transcription of L genes. The translated L proteins downregulate the expression of the IE and the E genes (Gray, Baumann et al. 1987; Lewis, Thompson et al. 1993; O'Callaghan and Osterrieder 2008).
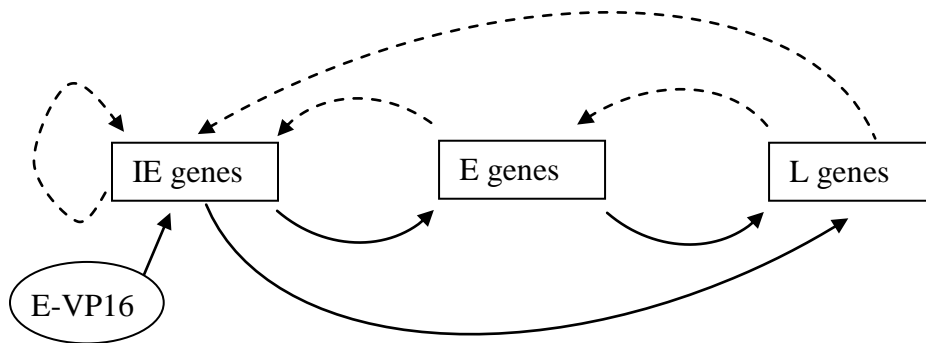
Figure 1.2 EHV-1 gene expression during lytic infection. The IE gene transcription is activated by E-VP16. The IE protein activates transcription of E and L genes. The E proteins activate transcription of L genes while suppressing expression of the IE gene. The L proteins suppress expression of E and IE genes. The IE gene auto-regulates its own expression. Dashed arrows represent suppression activity; solid arrows represent stimulation. Adapted from (Sellon and Long 2007)

1.3.4 EHV-1 viral cycle and propagation

EHV-1 virions attach to the respiratory epithelium through the envelope glycoproteins and enter the cells. The equine major histocompatibility complex (MHC) class I molecules have been described as a gD receptor for viral entry (Sasaki, Hasebe et al. 2011). An initial replication at the point of entry takes place. This lytic cycle takes approximately 20 h and is conducted in the following order: 1) attachment to the host cell membrane 2) membrane fusion and penetration into the cell or entry through endocytic pathways (Hasebe, Sasaki et al. 2009) 3) translocation of viral DNA to the nucleus 4) egress of assembled nucleocapsid from the nucleus 5) envelopment of the virions 6) offspring virions released causing cell lysis. The initial replication enables the virus to infect neighbouring epithelial cells and also dermal cells, neuronal axons (from where it will access the trigeminal ganglion around two days post infection) (Slater, Borchers et al. 1994), dendritic cells and leukocytes in the lamina propria. These infected leukocytes transport the virus via the lymphatic circulation to the draining lymph nodes where a second viral multiplication takes place, which allows the virus to gain entry to the blood circulation in a process called leukocyte-associated viremia. The virus then establishes a non-productive infection of leukocytes, the majority of which do not express viral antigens on the cell surface and, therefore, are not recognized by the immune system as infected. The infected leukocytes reach distant organs where the virus can infect the vascular endothelium and replicate, causing clinical signs such as abortion or neurological syndrome. (Gibson, Slater et al. 1992) (Goehring, Hussey et al. 2011).

1.4 EHV-1 pathogenesis and clinical signs

1.4.1 EHV-1 virulence

Ab4 and V592 are two EHV-1 field strains that have been extensively studied. Ab4 was isolated in 1980 from a quadriplegic mare who had aborted during an outbreak of paresis in geldings and mares (Crowhurst, Dickinson et al. 1981). Ab4-like strains are characterized by a high virulence, high endothelial cell tropism, and high levels of viremia. The viremia levels remain elevated for extended periods of time in horses infected with Ab4-like strains. Ab4-like strains have been found in most EHM outbreaks (Goodman, Loregian et al. 2007). V592 was isolated from a fetus during an abortion storm in 1985 (Mumford, Rossdale et al. 1987). V592-like strains are characterized by low virulence, reduced endothelial cell tropism, low levels of viremia of short duration and have been found to be involved in most non-neurological outbreaks (Goodman, Loregian et al. 2007). The difference in the virulence of Ab4 and V592 variants is attributable to a dimorphism in the sequence of gene 30 (ORF 30) that codes for the DNA polymerase. The dimorphism at nucleotide position 2254 causes a substitution of amino acids at position 752: asparagine (N) in V592 for aspartic acid (D) in Ab4. The N_{752} variant has been found in almost 98% of the outbreaks associated with abortions and close to 25% of the outbreaks associated with EHM, accounting for most of the viral genotypes that are circulating in horses (Allen and Breathnach 2006). This means that neither clinical presentation is exclusively caused by a particular variant (Nugent, Birch-Machin et al. 2006; Goodman, Loregian et al. 2007). The infection with the D_{752} variants appear to show increased severity and number of lesions in the central nervous system (CNS) which could be attributed to the increased replicative capacity. This variant has been found to be more common in neurologic outbreaks (Allen and Breathnach 2006; Goodman, Loregian et al. 2007)

1.4.2 EHV-1 transmission

Respiratory secretions of infected animals shedding infective particles during lytic cycles of the virus, either from a primary infection or from reactivation episodes, are the main source of EHV-1. Fetal membranes or reproductive tract secretions immediately after abortion are also a source of highly infectious virus (Gibson, Slater et al. 1992); (Goehring, Hussey et al. 2011). One study, investigating the nasal shedding of EHV-1 by serially testing EHV-1 positive animals during an EHM outbreak, suggested that nasal shedding can take place for up to 9 days from the day the animal starts presenting with neurological signs (Burgess, Tokateloff et al. 2012). Perpetuation of EHV-1 in the population is due to reactivation from latency and continual introduction of susceptible animals. For example, latently infected mares that reactivate virus can infect foals before weaning, and transmission between foals then spreads the disease in the herd. Depending on the vaccination status, the age of the animals and the movement of horses in or out of the herd, the transmission of the disease could vary. In most cases, natural infection and reactivation episodes do not present with evident clinical signs (Edington, Bridges et al. 1985). Mild respiratory disease can occur but is usually not noticed by the caretaker of the animals (Allen 2004).

1.4.3 Clinical signs of EHV-1 infection

1.4.3.1 Respiratory disease

The presentation of clinical signs of respiratory disease can vary in duration and severity depending on different factors: age of the animal, immune status and virulence of the virus strain. After experimental infection, the incubation period can range from one to ten days. During the first 24 h of infection, the virus replicates in epithelial cells of the upper respiratory

tract, generating erosions in the mucosa. The virus then reaches the lamina propria, infecting endothelial cells and leukocytes that travel to the draining lymph nodes (Gibson, Slater et al. 1992) (Allen 2004). Fever usually begins and subsides within the first 24-48 hours post-infection (HPI). In many cases, the fever reappear a week after infection with enlargement of lymph nodes. The initial respiratory disease is in the upper respiratory tract and characterized by rhinopharyngitis and tracheobronchitis with the occasional presence of coughing. If the host has never encountered EHV-1 previously, the presentation of respiratory signs can be severe and secondary bacterial infections are common. In cases of uncomplicated viral respiratory infections, the nasal discharge is serous; however, it can change to mucopurulent with secondary infections. An uncomplicated upper respiratory tract infection with EHV-1 will typically resolve within three weeks (Allen 2004).

1.4.3.2 Abortion and neonatal disease

When initial infection with EHV-1 or reactivation from latency takes place during pregnancy, a late-term abortion without premonitory signs or delivery of a weak or stillborn term foal can occur (Reed and Toribio 2004). Most abortions occur in the last trimester of gestation while the fetus and placenta do not seem to be affected during the first and second trimester (Smith, Mumford et al. 1996). The virus can reach the pregnant uterus by leukocyte-associated viremia or by reactivation in-situ of latent virus (Bryans 1969; Gleeson and Coggins 1980; Allen 2004). It affects primarily the small arteriolar branches in the glandular layer of the endometrium, predominantly at the site where highly vascularized chorionic villi (microcotyledons) extend into elaborate invaginations of the endometrium, causing vasculitis, ischemia and thrombosis. Depending on the severity and extent of these lesions, the infection can result in separation of the placenta, leading to asphyxia of the fetus and abortion. In most cases,

virus can be recovered from the aborted fetus showing that the virus crosses the fetal-placental barrier. Not all of the mechanisms of EHV-1 infection in the pregnant uterus are fully understood. The highly virulent strains of EHV-1 have a high affinity for endothelial cells (Goehring, Hussey et al. 2011), which may explain the severity and widespread nature of the lesions in the endometrium. However, this does not fully explain the pathogenesis of abortion because low virulence strains with lower affinity for endothelial cells should cause less severe inflammation, but can also cause abortion (Smith 1999; Allen 2004). In those cases where the virus does not cause abortion, foals are born with or rapidly develop acute respiratory disease, intractable diarrhea, pyrexia and lethargy that lead to death within a few days (Allen 2004).

1.4.3.3   Equine herpesvirus myeloencephalopathy

Viremia is a pre-requisite for the development of neurological signs as leukocyte-associated viremia transports EHV-1 infected cells throughout the circulatory system, providing the virus an opportunity to reach endothelial cells in the CNS tissue (Goehring, van Maanen et al. 2010). There is insufficient evidence to support the hypothesis for lytic EHV-1 infection of neurons. The magnitude of post-exposure viremia has been identified as a risk factor for the development of neurological disease in experimental EHV-1 infection (Allen 2008), suggesting that endothelial exposure to high viral loads raises the risk of EHM (Allen 2008; Goehring, van Maanen et al. 2010; Wilsterman, Soboll-Hussey et al. 2011). Although not proven, it is suspected that sustained exposure to infected PBLs leads to higher rates of infection in the endothelial cells. The virus can be detected in endothelial cells in the CNS by immunofluorescence (Edington, Bridges et al. 1986). The pathogenesis of EHM includes vasculitis, thrombosis and ischemic necrosis of the affected tissue in the brain, in the spinal cord or in both sites, which may be accompanied by hemorrhage. Death of nervous tissue as a consequence of the endothelial

damage by EHV-1 infection causes myeloencephalopathy and generates the neurological signs (Reed and Toribio 2004). Endothelial cell infection by direct contact with EHV-1 infected leukocytes appears to be one way by which the virus evades the immune system (Goehring, Hussey et al. 2011). Conversely, it has been proposed that the horse's immune response may exaggerate the virally induced vascular lesions, thereby intensifying the pathogenesis of EHV-1 neurological syndrome (Allen 2004). The neurological signs are diverse and are correlated with the lesion location within the CNS and severity of the lesions. Generally, signs start between six and ten days after the initial infection. This initial infection may be associated with fever and respiratory signs (Edington, Bridges et al. 1986). Possible clinical signs are: edema in the limbs or abdomen, limb weakness, ataxia, proprioceptive deficits, stumbling, complete paralysis with subsequent recumbency, bladder atony followed by urine retention or incontinence, poor tail and anal tone and fecal incontinence or inability to pass feces. Urine scalding as a consequence of bladder paralysis, bladder rupture, colic and pneumonia are possible sequellae to CNS infection. Prognosis for recumbent animals is generally considered poor (Allen 2004).

1.5   Latency and reactivation of alphaherpesviruses

This section will cover latency and reactivation in alphaherpesviruses, with focus on HSV-1 as it has been the most studied virus of this subfamily.

One of the most remarkable characteristics of herpesviruses is the ability after initial infection to remain latent within that host. Viral genomes can persist in infected cells without production of viral progeny and hidden from the host immune system. Two theories concerning the establishment of latency were proposed in the past: the "static theory" and the "dynamic

theory". According to the static theory, there is a failure of the virus' ability to express viral genes with the exception of the latency associated transcripts or LAT and, hence, there is no viral genome replication. Changes that have not yet been identified in the infected cell's intracellular environment allow reactivation and viral replication. According to the dynamic theory, the host immune system is in control of viral replication, keeping it at a minimum. Reactivation is a consequence of a weakened immune system. Neither of these theories has proven correct although they have not been proven wrong. The molecular mechanisms of latency and reactivation have not yet been clarified completely (Stevens 1989; Preston 2008).

The initial infection site for HSV-1 is the epithelium in mucosa, cornea and skin after direct contact, usually without viremia. For VZV, the route of infection is inhalation of infected respiratory tract secretions with the initial infection taking place in epithelial and immune cells followed by a cell-associated viremia and a secondary cell infection in the subdermis. Both viruses infect neurons by accessing the axons in the skin. However, HSV-1 infects neurons that innervate the site of the initial replication and establishes latency in neurons of the trigeminal ganglia while VZV, thanks to the cell-associated viremia, can infect and establish latency in sensory neurons and autonomic ganglia across the entire neuraxis (Kinchington, St Leger et al. 2012). In contrast, EHV-1-induced leukocyte-associated viremia transports the virus to distant locations where it can infect more leukocytes or endothelial cells. There is no definitive or clear evidence that EHV-1 replicates in equine neurons (Allen 2004) and there is only anecdotal evidence showing infection of neurons in locations other than the trigeminal ganglia (Patel, Edington et al. 1982; Slater, Borchers et al. 1994).

1.5.1   Establishment and maintenance of alphaherpesvirus latency

Different ideas about the mechanisms by which HSV-1 establishes latency in neurons have been proposed. One hypothesis, that could support the static theory of latency establishment, proposes that during the viral infection of neurons, the early viral cycle is interrupted due to a lack in the transport of the transactivator VP16 into the neuronal nucleus or due to a reduced interaction of VP16 with cellular factors such as HCF and Oct1 that start IE gene transcription (Lakin, Palmer et al. 1995; Kristie, Vogel et al. 1999; Kolb and Kristie 2008). Both scenarios suggest insufficient IE gene expression and repression of the lytic cycle in neurons; consequently there is no expression of viral proteins and the viral genome remains in the infected cell. Another hypothesis proposes that during the primary infection, the virus can either induce productive infection or latent infection, suggesting that productive infection is not a requirement for establishment of latency (Nicoll, Proença et al. 2012).

The linear genome of all alphaherpesviruses adopts a circular conformation and is assembled into nucleosomes in latently infected cells (Roizman 2007). In the case of HSV-1 lytic infection, it has been shown that viral proteins and LAT are expressed in less than 1% of infected neurons (Speck and Simmons 1992). It has also been proposed that the number of latent viral genomes in latently infected neurons could be related to the establishment, maintenance and reactivation of latency, as an enormous difference between the numbers of genomes in the infected neurons has been found (Sawtell 1997). How the virus genome can be present within cells in such variable numbers and how the cell can survive the initial infection without damage could be explained by the presence of $CD8^+$ T cells surrounding latently infected tissues. These immune cells can restrain the viral replication in a non-cytolytic fashion by recognising viral gB,

and inhibit viral gene expression at the last stage of the lytic cycle (Decman, Kinchington et al. 2005).

It is still not clear which mechanisms are more relevant to establishment of HSV-1 latency, but there is a possibility that it requires expression of viral proteins and does not depend on a total inhibition of the viral gene expression (Roizman 2007; Nicoll, Proença et al. 2012). The only transcripts consistently detectable in some latently infected neurons are the LAT. These transcripts do not encode proteins (Stevens, Wagner et al. 1987; Wagner and Bloom 1997). The direct role of the LAT in the establishment of latency is controversial. In a mouse neuronal cell model, using HSV-1 mutants deficient for LAT, it was shown that LAT are not essential for establishment, maintenance and reactivation from latency (Javier, Stevens et al. 1988). A later study, also using HSV-1 mutants deficient for LAT, showed that the efficiency of reactivation from latency in this mouse model was reduced and that these mutants had an increased expression of E genes. LAT may play a role in suppressing the expression of IE transcripts (Chen, Kramer et al. 1997). In contrast, a study performed using a rabbit neuronal cell model showed that HSV-1 mutants deficient for LAT had a decreased expression of lytic transcripts, suggesting that LAT increase expression of these transcripts (Giordani, Neumann et al. 2008). A clear function regarding regulation of the expression of lytic genes cannot yet be attributed to LAT. The role of LAT in the maintenance of latency in HSV-1 may be related to the ability to inhibit apoptosis in the latently infected neurons, as cell lines are more resistant to cell death when LAT are expressed (Nicoll, Proença et al. 2012). It has been demonstrated that BoHV-1 encodes a latency related protein with anti-apoptotic functions (Perng, Maguen et al. 2002). Aside from the LAT expression during latency, the idea that latently infected cells do express some viral proteins could also account for the maintenance of latency. There is evidence of the

21

presence of HSV-1-specific activated CD8$^+$ T cells surrounding latently infected tissues (Decman, Kinchington et al. 2005). This evidence could support the dynamic theory of establishment of latency.

Another line of investigation discovered a novel neuronal expressed protein called Zhangfei that has the ability to inhibit HSV-1 VP16 activation of IE genes (Akhova, Bainbridge et al. 2005). The same group had previously established that an ER related protein, Luman, could interact with the cellular protein HCF that is required in the cell nucleus to bind to VP16 for IE gene activation. They showed that in cells expressing Luman, HCF was relocated to the cytoplasm and that those cells were resilient to HSV-1 lytic infection, suggesting a role for Luman in latency establishment. Interestingly, it was also shown that Luman can activate the ICP0 promoter and the LAT promoter, suggesting an important role for Luman in reactivation of HSV-1 from latency (Lu and Misra 2000).

1.5.2   Reactivation from latency

As for establishment of latency, several theories have been proposed for virus reactivation from latency. The exact origin of the molecular process that triggers reactivation from latency is still uncertain, but the broad term "stress" has been applied for years (Preston 2008). When referring to human alphaherpesviruses, reactivation of HSV-1 by stress is a well know example. Latent HSV-1 infection reactivates frequently in humans under psychological or physiological stress and the reactivation is detected as a proliferative/ulcerative epithelial lesion, usually in or around the oral cavity. The molecular events leading to the clinical sign lesions of reactivation have not been completely elucidated. Using *in vivo* mouse and rabbit models, a number of triggers, such as hyperthermia, tissue damage, immunosuppression, UV light

22

exposure and physiological stress have been used to induce reactivation of HSV-1 but not reactivation of VZV (Nicoll, Proença et al. 2012). Dexamethasone treatment at higher than therapeutic doses has been used to reactivate BoHV-1 and EHV-1 in rabbits and horses, respectively (Edington, Bridges et al. 1985; Rock, Lokensgard et al. 1992). At the cellular level, the stimuli that have been shown to reactivate HSV-1 in an *in vitro* neuronal model include hyperthermia, deprivation of nerve growth factor, inhibitors of histone deacetylase, and dexamethasone (Nicoll, Proença et al. 2012).

One theory of reactivation proposes that stress causes, at the neuronal level, a shift in the transcription factor pattern with stimulation of the ICP0 promoter, synthesis of ICP0 protein and consequent deactivation of repression of the entire viral genome. In this context, ICP0 is proposed as a key player in the reactivation process *in vitro* (Nicoll, Proença et al. 2012). However, studies in mice infected with ICP0 mutants or wild type HSV-1 showed that the efficiency of reactivation following a stimulus such as hyperthermia was similar, proving that ICP0 is not required to trigger reactivation *in vivo* (Thompson and Sawtell 2006). A recent study by Kim and colleagues presented novel insights regarding reactivation *in vitro* (Kim, Mandarino et al. 2012). Using cultures of latently HSV-1-infected primary superior cervical ganglion (SCG) sympathetic neurons, the authors characterized the expression of lytic genes after pharmacological induction of reactivation. A reactivation program divided into 2 phases was described. In a first phase, around 20 h after induction, there was expression of all lytic gene classes (IE, E and L) simultaneously, suggesting that deactivation of repression of the viral genome occurs without the need for viral proteins. At 48 h after induction a second phase with viral genome and virion production took place. Interestingly, the authors found that the

23

transactivating activity of VP16 was required for the second phase, suggesting that VP16 is a determining factor for reactivation.

The other theory proposed for reactivation is related to the immunosuppression imposed by stress. As stated previously, the presence of HSV-1 specific CD8$^+$ cells surrounding latently infected tissues has been described (Kinchington, St Leger et al. 2012). If those cells normally prevent reactivation of latent virus, events leading to a temporary inhibition of the CD8$^+$ cell function could allow the propagation of the reactivated infection that normally would be restrained (Preston 2008; Kinchington, St Leger et al. 2012).

1.6   Latency and reactivation of EHV-1

1.6.1   Prevalence of latent EHV-1 infection

The first studies that attempted to detect viral genomes in asymptomatic horses estimated that 30% of horses were latently infected (Edington, Welch et al. 1994; Allen 2006; Allen, Bolin et al. 2008). Two studies conducted in Kentucky (Allen, Bolin et al. 2008) and California (Pusterla, Mapes et al. 2010) obtained estimates of latent infections of 54% and 15%, respectively, by detecting viral DNA using two different techniques with comparable analytic sensitivity. In the Kentucky study, submandibular lymph nodes (SMLN) from 132 Thoroughbred broodmares were analyzed using an ultrasensitive magnetic bead-based, sequence capture, nested PCR technique. The California study used 2 sources of tissue (SMLN and trigeminal ganglia) from 147 horses of different breeds, four mules and two donkeys and the detection technique was a real-time TaqMan® PCR. The second study commented that when using highly sensitive detection techniques, contamination with EHV-1 DNA of the equipment and samples can lead to false positives and needs to be prevented. A previous publication from an author of the first

24

study (Allen 2006) had reported the high efficacy of the magnetic bead, sequence-capture, nested PCR to detect latent EHV-1 from mandibular lymph nodes samples collected antemortem, compared to conventional nested PCR and real time PCR. Using the new PCR technique, EHV-1 DNA was detected in 8 out of 12 horses with unknown EHV-1 status and in 18 out of 24 experimentally infected animals. For all three studies, (Allen 2006; Allen, Bolin et al. 2008; Pusterla, Mapes et al. 2010) negative controls were used to assure that no active infection was present in the samples. The discrepancy in the reported prevalence of latent EHV-1 infection could be explained by differences in geographical locations, management practices, horse breeds, PCR assays and more factors that may have not yet been considered (Lunn, Davis-Poynter et al. 2009). Considering that there are limitations in the techniques to detect latent infection and an estimated 85% of horses have been exposed to EHV-1 by two years of age (Allen 2004), some reported figures are also probably an underestimation and most horses should be considered to be latently infected (Lunn, Davis-Poynter et al. 2009).

1.6.2 Establishment and maintenance of EHV-1 latency

There is little information about the molecular events related to establishment and maintenance of latency of EHV-1. It has been suggested that after viral entry into host cells, EHV-1 can establish either a productive infection or enter a latent state, and both states apparently occur in parallel as viral replication does not seem to be a requirement for latency establishment in the neurons of the trigeminal ganglion (Sellon and Long 2007). Latency establishment in PBLs has also been described (Slater, Borchers et al. 1994; Sellon and Long 2007). Within PBLs populations, the most frequent cell type harboring viral genome (detected by LAT expression) is the CD5$^+$/CD8$^+$ T lymphocyte (Chesters, Allsop et al. 1997). During latency, viral DNA adopts a circular conformation like other alphaherpesviruses, and expression of all

25

EHV-1 genes is repressed except for LAT that are located in an antisense region within the IE gene. There is no production of viral proteins, and latently infected cells remain "hidden" from the immune system. The molecular events that lead to establishment and maintenance of latency in EHV-1 have not yet been elucidated. The proposed theories for other alphaherpesviruses could potentially apply to EHV-1, taking into account the similarities and differences among the viruses. However, an *in vitro* model of equine origin to study establishment and maintenance of EHV-1 latency is still needed.

1.6.3 Reactivation of EHV-1 from latency

It is accepted that EHV-1 can establish latency in neurons of the trigeminal ganglion. Although establishment of latency in lymphoreticular tissues and PBLs has been proposed, *in vitro* experiments to detect EHV-1 reactivation from these sites have not produced conclusive results. (Gleeson and Coggins 1980; Scott, Dutta et al. 1983; Edington, Welch et al. 1994; Slater, Borchers et al. 1994; Chesters, Allsop et al. 1997; Smith, Iqbal et al. 1998).

Between 1985 and 1994, Welsh Mountain ponies from closed herds or specific pathogen-free pony foals were used for four EHV-1 latency and reactivation experiments. In the first study, eight Welsh Mountain ponies (five yearlings and three adults over 12 years of age) were infected and kept in isolation (as a group) for three months. Following the isolation period, the absence of active infection was demonstrated by a decrease in antibody titer for all animals and absence of virus in nasal secretions and leukocytes. All animals were then treated with immunosuppressive doses of dexamethasone (1mg/kg,) and prednisolone (2mg/kg). The authors reported viral recovery from nasal swabs and blood samples by co-cultivation with cell lines permissive to EHV-1 infection (EEK and RK-13) in six out of eight ponies. Three animals had

viremia without nasal shedding and two had viremia followed by nasal shedding. In one pony, virus was recovered from only one nasal swab. The authors suggested that the presence of viremia without nasal shedding could be interpreted as reactivation and that the detection of nasal shedding with or without the presence of viremia could be interpreted as potential EHV-1 re-infection from other horses (Edington, Bridges et al. 1985). In the second study, two pathogen-free pony foals were inoculated twice, 61 days apart, and an additional two pathogen-free pony foals were inoculated once at the same time as the second inoculation for the first group took place. Approximately 40 days after the second or only inoculation, respectively, all foals were given intravenous injections of dexamethasone (2mg/kg daily) for 3 consecutive days. Nasal mucus samples and blood samples were collected daily for 17 days after dexamethasone administration, co-cultivated with RK-13 cells for up to 1 week (nasal samples) or 2 weeks (isolated white blood cells) and re-plated if no viral plaques were observed. Virus was isolated from nasal mucus samples after dexamethasone treatment as follows: from the single inoculation group, one foal yielded virus on day 10 while virus was recovered from the other foal on days 5, 8 and 10; from the double inoculation group, one foal yielded virus on day 12 while virus was recovered from the other foal on days 8, 10 and 12. In all positive cases, cell cultures needed to be re-passaged before cytopathic effects (CPE) were observed. Virus was not isolated from white blood cell samples (Gibson, Slater et al. 1992).

In the third study, successful detection of viral DNA by PCR and reactivation of EHV-1 by co-cultivation of lymphoid tissues collected post-mortem from five latently infected ponies was reported. The ponies, originally from a closed herd, had been experimentally infected 10 weeks prior to euthanasia. Virus was grown by co-cultivation with EEK or RK-13 cells from the following tissue samples: PBLs, thymus, spleen, tonsil and retropharyngeal, submandibular, and

27

bronchial lymph nodes. Viral DNA was detected by PCR in all of the same tissues and, in addition, in the popliteal lymph nodes, lung, bronchoalveolar lavage fluid and ganglia of the trigeminal nerve. In three instances, samples positive by co-cultivation were negative by PCR, but in general, PCR was more sensitive for virus detection than co-cultivation. The authors determined that all ponies were latently infected only by PCR detection of viral genome, while one pony was consistently negative by co-cultivation. It is interesting that these authors were not able to reactivate EHV-1 from neuronal ganglia while they did detect viral DNA in neuronal ganglia in one pony by PCR (Welch, Bridges et al. 1992).

The fourth study showed that latent EHV-1 could be reactivated from the trigeminal ganglion (Slater, Borchers et al. 1994). Four EHV-free SPF ponies were experimentally infected with EHV-1. Eight weeks later, reactivation of the virus from latency was induced by treating one pony with dexamethasone, one pony with cyclophosphamide (5mg/kg/day) and two ponies with cyclosporine A (10mg/kg/day) intravenously for three days. The ponies treated with cyclosporine A did not show reactivation and were treated 25 days later with dexamethasone (2mg/kg/day for three days). Viral reactivation in all four ponies was demonstrated by detection of high viral titers of EHV-1 in nasal mucus via co-cultivation with RK-13 cells. Although leukocyte associated viremia could not be detected by co-cultivation of PBMC samples, three ponies tested positive for EHV-1 by nested PCR in PBMC samples. The nested PCR used in this study, which differentiates between EHV-1 and EHV-4, was more sensitive than other PCRs available at the time this study was conducted (Borchers and Slater 1993). Six weeks after the viral shedding induced by the reactivation stimulus had ceased and no virus was recovered from nasal mucus or PBMC samples, all four ponies were euthanized and necropsied. Samples were collected from a large number of tissues: submandibular, retropharyngeal, bronchial and

28

mediastinal lymph nodes, tonsils, spleen, thymus, conjunctiva, liver, nasal epithelium, trachea, lung, olfactory nerve (cranial nerve I), optic nerve (cranial nerve II), trigeminal nerve (cranial nerve V) and trigeminal ganglion, and the facial nerve (cranial nerve VII). Although horses were assumed to be latently infected because of successful virus reactivation six weeks earlier, post-mortem samples (nasal tissue and trigeminal ganglion) from only two ponies showed virus reactivation by co-cultivation. However, samples of the trigeminal ganglion and the submandibular lymph nodes from all four ponies tested positive for EHV-1 by PCR. Viral detection by PCR in the rest of the lymphoid tissues was not consistent in the four ponies, but three out of four PBMC samples tested positive (Slater, Borchers et al. 1994).

In addition to the *in vivo* reactivation studies in Welsh Mountain ponies, other studies have examined the prevalence of latent equine herpesvirus (1 and 4) infection in samples from 40 horses collected from abattoirs (Edington, Welch et al. 1994). Samples were analyzed by PCR, differentiating between EHV-1 and EHV-4 (Welch, Bridges et al. 1992), and by co-cultivation without discrimination between viruses. The tissues that showed the highest prevalence of latent EHV-1 infection by co-cultivation were the submandibular (35/40), retropharyngeal (25/40) and bronchial (22/40) lymph nodes. All positive co-cultivated samples were positive after the second passage. The nasal mucosa and trigeminal ganglion samples did not show viral reactivation in any sample. The authors commented that circulating mononuclear cells were collected from 20 horses; however, no results were published in this report. Further differentiation between viruses by indirect immunofluorescence in 22 samples from the same study showed that EHV-1 alone was present in 14% of the samples, EHV-4 alone was present in 36% of the samples, and both viruses were present in 50% of the samples. The authors also reported that 87.5% of bronchial lymph nodes were positive by PCR detection, of which 32.5%

contained both viruses, 30% contained only EHV-1 and 25% contained only EHV-4. Of the nine samples of trigeminal ganglion analyzed by PCR, four tested positive to EHV-4 only, one tested positive to EHV-1 only and three tested positive to both viruses. Overall, the authors concluded that the main sites of latency for both EHV-1 and EHV4 are the lymph nodes of the respiratory tract and that the prevalence of latent equine herpesviruses is high in the equine population (Edington, Welch et al. 1994).

The same authors, in 1997, reported the detection of EHV-1 LAT in equine leukocytes collected from bronchial lymph nodes and peripheral blood but they were not able to detect these transcripts in trigeminal ganglia. The samples used to detect LAT were from abattoir horses and from known latently infected ponies used in the reactivation experiment that was published in 1985 (Chesters, Allsop et al. 1997).

In 1998, Smith and colleagues reported the first chemically induced *in vitro* reactivation of latent EHV-1 from peripheral blood leukocytes and also identified the main type of latently infected leukocyte to be $CD5^+/CD8^+$. These authors investigated *in vitro* induction of reactivation following treatment of PBLs with mitogens, hormones and cytokines. They found that treatment of PBLs isolated from whole blood samples with phytohaemagglutinin (PHA), pokeweed mitogen (PWM), human interleukin 2 (IL-2) and equine chorionic gonadotrophin (eCG) for 24 h could induce EHV-1 reactivation. IL-2 is an immune cytokine secreted by the T-lymphocytes and eCG is a hormone released by the gravid uterus, while PWM and PHA are mitogens derived from plants. Reactivation was detected by indirect immunofluorescence (IIF) targeting EHV-1 gB and by co-cultivation with EEK and RK-13 cells. The authors described that detection of reactivating virus by co-cultivation required at least 10^6 leukocytes. RT-qPCR targeting EHV-1 gB transcripts was performed on RNA isolated from leukocytes exposed for 24

h to IL-2 or PHA, and showed positive results. The authors further identified by IIF that the cells harbouring reactivated virus were 80% $CD5^+/CD8^+$ and 20% $CD5^+/CD8^-/CD4^-$. The mechanisms by which IL-2 and eCG can induce reactivation are independent. Reactivation after treatment with IL-2, PHA or PWM was blocked after addition of IL-2 antibodies, and eCG antibodies could block reactivation induced by eCG. IL-2 antibodies did not block eCG induced reactivation and eCG antibodies did not block IL-2 activation. Reactivation was achieved after treatment of whole leukocyte preparations with those chemicals, but could not be achieved in purified T-lymphocytes ($CD3^+$ cells). However, virus reactivation in purified $CD3^+$ cells could be accomplished if supernatant from stimulated whole leukocyte preparations was added to the culture. This showed that viral reactivation induced by eCG and IL-2 was accomplished by stimulation of $CD5^+/CD8^+$ cells and that activated $CD5^+/CD8^+$ cells produced substances that induced reactivation in other cell types.

It has been suggested (Scott, Dutta et al. 1983; Edington, Bridges et al. 1985) that, as a result of sporadic reactivation of EHV-1 in latently infected PBLs, the virus reaches the respiratory epithelium, the pregnant uterus and endothelial cells in the CNS, establishing infection, viral shedding, viremia and, in some cases, clinical disease (Smith, Iqbal et al. 1998; Allen 2004). A shift from a non-productive to a productive infection, meaning reactivation from latency, is prompted in response to specific triggers that involve viral, endogenous and exogenous factors (Chesters, Allsop et al. 1997). Different types of stress have been proposed to be involved in reactivation from latency. The use of drugs, such as high doses of dexamethasone has been proved to cause reactivation (Edington, Bridges et al. 1985).

While various physiological and physical stressors can reactivate EHV-1, it is still unclear how reactivation results in the rapid spread of the virus to susceptible horses within the

31

herd. In a more recent attempt to reactivate EHV-1 in latently infected animals using dexamethasone, it was found that one out of four horses showed fever while all four treated animals allowed low grade and transient molecular-based detection of EHV-1 in blood and nasal secretions, with negative viral isolation by co-cultivation (Pusterla, Hussey et al. 2010). However, housing the treated horses with EHV-1 seronegative sentinel horses did not result in infection of the sentinel horses. There was no development of clinical signs, viral recovery from any sample, viremia detection or seroconversion in any of the sentinel horses. For the dexamethasone treatment of these animals, although longer in time (5 consecutive daily treatments), the doses used (0.2-0.3mg/kg) were considerably lower compared with previous studies. Other postulated stressors such as hospitalization (Carr, Schott et al. 2011) or transport over long distances (Pusterla, Mapes et al. 2009) have failed to result in reactivation. To date, the molecular mechanisms of EHV-1 reactivation have not been elucidated.

1.7 The unfolded protein response

1.7.1 Protein folding in eukaryotic cells

Proteins are macromolecules that depend on the final three-dimensional conformation to become biologically active (Anfinsen 1973). Synthesis of proteins involves transcription of genes in the nucleus and ribosomal translation either in the cytoplasm or in the endoplasmic reticulum (ER) (Warner 1963), an intricate phospholipid membrane system that is present in all eukaryotic organisms (Healy, Gorman et al. 2009). Synthesis of soluble proteins to be secreted or directed to the cellular membrane takes place in the ER membrane, where the secretory pathway

starts (Caro and Palade 1964). Such polypeptides must express a targeting signal to be directed to the ER (Allison and Young 1989).

The product of translation is a linear polypeptide chain in its primary conformation, meaning that it is not biologically active. The nascent polypeptide still attached to the ribosome or the completely translated polypeptide must be translocated into the ER lumen in order to be folded, processed and glycosylated. The oxidizing luminal environment of the ER is characterized by a high concentration of chaperone proteins and high calcium concentration, as most of these chaperone proteins are calcium dependent (Lièvremont, Rizzuto et al. 1997). Chaperone proteins assist the transport of the nascent polypeptide chain into the ER, and they also assist the folding process by stabilizing the peptide, preventing premature folding and avoiding protein aggregation (Beckmann, Mizzen et al. 1990). The glucose regulated protein 78KDa/ immunoglobulin heavy chain binding protein (GRP78 or BiP) is a chaperone protein present in the ER of all cells, which plays a fundamental role in protein folding, quality control and some translocational processes (Haas and Wabl 1983; Munro and Pelham 1986; Hendershot, Ting et al. 1988). The folding process shapes polypeptides into an active/functional state that is then targeted for transportation and finally delivered to its definitive location. During the folding process, misfolding of polypeptides can occur under normal conditions. Misfolded proteins can either be refolded in the ER lumen or targeted for degradation. ER processes only take place under normal and optimal conditions; if these conditions are distorted, the influx of new unfolded, immature proteins into the ER is reduced, primarily to ensure the cell's survival and eventually to re-establish the normal post-translational modification process (Kim, Xu et al. 2008).

1.7.2 ER stress signaling: activation of the unfolded protein response

Under normal, homeostatic conditions the chaperone protein GRP78 is bound to the luminal portion of an ER group of transmembrane proteins, and also binds to the incoming unfolded or misfolded proteins that will be modified in the ER. When the influx of unfolded proteins into the ER lumen is higher than the capacity of the ER to process and modify those proteins, GRP78 is forced to bind to the increasing number of unfolded proteins, consequently releasing its bond to the luminal portion of transmembrane regulatory proteins and initiating a signaling cascade known as the unfolded protein response (UPR)(Healy, Gorman et al. 2009).

The UPR is an evolutionarily conserved response triggered by the accumulation of unfolded and misfolded proteins in the ER. Different stimuli can alter cellular homeostasis, induce ER stress and, consequently, interrupt the protein folding process generating accumulation of unfolded and misfolded proteins. Conditions such as hypoxia, glucose deprivation, oxidative stress, disequilibrium in calcium concentration, viral infection and neoplastic cellular modifications can induce ER stress and UPR activation. The UPR communicates to the nucleus the folding status of proteins in the ER lumen to orchestrate a highly regulated response. Depending on the duration, intensity and origin of the stress stimulus, the UPR can proceed to different outcomes. There is an initial adaptive response that can be separated into an immediate reaction and a secondary reaction. The immediate reaction's purpose is to decrease the translation and transport of new polypeptides into the ER. The secondary reaction's objective is to induce the expression of genes that either help in the adaptation to stress or, failing that, induce apoptosis. When the ER fails to adapt to the stress or the UPR fails to restore normal ER function, an alarm response is activated to notify the cell and its surrounding of the abnormal situation by linking with several cellular pathways (Yoneda,

Imaizumi et al. 2001; Kato, Nakajima et al. 2011). Eventually, if this response fails to restore ER function, the apoptotic phase is triggered, leading to programmed cell death (Hetz 2012) (Kim, Xu et al. 2008). The activation of the UPR results in a number of mechanisms to decrease the presence of unfolded proteins in the ER lumen: prevention of the influx of more unfolded/misfolded proteins into the ER, induction of the expression of UPR-related genes, and physical expansion of the ER membrane (Shaffer, Shapiro-Shelef et al. 2004) that ultimately enhances the folding capacity, the quality control system and the ER-associated degradation capacity. The UPR signaling mechanisms are based mainly on the release of GRP78 from three transmembrane proteins: Inositol–requiring kinase 1 α (IRE1α), Pancreatic ER kinase (PKR)-like ER kinase (PERK) and Activating transcription factor 6 (ATF6) (Figure 1.3).
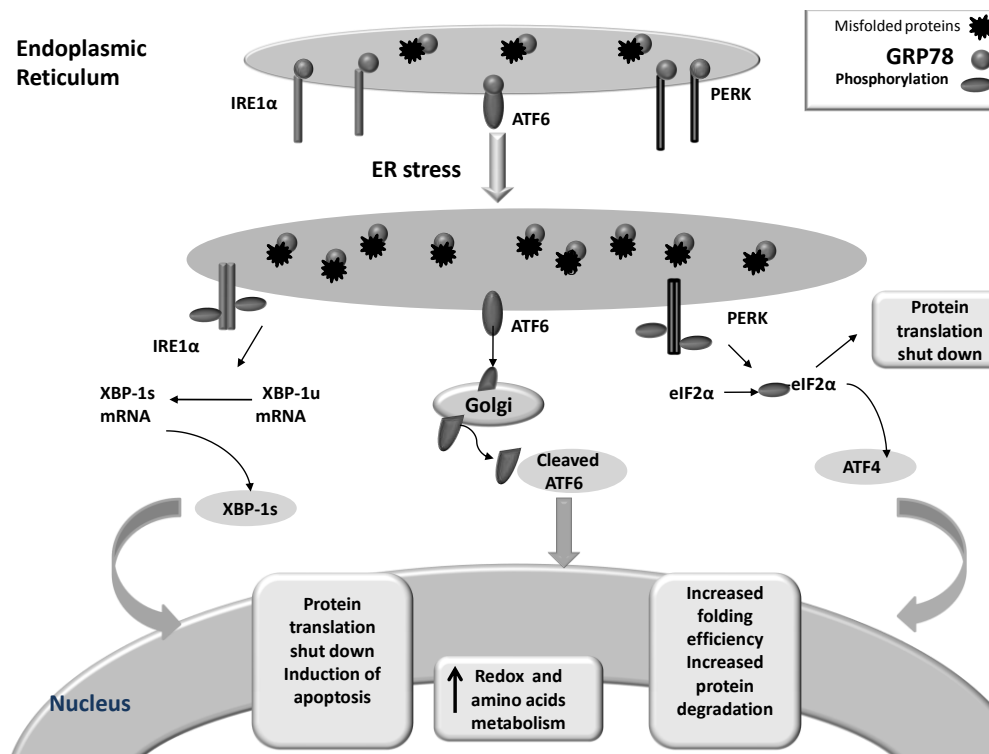
Figure 1.3 Activation of the UPR upon ER stress. During homeostatic conditions, GRP78 binds IRE1α, ATF6 and PERK, preventing their activation. Upon ER stress, the increased influx of misfolded proteins displaces GRP78 from the membrane position and activates the signaling cascade. IRE1α homodimerization induces its own autophosphorylation and activates its endoribonuclease activity that splices XBP1 mRNA. ATF6 undergoes translocation to the Golgi complex and cleavage. PERK homodimerization induces its own autophosphorylation, phosphorylation of eIF2α and induction of ATF4. Activation of the 3 arms of the UPR induces transcription of UPR related genes in the nucleus.

1.7.2.1 Activating transcription factor 6 (ATF6)

ATF6 is a protein from the family of cAMP response element binding (CREB) transcription factors (Yoshida, Haze et al. 1998; Hai and Hartman 2001). It is localized in the ER membrane. ATF6 possesses a basic leucine zipper (bZIP) and a transcription activation domain in its cytosolic portion (Asada, Kanemoto et al. 2011). After dissociation of GRP78-ATF6 binding, ATF6 is released from the ER membrane and is translocated into the Golgi, where it is cleaved by the Site 1 and Site 2 protease system and the bZIP domain is translocated to the nucleus (Shen, Chen et al. 2002). The bZIP domain binds to ER stress response elements (Yoshida, Haze et al. 1998) and induces transcription of UPR related genes. Some of the downstream targets of ATF6 activation are: ER chaperone proteins GRP78, GRP94 and calreticulin, XBP1, and Protein disulfide isomerase (PDI) (Hsu, Hsieh et al. 2005; Kim, Xu et al. 2008).

A number of ER localized, ER stress-related proteins with high ATF6 homology have been described: Luman (CREB3) (Lu, Yang et al. 1997); OASIS (CREB3L1) (Kondo, Murakami et al. 2005); CREB3L2 (Kondo, Saito et al. 2007); CREB3L3 (Zhang, Shen et al. 2006) and CREB4 (CREB3L4) (Nagamori, Yabuta et al. 2005). Many of these proteins have been shown to activate the UPR and are believed to replace or supplement the activity of ATF6 in some cells. Most of these transcription factors are expressed in specific cells. For instance, Luman mRNA has been found in various cell types but Luman protein translation appears to be specific to neuronal tissues (Asada, Kanemoto et al. 2011). It was shown that Luman induces HERP (homocystein-inducible ER stress inducible ubiquitin like domain member 1) and ER-associated protein degradation (Liang, Audas et al. 2006). Luman has been found to contribute to neuronal repair upon sensory nerve injury (Ying, Misra, Verge, submitted). Knowledge of the

37

role of Luman as a stress response protein and its involvement in the UPR has increased enormously in the last years, as studies in this area progress. Luman activation is inhibited by another neuronal protein, Zhangfei (Misra, Rapin et al. 2005). Zhangfei also has the ability to suppress the UPR activation by other means independently of Luman (Bergeron, Zhang et al. 2012).

1.7.2.2 Inositol requiring kinase 1 α (IRE1α)

IRE1α is a transmembrane protein localized in the ER membrane. It possesses a kinase domain and mRNA endoribonuclease activity in its cytosolic domain (Tirasophon, Welihinda et al. 1998). After GRP78 dissociation, IRE1α homodimerization activates the kinase domain that induces autophosphorylation and transphosphorylation that further activates its mRNA endoribonuclease activity. Activation of IRE1α endoribonuclease activity splices XBP1 (X-box binding protein) mRNA, giving rise to the XBP1 spliced form, which is now translated to XBP1s protein, a bZIP transcription factor that binds to the ER stress response element and promotes the transcription of target UPR genes (Bertolotti, Zhang et al. 2000; Yoshida, Matsui et al. 2001). IRE1α also has the ability to degrade specific mRNAs different from the XBP1 mRNA in a regulated IRE1 dependant decay (RIDR) (Hollien and Weissman 2006), and it is a key player in the alarm response and in the cross talk with cellular pathways by activating JNK (c-Jun N-terminal kinase) and NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) (Hetz 2012). Some of XBP1s targets are: CHOP (DNA damage inducible transcript 3), a pro-apoptotic gene; HERP and EDEM (ER degradation enhancing α mannosidase like protein) genes related to the ER associated protein degradation, and p58IPK, an inhibitor of PERK (Ma and Hendershot 2004; Hetz 2012).

1.7.2.3 Pancreatic ER kinase (PKR)-like ER kinase (PERK)

PERK, as IRE1α, is an ER resident transmembrane protein with a serine/threonine kinase domain. Under ER stress it dissociates from ER luminal binding to GRP78. Homodimerization and auto trans-phosphorylation activates its kinase activity. PERK phosphorylates the eukaryotic translation initiation factor 2α (eIF2α), thereby inactivating it (Bertolotti, Zhang et al. 2000). Phosphorylation of eIF2α causes the inhibition of cap-dependant mRNA translation, generating a change to a cap-independent translation of mRNA containing the internal ribosome entry site (IRES) sequence (Hanson, Zhang et al. 2013), therefore reducing the load of incoming proteins into the ER. However, there is a selective translation of some mRNA, including the activating transcription factor 4 (ATF4) mRNA. ATF4 increases chaperone levels by inducing XBP1 expression, and also induces genes related to redox balance, amino acid metabolism and autophagy, seeking to restore ER homeostasis. PERK activation also induces and up-regulates apoptotic signals such as CHOP (Healy, Gorman et al. 2009) and induces the expression of growth arrest and DNA damage-inducible protein (GADD34) which associates with a protein phosphatase 1(PP1) to dephosphorylate eIF2α as a feedback loop auto-regulating PERK activation (Brush, Weiser et al. 2003).

It is important to note that upon activation of the UPR, the two different phases that take place in a cell, namely the adaptive phase and the apoptotic phase, are programmed to occur in temporal fashion, one after the other. It would be expected that expression of UPR related genes followed that same pattern. However, when gene expression assays are performed, the adaptive phase genes and the pro-apoptotic genes are found to overlap in time. In most of the *in vitro* experiments performed to study the UPR, chemical inducers of ER stress are used in concentrations and durations that are detrimental to cells in such a way that the cells are

condemned to death. In contrast, cells exposed to physiological stress can regulate the UPR and survive, or even adapt to UPR activation for prolonged periods of time, as is the case with secretory cells (Hetz 2012).

1.8   Regulation of the UPR during viral infection

When a virus infects a cell, it depends on the cellular machinery to synthesize viral proteins. This means that a virally infected cell will be suddenly forced to increase its protein production. As described earlier, one of the stressors that can trigger activation of the UPR is viral infection. Activation of the UPR is a major obstacle for viral infection as it leads to shutdown of protein translation. Also, prolonged activation of the UPR leads to apoptosis of the host cell, which would also stop virus production. To get around these obstacles, viruses have a number of strategies to manipulate the UPR. Several of these methods have been described in the literature for different viruses.

Hepatitis C virus (HCV) is a member of the *Flaviridae* family and possesses 2 envelope proteins (E1 and E2) that have been found to induce expression of ER chaperone proteins GRP78 and GRP94 (Liberman, Fong et al. 1999). Later, it was described how replication of HCV triggered activation of the UPR but selectively decreased the phosphorylation of eIF2α by PERK inhibition, mediated by binding of the E2 protein (Tardif, Mori et al. 2002; Pavio, Romano et al. 2003). The HCV proteins E1 and E2 were found to induce the CHOP promoter and the splicing of XBP1, probably by IRE1α activation (Chan and Egan 2005). In contrast, one study showed that HCV can suppress the activation of IRE1 (Tardif, Mori et al. 2004), while another study described how an ER membrane associated viral protein, NS4B, induces UPR activation, cleavage of ATF6 and splicing of XBP1 (Li, Ye et al. 2009).

Another member of the *Flaviridae* family, West Nile virus (WNV), regulates UPR activation as it strongly induces the IRE1α pathway while regulating activation of the PERK pathway (Ambrose and Mackenzie 2011). However, there is disagreement with previous studies that proposed that WNV infection triggered activation of the UPR with a strong induction of CHOP (a downstream target of PERK activation) and subsequent UPR driven apoptosis (Medigeshi, Lancaster et al. 2007). Differences could be attributable to the viral strains used in the studies. The hypothesis of apoptosis induction by WNV seems plausible as it has been described that a member of the *Picornaviridae* family, Coxsackievirus B3 (CVB3), induces strong CHOP expression and apoptosis (Zhang, Ye et al. 2010) and a member of the *Togaviridae* family, Semliki Forest virus (SFV), also induces UPR driven apoptosis by up-regulation of CHOP expression (Barry, Fragkoudis et al. 2010).

Hepatitis B virus (HBV) a member of the *Hepadnaviridae* family has been shown to induce expression of UPR chaperones by the over expression of the viral Large Surface Protein, and hepatitis B virus X protein (HBx) has been shown to activate the ATF6 and IRE1α pathways of the UPR (Xu, Jensen et al. 1997; Li, Gao et al. 2007).

In the *Herpesviridae* family, Cytomegalovirus (CMV) (a member of the subfamily *Betaherpesvirinae*), has been described to selectively regulate the UPR to its benefit. The viral protein pUL38 was found to strongly activate the PERK pathway, resulting in elevated ATF4 expression and phosphorylation of eIF2α, while suppressing activation of the JNK pathway mediated by IRE1α, both resulting in rescue of the cell from apoptosis (Xuan, Qian et al. 2009). At the same time that CMV infection induces expression of GRP78, inhibition of GRP78 was shown to stop the assembly of CMV (Buchkovich, Maguire et al. 2009).

Kaposi's sarcoma-associated herpesvirus (KSHV or Human herpesvirus 8), a member of the subfamily *gammaherpesvirinae,* has been found to increase expression of ATF4 upon infection of cells. As both ATF4 and XBP1 are inducers of viral replication, induction of ER stress and UPR activation could play a role in viral reactivation from latency (Wilson, Tsao et al. 2007; Caselli, Benedetti et al. 2012). In contrast to these reports, one study showed that treatment of KSHV-infected cells with non-toxic doses of the glucose analog 2-deoxy-D-glucose (2-DG) led to inhibition of N-linked glycosylation with further UPR activation. Interestingly, PERK-mediated UPR activation caused the shutdown of protein translation, inhibition of lytic gene expression and inhibition of viral reactivation (Leung, Duran et al. 2012).

The interaction between alphaherpesviruses and the UPR has been evaluated in VZV and HSV-1. Regarding VZV, Carpenter and colleagues described the enlargement of the ER as a sign of ER stress with a 10-fold increase occurring in infected as opposed to uninfected cells. Increased splicing of XBP1 and increased expression of CHOP in virus-infected cells was described. UPR activation appears to modulate ER stress in VZV infected cells and spare them from apoptosis. It is interesting to note that in this study, it was proposed that autophagy in VZV infected cells is a pro-viral event, which is in contrast to the viral inhibition of autophagy in HSV-1 infection that serves to ensure virus survival (Carpenter, Jackson et al. 2011). It has been suggested that while HSV-1 activates PERK, this activation is counteracted by indirect dephosphorylation of eIF2α mediated by the viral protein $\gamma_1 34.5$ and the consequent derepression of protein translation (Cheng, Feng et al. 2005). A later report showed that HSV-1 glycoprotein B physically interacts with PERK, thereby inhibiting its activation and regulating the PERK pathway (Mulvey, Arias et al. 2007). A more recent study investigated the expression of the UPR during early HSV-1 infection (Burnett, Audas et al. 2012). It was shown that during the first 24 h

after infection, HSV-1 induces transcription of UPR genes through ER stress-responsive elements in their promoters. It was also confirmed that the ATF6 arm of the UPR was active between 2 and 8 h after infection and was later suppressed. Phosphorylation of eIF2α was shown to be decreased from 2 to 8 h and to increase at 24 h after infection. ATF4 and CHOP showed a similar expression pattern with suppression in the early stages and induction in the late stages. The IRE1α arm was also suppressed during the first 24 h after infection. Interestingly, the viral gene ICP0 promoter is responsive to ER stress and it may act as a stress detector on behalf of the virus. It was determined in this study that HSV-1 can silence the UPR during early infection and modulate it in its favour (Burnett, Audas et al. 2012).

1.8.1   UPR involvement in herpesvirus latency and reactivation (rationale for examining the UPR as a potential trigger for EHV-1 reactivation)

A variety of seemingly unrelated stressors are known to trigger the reactivation of herpesviruses from latency. However, a common molecular pathway that would focus the effects of these stressors to the latent herpesvirus genome has not been identified. Since the UPR is activated by a variety of stressors, such as inflammation, hypoxia, an increase in protein synthesis, neuronal injury etc. (many of these are known herpesvirus reactivators) I speculate that herpesviruses use the UPR as a means of sensing stress in their hosts. Indeed, Wilson and others (2007) have linked Xbp1s, an important UPR mediator, to the reactivation of Kaposi sarcoma herpesvirus from latently infected plasma cells. In addition, our laboratory has discovered two basic-leucine motif containing transcription factors that can regulate the UPR as well as the HSV lytic cycle. Luman/CREB3 (Lu et al, 1997, 1998) is an ER-associated protein that is released from the ER in response to stress (Raggo et al. 2002), such as damage to peripheral nerves (Ying, Misra, Verge, submitted). It then enters the nucleus to activate gene

expression. Luman resembles the UPR mediator, ATF6 and can transactivate the expression of UPR genes (DenBoer et al, 2005; Liang et al. 2006). Luman can also substitute for the HSV transactivator VP16 for initiating the HSV lytic cycle (Lu and Misra, 2000). In contrast, the other neuronal protein, Zhangfei/CREBH (Lu and Misra, 2000), can very effectively shut off the UPR (Zhang et al, submitted), Luman (Misra et al. 2005) as well as the initiation of the HSV lytic cycle by VP16 or Luman (Akhova et al. 2005). These observations, that Luman and Zhangfei can influence the UPR as well as HSV replication in sensory neurons, the seat of HSV latency, have led us to suggest that HSV regulates latency and reactivation by using the UPR (specifically Luman and Zhangfei) as sensors of stressful and non-stressful conditions (Fig 1.4).

Since Luman (Kang et al. 2009; Kim et al, 2010; Fox, 2010) and Zhangfei (Misra et al, 2012; Zhang et al., 2010; Lopez-Mateo et al. 2012) are also thought to regulate the UPR and stress responses in tissues other than neurons, I extended our proposed model to PBLs, the proposed site of EHV-1 latency.
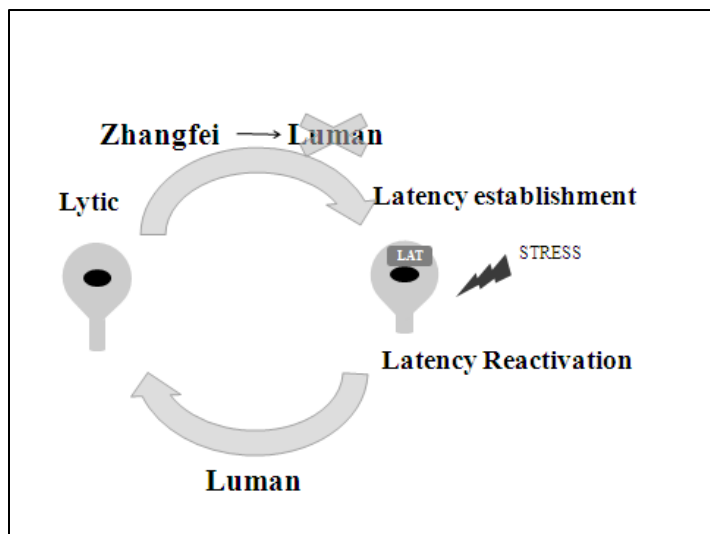
Figure 1.4. A model proposing the involvement of the UPR, specifically Luman and Zhangfei, in the establishment of herpesvirus latency and reactivation from it. Both Zhangfei and Luman (anchored in the ER in an inactive state) are present in unstressed differentiated cells such as sensory neurons and as yet unidentified lymphoid cells in the peripheral blood. Zhangfei, in this state modulates the UPR (which, if unrestrained, can trigger apoptosis) and inhibits the replication of herpesviruses that might infect the cell. The suppression of the herpesvirus lytic cycle leads to latency, which is characterized by the expression of LAT. Stress (in many of its forms) releases Luman from the ER and the protein then activates the UPR. In cells harbouring latent herpesviruses Luman also initiates the herpesvirus lytic cycle leading to reactivation.

1.9 Hypothesis

My overall hypothesis states that latent EHV-1 can be reactivated by activating the UPR in equine peripheral blood leukocytes (PBLs). This hypothesis is based on the assumption that EHV-1 establishes latency in PBLs, and that EHV-1 relies on the UPR as a pluripotent cellular stress sensor.

1.9.1 Objectives

To test this hypothesis I had the following objectives:

- **Objective 1:** To establish a RT-qPCR assay that could be used to detect and quantitate down-stream UPR gene expression in equine cells, examine the UPR activation over a period of time in equine dermal cells and determine if the UPR can be activated in equine PBLs.

- **Objective 2:** To characterize lytic EHV-1 infection in equine dermal cells and detect the expression of EHV-1 genes over time.

- **Objective 3:** To investigate the relationship between EHV-1 gene expression and UPR gene expression during lytic infection of equine dermal cells.

- **Objective 4:** To detect latent EHV-1 in PBLs from horses presumed to be latently infected and determine if stimulation of the UPR in these cells leads to virus reactivation.

2   DETECTION OF UPR GENE EXPRESSION

My first objective was to establish a RT-qPCR assay that could be used to detect and quantitate down-stream UPR gene expression in equine cells, examine the UPR activation over a period of time in equine dermal cells and determine if the UPR can be activated in equine PBLs.

Primers targeted to selected UPR related genes were designed and tested in equine dermal cells (E.Derm) treated with thapsigargin, a chemical compound that produces a calcium imbalance in the cells and induces ER stress. Genes that had been reported to respond to perturbations of ER function in other species such as humans and dogs, and that represented the 3 arms of the UPR were selected (Table 2.1). These included the genes for: X-box binding protein 1 spliced (XBP1s), 78 kDa glucose regulated protein-like (GRP78), DNA damage-inducible transcript 3 protein-like (CHOP), and homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 2 protein-like (HERP). The equine-specific *(Equus caballus)* mRNA sequences for these protein products are available in GenBank; the accession numbers are listed in Table 2.1. 18s ribosomal RNA, which is a basic constituent of eukaryotic cells and has been described as a reliable reference gene for RT-qPCR (Allen, Payne et al. 2007) was selected as the reference gene for this project. .

The published guidelines for minimum information for the publication of data from RT-qPCR experiments (MIQE) require that the PCR reactions be efficient and highly specific. The analytical specificity of the reaction is defined as the PCR assay detecting the appropriate target sequence rather than nonspecific targets that are also present in a sample (Bustin, Benes et al. 2009). To confirm the specificity of the PCR reactions, I established the identity of the PCR products by determining their size, the kinetics of their dissociation over a range of temperatures and, ultimately, by determining their nucleotide sequence. I also strived to optimize the

efficiency of the reaction to ensure that the product doubled in quantity at each cycle of the reaction.

2.1 Materials and Methods

2.1.1 Equine dermal cell culture

A cell line of equine dermal (E.Derm) cells with fibroblast morphology (NBL-6, ATCC® CCL-57™) was generously provided by Elaine Van Moorlehem from the Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan. E.Derm cells were grown and maintained in Minimum Essential Medium (MEM) supplemented with 2mM L-Glutamine (Fisher Scientific, Waltham, Massachusetts, USA), 1% MEM Non-Essential Amino Acids (NEAA, Invitrogen), 10mM HEPES (Invitrogen) and 0.1% gentamicin (Invitrogen). E.Derm cells were cultured in 6-well or 12-well culture plates (BD Falcon) and incubated in a 5% CO_2 humidified atmosphere at 37°C. Cells were allowed to grow for 2 days or until 80% confluence.

2.1.2 Horses

Six mares from the WCVM teaching herd were used to collect blood samples. These animals are used for teaching purposes in clinical laboratories and classes, and have no recorded history of EHV-1 infection. Procedures were approved by the Animal Research Ethics Board, University Committee on Animal Care and supply (UCACS), University of Saskatchewan, and followed the guidelines of the Canadian Council on Animal Care (CCAC).

2.1.3 Blood Sampling, PBLs isolation and culture

Blood samples (30 or 60ml) were collected in 10 ml Vacutainer® K2 EDTA Tubes (BD Falcon, New Jersey, USA). The blood was diluted 1:1 with Dulbecco's Modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA). The PBLs were isolated using Lymphocyte separation medium (LSM, MP Biomedicals, California, USA) by layering 2.5 ml of LSM and 5ml of diluted blood in 15 ml centrifuge tubes (BD Falcon) followed by 30 minutes of centrifugation at $800 \times g$ in a Sorvall Legend RT centrifuge (Thermo Scientific, Waltham, Massachusetts, USA). All the centrifugation steps were carried out at room temperature. After centrifugation, three clear layers could be distinguished. The middle layer was transferred to a fresh 15 ml tube containing 10 ml of DMEM followed by 7 minutes of centrifugation at $275 \times g$. The supernatant was discarded and the pellet washed with 8 ml of DMEM. After 7 minutes of centrifugation at $150 \times g$, the supernatant was discarded and the pellet resuspended in Roswell Park Memorial Institute Medium (RPMI 1640, Invitrogen). Trypan blue dye 0.4% in D-PBS (BDH Inc, Toronto, Canada) was added to the cell suspension to a final concentration of 0.08% to assess cell viability, and a haemocytometer chamber was used to count cells. Dead cells absorb the blue dye, while viable cells with intact membranes do not absorb it. PBLs were maintained in RPMI 1640 with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). PBLs were maintained in 6 well culture plates or in 25 cm^2 cell culture flasks (BD Falcon) and incubated in a 5% CO_2 humidified atmosphere at 37°C.

2.1.4 Polymerase Chain Reaction (PCR)

All primers were purchased from Integrated DNA technologies (Coralville, Iowa, USA.). Primers for equine GRP78 and XBP1s genes were designed using the Primer3 (v.0.4.0) web site

(http://frodo.wi.mit.edu/) using the nucleotide sequences found in GenBank. General primer selection default conditions were maintained according to the software requirements with product size between 200 and 400 base pairs and primer size between 18 and 22 base pairs (Table 2.2). For assessing transcripts from equine HERP and CHOP genes, I used primers originally designed in Dr. Misra's lab to amplify targets in the canine homologues of these genes.

RNA (from E.Derm cells and PBLs) was purified using the RNeasy plus minikit (Qiagen) according to manufacturer's instructions. The concentrations of DNA and RNA were measured using a Nanodrop 2000C spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) synthesis was performed using the QuantiTect® reverse transcription kit (Qiagen) with integrated removal of genomic DNA contamination, according to instructions provided with the kit.

Quantitative real time PCR (RT-qPCR) was performed in a Mx3005P QPCR thermal cycler (Stratagene, La Jolla, CA, USA). To quantify transcripts of interest, the Brilliant II SYBR® Green QPCR Master Mix protocol (Agilent technology, Santa Clara, California, USA) was used. Samples were amplified using the following thermal cycler conditions: 95°C for 10 min followed by 40 cycles of: 95°C for 30 s, 60°C for 1 min, 72°C for 1 min. Data were analyzed using the thermal cycler-associated software. The UPR (Table 2.2) and the TNF α (Table 2.4) primer sets for transcript amplification were used at a final concentration of 1.875 μM. The concentration of transcripts in each sample was expressed as the quantification cycle (Cq).

The efficiency of RT-qPCR reactions for each set of primers was determined from the slope of the standard curve developed with serial 4 fold dilutions of cDNA template. The PCR efficiency was calculated from the following equation(1).

(1) Efficiency (E) = (10^(-1/slope)-1)*100

The theoretical maximum of 100% indicates that the amount of product doubles with each cycle and an efficiency of between 95-150% is considered acceptable (Bustin, Benes et al. 2009). Efficiency values greater than 150% could indicate that more than one product is amplified in the reaction.

To assess the effect of cell stimulation, gene expression levels were normalized to the reference gene 18s and differences between treated and control samples expressed as fold change. Fold changes were calculated according to the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen 2001) as follows: The Cq value of the reference gene (18s) was subtracted from the Cq value of the gene of interest in the control sample (DMSO) to generate the ΔCq value of the control and the same procedure was repeated for the treatment sample (thapsigargin) generating the ΔCq of the sample. Then, the ΔCq control was subtracted from the ΔCq sample (ΔCq sample-ΔCq control) to generate the $\Delta\Delta$Cq value, to finally calculate the fold change as $2^{-\Delta\Delta Cq}$. A minimum fold change value of 2 was arbitrarily assigned as a cut-off to demonstrate gene induction in the stimulated cells.

2.1.5   PCR product purification and sequencing

Double stranded DNA fragments from PCR reactions (the PCR products) were purified using the MinElute PCR purification kit (Qiagen) as per manufacturer's instructions. Purified RT-qPCR products were mixed with 6x stop solution (1 part of 2.5% bromophenol blue (Sigma

Aldrich), 1 part of 2.5% xylene cyanol (Bio-Rad Laboratories), 3 parts of glycerol (Sigma Aldrich) and 5 parts of distilled water) and loaded onto a 2% agarose gel prepared with ultra-pure agarose (Invitrogen) in 0.5X TBE buffer (Tris base, boric acid and EDTA in ultrapure water) and 5 µl of SYBR® Safe DNA free stain (Invitrogen). Gels were electrophoresed at 110 volts until the dye front was within a few centimeters of the end of the gel. A 1kb plus DNA ladder (Invitrogen) was electrophoresed alongside the samples to estimate the size of the PCR products. DNA bands were visualized in an Alphaimager (Alpha Innotech Cell Biosciences Santa Clara, California, USA).

Sanger sequencing of the PCR products using the UPR primer sets (Table 2.2) was performed by the Plant Biotechnology Laboratory, National Research Council, University of Saskatchewan, Saskatoon, SK, Canada. The sequenced products were matched to sequences published in GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul, Gish et al. 1990) software available from the NCBI website (Table 2.3).

2.1.6   UPR activation in E.Derm Cells

Chemicals used for cell treatment were obtained from Sigma Aldrich (St. Louis, Missouri, USA). DMSO (Dimethyl sulfoxide) was used at a final concentration of either 0.4 µl or 0.8 µl per ml and thapsigargin (750µM in DMSO) was used at a final concentration of either 300nM or 600nM.

Experimental design diagram
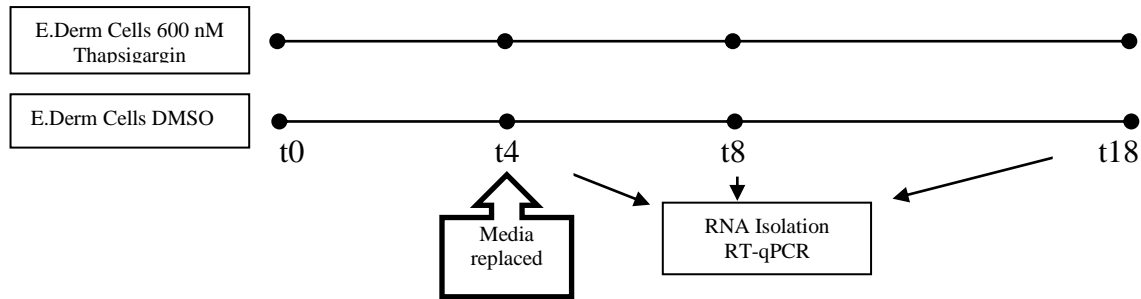
E.Derm cells were plated at $2x10^5$ in 6-well plates and grown until 80% confluence. The general design for this experiment was to induce ER stress in cultured E.Derm cells by treatment with thapsigargin for 4 h (Bergeron, Zhang et al. 2012), followed by RNA isolation and RT-qPCR to detect UPR gene expression. Cells incubated with DMSO were used as a negative control. Thapsigargin was the drug selected in this project to activate the UPR, because it was currently in use in our laboratory for other projects with successful results (Bergeron, Zhang et al. 2012). It has also been stated in the literature that "thapsigargin induces a more rapid and stronger UPR than tunicamycin" (Gülow, Bienert et al. 2002).

To initially test the primers and validate the PCR products by sequencing, the treatment was 4h incubation with 300nM thapsigargin compared to DMSO control. In a second experiment, sample collection was modified to obtain information about the expression of the UPR genes over a period of time after inducing ER stress as one of the later objectives (objective 3) of this project was to evaluate the effect of UPR activation on viral gene expression during replication of EHV-1. Considering that during lytic EHV-1 infection, transcripts of some viral genes can be detected starting at 4 h post infection while others can be detected as late as 18 h post infection, the UPR was evaluated over a period of 18 h. Cells were treated at time 0 by

adding 600 nM of thapsigargin or DMSO, treatment was removed and fresh media added at 4h, and cells were harvested at 4h, 8h or 18h following treatment for total RNA collection and RT-qPCR to detect UPR gene expression. At each time point, the expression of each UPR gene after thapsigargin treatment was compared to the expression of the same gene in cells treated with DMSO, and expressed as fold change. The experiments were performed in duplicate and repeated on 9 different occasions. A higher concentration of thapsigargin was chosen for the second experiment because I found that using a higher dose did not have detrimental effects on the E.Derm cells.

2.1.7   UPR activation in PBLs

Experimental design diagram:
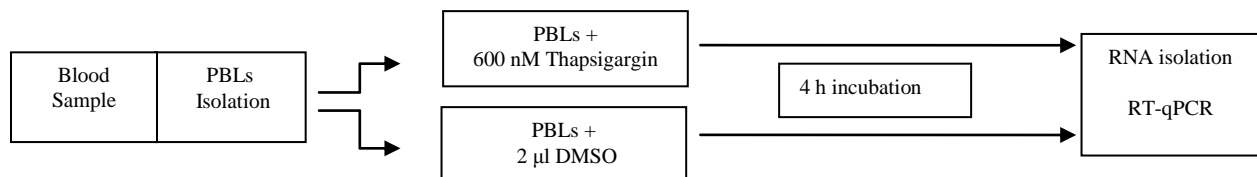
In order to evaluate if UPR gene expression could be activated and measured in equine PBLs, the same UPR primers used in the E.Derm cell experiments plus a set for amplification of TNFα mRNA were used (Table 2.4). Amplification of tumor necrosis factor α (TNFα) was used as described previously (Figueiredo, Salter et al. 2009) to evaluate a common PBLs gene transcript that is induced by cellular stress (Table 2.4). It was used as a positive control of PBLs activation in response to the thapsigargin treatment and to ensure that DMSO treatment did not induce PBLs activation.

Induction of ER stress in the PBLs was achieved   by treatment with 600nM thapsigargin or DMSO (negative control) for 4 h. Cells were harvested, RNA isolated and RT-qPCR

performed. The expression of each gene after thapsigargin treatment was compared to the expression of the same gene in cells treated with DMSO, and expressed as fold change. The experiments were performed in duplicate and repeated on 4 different occasions.

2.1.8 Statistical analysis

UPR gene expression (as ΔCq) in thapsigargin stimulated E.Derm cells at individual time points was compared to UPR gene expression (as ΔCq) in DMSO treated E.Derm cells. Replicate values within each experiment were averaged. Data from all experiments was analyzed by paired T-test with a rejection level of p value > 0.05, using StataIC 10 software (StataCorp. 2007. Stata Statistical Software: Release 10. College Station, TX: StataCorp LP). For graphing purposes in the figures, data are expressed as the fold change of stimulated versus unstimulated cells.

2.2 Results

2.2.1 Primer specificity

Primer sequences and characteristics for all genes are shown in table 2.2. E.Derm cells stimulated for 4h with 300 nM thapsigargin were used to validate the primers for the UPR genes and 18s ribosomal RNA. The size of the PCR products, estimated from agarose gels, agreed with the expected product sizes (Figure 2.1). The nucleotide similarities for the analyzed sequences (Table 2.3) were as follows: GRP78 and CHOP 99% and HERP 98% similarity without gaps; XBP1s 98% similarity with 1 gap. The 18s amplified PCR product agreed 100% with the

compared database sequence for that gene. Expression of the 18s ribosomal RNA was not modified by the treatments and remained stable for at least 18 h, as is shown in Figure 2.2.

2.2.2   Primer characteristics:

Primer efficiency: Using cDNA dilutions obtained from E.Derm cells treated with 300 nM thapsigargin for 4 h, the following efficiency values were found to be acceptable (Figure 2.3 A): reference gene 18s primers (105%), GRP78 gene primers(143%) and XBP1s gene primers (108%). On the other hand, HERP and CHOP gene primers showed efficiency values higher than 200% (520% and 366%, respectively) and were considered not ideal. The dissociation curves for CHOP and HERP genes suggested that mispriming or primer-dimer artifact occurred during the PCR amplification (described later in this section). However, the identity of the amplification product for both primer sets was established after thapsigargin treatment of the cells and an increase of the expression of both CHOP and HERP transcripts was consistently determined. I concluded that even though the efficiency for these primer sets (CHOP and HERP) was not ideal, and accurate quantification of gene expression was not possible, the primers were specific as the product of amplification was identical to the expected product.

An amplification plot depicts the fluorescence signal, an indicator of product concentration, at each cycle number (Figure 2.3 B). A horizontal line represents the threshold, and the cycle at which the fluorescence signal first exceeds the threshold is the Cq or quantification cycle value (this is often also referred to as the cycle threshold or Ct). A Cq is determined for each target. There is an inverse relationship between the Cq value and the quantity of product. As can be seen in Figure 2.4, treatment of E.Derm cells with thapsigargin

resulted in induction of gene expression for all UPR-related genes as shown by a lower Cq value (i.e. the curve shifts to the left) when compared to the Cq value in DMSO-treated control cells.

The temperature at which the two strands of a double-stranded PCR product disassociate is a reflection of the nucleotide sequence and length of the product. Following a RT-qPCR reaction, the product is heated and its fluorescence, measured at each degree rise in temperature, is expressed as a change in fluorescence per degree. The resulting dissociation curve provides an indication of the homogeneity of the product (Figure 2.3 C). Mixed products yield multiple or aberrant dissociation profiles. Figure 2.3 C shows the dissociation curves for PCR products for each gene. For 18s, XBP1s and GRP78, the dissociation curves represent homogeneous PCR products as each is composed of single peak. The melting curves for CHOP and HERP were different. It can be noted that there is a "shoulder" in the CHOP curve, before it reached the peak at the temperature described for that product. The melting curve for HERP also shows a peculiarity. There is a primary curve followed by the expected melting curve with the characteristic peak. This primary curve could correspond to a product different than the expected product. Such products are the results of mispriming, i.e. the annealing of primers to sequences on non-target DNA that are partially complementary to the target DNA. Primers can also form primer dimer artifacts by annealing to themselves and creating small templates for PCR amplification. The presence of any of these structures could explain the increased apparent efficiency values calculated. However, as described previously, the specificity of the primers was confirmed as the identity of the product being amplified in the PCR reaction corresponded to the target genes.

2.2.3 UPR activation in equine dermal cells

To examine the UPR activation over an 18-hour period, E.Derm were treated with thapsigargin or DMSO for 4h, and total RNA was collected for detection of UPR gene expression by RT-qPCR at 4h, 8h or 18h (Figure 2.5). At each time point, the expression of each gene after thapsigargin treatment was compared to the expression of the same gene in cells treated with DMSO, and expressed as fold change.

For the GRP78 gene, CHOP gene and HERP gene there was an increase in gene expression in thapsigargin-treated cells at each time point compared to DMSO treated cells). The fold changes for GRP78 expression at t4, t8 and t18, respectively, were: 5.957 (p=<0.0001); 13.913 (p=0.02) and 20.710 (p=0.004). The fold changes for CHOP expression at t4, t8 and t18, respectively, were: 25.789 (p=<0.0001); 23.761 (p=0.019) and 26.598 (p=0.0008). The fold changes for HERP expression at t4, t8 and t18, respectively, were 42.459 (p=<0.0001); 30.688 (p=0.0003) and 35.149 (p=0.008). The fold change for XBP1s gene expression at t4 was 1.768 (p=0.278), at t8 was 3.319 (p=0.051) and at t18 was 3.265 (p=0.047) (Figure 3.5.). XBP1s gene expression was therefore not induced at t4, and at t8 and t18 the increase in gene expression was not as marked as it was for the other 3 genes. For that reason I decided to focus on the genes (HERP, CHOP and GRP78) that showed higher induction after treatment with thapsigargin. Unfortunately, efficiency for all the primer sets was not calculated until after these experiments were performed. Even though the efficiency for the CHOP and HERP primer sets was considered less than ideal, I decided to continue working with these primer sets as induction of gene expression after thapsigargin treatment was consistently detected and the correct PCR products were amplified.

2.2.4 UPR activation in equine peripheral blood leukocytes

Figure 2.6 shows that the UPR genes CHOP, GRP78 and XBP1s were consistently induced by cell treatment with thapsigargin, presenting fold change values of 13.4 (P=0.006), 31.1 (P=0.004) and 13.7 (P=0.01), respectively. The expression of HERP was not induced in PBLs by thapsigargin treatment (fold change value 0.09, p=0.60). Expression of the TNFα gene was induced by thapsigargin treatment presenting a fold change value of 41.8 (p=0.01). I concluded that the UPR was activated by the use of thapsigargin, although the profile of the genes being induced did not follow the same pattern as in E.Derm cells: HERP expression was not induced in PBLs while XBP1s expression was induced in PBLs. This may be a consequence of the different nature of the cells.

2.3   Discussion

To study UPR activation in equine cells, primers were designed and tested in equine dermal cells (E.Derm) treated with thapsigargin, a chemical compound that produces a calcium imbalance in the cells and induces ER stress. Expression of the 18s gene remained stable for the full 18h of the experiment and was not affected by thapsigargin treatment, which confirmed that it was a useful reference gene for the gene expression experiments. The primers that were designed to identify the expression of the UPR genes HERP, CHOP, GRP78 and XBP1s were confirmed to amplify the correct targets in E.Derm cells. Although primer characteristics were not ideal for all the primers, the RT-qPCR assay was considered to be sufficiently useful for use in further experiments. Mispriming or primer dimer artifacts may have occurred when using the

CHOP and HERP primers, however, this did not interfere with amplification of the correct product.

I concluded that the primers correctly amplified horse UPR gene transcripts and that the UPR in equine cells was activated by the use of thapsigargin. This is the first report addressing UPR activation in cells of equine origin. The UPR remained activated in E.Derm cells for an 18-hour period, evaluated at 3 different time points, after thapsigargin treatment. The profile of the genes induced in PBLs was not the same as in E.Derm cells: HERP expression was induced in E.Derm cells but not in PBLs, and conversely, XBP1s expression was induced in PBLs but not in E.Derm cells. This difference in gene expression between the E.Derm and PBLs was not expected and may be a consequence of the different nature of the cells. Dermal cells are cells with higher metabolic activity compared to PBLs, and it is possible that some cellular pathways related to the UPR present different activation profiles in these 2 cell types.

Table 2.1 Selected UPR genes: names, abbreviations and NCBI accession numbers.

Name	NCBI Accession number
Equus caballus similar to X-box binding protein 1 isoform XBP1s	(LOC100059209), mRNA XM_001916129
Equus caballus 78 kDa glucose-regulated protein-like GRP78	(LOC100067235), miscRNA XR_131575.1
Equus caballus DNA damage-inducible transcript 3 protein-like CHOP	(LOC100053417), mRNA XM_001488999.1
Equus caballus homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 2 protein-like HERP	(LOC100055464), mRNA XR_036041.3
Equus caballus 18S ribosomal RNA (18s) gene	AJ311673.1

Table 2.2 Details of primer pairs as obtained from the Primer3 (v.0.4.0) software

18S rRNA	Sequence (5'->3')	Template strand[1]	Length[2]	Start[3]	Stop[4]	Tm[5]	GC%[6]
Forward primer	AAACGGCTACCACATCCAA	Plus	19	426	444	58.99	47.37
Reverse primer	TCGGGAGTGGGTAATTTGC	Minus	19	479	461	60.85	52.63
Product length[7]	54						
HERP	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	CCGAGCCTGAGCCCGTCACG	Plus	20	102	121	67.61	75.00
Reverse primer	CTTTGGAAGCAAGTCCTTGA	Minus	20	313	294	55.87	45.00
Product length	212						
CHOP	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	TGGAAGCCTGGTATGAGGAC	Plus	20	149	168	58.80	55.00
Reverse primer	TGCCACTTTCCTCTCGTTCT	Minus	20	489	470	58.95	50.00
Product length	341						
XBP1 s	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	GCGGGTCTGCTGAGTCTGCAGC	Plus	22	486	507	71.13	68.18
Reverse primer	GGGTGCTTCCTCAATTTTC	Minus	19	867	849	57.16	47.37
Product length	383						
GRP78	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	GGCTTGGATAAGAGGGAAGG	Plus	20	844	863	57.35	55.00
Reverse primer	GGTAGAACGGAACAGGTCCA	Minus	20	1230	1211	59.03	55.00
Product length	387						

---

[1] Refers to the direction of the RNA strand to which the primer anneals
[2] Primer length (in bp)
[3] Nucleotide position where the primer starts
[4] Nucleotide position where the primer stops
[5] Melting temperature (in °C) of the primer, the temperature at which the primer disassociates from the template.
[6] Guanine-cytosine content of the primer (in percent)
[7] Product length (in bp)

Table 2.3 Sequence alignments between the RT-qPCR amplification products (top) and the published equine mRNA sequences (bottom). Percentage of identity and percentage or number of gaps is indicated. The black arrow shows a gap; bold underlined letters show mismatches.

<u>**GRP78**</u>

Identities = 343/345 (99%), Gaps = 0/345 (0%)

```
GRP78        16   CGAGTCAGGGTCTCAGAGAAGTCTTCTCCTTCATAGAAGGATTCAATTTCAATTCTTGCT 75
XR_131575.1 1277  CGAGTCAGGGTCTCAGAGAAGTCTTCTCCTTCATAGAAGGATTCAATTTCAATTCTTGCT 1218

GRP78        76   TGATGTTGAGAAGACAGGGCCCGTTTGGCCTTTTCTACCTCACGCCGGAGTTTCTGCACA 135
XR_131575.1 1217  TGATGTTGAGAAGACAGGGCCCGTTTGGCCTTTTCTACCTCACGCCGGAGTTTCTGCACA 1158

GRP78        136  GCTCTGTTGTCTTTCCGAACATCTTTGCCAGTCTTCTTTTTGTAGAGCTTGATGAAGTGT 195
XR_131575.1 1157  GCTCTGTTGTCTTTCCGAACATCTTTGCCAGTCTTCTTTTTGTAGAGCTTGATGAAGTGT 1098

GRP78        196  TCCATGACACGCTGGTCGAAGTCTTCTCCCCCCAGATGAGTATCTCCATTAGTGGCCACA 255
XR_131575.1 1097  TCCATGACACGCTGGTCGAAGTCTTCTCCCCCCAGATGAGTATCTCCATTAGTGGCCACA 1038

GRP78        256  ACTTCGAAGACACCATTATCAATGGTGAGAAGAGACACATCGAAGGTTCCACCACCCAGG 315
XR_131575.1 1037  ACTTCGAAGACACCATTATCAATGGTGAGAAGAGACACATCGAAGGTTCCACCACCCAGG 978

GRP78        316  TCAAACACCAGGATGTTCTTCTCCCCTTCCCTCT**A**ATCCA**A**GCCA 360
XR_131575.1 977   TCAAACACCAGGATGTTCTTCTCCCCTTCCCTCT**T**ATCCA**G**GCCA 933
```

<u>**CHOP**</u>

Identities = 297/298 (99%), Gaps = 0/298 (0%)

```
CHOP           17  CGTTGCTTT**T**CAGCCCGGGCTGGGGACTGGCCACTCTGTTTCCGTTTCCTAGGTCTTACT 76
XM_001488999.1 353 CGTTGCTTT**C**CAGCCCGGGCTGGGGACTGGCCACTCTGTTTCCGTTTCCTAGGTCTTACT 294

CHOP           77  TGGTCTTCCTCTTCTTCCTCCTGAGCCAGGGAGCTCTGACTGGAATCTGGAGAGCGAGGG 136
XM_001488999.1 293 TGGTCTTCCTCTTCTTCCTCCTGAGCCAGGGAGCTCTGACTGGAATCTGGAGAGCGAGGG 234

CHOP           137 CTCTGGGAGGTGCTCGTGACCTCTGCTGGCCCTGGTTCCTCGGTCAGCCACGCCAGAGAG 196
XM_001488999.1 233 CTCTGGGAGGTGCTCGTGACCTCTGCTGGCCCTGGTTCCTCGGTCAGCCACGCCAGAGAG 174

CHOP           197 GCGGGGTCAAGAGTGGTGAAGGTTTTTGATTCTTCCTCCTCGTTTCCAGGGGGTGAGACA 256
XM_001488999.1 173 GCGGGGTCAAGAGTGGTGAAGGTTTTTGATTCTTCCTCCTCGTTTCCAGGGGGTGAGACA 114

CHOP           257 TAGGTACCCCCATTTTCATCTGAGAACAGCACCTCCTGCAGGTCCTCATACCAGGCTT 314
XM_001488999.1 113 TAGGTACCCCCATTTTCATCTGAGAACAGCACCTCCTGCAGGTCCTCATACCAGGCTT 56
```

HERP

Identities 166/169(98%) Gaps 0/169(0%)

```
HERP          14  GCGCCACCGCGACTTGGAGCTGAGCGGCGACCGCAGCTGGAGCGTGGGCCGCCTCAAGGC  73
XR_036041.3  201  GCGCCACCGCGACTTGGAGCTGAGCGGCGACCGCAGCTGTAGCGTGGGCCGCCTCAAGGC  260

HERP          74  TCATCTGAGCCGCGTCTACCCCGAGCGCCCGCGGCCGGAGGACCAGAGGTTAATTTATTC  133
XR_036041.3  261  TCATCTGAGCCGCGTCTACCCCGAGCGCCCGCGGCCGGAGGACCAGAGGTTAATTTATTC  320

HERP         134  CGGGAAGCTCTTGTTGGATCACCAGTGTCTCAAGGACTTGCTTCCAAAG  182
XR_036041.3  321  CGGGAAGCTCTTGTTGGATCACCAGTGTCTCAGGGACTTGCTCCCAAAG  369
```


XBP1s

Identities = 371/377 (98%), Gaps = 1/377 (0%)

```
XBP1s            7  CTTGACTCTCTGTCT-AGAGGGTATCTCTAAGACTAGGGGTTTGGTATATACGTGGTCAA  65
XM_001916129.2  886  CTTGGCTCTCTGTCTCAGAGGGTATCTCTAAGACTAGGGGTTTGGTATATACGTGGTCAA  827

XBP1s           66  AACGAATCAGTTCATTAATGGCTTCCAGCTTGGCTGATGACGTCCCCAGTGATGGAGAAG  125
XM_001916129.2  826  AACGAATCAGTTCATTAATGGCTTCCAGCTTGGCTGATGACGTCCCCAGTGATGGAGAAG  767

XBP1s          126  GGGAGGCTGGTAAGGAACTGGGTCCTTCTGGGTAGACCTCTGGGAGTTGCTCCAGGCTGG  185
XM_001916129.2  766  GGGAGGCTGGTAAGGAACTGGGTCCTTCTGGGTAGACCTCTGGGAGTTGCTCCAGGCTGG  707

XBP1s          186  TCGATTCTGGGGAAGGACATCTGAAGAACATGACTGGGTCCAAGTTGAACAGAATGCCCA  245
XM_001916129.2  706  TCGATTCTGGGGAAGGACATCTGAAGAACATGACTGGGTCCAAGTTGAACAGAATGCCCA  647

XBP1s          246  ACAGGATATCAGACTCAGAGTCTGAAGAGTCCACACTGTCAGAATCCATGGGGAGAGGTT  305
XM_001916129.2  646  ACAGGATATCAGACTCAGAGTCTGAAGAGTCCACACTGTCAGAATCCATGGGGAGAGGTT  587

XBP1s          306  CTGGAGGGGTGACAACTGGGCCTGCACCTGCTGCAGAGGCGCACGTACCCCGAGTGCTGC  365
XM_001916129.2  586  CTGGAGGGGTGACAACTGGGCCTGCACCTGCTGCAGAGGCGCACGTAGACTGAGTGCTGC  527

XBP1s          366  AGACTCAGCAGACCCGC  382
XM_001916129.2  526  GGACTCAGCAGACCCGC  510
```


18s

Identities = 54/54(100%), Gaps = 0/54(0%)

```
18s            1  AAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCACTCCCGA  54
AJ311673.1   426  AAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCACTCCCGA  479
```

Table 2.4 Primer set for amplification of equine TNFα.

TNFα	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	AAAGGACATCATGAGCACTGAAAG	Plus	24	26	49	59.54	41.67
Reverse primer	GGGCCCCCTGCCTTCT	Minus	16	106	91	60.69	75.00
Product length	81 bp						
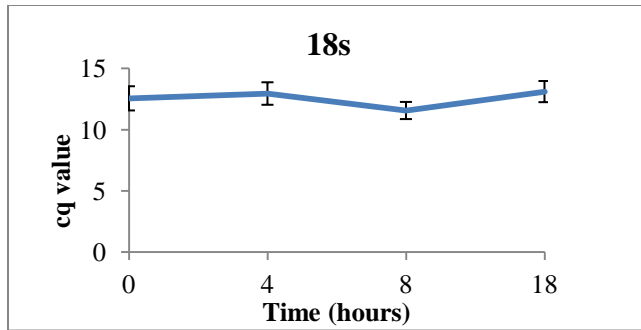
Figure 2.1 Expression of 18s over an 18 hour period after thapsigargin treatment. RT-qPCR was performed on E.Derm cells treated for 4 h with thapsigargin. Cells were harvested and RNA isolated at 0, 4, 8 and 18 h. Time 0 is the time when treatment was started. Time 4 is the time when treatment was removed. Cq values at each time point represent averages from 4 experiments, and standard error bars are presented.

A)

Gene	Expected Product size (bp)
XBP1s	383
HERP	212
CHOP	341
GRP78	387
18s	54

B)

Figure 2.2 PCR product identification. A) Table showing expected sizes of RT-qPCR products XBP1s, HERP, CHOP, GRP78 and 18s. B) Agarose gel electrophoresis of RT-qPCR purified products and 1Kb ladder marker (bp).

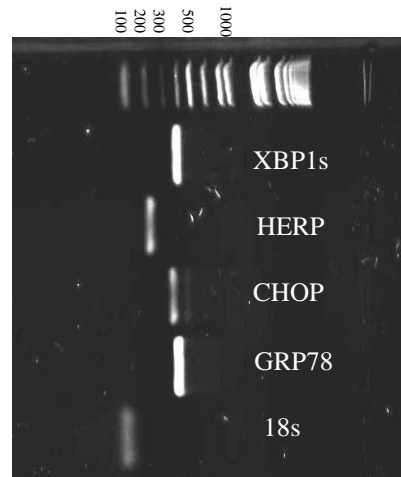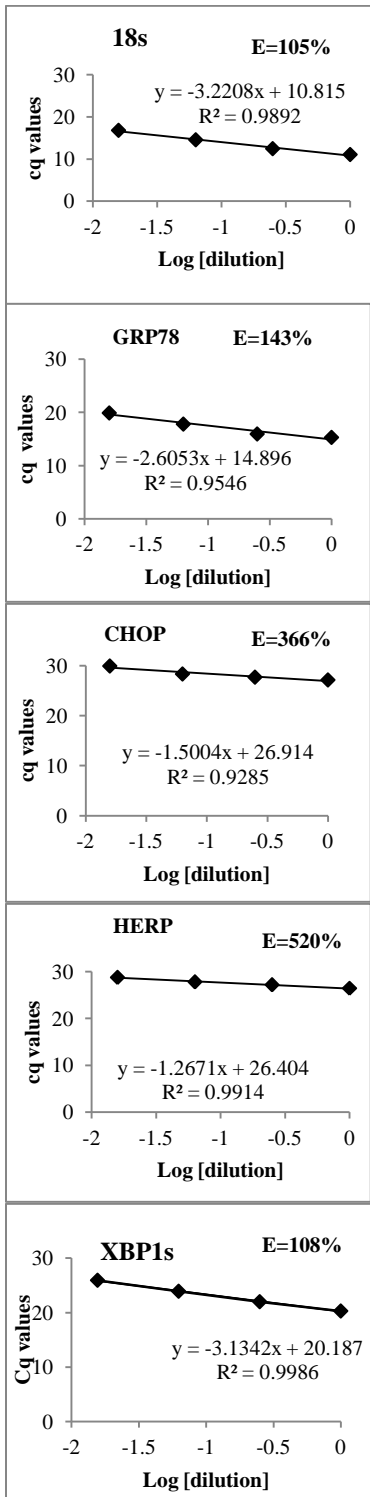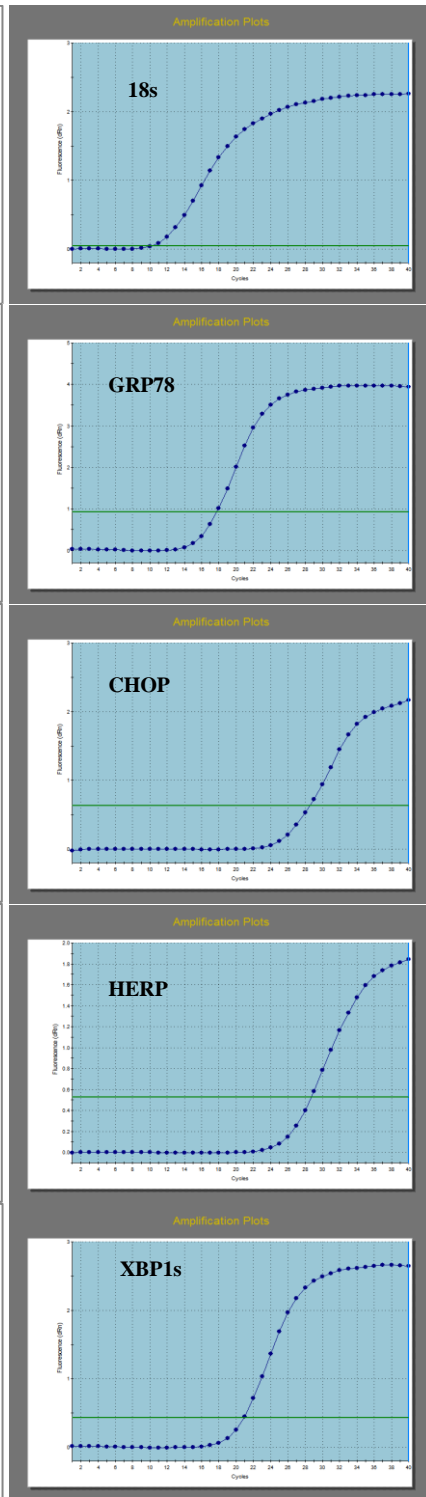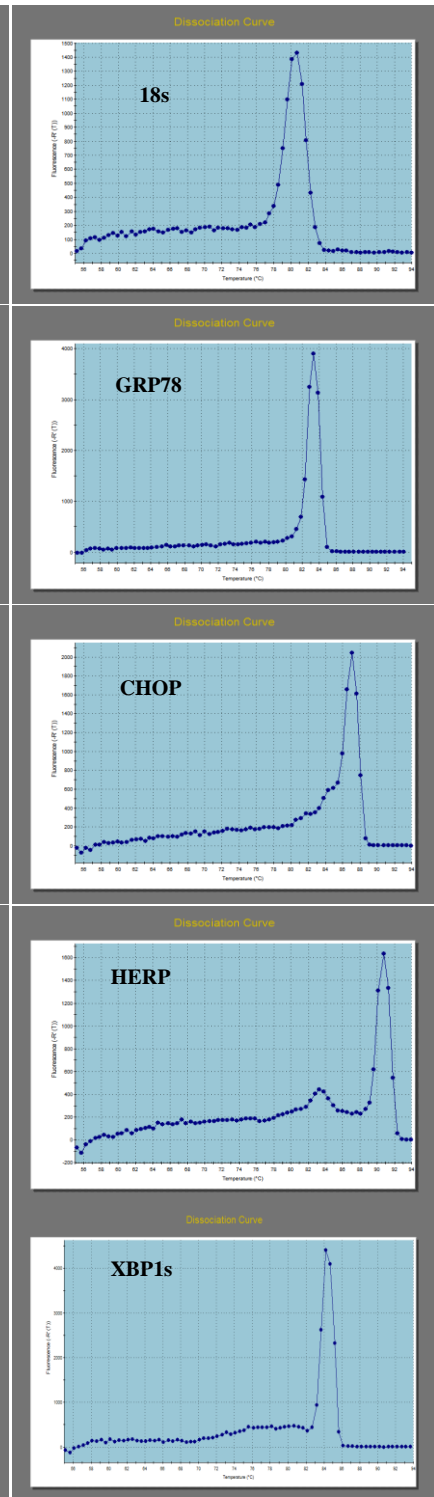**Figure 2.3 UPR primer and product characteristics.** A) Calibration curves for each primer set. Amplification results for serial 4-fold dilutions of E.Derm cDNA were plotted as Cq values versus Log [dilution]. The trendline, the equation of the curve, the slope of the curve, the R squared value (R^2) and the efficiency (E), in percent, are presented. B) Amplification plot curves for each primer set. A sigmoid curve represents the plotting of the fluorescence signal versus the cycle number. There is an inverse correlation between the Cq value and the quantity of product. C) Dissociation curves for each primer set. The fluorescence signal emitted by dissociating double stranded cDNA is plotted as a function of rising temperature. Figures are from one representative experiment.
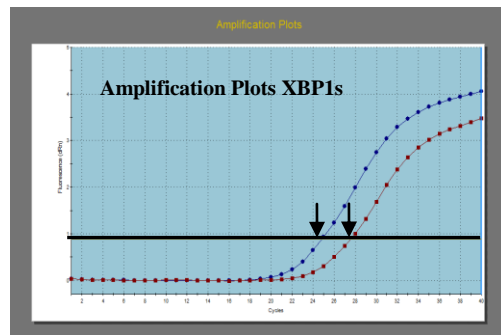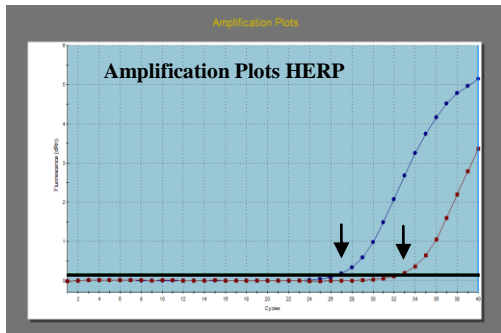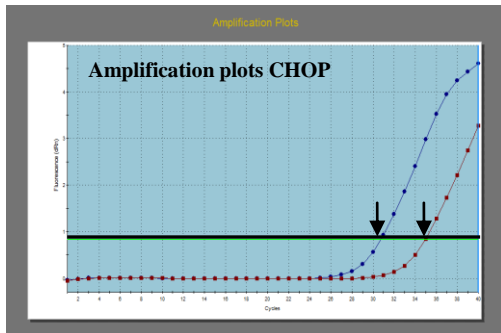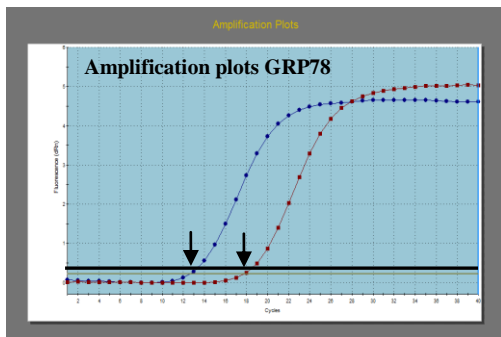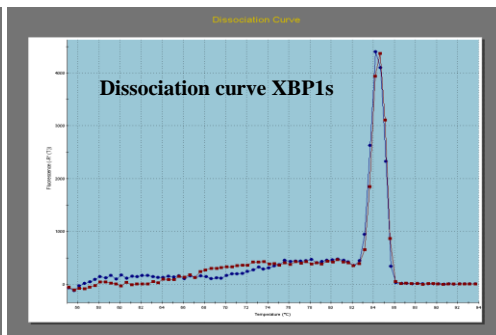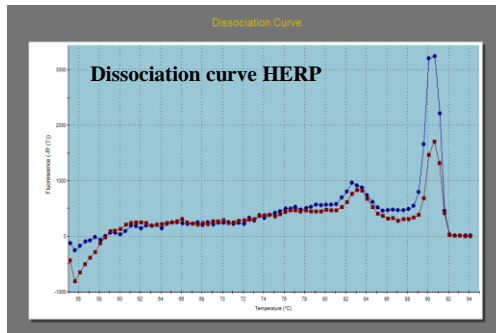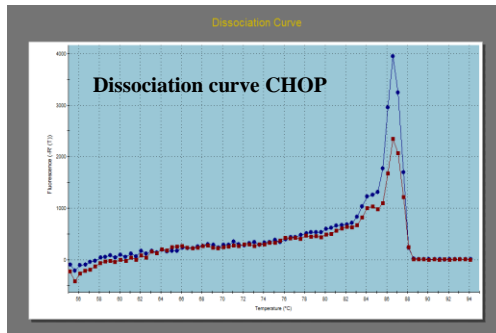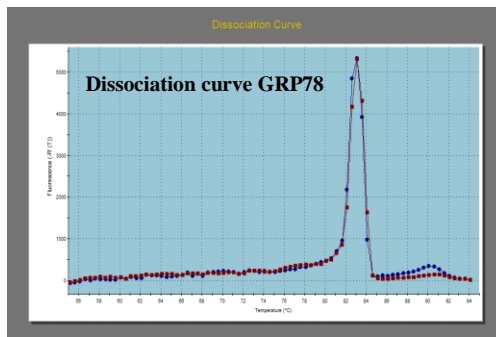
Figure 2.4 Amplification plots and dissociation curves for GRP78, CHOP, HERP and XBP1s. Blue lines represent thapsigargin treated E.Derm samples. Red lines represent DMSO treated control samples. A) Amplification plots. Thapsigargin treated samples (blue lines) shift to

the left with respect to DMSO treated samples (red lines) as transcripts for that gene increase in the sample and correspondingly, Cq values decrease. Black arrows indicate the Cq value as the point where the curve crosses the horizontal threshold (black line) B) Dissociation curves showing melting characteristics of the PCR products. Note that the red curves for CHOP and HERP follow the same pattern as the blue curves. The difference in height may be due to the quantity of product present in DMSO treated samples (red line), which is less than that in thapsigargin treated samples (blue line). Figures are from one representative experiment.
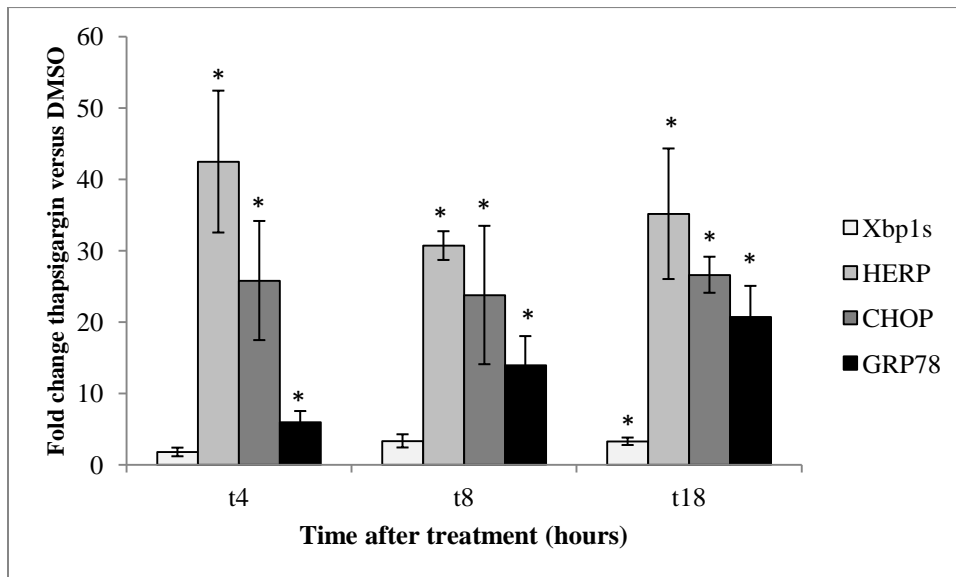
Figure 2.5 Expression of UPR genes in E.Derm cells after thapsigargin treatment. E.Derm cells were treated with 600 nM of thapsigargin or with 2 µl of DMSO (control). At t4, t8 and t18 (hours after treatment was added), cells were harvested, cellular RNA was isolated, and RT-qPCR was performed. Gene expression was calculated as fold change (see materials and methods) of thapsigargin treated cells versus DMSO treated cells. Error bars represent standard error, n=9. * $p < 0.05$
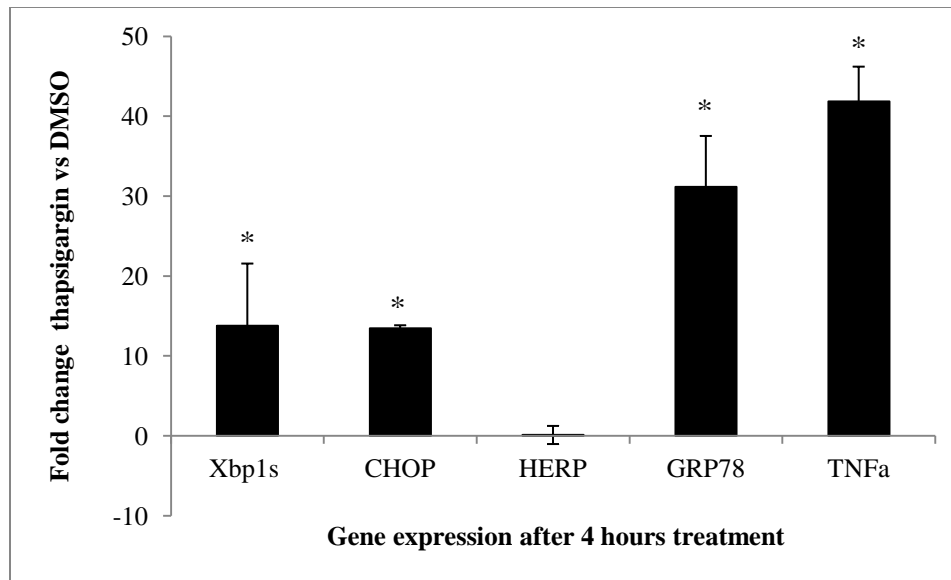
Figure 2.6 Expression of UPR genes and TNF alpha in PBLs after thapsigargin treatment.
PBLs were isolated from a whole blood sample and treated for 4 h with 600 nM of thapsigargin
or with 2 µl of DMSO as a control. After 4 h, PBLs RNA was isolated and RT-qPCR performed.
Gene expression was calculated as fold change (see materials and methods) of thapsigargin
treated cells versus DMSO treated cells. n=4 Error bars represent standard error. * p<0.05

3 LYTIC EHV-1 INFECTION IN EQUINE CELLS

The second objective of this project was to characterize lytic EHV-1 infection in equine dermal cells and detect the expression of EHV-1 genes over time. Toward the first goal, E.Derm cells were inoculated with EHV-1 and viral replication was assessed by visual inspection of the cells and a single step growth curve was produced in order to determine the dynamics of EHV-1 replication. The replication cycle of viruses can be divided into three basic components when assessed by a single step growth curve: the attachment period, the eclipse period and the period of release of virus particles. The attachment and penetration period corresponds to the initial contact of the virus with the host cell. During the eclipse period, infectious virus cannot be detected as synthesis, assembly and maturation of virions is taking place. During the release of mature virions, an exponential increase in the concentration of infectious virus occurs. The elapsed time from the attachment period to the release of infectious mature virions is characteristic for each virus. I wanted to investigate the time frame of EHV-1 growth in cultured cells to correlate the landmarks in the replication cycle with the gene expression during lytic infection.

Toward the second goal, a RT-qPCR for detection of EHV-1 genes representing the different temporal gene classes was designed, and the expression of EHV-1 genes of different temporal classes was determined over an 18-hour time period following infection of E.Derm cells. The process of reactivation from latency is thought to resemble the lytic cycle, as both processes require expression of lytic viral proteins. The molecular events involved in the establishment, maintenance and reactivation from latency for EHV-1 have not been elucidated, but in order to understand them, it is first necessary to comprehend the molecular events during the lytic cycle. Since there are relatively little comparative data on the regulation of viral gene

expression during the lytic cycle of EHV-1, I examined viral gene expression during EHV-1 infection of equine dermal cells (E.Derm). The experiments were also intended to confirm that I could reliably detect lytic EHV-1 infection by CPE detection and by viral gene expression in culture.

EHV-1 genes can be classified as Immediate Early (IE), Early (E) or Late (L) genes based on the order of their expression. Genes representing each temporal class were selected: the immediate early gene from the IE class, the ICP0 gene from the E class, and the gB and E-VP16 genes from the L class (Gray, Baumann et al. 1987). During lytic EHV-1 infection, transcripts of the IE gene and some E genes can be detected starting at 4 h post infection (HPI) while the late genes can be detected as late as 18 HPI (Caughman, Staczek et al. 1985; Gray, Baumann et al. 1987). For the gene expression experiments described here, time points were therefore selected to represent the early events before infectious virus is detected (t4), the beginning of infectious virus detection (t8) and the advanced cell infection (t18, see diagram in materials and methods). Cells were also harvested immediately upon removal of viral inocula (t0) for comparison to uninfected cells.

3.1 Materials and methods

3.1.1 Equine dermal cell culture

E.Derm cells were cultured as described in chapter 2.

3.1.2 Virus

A neuropathogenic (D_{752}) field isolate of EHV-1 was obtained from Prairie Diagnostic Services Inc. (PDS, Saskatoon, Saskatchewan, Canada). The virus was isolated from an affected horse during a 2008 EHM outbreak in Saskatoon and confirmed to be a neuropathogenic strain of EHV-1 by PCR testing (Animal Health Laboratory, University of Guelph, ON). Virus stock was originally grown in Vero cells and viral DNA was purified. Subsequent viral stocks were propagated in E.Derm cells. Briefly, E.Derm cells were washed once with DMEM without serum and virus, diluted 1:25 in DMEM without serum, was added. Cells with viral inocula were incubated for 1 h at 37°C. Complete DMEM was added to each flask and cells returned to the incubator at 37°C until CPE was detected.

For the plaque assay to determine virus titers (see below) serum containing neutralizing antibodies from one client-owned horse (an Arabian gelding, 22 years of age, that tested positive to EHV-1 during an EHM outbreak, referred as horse 2 in section 5.1.1) was used; a serum neutralization test was performed to determine levels of anti-EHV-1 antibodies in the serum. Forty thousand E.Derm cells were plated per well in a 24 well plate. One thousand plaque forming units (pfu) of EHV-1 were incubated with serial five-fold dilutions of the serum for 1 hour at 37°C before addition to each well containing E.Derm cells. Following incubation for 24 h at 37°C in a 5% CO_2 humidified atmosphere, plates were observed for cytopathic effect (CPE) to

determine the serum dilution that neutralizes viral growth. The neutralizing antibody titer was determined as the inverse of the highest dilution at which no plaques were detected. The neutralizing titer was 5, corresponding to the dilution 1:5.

Virus titers were determined by plaque assay as follows: E.Derm cells were plated in 24 well plates at 1×10^5 cells per well. Five hundred µl of serially diluted virus (10^{-2} to 10^{-7}) were added and cells incubated for 1 h at 37°C while plates were swayed every 15 minutes. Inocula were removed from wells and replaced with DMEM containing EHV-1 neutralizing antibody serum (see above). Plates were incubated at 37°C for 4 days until plaques were visible. Medium was removed; cells were fixed in methanol and stained with Giemsa stain (BDH Inc.) to visualize and count plaques. Giemsa stain was prepared as a 0.8% dilution of Giemsa powder in equal parts of methanol and glycerol. Plaques per well were counted and virus concentration calculated using the following formula:

$$\frac{\text{\# of plaques per well}}{d \times V} = \text{pfu/ml}$$

Where d= dilution factor and V= volume of diluted virus added to each well.

3.1.3   Lytic EHV-1 infection in equine dermal cells

E.Derm cells (1×10^6/well) were plated in 6 well plates and inoculated with EHV-1 at a multiplicity of infection (moi) = 0.01pfu/cell. The low moi allowed me to observe the infected culture as the virus spread from cell to cell. Two 6 well plates were inoculated and maintained for 6 days. The cultures were visually examined and photographed every day for characteristic alphaherpesvirus cytopathic effect (CPE). This CPE is characterized by focal rounding up of cells followed by an increase in refractility and detachment from the cell monolayer.

3.1.4   Single step growth curve

E.Derm cells were plated (1×10^5 cells/well) in 6 well plates and inoculated with EHV-1 at a moi of 1pfu/cell. After one hour of incubation at 37°C (5% CO2) to allow for virus attachment, the inocula were removed, fresh media was added and cells return to the incubator. Time 0 (t0) was defined as the time when inocula were removed and fresh media was added to the wells. Cells were harvested at t0, t4, t8, t12, t24 and t36. Plaque assays were conducted to determine the number of pfu per cell associated with the cultures at different times after infection. The experiment was performed in duplicate and repeated on 3 different occasions.

3.1.5   Polymerase chain reaction (PCR)

PCR reactions, purification of PCR products and sequencing were performed as described in chapter 2. Purified EHV-1 DNA from our viral stock was used to determine the specificity of the primers by RT-qPCR. EHV-1 DNA was isolated as described previously (Gray, Baumann et al. 1987). Viral genes were selected to represent the three classes of IE, E and L genes and GenBank accession numbers for the selected genes are shown in table 3.1. Primers for equine glycoprotein B and E-VP16 transcripts were designed as described in chapter 2 while primers for IE and ICPO transcripts were as described previously (Hasebe, Kimura et al. 2006). Characteristics of the latter two primer pairs were corroborated with the Primer 3 software. Gene expression at each time point was normalized to the 18s ribosomal RNA. By convention, RT-qPCR data is expressed as fold changes compared to control. For example, a sample treated with a drug may have 5 fold more RNA for a particular gene when compared to an untreated sample. For cells infected with a virus, this type of comparison is not possible since the obvious control – mock-infected cells – would contain no viral transcripts and would therefore have no Cq values

for any of the viral gene products. Viral gene expression at individual time points was therefore quantified with respect to gene expression at the time of infection (t0), i.e. the normalized value for a gene (ΔCq) at t0, when only background levels of transcripts would be present, was subtracted from the normalized value at each time point after infection. Gene expression was then reported as the minus maximum ΔCq value versus time zero. It should be noted that herpesviruses have the ability to shut down translation of cellular host mRNA and also have the ability to degrade mRNA. In contrast, levels of 18s ribosomal RNA remain constant (Figure 3.5 B) after herpesvirus infection (Nishioka and Silverstein 1977).

3.1.6   Expression of EHV-1 genes during lytic infection in E.Derm cells

Experimental design diagram:
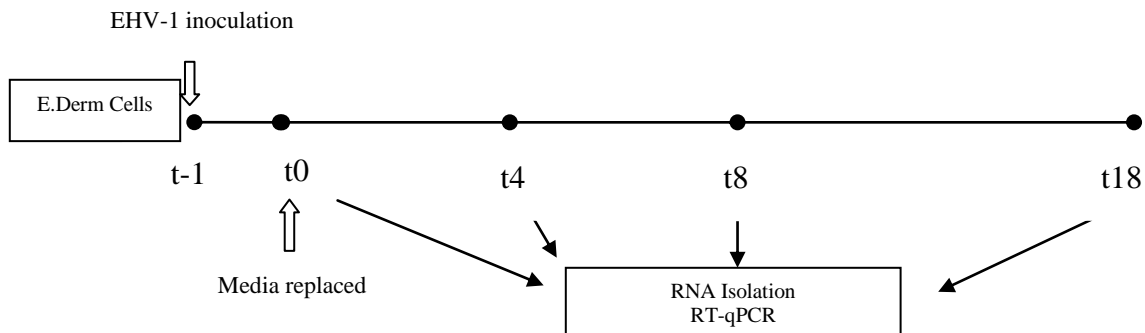
E.Derm cells were plated (2×10^5 cells/well) in 6 well plates and inoculated with EHV-1 at a moi of 5 pfu/cell (t -1). A relatively high moi was used to increase the probability of simultaneous infection of all cells in the culture. After one hour of incubation at 37°C, the inoculum was removed, fresh media was added and cells were returned to the incubator. Cells were harvested immediately after fresh media was added (t0), and at t4, t8 and t18 (see diagram). RNA was extracted as described in chapter 2 and EHV-1 gene expression was analyzed using

RT-qPCR. As a precaution, a DNAse treatment step was added during RNA extraction to assure that viral DNA was not amplified during the PCR reaction. Absence of DNA was confirmed by performing a qPCR without previous synthesis of cDNA in the sample. The experiment was performed in duplicate and repeated on 7 different occasions.

3.2 Results

3.2.1 Lytic EHV-1 infection in equine dermal cells

One day post inoculation CPE were detected in all wells. By day 6, almost 80% of all cells showed CPE. Figure 3.1 shows images representative of days after inoculation.

3.2.2 Single step growth curve

The growth of EHV-1 in E.Derm cells resembled that described previously (Hussey, Hussey et al. 2011). It should be noted that the virus concentration in Figure 3.9 at t0 represents the input virus concentration of 1 pfu/cell. No virus could be detected t4, corresponding to the eclipse period in virus replication (Dubovi and Maclachlan 2011). After the eclipse period, viral pfu increased between t8 and t12 from 3.35×10^{-3} to 0.6 pfu/cell. Viral production at t24 corresponded to 1.5 pfu/cell, approaching a maximum plateau level of 16 pfu/cell at t36. These values represent relatively poor yields of virus and suggest that although cells showed cytopathic effect relatively few infected cells produced infectious virus. Increase in the infectious virus concentration was detected from t8 and by t12, the lytic cycle was completed.

3.2.3   Primer specificity

The primer pairs used for detection of EHV-1 gene transcripts are detailed in Table 3.2.

QPCR was performed on purified EHV-1 DNA and the product sizes estimated by gel electrophoresis matched the expected sizes (Figure 3.3). PCR product were sent for sequencing and the nucleotide sequences for the ICP0, IE, E-VP16 and gB amplified PCR products agreed 100% with the compared database sequence for each gene without gaps (Table 3.3).

3.2.4   Primer characteristics

Using serial 4 fold dilutions of viral DNA for each gene, the following efficiency values were found to be acceptable (Figure 3.4 A): the ICP0 gene primer set showed an efficiency of 112%; the E-VP16 gene primer set showed an effciency of 127%; the gB gene primer set showed an efficiency of 115% and the IE gene primer set showed an efficiency of 87%. The amplification plots and dissociation curves generated by the qPCR software for each gene are shown in Figure 3.4 B and C. The PCR products' characteristics suggested the correct identity for all the amplified targets.

3.2.5   Expression of EHV-1 genes during lytic infection in E.Derm cells

As shown in Figure 3.5 A, transcripts for the immediate early and early class of genes (IE and ICP0, respectively) were present at relatively higher levels at t4 than those for the late genes (E-VP16 and gB). However, transcripts for all genes continued to increase and expression of the IE, E and L genes was more similar at t8 and t18.

3.3   Discussion

Lytic EHV-1 infection in equine cells was detected by observation of CPE, infectious virus was detected at t8 and by t12 the replication cycle was complete. The growth of EHV-1 in E.Derm cells resembled that described previously (Hussey, Hussey et al. 2011).

EHV-1 gene expression was determined by RT-qPCR. Primers were designed and tested by qPCR in EHV-1 purified DNA. The primers that were designed to identify the EHV-1 genes IE, ICP0, E-VP16 and gB were confirmed to amplify the correct target in infected E.Derm cells by RT-qPCR. The expression of EHV-1 genes in E. Derm cells was assessed over the 18 h following an hour of incubation with the virus and replacement of media. At t4, expression of the IE and E genes was relatively higher than the expression of the L genes while this discrepancy disappeared at t8 and t18. The increased expression of all gene classes simultaneously can be explained because in culture, we can find infected cells that are at the first stages of infection expressing the IE gene and other cells that are in a later stage of infection expressing the E or the L genes in the same well. These findings do not represent a different temporal pattern of viral gene expression; it is a consequence of the experiment itself.

Table 3.1 Selected EHV-1 genes: names, abbreviations, NCBI accession numbers and gene classification.

Name	NCBI Accession number	Gene class
Equine herpesvirus type 1 immediate-early protein gene, *IE*	J04366.1	Immediate early α
Equine herpesvirus 1 ORF63 gene for transactivator protein, (homologue of HSV ICP0) *ICPO*	AB158517.1	Early β
Transactivating tegument protein, Equid herpesvirus 1 ORF12 (homologue of HSV VP16) *E-VP16*	YP_053057.2 NC_001491.2 From 13595 to 14953	Late γ_1
Equid herpesvirus 1 ORF33 gene for glycoprotein B (homologue of HSV glycoprotein B) *gB*	AB279609.1	Late γ_2

Table 3.2 Details of primer pairs as obtained from the Primer3 (v.0.4.0) software.

IE	Sequence (5'->3')	Template strand[1]	Length[2]	Start[3]	Stop[4]	Tm[5]	GC%[6]
Forward primer	CGACAGCGATACCGAGACCT	Plus	20	1434	1453	61.44	60.00
Reverse primer	TTCGTCGCTGTCGCTGTCGT	Minus	20	1647	1628	64.77	60.00
Product length[7]	214						

E-VP16	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	CGGGGTAGTGATCTTGGAAA	Plus	20	14446	14465	56.93	50.00
Reverse primer	TCGGTCGTTGTACCGTATGA	Minus	20	14751	14732	58.55	50.00
Product length	306						

ICPO	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	TTTTTGGCCGTGGATTCTGG	Plus	20	259	278	59.03	50.00
Reverse primer	AGTTCTGCTTGGACGATGAG	Minus	20	566	547	57.62	50.00
Product length	308						

Glycoprotein B	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	GGCAGACCACCAACGATACT	Plus	20	812	831	59.75	55.00
Reverse primer	CGACTTTCTTCTCGGTCCAG	Minus	20	1159	1140	58.02	55.00
Product length	348						

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[1] Refers to the direction of the RNA strand to which the primer anneals
[2] Primer length in bp
[3] Nucleotide position where the primer starts
[4] Nucleotide position where the primer stops
[5] Melting temperature (in °C) of the primer
[6] Guanine-cytosine content (in percent) of the primer
[7] Product length in bp

Table 3.3 Sequence alignment between the RT-qPCR amplification products (top) and the published EHV-1 genes (bottom). Percentage of identity and percentage or number of gaps.

ICP0

Identities = 203/203 (100%), Gaps = 0/203 (0%)

```
ICP0        1    CGAGTGAGCTCTTCCTCGTTACAGCCATGCTCGCACAGTGTATCCATAACAATGTTCCGC  60
U81154.1    755  CGAGTGAGCTCTTCCTCGTTACAGCCATGCTCGCACAGTGTATCCATAACAATGTTCCGC  696

ICP0        61   ATCACCAACGCTAGCTCTGGGGTCTCGAATAGCTGGTCGAGCCTTTCGACCAGCCAGTCC  120
U81154.1    695  ATCACCAACGCTAGCTCTGGGGTCTCGAATAGCTGGTCGAGCCTTTCGACCAGCCAGTCC  636

ICP0        121  ACCAGTGGCTGCAGTCGGGGAGCCCCGGCAGTCCCATTAGCGTTGAGGGGCACAAATGCC  180
U81154.1    635  ACCAGTGGCTGCAGTCGGGGAGCCCCGGCAGTCCCATTAGCGTTGAGGGGCACAAATGCC  576

ICP0        181  ATGGGTCCGTTCCACGCAGAGAT  203
U81154.1    575  ATGGGTCCGTTCCACGCAGAGAT  553
```

IE

Identities = 162/162 (100%), Gaps = 0/162 (0%)

```
IE          1     CCCCGGCACCATCCGCCTGGTCGTCCTCGTCCATCGAGGACGAGGACGAGGACGACGATG  60
J04366.1    1600  CCCCGGCACCATCCGCCTGGTCGTCCTCGTCCATCGAGGACGAGGACGAGGACGACGATG  1541

IE          61    AGATGGAGATGCTCCGGACCCGGGGTGCCGGGGACCCTCCGCCCGGGGAGGCCGAGGATG  120
J04366.1    1540  AGATGGAGATGCTCCGGACCCGGGGTGCCGGGGACCCTCCGCCCGGGGAGGCCGAGGATG  1481

IE          121   GAAACTCGGGCTGCGGGGACCCCGGGCAGGTCTCGGTATCGC  162
J04366.1    1480  GAAACTCGGGCTGCGGGGACCCCGGGCAGGTCTCGGTATCGC  1439
```

E-VP16

Identities = 295/295 (100%), Gaps = 0/295 (0%)

```
E-VP16      1      ACCGTATGACAGGCGTCCCCCTATCCGTTTACTGTAGGAGTGCTCCGCCAGAGGAAAGAG  60
AY665713.1  14740  ACCGTATGACAGGCGTCCCCCTATCCGTTTACTGTAGGAGTGCTCCGCCAGAGGAAAGAG  14681

E-VP16      61     CGGGGTCGCCGGGTGCGCGTCCGAGTATGCCTCCATTTTGGTTCTTATCTGGTGCGTCAG  120
AY665713.1  14680  CGGGGTCGCCGGGTGCGCGTCCGAGTATGCCTCCATTTTGGTTCTTATCTGGTGCGTCAG  14621

E-VP16      121    AAAGCCAATAGTCCTGGGTAGCTTTCCCGAAAACAGAGGCTCAGCCTCGAGGGGGCTGTT  180
AY665713.1  14620  AAAGCCAATAGTCCTGGGTAGCTTTCCCGAAAACAGAGGCTCAGCCTCGAGGGGGCTGTT  14561

E-VP16      181    TTCTTCCTCGATGAGACCAGCGCGAATCAACGGTAGGCCAAGCTCGCGTCGGCGATAGTT  240
AY665713.1  14560  TTCTTCCTCGATGAGACCAGCGCGAATCAACGGTAGGCCAAGCTCGCGTCGGCGATAGTT  14501

E-VP16      241    TATCCGCTGTAGATCGTGGAACTCTAGGGGGTCGTTTTCCAAGATCACTACCCCG  295
AY665713.1  14500  TATCCGCTGTAGATCGTGGAACTCTAGGGGGTCGTTTTCCAAGATCACTACCCCG  14446
```

gB

Identities = 304/304 (100%), Gaps = 0/304 (0%)

gB   1   GTTCCAGCTGACGGTGACATGCGGGGTAGTGATAAAATTTTTGGTAACCGGCTCTTCGGC 60
AB279609.1   1140   GTTCCAGCTGACGGTGACATGCGGGGTAGTGATAAAATTTTTGGTAACCGGCTCTTCGGC 1081

gB   61   TTGTAGTTTACTGTCTAAGTCGCGGGGCCTGTACCCTTCAACTTGCCTGAAACGCTCCTG 120
AB279609.1   1080   TTGTAGTTTACTGTCTAAGTCGCGGGGCCTGTACCCTTCAACTTGCCTGAAACGCTCCTG 1021

gB   121   CGCGTAGCTATTGTGCTCTATGCGAGCGGCAGCCCTCAGGCCGTAAAACGGAGACGCGTA 180
AB279609.1   1020   CGCGTAGCTATTGTGCTCTATGCGAGCGGCAGCCCTCAGGCCGTAAAACGGAGACGCGTA 961

gB   181   CACAATATCACCGGTGGACAGGGCGAAGGAGTCGTAGGGGTAGACGGACCGCGCCTCCAC 240
AB279609.1   960   CACAATATCACCGGTGGACAGGGCGAAGGAGTCGTAGGGGTAGACGGACCGCGCCTCCAC 901

gB   241   CTCCTCGACGATGCAGTTGACAGACGTTGACGTGTAGTGCCTCCATGGCATCCACCCCAC 300
AB279609.1   900   CTCCTCGACGATGCAGTTGACAGACGTTGACGTGTAGTGCCTCCATGGCATCCACCCCAC 841

Day 1	Day 2
Day 3	Day 4
Day 4b	Day 6
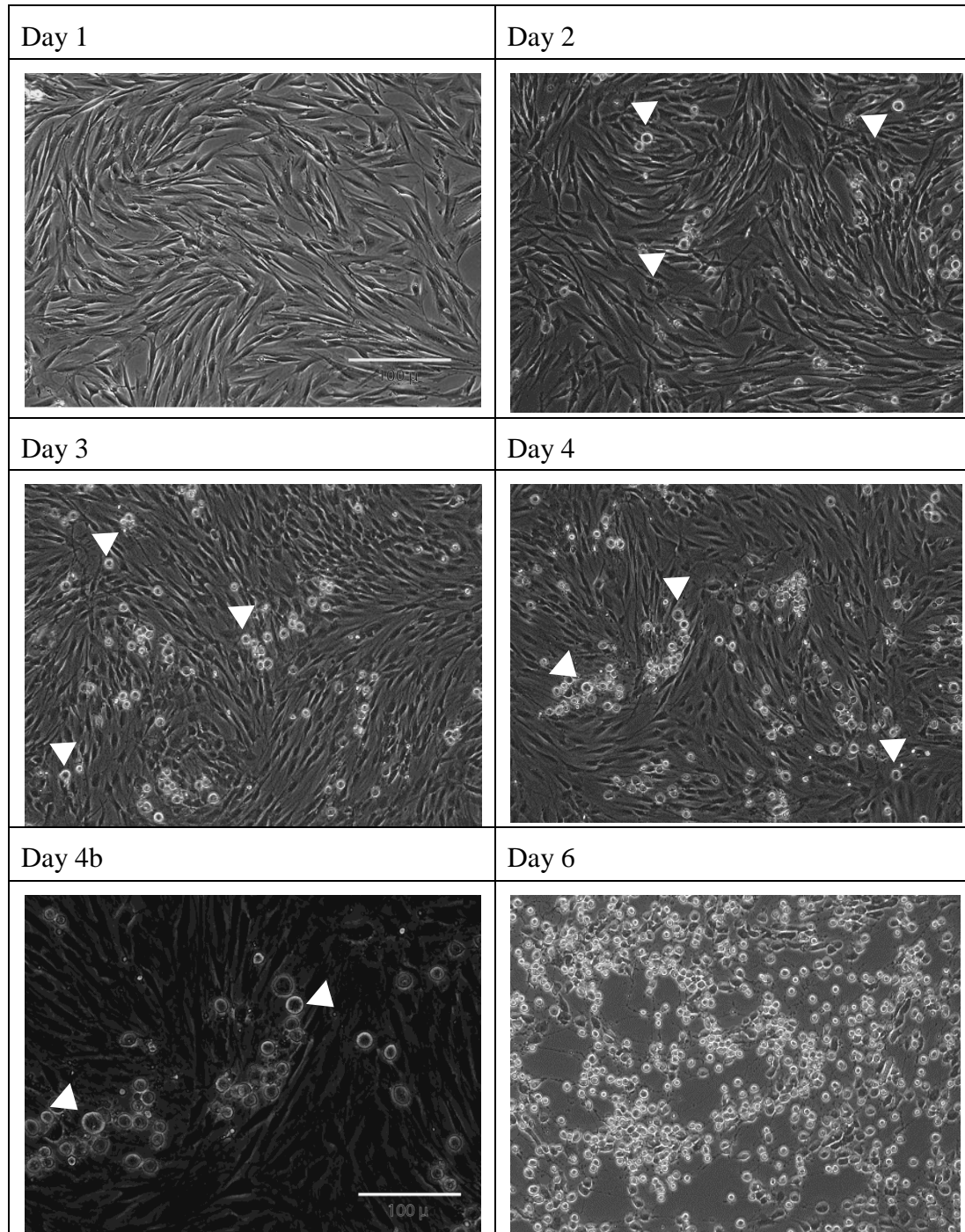
Figure 3.1 E.Derm cells infection with EHV-1 neuropathogenic strain. Photographs (10X magnification) were taken daily after infection. Day 1: Normal E.Derm cells. Day 2: CPE in cells are detectable (Arrow heads showing CPE as examples). Day 3: Increase in the number of CPE

and small areas of infected cells are visible. Day 4: further increase in the number of CPE and enlarged areas of infected cells. 4b) same as 4a with 20X magnification. Note the rounding up of the infected cells and increase in refractility. Day 6: almost 80% of E.Derm cells show CPE.
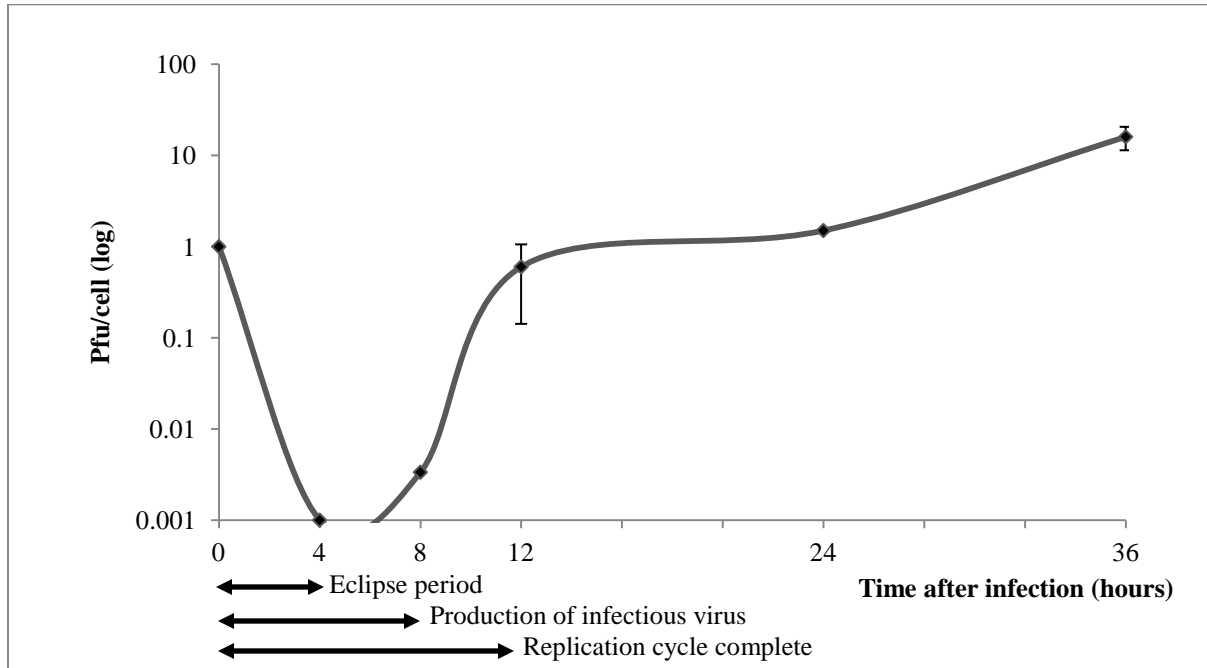
Figure 3.2 Single step growth curve of EHV-1. A single step growth curve was performed. Virus content was measured in E.Derm cells after infection with a moi of 1 pfu/cell. The virus titer at each time point was determined by plaque assay in E.Derm cells at t0, t4, t8, t12, t24 and t36 after the inoculum was removed and media replaced. The virus concentration at t0 (1pfu/cell) represents the input virus. At t4, no virus could be detected corresponding with the eclipse period. At t8, infectious virus could be detected. By t12, the replication cycle was complete. Error bars represent standard error n=3.

A)

Gene	Expected Product size (bp)
IE	214
ICPO	308
E-VP16	306
gB	348

B)
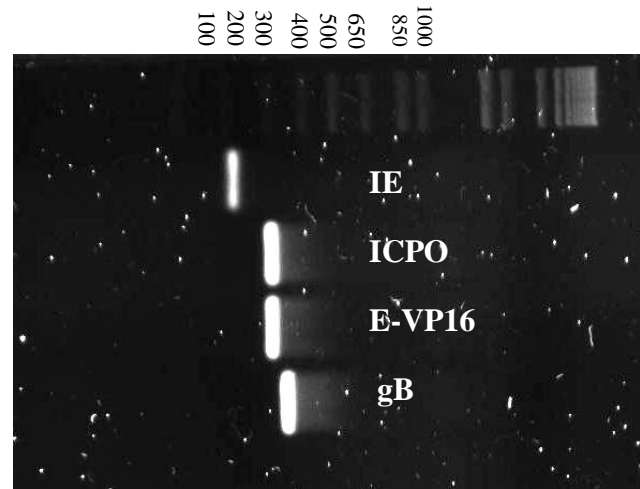
Figure 3.3 PCR product identification. A) Table showing expected sizes of PCR products IE, ICP0, E-VP16 and gB. B) Agarose gel electrophoresis of qPCR purified products and 1Kb ladder marker. Labeling for the ladder marker size is in bp.

A)

E=112% **ICP0**

$y = -3.065x + 9.379$
$R^2 = 0.9558$

E=127% **E-VP16**

$y = -2.8117x + 9.602$
$R^2 = 0.9671$

E=115% **gB**

$y = -3.0183x + 8.841$
$R^2 = 0.9965$

E=87% **IE**

$y = -3.8633x + 11.733$
$R^2 = 0.9802$
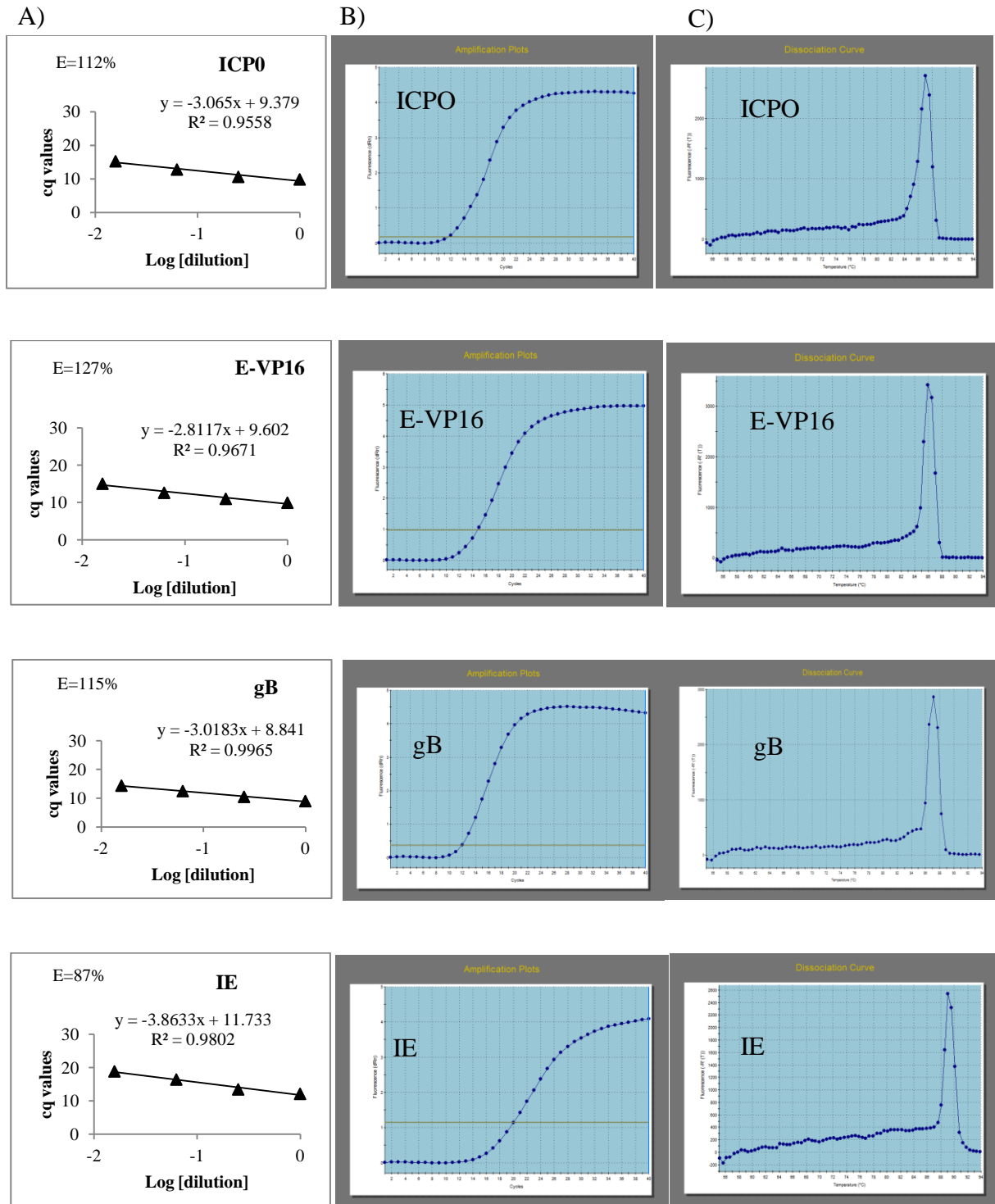
Figure 3.4 Characteristics of EHV-1 primers and products. A) Calibration curves from one experiment for each primer set. Serial 4-fold dilutions of DNA were plotted as Cq values versus Log [dilution]. The trend line, the equation of the curve, the slope of the curve, the R squared

88

value (R^2) and the efficiency (E) in percentage, are presented. B) Amplification plot curve for each primer set. A sigmoid curve represents the plotting of the fluorescence signal versus the cycle number. There is an inverse relationship between Cq value and quantity of product. C) Dissociation curve for each primer set. The fluorescence signal emitted by dissociating double stranded cDNA is plotted as a function of rising temperature. Figures are from one representative experiment.
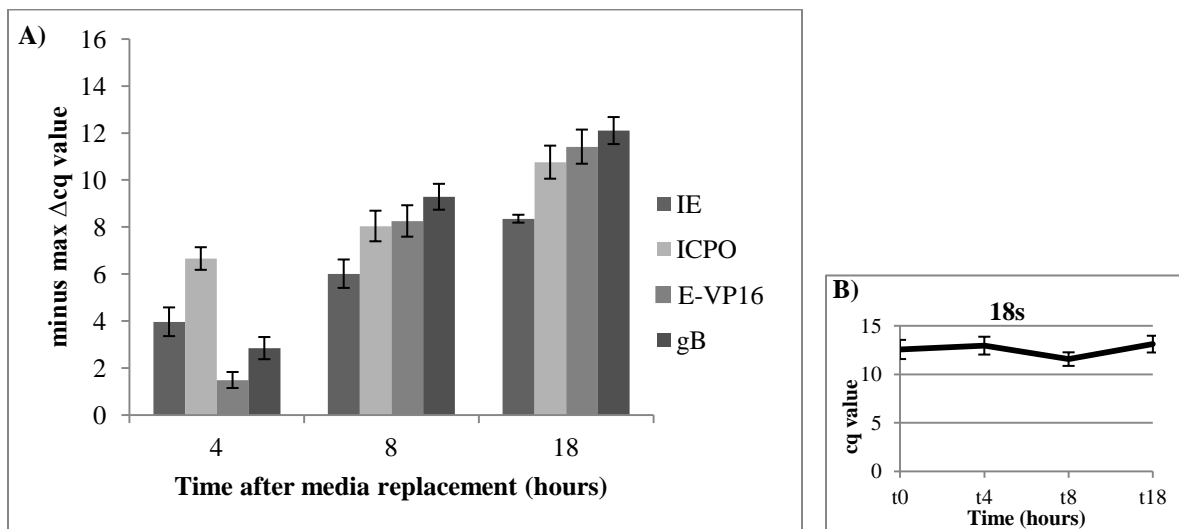
Figure 3.5 Expression of EHV-1 genes during lytic infection in E.Derm cells. A) Following infection with EHV-1 at time -1h and replacement of the media at time 0 (t0), cells were harvested at t0, t4, t8 and t18, cellular RNA was isolated, and RT-qPCR was performed. Gene expression was calculated as "minus maximum ΔCq value versus time zero". B) Expression of 18s over an 18 hour period. Average Cq values at each time point are shown. Error bars represent standard error, n=7.

4   INTERACTION BETWEEN EHV-1 INFECTION AND THE UPR IN EQUINE CELLS

The third objective of this project was to investigate the relationship between EHV-1 gene expression and UPR gene expression during lytic infection.

The tools developed in addressing the first and second objective in chapters 2 and 3 were used for the following: first, to determine if EHV-1 could influence expression of UPR genes during lytic infection and second, to evaluate if components of the UPR could influence the expression of EHV-1 genes of various temporal classes. In the previous chapters, I described how equine cells responded to thapsigargin, an inducer of the UPR, by increasing intracellular levels of transcripts for some UPR-related genes. The expression of EHV-1 genes in the same cells after viral infection was also described. Viral infection imposes ER stress by vastly increasing the amount of protein produced by the infected cell. In the case of enveloped viruses this may overwhelm the capacity of the ER for processing membrane-associated proteins (Kim, Xu et al. 2008), triggering activation of the UPR. This effect of virus infection on the UPR has been studied in different viruses including HSV-1, a member of the same subfamily as EHV-1. To date, no data concerning interactions between EHV-1 and the UPR in equine cells have been published. Prior to testing the hypothesis that activation of the UPR in latently EHV-1 infected peripheral blood leukocytes will trigger viral reactivation, I wanted to first determine if UPR activation could influence the temporal pattern of EHV-1 gene expression. Data from experiments with other alphaherpesviruses such as HSV (Kim, Mandarino et al. 2012) suggests that viral gene expression is not temporally regulated during reactivation from latency, but rather that all classes of genes are expressed simultaneously. A change in the EHV-1 gene expression during lytic infection of cells with an activated UPR would therefore support an association

90

between UPR activation and reactivation from latency. To evaluate if activation of the UPR influences the expression of selected EHV-1 genes during lytic infection, I examined EHV-1 gene expression in cells in which the UPR had been activated.

4.1 Materials and methods

4.1.1 Cell cultures and reagents, virus and polymerase chain reaction (PCR)

These were as described in chapters 2 and 3.

4.1.2 Influence of EHV-1 infection on the UPR

Experimental design diagram:
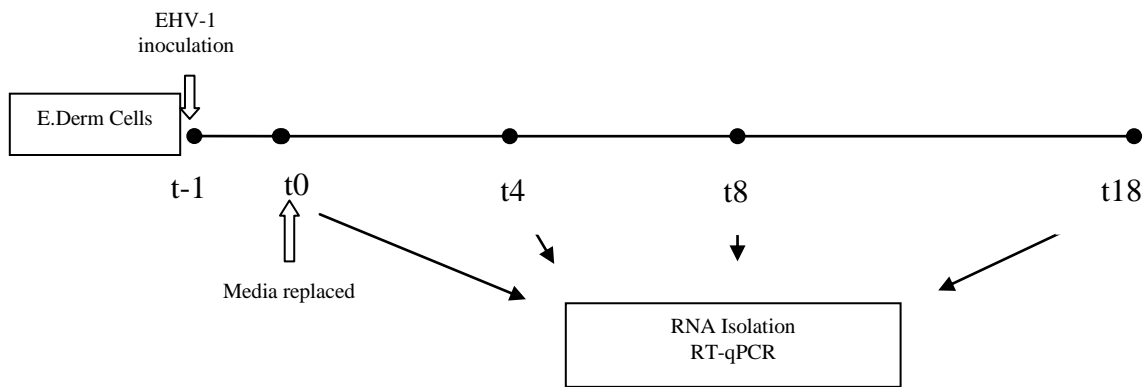
To determine if lytic EHV-1 infection induced UPR activation, E.Derm cells ($2x10^5$ cells/well in 6 well plates) were inoculated with EHV-1 at a moi of 5 pfu/cell (see diagram below). A relatively high moi was used to increase the probability of simultaneous infection of all cells in the culture. After 1h of incubation at 37°C (5% CO_2), the inoculum was removed, fresh media was added and cells were returned to the incubator. Cells were harvested over the following 18 h: immediately (t0), and at t4, t8, and t18. Expression of UPR genes (HERP, CHOP

and GRP78) was analyzed using RT-qPCR. The experiment was performed in duplicate and repeated on 7 different occasions.

The reference gene 18s was used as a normalizer, as in previous experiments. Fold change was calculated compared to t0 samples instead of comparing to a control sample. I arbitrarily decided to regard 2 fold changes in gene expression (either increase or reduction) as biologically relevant.

4.1.3   Influence of the UPR activation on EHV-1 gene expression

Experiment design diagram
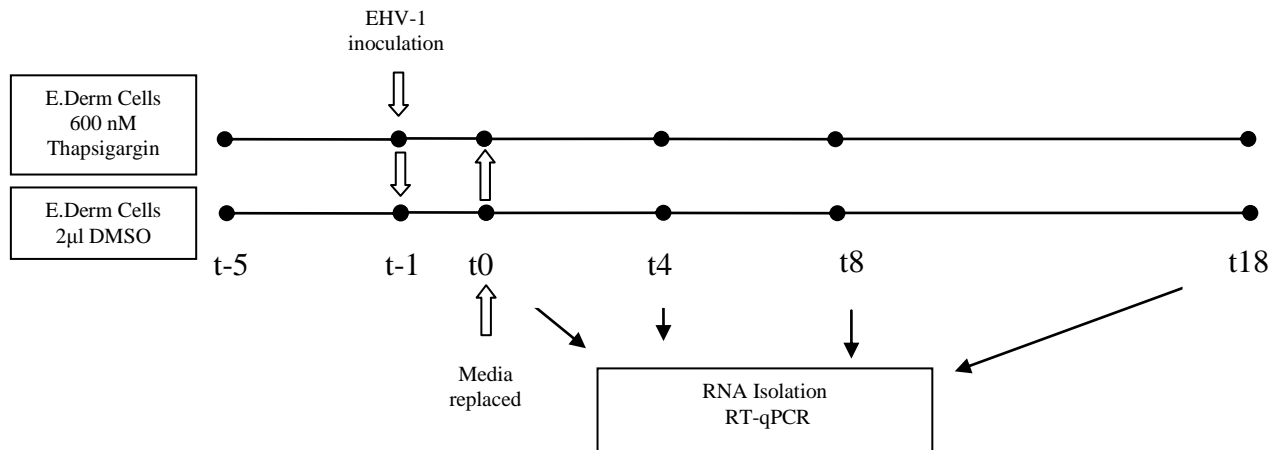
E.Derm cells ($2x10^5$ cells/well in 6 well plates) were first treated with either 600nM of thapsigargin or DMSO as control. After 4 h of treatment (t-5 to t-1, see diagram below), medium was removed and cells were inoculated with EHV-1 at a moi of 5 pfu/cell. Following 1 hour of incubation at 37^0C to allow adhesion of virus (t-1 to t0 in diagram), inocula were removed and cells were harvested at t0, t4, t8 and t18 after media was replaced. Expression of EHV-1 genes (IE, ICP0, E-VP16 and gB) was analyzed using RT-qPCR. The experiment was performed in

duplicate and repeated on 4 different occasions. The reference gene 18s was used as a normalizer and the strategy used to calculate gene expression was as described in section 3.1.5. This analysis allowed comparison of gene expression over time with respect to t0.

4.2 Results

4.2.1 Influence of EHV-1 infection on the UPR

As shown in Figure 4.1, the expression of the UPR-related genes (HERP, CHOP and GRP78) was not increased or decreased in response to EHV-1 infection.

4.2.2 Influence of the UPR activation on EHV-1 gene expression

Viral gene expression appeared to follow the pattern observed in untreated cells (compare the pattern in Figure 3.5 in the previous chapter with Figure 4.2 below). Transcripts for the IE and ICP0 (early) genes could be detected at t4 with a steady increase in gene expression at t8 and t18. Expression of the late genes (E-VP16 and gB) at t4 was lower compared to the expression of the immediate early and early genes but showed an increased expression at t8 and t18 compared to those genes. The levels of expression of each gene under the two conditions (treatment with thapsigargin versus untreated) were further compared as a ratio (Figure 4.3) to determine if treatment with thapsigargin altered the level of expression of the four indicator genes. While the ratio for all genes was close to 1 at t8 and t18, the ratios were all less than 1 at t4. This suggested that UPR activation transiently suppressed expression of EHV-1 genes in the first 4 h after the inoculum was removed.

4.3 Discussion

Infection of E.Derm cells with EHV-1 had no effect on UPR gene expression in the 18h period of evaluation that started after the inocula were removed and media was replaced. This suggests that EHV-1 does not influence UPR gene expression during lytic infection of E.Derm cells.

Activation of the UPR in E.Derm cells before infection with EHV-1 apparently induced a reduction in EHV-1 gene expression. This was suggested by the observation that the UPR activation suppressed the expression of all four indicator genes at t4; this inhibitory effect was not apparent later in infection and viral gene expression was restored at t8 and t18.

In summary, the fact that lytic EHV-1 infection did not influence the expression of the UPR genes and that activation of the UPR suppressed the expression of EHV-1 genes in a temporary and reversible manner, these experiments did not provide convincing evidence to support the hypothesis that UPR activation plays a role during EHV-1 reactivation from latency.
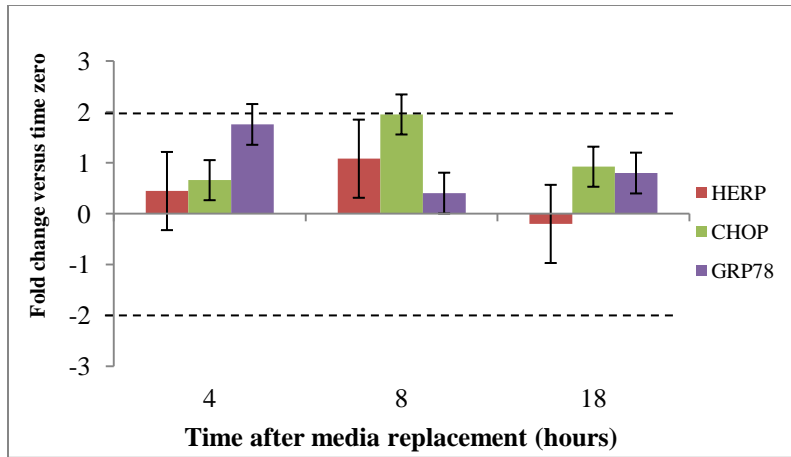
Figure 4.1 Expression of UPR genes during lytic EHV-1 infection in E.Derm cells. Cells were harvested at t0, t4, t8 and t18, cellular RNA was isolated, and RT-qPCR was performed. Expression of the UPR genes was measured and calculated as fold change compared to time zero (t0) (see text above). Dotted lines represent the 2 fold change values considered as biologically relevant. Error bars represent standard error, n=7
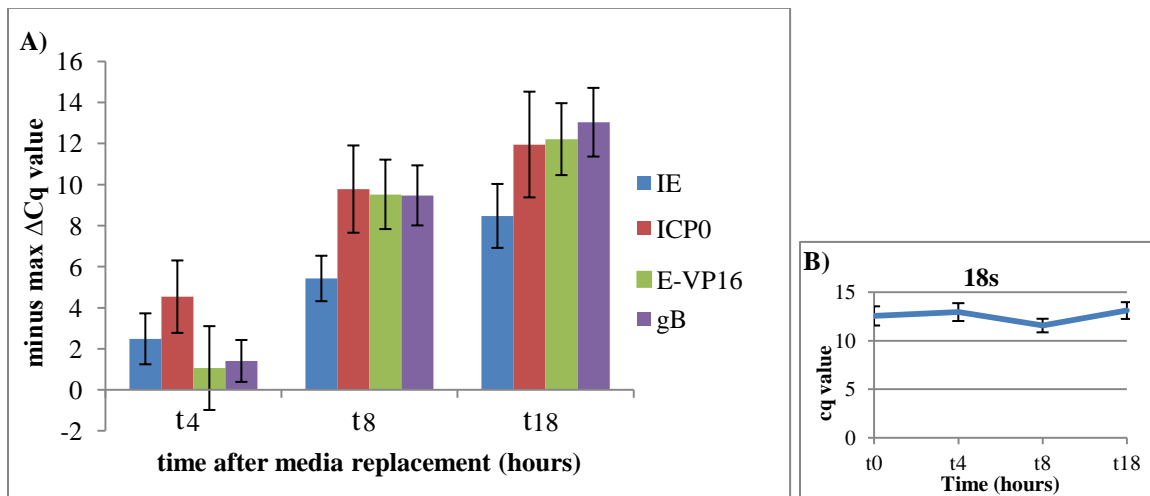
Figure 4.2 Expression of EHV-1 genes during lytic infection in E.Derm cells treated with thapsigargin for 4 h. A) E.Derm cells were treated with 600 nM of thapsigargin prior to infection. At t0, t4, t8 and t18 (see diagram), cells were harvested, cellular RNA was isolated, and RT-qPCR was performed. Gene expression was calculated as "minus maximum ΔCq value

versus time zero". B) Expression of 18s over an 18-hour period. RT-qPCR was performed on RNA isolated from E.Derm cells at t0, t4, t8 and t18. Average Cq values at each time point are shown. Error bars represent standard error, n=4.
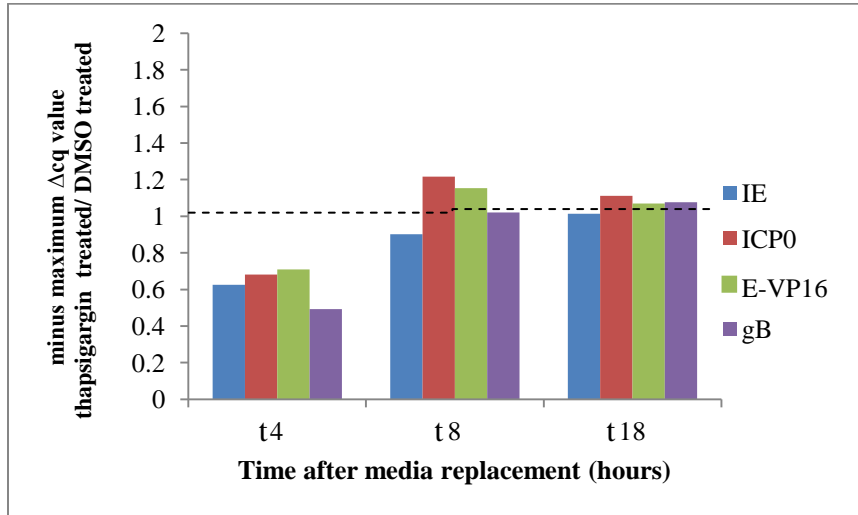
Figure 4.3 Expression of EHV-1 genes during lytic infection of E.Derm cells after 4 h treatment with thapsigargin compared to that in DMSO-treated cells. E.Derm cells were either treated with 600 nM of thapsigargin or treated with 2 μl of DMSO as a control and were subsequently infected with EHV-1. At t0, t4, t8 and t18 (see diagram), cells were harvested, cellular RNA was isolated, and RT-qPCR was performed. Gene expression was calculated as "minus maximum ΔCq value versus time zero" (see section 3.2.5) and ratios of gene expression between thapsigargin-treated and control cells determined. The dotted line at value y=1 represents no change in gene expression. The ratios were calculated from the average of 4 experiments.

5 DETECTION AND ATTEMPTED REACTIVATION OF LATENT EHV-1 INFECTION IN EQUINE PERIPHERAL BLOOD CELLS

The fourth objective of this project was to detect latent EHV-1infection in PBLs obtained from horses presumed to be latently infected and to investigate whether stimulation of the UPR in these latently-infected PBLs would lead to reactivation of EHV-1 virus. Two approaches were chosen to detect latent EHV-1 from PBLs: First, the sensitivity of a nested PCR to detect and distinguish latent EHV-1 and EHV-4 was determined and second, co-cultivation of stimulated PBLs with cells permissive to EHV-1 infection was assessed. Co-cultivation refers to the culture of 2 different cell types in the same medium, and *in vitro* reactivation of latent EHV-1 by co-cultivation of PBLs with cells permissive for EHV-1 replication has been described previously (Smith, Iqbal et al. 1998). In this chapter, presumed latently infected PBLs were co-cultivated with E.Derm cells permissive for EHV-1 replication. If EHV-1 reactivation from latently infected PBLs occurred and infectious virus was produced, the E.Derm cells would function as a detection system by amplifying the virus. Lytic infection in the E.Derm cells could then be detected visually by CPE and presence of viral DNA by qPCR.

In the previous sections it was established that equine cells respond to cellular stress induced by thapsigargin by activating the UPR and that it was possible to detect activation of the UPR by using qPCR. It was also established that expression of four EHV-1 genes (IE, ICP0, E-VP16 and gB) could be detected during lytic infection using qPCR. To achieve the objective of this chapter, PBLs isolated from blood samples of horses presumed to be latently infected with EHV-1 were treated with different compounds. IL-2 was used as previously described to induce reactivation of latent EHV-1 in PBLs *in vitro*, and thapsigargin was used to induce the UPR.

5.1 Materials and methods

5.1.1 Horses

Two mares from the WCVM teaching herd were used to collect blood samples and isolate PBLs for the initial set-up of co-cultivation experiments. These animals are used for teaching purposes in clinical laboratories and classes, and had no recorded history of EHV-1 infection. Four client-owned horses known to have been exposed to and infected with EHV-1 during an outbreak of EHM in Saskatoon in 2008 were used as presumed EHV-1 latently infected horses. During the outbreak, all four horses tested positive to EHV-1 by PCR (nasal swabs) and 2 presented with neurological clinical signs. Specifically, one Quarter Horse gelding (17 years of age, horse 1) presented uveitis and mild respiratory signs; one Arabian gelding (22 years of age, horse 2) presented uveitis and ataxia; and one Arabian mare (19 years of age, horse 3) presented ataxia, limb weakness and recumbency during the outbreak. The fourth horses, an Arabian/Standardbred mare (11 years of age, horse 4) showed no clinical signs during the outbreak. At the time of the study, horse 1, 2 and 4 did not show clinical sequellae and were being actively ridden. The Arabian mare presented sporadic limb weakness. All procedures were approved by the Animal Research Ethics Board, University Committee on Animal Care and Supply (UCACS), University of Saskatchewan, and followed the guidelines of the Canadian Council on Animal Care (CCAC).

5.1.2 Blood collection, PBLs isolation and cell culture

Blood collection, isolation and culture of PBLs, and culture of E.Derm cells were performed as described in chapter 2. Compounds used to stimulate PBLs and the final concentrations used in the experiments are shown in the table below:

Reagents	Final concentration
DMSO (Dimethyl sulfoxide)	0.4µl or 0.8 µl per ml
Thapsigargin 750µM in DMSO	300nM or 600nM
Recombinant human Interleukin 2 (IL-2)*	20IU/ml

* R&D systems (Minneapolis, USA)

5.1.3 Virus

The neuropathogenic field strain of EHV-1 used for establishment of the co-cultivation experiments was as described in chapter 3.

5.1.4 DNA extraction

DNA (from E.Derm cells and PBLs) was purified using DNeasy kits (Qiagen, Maryland, USA) according to manufacturer's instructions.

5.1.5 Nested polymerase chain reaction (PCR)

Conventional (nested) PCR for selective detection of EHV-1 and differentiation from EHV-4 was developed previously in Dr. Misra's laboratory (Table 5.1) (Wagner, Bogdan et al. 1992). Conventional PCR was performed in a MJ Research PTC-200 peltier thermal cycler (Bio-Rad Laboratories Inc. Hercules, CA, USA). Nested PCR reactions were performed with the following thermal cycler conditions: 94°C for 1 min, followed by 30 cycles of: 4°C for 30 s, 60°C

for 30 s, 72°C for 1 min, followed by 10 min at 72°C with a final step at 4°C. Briefly, this nested PCR works in 2 steps (Figure 5.1): in the primary reaction, there is amplification with a set of primers that recognizes a sequence of the glycoprotein B (gB) gene common to both viruses. The product of this primary reaction is a 414 bp "common" product that is used as a template for the secondary reaction. In the latter, the same reverse primer as in the primary reaction is used but 2 different forward primers are used: one specific for EHV-1 gB and one specific for EHV-4 gB. The products of this secondary reaction can now be differentiated: a 335bp product is specific for EHV-4 and a 129 bp product is specific for EHV-1. Product identification by agarose gel electrophoresis was as described in chapter 2.

To determine the sensitivity of the nested PCR assay, serial 10 fold dilutions of purified EHV-1 DNA, ranging from 10 ng/µl to 1 fg/µl, were prepared. Nested PCR was performed on all the dilutions and the lowest concentration of DNA that consistently provided positive PCR results determined as the limit of detection of the assay. Further, to determine if high concentration of cellular (PBLs) DNA would interfere with detection of low viral DNA concentrations, decreasing amounts of viral DNA (1 ng, 100 pg, 10 pg, 1 pg) were added to increasing amounts of extracted PBLs DNA (250 ng, 500 ng, 1 µg) and nested PCR performed.

For detection of latent EHV-1 in one presumed latently infected horse (Horse 1 in section 5.1.1), PBLs were isolated from whole blood and DNA was extracted. Nested PCR was performed using 500 ng of input DNA as described above. The horse was tested on 3 separate occasions for presence of latent virus.

5.1.6 Quantitative real time PCR

Quantitative real time (qPCR) targeting the EHV-1 gB gene was performed in a Mx3005P QPCR thermal cycler (Stratagene, La Jolla, CA, USA). To determine the presence of viral DNA the protocol used was the Quantifast SYBR$^®$ green PCR kit protocol, (Qiagen). Samples were amplified using the following thermal cycler conditions: 95°C for 5 min, followed by 40 cycles of: 95°C for 10 s, 60°C for 30 s, followed by 1 cycle at 95°C for 1 min, 55°C for 30 s and 95°C for 30 s. The primer sets for transcript amplification were used at a final concentration of 12.5 μM.

5.1.7 Detection of EHV-1 infection in E.Derm cells co-cultivated with isolated PBLs

Experimental design diagram:
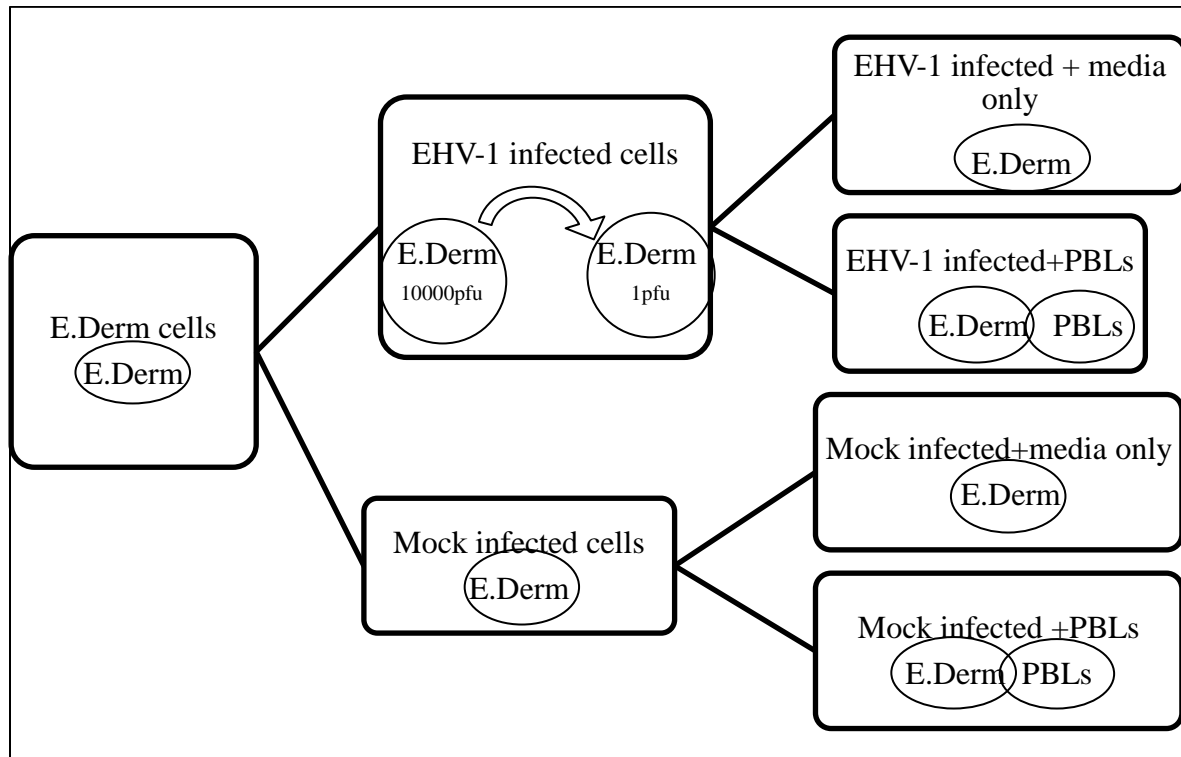
A pilot experiment was designed to study the interaction of EHV-1 infected cultured E.Derm cells and PBLs isolated from equine blood samples, and to determine the ability of the qPCR assay to detect viral DNA in the total DNA isolated from the co-culture (PBLs+ E.Derm DNA). The experiment was performed in duplicate and repeated 3 times (see diagram). E.Derm cells ($1x10^5$cells/well in 6 well plates) were inoculated with serial 10 fold dilutions of EHV-1 to result in a final virus concentration of 10,000, 1,000, 100, 10 and 1 pfu/well, respectively. After a 1-hour incubation at 37^0C (5% CO2) for adhesion of virus, inocula were removed. One set of wells was maintained in RPMI 1640 medium alone (media only) while the other set had $1x10^6$ PBLs suspended in RPMI 1640 medium added to the each well. Mock-infected control wells were inoculated with media only and cultured with or without the addition of PBLs.

Wells were visually observed and photographs taken every day for 5 days post-infection (DPI). By 5 DPI, cells (both floating cells and attached, E.Derm and PBLs) were harvested as follows: supernatant was collected and centrifuged for 5 minutes at 500g and attached cells were scraped from the well. DNA was extracted from floating and adherent cells together. The qPCR assay targeting the EHV-1 gB gene was performed for all samples.

5.1.8 Attempted reactivation of latent EHV-1 and detection by co-cultivation

Experimental design diagram:
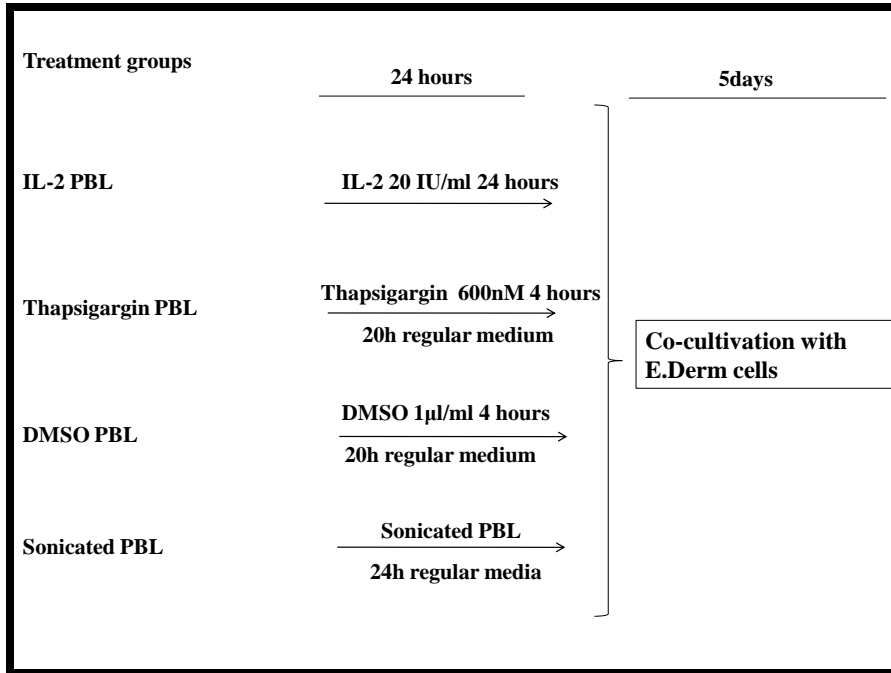
Blood samples from horses presumed to be latently infected with EHV-1 (n=3) were taken and isolation of PBLs was performed. Considering that 1 in 50,000 PBLs had been described to be latently infected with EHV-1 (Smith, Iqbal et al. 1998), 5×10^6 PBLs/well were used as this would ensure presence of at least 100 latently infected PBLs per well. Experiments were performed in triplicate and PBLs were treated as follows: the IL-2 PBLs group cells were treated with 20 IU/ml of IL-2 in RPMI1640 medium for 24 h; the thapsigargin PBLs group cells were treated with 600nM of thapsigargin in RPMI 1640 medium for 4 h and maintained in RPMI 1640 medium for the following 20 h; the DMSO PBLs group cells served as a control and were treated with 1μl/ml of DMSO in RPMI 1640 medium for 4 h and maintained in RPMI 1640 medium for the following 20 h; and the sonicated PBLs group cells were sonicated 3 times for 7

s and maintained in RPMI 1640 medium for 24 h. Sonicated cells were used to control for the presence of active viral infection in the samples. With active viral infection, CPE would be detected in co-cultivated E.Derm cells because infectious particles (virions) would already be present and their production would not depend on cell integrity of the PBLs. Conversely, latent virus depends on PBLs integrity to produce infectious particles.

After 24 h of treatment, PBLs from all groups were co-cultivated with $2x10^5$ E.Derm cells/well and maintained in RPMI 1640 medium for 5 days post-exposure (DPE). One group of E.Derm cells, cultured in RMPI 1640 medium without addition of PBLs, was added as a control and maintained for 5 days. The media was not changed during the 5 day co-cultivation period.

Photographs were taken every day for 5 days. Five days after the beginning of co-cultivation, cells were harvested and DNA extracted from both floating and attached cells (E.Derm and PBLs). QPCR for detection of EHV-1 DNA was performed on all samples. Three different dilutions of the sample DNA were tested (10ng/PCR reaction; 1ng/PCR reaction; 0.1ng/PCR reaction) to assure that high total DNA concentration was not interfering with viral DNA detection. Purified EHV-1 DNA dilutions were used as a positive control with 14.7ng/PCR reaction; 1.47ng/PCR reaction and 0.147ng/PCR reaction in each PCR run.

5.2   Results

5.2.1   Evaluation of a nested PCR to detect viral genomes in PBLs

A detection limit for the nested PCR of 1 pg of purified viral DNA in up to 500 ng of cellular (PBLs) DNA was determined (data not shown). Based on an equine genome size of

2.7×10^9 bp (Wade, Giulotto et al. 2009) and an EHV-1 genome size of 1.5×10^5 bp (Telford, Watson et al. 1992), the necessary frequency of latent infection to ensure presence of 1 pg of viral DNA in 500 ng of cellular DNA could be calculated. It was determined that 1pg of viral DNA in 500 ng of PBLs DNA was equivalent to 1 viral genome in 27 PBLs, i.e. 1 in 27 PBLs would need to be latently infected in order to make the nested PCR a useful and reliable tool for detection of latent EHV-1 infection. In comparison, the frequency of latent infection in equine PBLs has been described as 1 in 50,000 cells (Smith, Iqbal et al. 1998). Blood samples from one presumed latently infected horse were tested with this PCR technique 3 times and it tested positive to EHV-1 on only one occasion. The sensitivity of the assay in relation to the frequency of the latent virus therefore represents a limitation for the use of this detection technique.

5.2.2 Detection of EHV-1 infection in E.Derm cells co-cultivated with isolated PBLs

Figure 5.2 shows photographs of the co-cultivated wells of the pilot experiment on day 1 and day 4. Starting from the left, the first column shows images of wells inoculated with the lowest dilution of virus (1×10^5 pfu/well). In wells cultured with media alone, presence of CPE was clearly detected over large areas on 1D PI. By 4 DPI, no E.Derm cells remained attached to the wells and clumps formed by detached cells were visible. In wells cultured with PBLs, CPE could be detected but visualization was not as clear as in wells without PBLs. By 4 DPI, only few E.Derm cells remained attached to the well and large clumps of detached E.Derm cells aggregated with PBLs were visible.

The second column in Figure 5.2 represents images of wells inoculated with a media containing diluted virus (1×10^3 pfu/well). In wells cultured with media alone, presence of CPE was detected on 1 DPI in certain areas across the well as plaques and rounded, refringent cells

were visible surrounding these plaques. By 4 DPI, almost all E.Derm cells looked rounded and more refringent but many of those cells were still attached to the well. In wells cultured with PBLs, presence of CPE was detected on 1 DPI in isolated E.Derm cells but detection of CPE on 4 DPI was difficult. There were individual cells with CPE characteristics and areas with detached E.Derm cells. However, there was no clear visualization of plaques or CPE.

The third column in Figure 5.2 represents images of wells inoculated with the lowest concentration of virus (1 pfu/well) used for this experiment. In wells cultured with and without PBLs, no CPE were detected on 1 DPI. In wells cultured with media alone, 33% of the wells presented CPE on 4 DPI, while in wells cultured with PBLs, there were no detectable CPE. In wells without distinct CPE, E.Derm cells with PBLs looked different from those without PBLs on 4 DPI.

In the last column in Figure 5.2, images of mock infected E.Derm cells are shown. In wells cultured with media alone, cells had the appearance of normal dermal cells on day 1 and 4. In wells cultured with PBLs, the high number of PBLs hampered correct visualization of the E.Derm cells on day 1. On day 4, E.Derm cells cultured with PBLs showed different morphology compared to those cultured without PBLs. This was consistent with the findings of infected cells in wells without distinct CPE (see above). Therefore, integrity of E.Derm cells was compromised after 4 days of co-cultivation with PBLs. Visualization of CPE in wells containing PBLs was difficult because of the high total number of cells per well. In wells with a higher concentration of virus, the presence of PBLs hindered the detection of CPE on 4 DPI but it did not impede detection of CPE entirely.

The results of CPE detection were corroborated by qPCR. All wells that had CPE detected visually tested positive for presence of viral DNA by qPCR (Figure 5.3). Wells in which CPE were not detected visually (wells inoculated with the highest dilution of virus and cultured with PBLs, mock infected E.Derm cells cultured with PBLs and mock infected E.Derm cells cultured in media alone) tested negative for viral DNA presence by qPCR.

5.2.3   Attempted reactivation of latent EHV-1 and detection by co-cultivation

E.Derm cells and treated PBLs from horses presumed to be latently infected were co-cultivated for 5 DPE. During the co-cultivation period, CPE could not be detected in any group (Figure 5.4). Co-cultivation of E.Derm cells with PBLs for more than 3 DPE induced changes in morphology in the E.Derm cells. Normal E.Derm are adherent large, elongated, spindle shaped cells (Leeson, Leeson et al. 1985) while the co-cultivated E.Derm cells looked shorter, rounded or oval shape, detached from the well and in some cases more refringent. A reduction in the number of both PBLs and E.Derm cell types over time was appreciated visually but not confirmed by cell counting. For 3 treatment groups (IL-2, Thapsigargin and DMSO treated PBLs), there was a clear reduction in cell number and by 5 DPE, it was extremely difficult to identify E.Derm cells in each well. The co-cultivated wells from the sonicated PBLs group were the only wells not showing morphological changes in the E.Derm cells or visible reduction in the number of cells by day 5 of co-cultivation. Viral DNA was not detected by qPCR in DNA samples isolated from any of the wells (data not shown).

5.3 Discussion

Conventional nested PCR does not appear useful for reliable detection of the latent EHV-1 genome if, as estimated in the published literature, only one latent viral genome per 50,000 PBLs is present.

From the pilot experiment I concluded that the presence of PBLs in the co-cultivated wells interfered with the visualization of CPE only at the highest dilution of the virus. Wells where CPE was present tested positive by PCR targeting EHV-1 gB.

The attempted reactivation of latent EHV-1 from PBLs and detection by co-cultivation was not achieved because either the reactivation stimulus was not the correct one or the virus was not present in the examined samples.

Table 5.1 Primers for detecting and differentiating EHV-1 and EHV-4 DNA. Four different primers targeting EHV gB are used in this nested PCR. EHV1P1 is the common forward primer and EHV1P2 is the common reverse primer. The product obtained from the amplification with these primers is a 414 bp product "common" to both viruses. This product is then used as a template for the secondary reaction. In the secondary reaction, EHV1-2P1 is used as the forward primer specific for EHV-1 detection, EHV4-2P2 is used as the forward primer specific for EHV-4 detection and EHV1P2 is used as the reverse primer common to both sequences.

	Sequence	Template strand	Length (in bp)	Tm (in ºC)	GC%
EHV1P1 common to both viruses Forward primer	ATGTCCWCTKGTTGCCG (w=a/t, k=t/g)	Plus	17	47-49	52-58
EHV1-2P1 EHV-1 specific Forward primer	TACTCCCACTTCCATGTCAACG	Plus	22	55	50
EHV4-2P2 EHV-4 specific Forward primer	CGTGTTCTCGCTTCTATACACC	Plus	22	55	50
EHV1P2 common to both viruses Reverse primer	TGTAAAAGTCTCCATCC	Minus	17	42	41
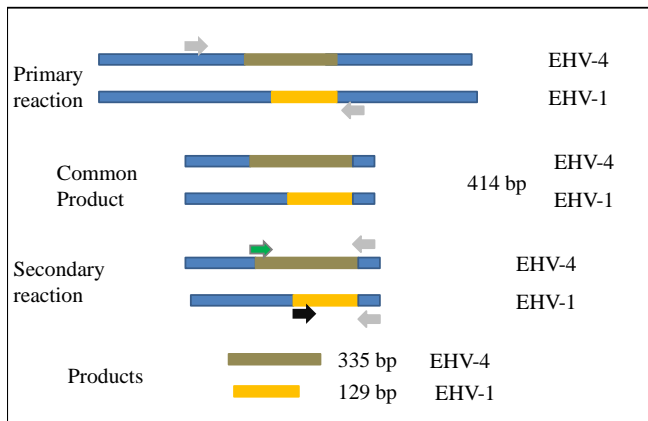
Figure 5.1 Nested PCR for detecting EHV-1 and EHV-4. In the primary reaction, the light grey arrows represent primers common to EHV-1 and EHV-4. The product from the primary reaction is a 414 bp product "common" to EHV-1 and EHV-4. In the secondary reaction, the green arrow represents the forward primer specific for EHV-4 glycoprotein B and the black arrow represents the forward primer specific for EHV-1. The products of the secondary reaction are specific for each virus. For EHV-4, it is a 335 bp product and for EHV-1 it is a 129 bp product.

		1x10^5 EHV-1 pfu/well	1x10^3 EHV-1 pfu/well	1EHV-1 pfu/well	Mock infected
DAY 1	Media only				
	With PBLs				
DAY 4	Media only				
	With PBLs				
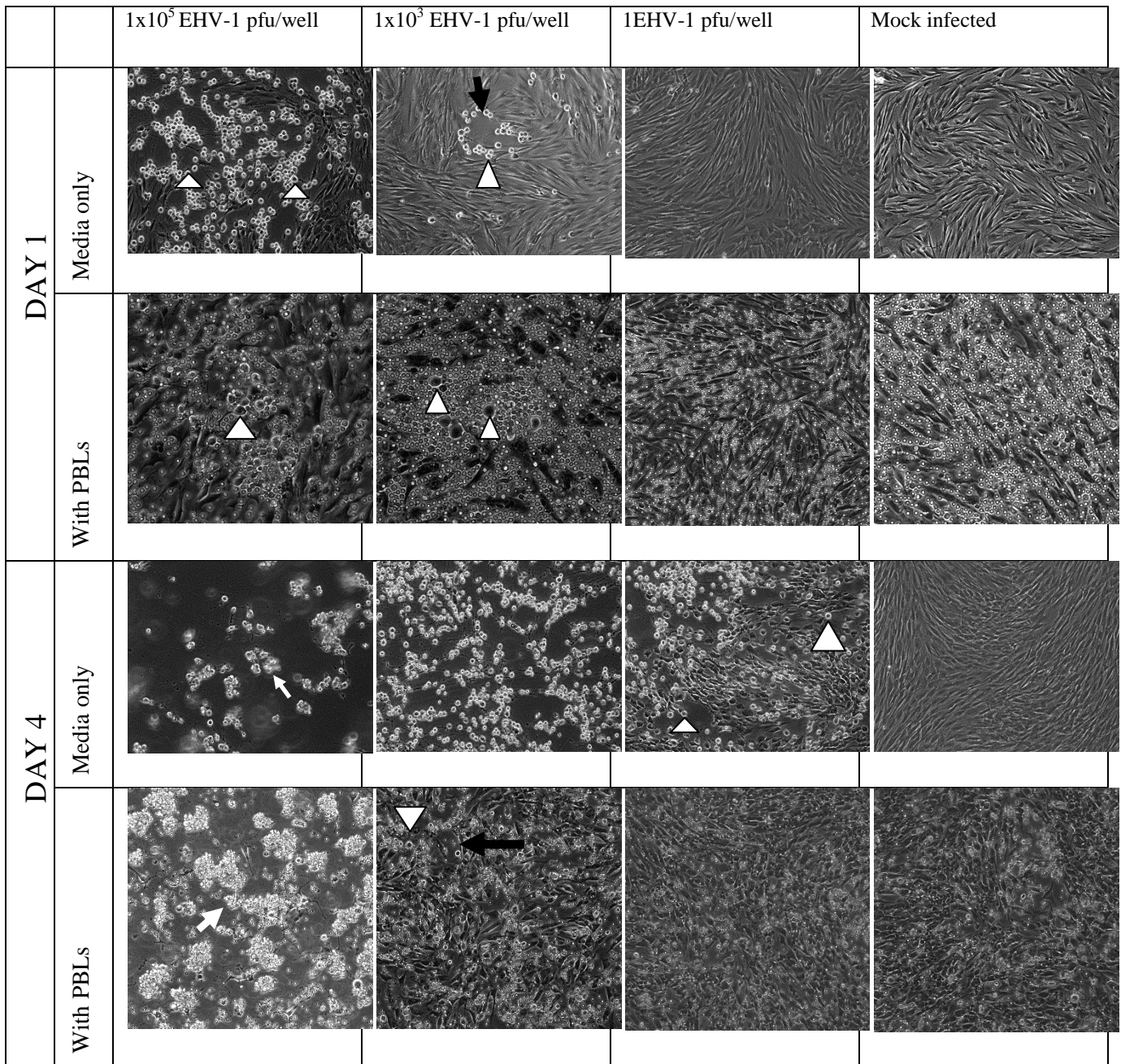
Figure 5.2 Co-cultivation of EHV-1 infected E. Derm cells and equine PBLs. Photographs of representative co-cultivated wells were taken on 1 DPI and 4 DPI. CPE are detectable as rounded, detached and refringent cells (white arrow heads). Viral infection produces detachment of cells from the well with plaques appearing as clear areas between infected cells (black

arrows). On 4 DPI, wells containing high viral concentration showed clumps of cells (white arrows). Presence of PBLs made visual detection of CPE difficult. 10X magnification.
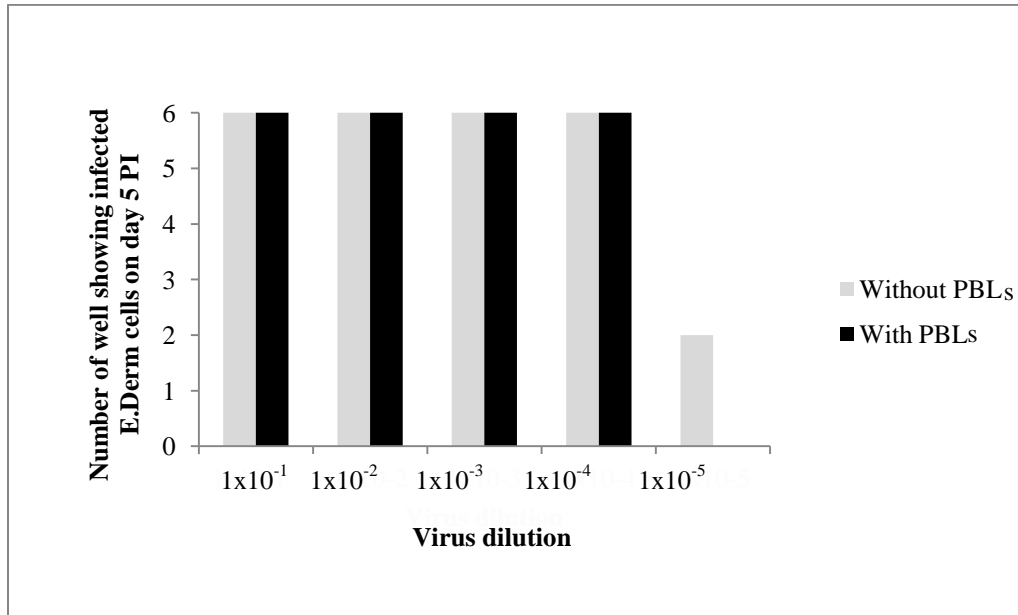
Figure 5.3 In vitro detection of EHV-1 CPE 5 DPI. E.Derm cells ($1x10^5$ cells/well) were inoculated with 10 fold dilutions of EHV-1. Six wells per viral dilution were co-cultivated with PBLs and six without PBLs. The number of wells showing CPE on 5 DPI is plotted.
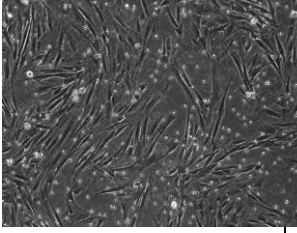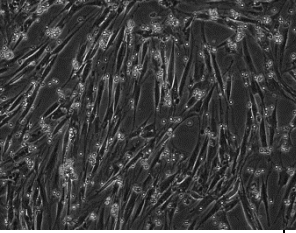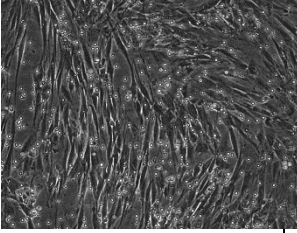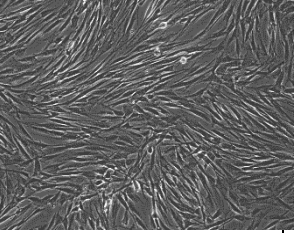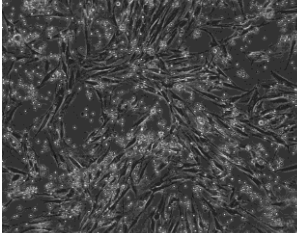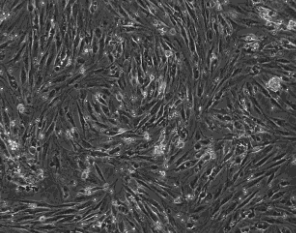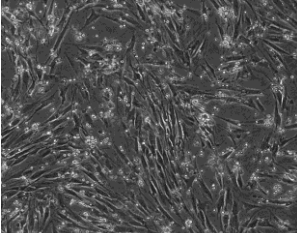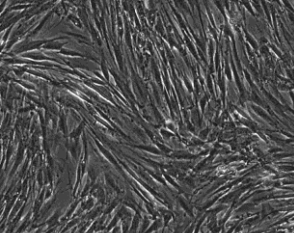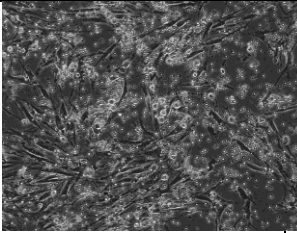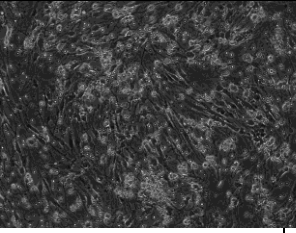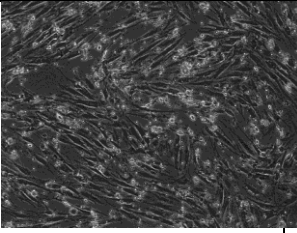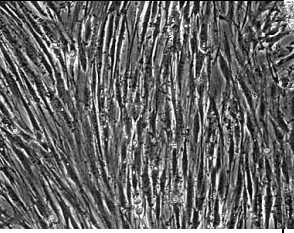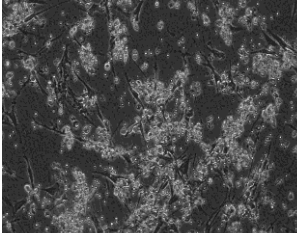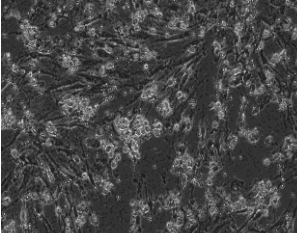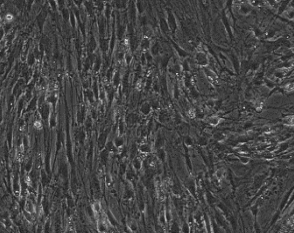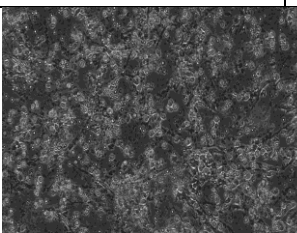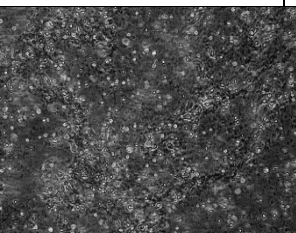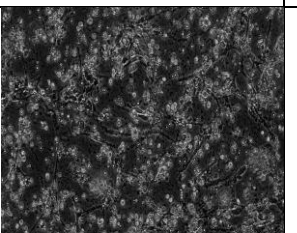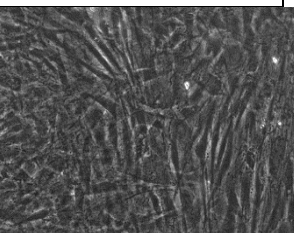
	IL-2 PBLs	Thapsigargin PBLs	DMSO PBLs	Sonicated PBLs
DAY 1				
DAY 2				
DAY 3				
DAY 4				
DAY 5				

Figure 5.4 Co-cultivation of E.Derm cells and presumed latently EHV-1 infected equine PBLs. Photographs of representative co-cultivated wells were taken every day for 5 days. After day 3 of co-cultivation with IL-2 PBLs, thapsigargin PBLs and DMSO PBLs, E.Derm cells showed different morphology than E.Derm cells co-cultivated with sonicated PBLs. Distinct CPE indicative of viral reactivation were not observed in any of the wells. 10X magnification.

6   DISCUSSION

EHV-1 appears to be very different from the other, more extensively studied, members of the *Alphaherpesvirinae* such as HSV-1 and VZV. EHV-1 has been described to establish latency in PBLs as well as sensory ganglia, whereas HSV-1 and VZV are latent in sensory neurons alone. EHV-1 may therefore allow the study of stress stimuli and signalling pathways that influence latency of an alphaherpesvirus in its natural host without having to sacrifice the host to remove its sensory ganglia. The collection of relatively large amounts of blood from latently infected horses is simple and feasible.

The UPR is a sensor of the stress level in the cell. Physiological activation of the UPR in normal cells is not detrimental for the cells and increases the cell protein folding capacity. When the stressful stimulus that induces the UPR is prolonged in time and of such intensity that it cannot be mitigated by the adaptive phase of the UPR, the cell undergoes apoptosis. It has been shown that during early lytic HSV-1 infection, the virus can modulate the activation of the UPR to avoid the detrimental aspects, while taking advantage of the positive aspects of UPR activation (Burnett, Audas et al. 2012). It has been proposed that ER stress related factors can be involved during HSV-1 latency establishment and reactivation (Figure 1.4). As there is still no proposed molecular model for establishment of EHV-1 latency and reactivation, I wanted to test the hypothesis that induction of ER stress with the consequent UPR activation in latently EHV-1 infected equine peripheral blood leukocytes (PBLs) could be a sufficient stimulus to trigger reactivation from latency.

To test this hypothesis, I first needed to be certain that the UPR could be consistently activated in equine cells and that the activation was measurable. This work is described in chapter 2. As PBLs are highly demanding cells to culture and do not replicate under natural

culture conditions, the E.Derm cell line was selected for preliminary work in the laboratory. I determined that treatment of E.Derm cells for 4 h with 600nM of thapsigargin (a drug that has been shown to induce the UPR in a number of cell lines of different origins, but has been not tested in the E.Derm cell line) induced the expression of UPR related genes (GRP78, HERP and CHOP) as measured by RT-qPCR. The GRP78 and the XBP1s genes primer set were designed for equine cells; however, while the identity of the amplification product for both XBP1s primer sets was validated by sequencing, XBP1s expression was not consistently induced after thapsigargin treatment in E.Derm cells and hence, this primer set was not included in the subsequent experiments with this cell line. The remaining primer sets used to measure the gene expression of HERP and CHOP after UPR activation had been tested in canine and human cells and were validated to amplify the correct target when used in equine cells. As described in chapter 2, efficiency of amplification of the primers for the reference gene 18s, for GRP78 gene and XBP1s gene was adequate but the efficiency of amplification for CHOP and HERP was not adequate. Efficiency values of greater than 150% for those primers could indicate that more than one product were amplified in the reaction. The dissociation curves for CHOP and HERP suggested that mispriming or primer-dimer artifacts may have occurred during the PCR amplification. However, the identity of the amplification products for both primer sets was established after thapsigargin treatment of the cells and the increase in the expression of both CHOP and HERP transcripts was consistently determined. I was also able to induce and measure the UPR gene expression in PBLs isolated from horse blood samples following the same protocol of UPR activation as for E.Derm cells. I therefore established that the UPR could be activated and measured in both cell types (E.Derm and PBLs) but the activation profile in both cell types differed to some extent: expression of XBP1s was induced in PBLs but it was not

induced in E.Derm cells after thapsigargin treatment; in contrast, expression of HERP was induced in E.Derm cells but it was not induced in PBLs after thapsigargin treatment. This difference in gene expression between the E.Derm and PBLs was not expected and may be a consequence of the different nature of the cells. Dermal cells are cells with higher metabolic activity compared to PBLs, and it is possible that some cellular pathways related to the UPR present different activation profiles in these 2 cell types. Unfortunately, the published literature lacks evidence to support a potential specific cause of the observed difference in UPR gene expression in my experiments.

In chapter 3, I established that our neuropathogenic EHV-1 virus stock was able induce lytic infection in the E.Derm cells at a low moi (0.01 pfu/cell) and that I was able to detect that lytic infection. CPE due to EHV-1 could be detected 24 HPI in E.Derm cells (Figure 3.1) and the growth of the virus in E.Derm cells increased exponentially over 36 h as described in the single step growth curve (Figure 3.2). The growth of EHV-1 observed in this experiment was similar to what has been published previously for EHV-1 and consistent with the growth characteristics of other alphaherpesviruses.

Also in chapter 3, the expression of EHV-1 genes was successfully detected by RT-qPCR over an 18 h period (Figure 3.5A). The expression of the IE and E genes at t4 was comparatively higher than the expression of the L genes. The expression of all the gene classes was increased at t8 and t18. The increased expression of all gene classes simultaneously can be explained because in culture, we can find infected cells that are at the first stages of infection, expressing the IE gene, and other cells that are in a later stage of infection, expressing the E or the L genes, in the same well. The objective of this experiment was to determine the ability to detect viral

117

transcripts in infected cells at different time points by qPCR, not to describe the temporal expression of the viral genes.

In chapter 4, I explored the potential interaction of EHV-1 and the UPR during lytic infection to investigate if one might influence the other. Two experimental designs were performed. In the first experiment, I evaluated the influence of EHV-1 infection on UPR gene expression. To that end, E.Derm cells were infected with EHV-1 at a moi of 5pfu/cell, harvested at different time points over an 18 h period and RT-qPCR was performed to measure the expression of the UPR genes (Figure 4.1). The expression of the 3 UPR genes (GRP78, CHOP and HERP) was not altered at any of the investigated time points after EHV-1 infection. I concluded that lytic EHV-1 infection does not influence the expression of CHOP, HERP or GRP78 genes, partially refuting the hypothesis that EHV-1 reactivation from latency relies on the activation of the UPR.

In the second experiment in chapter 4, I evaluated the possible influence of an activated UPR on the EHV-1 gene expression. E.Derm cells were first treated for 4 h with thapsigargin or DMSO, and were then inoculated with EHV-1 at a moi of 5pfu/cell, harvested at different time points in an 18 h period and RT-qPCR was performed to measure the expression of the EHV-1 genes. The expression of all measured EHV-1 genes in UPR activated infected cells at different time points resembled the expression of EHV-1 genes in untreated infected cells (Figure 3.5 A and Figure 4.2 A). The levels of expression of each gene under the two conditions were compared as a ratio (Figure 4.3) and while the ratio for all genes was close to 1 at t8 and t18, the ratios were all less than 1 at t4. This suggested that although the UPR suppressed the expression of all three indicator genes at t4, this inhibitory effect was not apparent later in infection. Activation of the UPR could therefore temporarily influence viral gene expression or viral

protein expression, as immediate early and early proteins are required for expression of late viral proteins. Nonetheless, it was a temporary and reversible effect as gene expression was restored at t8 and t18.

In chapter 5, I attempted reactivation of latent EHV-1 from PBLs *in vitro*. To detect latent EHV-1 infection in PBLs, 2 different approaches were attempted. The first approach involved the use of a nested PCR, described in chapter 5. A detection limit of 1 pg of purified viral DNA in 500 ng total cellular DNA was determined. This corresponded to a required frequency of latent infection of approximately 1 viral genome per 27 cells. It has been described in the literature that the frequency of latently infected PBLs is 1 in 50,000 cells (Smith, Iqbal et al. 1998). This suggested that the PCR detection technique would not be sensitive enough to detect latent EHV-1 in PBLs and may explain why blood samples from a presumed latently infected horse yielded inconsistent results by the PCR technique.

The second approach involved the use of co-cultivation as the detection technique to identify reactivating virus when EHV-1 latently infected PBLs were treated with chemicals to induced viral reactivation. With co-cultivation, E.Derm cells would amplify infectious reactivated virus originating from the PBLs and allow CPE detection and detection of EHV-1 gene expression by PCR. The rationale for using co-cultivation was that over a million PBLs could be seeded onto permissive E.Derm cells and reactivation in a single PBLs would be amplified several thousand fold by replication in E.Derm cells. The reported low frequency of latent EHV-1 infection in PBLs (as mentioned above) would therefore, no longer represent a limitation for virus detection.

A pilot experiment was designed to evaluate the interaction of the amplifying system cells (E.Derm), the PBLs and the virus (EHV-1) in the co-culture environment, and to determine the efficiency of detection of lytic EHV-1 infection in this system. Lytic EHV-1 infection was detected by visualization of CPE and by qPCR to detect viral DNA. E.Derm cells were inoculated with serial dilutions of EHV-1 and incubated in the presence or absence of PBLs. From this pilot experiment, I concluded that the presence of PBLs in the co-cultivation wells interfered with the visualization of CPE and that, after 4 days of co-cultivation, the presence of the PBLs compromised the integrity of the E.Derm cells. I was also able to confirm that by co-cultivation of PBLs and E.Derm cells, I could consistently detect viral presence as low as 10pfu/well by visualization and qPCR. I was not able to consistently detect viral infection when the wells were inoculated with the viral dilution corresponding to 1 pfu/well. The serial dilutions from the stock virus were performed and the highest dilution tested should have contained 1 pfu per total volume. Considering that the inoculum was an aliquot of the total dilution volume, there is a chance that the 1 viral pfu was actually not introduced to the well. Usually, it would be expected that viral infection occurred if 1 pfu is present in the well.

Once the efficiency of co-cultivation for detection of lytic EHV-1infection was determined, I attempted *in vitro* reactivation of EHV-1 from latently infected PBLs by thapsigargin-induced activation of the UPR. IL-2 was used as a positive control stimulus to induce *in vitro* reactivation of EHV-1 from latently infected PBLs, as described by others (Smith, Iqbal et al. 1998). Blood samples from 3 different horses that were involved in an outbreak of EHM in the Saskatoon area in 2008 were used; 2 of those horses had clinical signs of EHV-1 infection when the outbreak occurred and all 3 horses had nasal swabs that tested positive by PCR to EHV-1 at the time of the outbreak. The PBLs were first incubated with thapsigargin or

IL-2 for the specified times (Figure 5.4), while control PBLs were incubated with DMSO or were sonicated and incubated in regular medium (as a control for the presence of active viral infection in the samples). Twenty four h after the beginning of the treatment, PBLs were co-cultivated with E.Derm cells for 5 days and qPCR performed on isolated DNA on day 5. During the co-cultivation period, CPE could not be detected in any group. There was also no detection of viral DNA by qPCR in any of the DNA samples isolated on day 5 of co-cultivation.

From these experiments, I concluded that the presence of the PBLs in the co-cultivation wells seemed to compromise the integrity of the E.Derm cells after 4 days of co-cultivation. Comparing the pilot experiment with the final experiments: the same cell types (E.Derm cells and PBLs) were co-cultivated using the same medium (RPMI 1640), for the same period of time, under the same incubation conditions. In the pilot experiment, cells were not treated with chemicals and the changes in the E.Derm cell morphology seemed less severe compared to the treated co-cultivated wells. The sonicated cells were not treated with any chemicals and those co-cultivation wells appeared to show less cellular damage compared to the treated co-cultivated wells. Treatment with IL-2, thapsigargin and DMSO may have had a detrimental effect on the PBLs cells that later induced a detrimental effect on the E.Derm cells. PBLs media after treatment were washed and replaced with fresh media before co-cultivation with E.Derm cells. However, compounds may still have been present and directly affect the E.Derm cells in the well. Nevertheless, if viral reactivation had occurred in any of the treated wells it would have been expected to correlate with CPE detection at any time point of the co-cultivation period and detection of viral DNA by qPCR.

Detection of latent EHV-1 genomes from PBLs of horses presumed to be latently infected was not achieved by using either PCR or co-cultivation. Reactivation of latent EHV-1 from

PBLs was not achieved because either the reactivation stimulus was not the correct one or virus was not present in the examined samples. As latent EHV-1 infection could not be reliably detected in PBLs, I could not draw definitive conclusions about the potential effect of thapsigargin and of UPR activation on latently EHV-1 infected cells. I was not able to reproduce the results obtained by Smith and others as the use of IL-2 did not result in viral reactivation from presumed latently EHV-1 infected PBLs *in vitro*.

Regarding the reasons why I was not able to detect latent EHV-1 in PBLs from blood samples of horses presumed to be latently infected with the virus, I propose the following hypotheses:

1) All the blood samples used in this project were from horses. Most of the literature involving EHV-1 latency and reactivation in PBLs has been described in Welsh Mountain ponies (Edington, Bridges et al. 1985; Gibson, Slater et al. 1992; Welch, Bridges et al. 1992; Slater, Borchers et al. 1994; Baxi, Efstathiou et al. 1995). There is published evidence that inflammatory processes are different in horses and ponies. Studies investigating the wound healing process revealed differences in leukocyte function and cytokine profiles of the local inflammatory response between horses and ponies (Wilmink, Stolk et al. 1999; Van Den Boom, Wilmink et al. 2002; Wilmink, Veenman et al. 2003). There is a lack of published evidence regarding the differences in the inflammatory response between horses and ponies; however, it is possible that interpretations made based on information gathered about EHV-1 latency and reactivation from ponies is not applicable to EHV-1 latency and reactivation in horses.

2) The attempts to induce EHV-1 reactivation by chemical induction in my project were performed *in vitro* using isolated PBLs. Identification of the specific cells harbouring

latent infection, before induction of reactivation *in vivo*, may be needed to determine if latent infection does occur in circulating PBLs. In the first reports of EHV-1 reactivation by co-cultivation of PBLs, the reactivation was induced in ponies *in vivo* with drugs (Edington, Bridges et al. 1985; Welch, Bridges et al. 1992). Blood samples were then collected and co-cultivated. Induction of viral reactivation *in vivo* can mislead the interpretation of the specific cell type harbouring the latent virus. Upon reactivation, the specific tissues latently infected with EHV-1 can amplify the viral infection to surrounding tissues from where the circulating PBLs can be productively infected with the virus. Hence, it is possible that the co-cultivation described in some of those reports is due to at least 1 productively infected PBLs in the blood sample. Interestingly, in the most recent report describing EHV-1 reactivation in latently infected horses *in vivo* (Pusterla, Hussey et al. 2010), the authors were able to determine reactivation by transient molecular detection of EHV-1 but they were not able to isolate the virus by co-cultivation. In a study published by Allen (Allen 2006), the author discussed the difference between "real" latency versus persistent infection in PBLs, as true latency of the viral genome was only detected in samples from mandibular lymph nodes but not in circulating leukocytes.

3) There is currently, to my knowledge, only one published report describing *in vitro* reactivation of EHV-1 from latently infected PBLs (Smith, Iqbal et al. 1998). In this study, the researchers used blood samples collected post-mortem from latently infected ponies (previously experimentally infected with EHV-1) and blood samples from abattoir horses of unknown EHV-1 infectious state. The concern that arises about that publication is that the authors did not specify how they assessed the infectious status of the animals

from which the samples were collected. If any of those animals had experienced a recent viral reactivation, the authors had no means to determine the difference in the origin of the active viral infection observed in culture. Whether the lytic infection detected in cell culture originated from reactivation *in vitro* or from reactivation *in vivo* was not conclusively proven. I attempted to reproduce the IL-2 induced *in vitro* reactivation of EHV-1 using PBLs collected from horses presumed to be latently infected as described by these authors and I was not able to obtain viral reactivation.

While the original hypothesis of this thesis was that activation of a cellular stress response, the UPR (shown to be related to molecular events in other viral infections), could induce reactivation of EHV-1 from latently infected PBLs, a broader discussion emerged from my results and the review of the literature related to EHV-1 latency.

There is still an enormous lack of information regarding the molecular aspects of EHV-1 latency, and most of the conclusions arrived at in the past were extrapolated from evidence obtained from HSV-1 models. Even though EHV-1 and HSV-1 are both members of the alphaherpesvirus subfamily, there is a clear difference in the proposed viral behaviour. The question arises then, why would an alphaherpesvirus like EHV-1 establish latency in leukocytes? Other members of this subfamily, like BHV-1 and PRV, have been proposed to establish latency in mammalian lymphoid tissue although this has not been conclusively proven. Both viruses have been shown to be present in the tonsils but latency in PBLs has been not reported (Balasch, Pujols et al. 1998; Winkler, Doster et al. 2000). EHV-1 has been suggested to establish latency in lymphoid tissues (Welch, Bridges et al. 1992; Edington, Welch et al. 1994) such as retropharyngeal, submandibular, bronchial and tonsillar lymph nodes. What is the biological

124

implication for this switch in the site to establish latency for these viruses? It is worth considering that EHV-1 establishes latency in lymphoid tissues related to the respiratory tract, as do BoHV-1 and PRV. In this location, the virus could gain an advantage by reactivation in a tissue related to a mucosa from where it can increase the transmission. Evolutionarily, does this feature confer any advantage for EHV-1 over other alphaherpesviruses?

The presence of viral genome in PBLs has been detected in several studies which should not be disregarded. Is it possible that detection of EHV-1 genome in a very small proportion of PBLs during latent infection is the result of a non-productive rather than latent infection in those cells? The mere presence of the EHV-1 viral genome in PBLs cannot be regarded as proof of latent infection. In the study conducted by Pusterla and collaborators, the authors characterized the viral loads, strain and state of EHV-1 using qPCR in horses following natural exposure at a racetrack. They tested nasopharyngeal secretions (NPS) and whole blood samples of more than 70 adult horses for three weeks after the confirmation of a neurological case of EHV-1 in a racetrack (Pusterla, Wilson et al. 2009). They assigned three possibilities for viral state (table 6.1) based on the molecular expression of LAT in cDNA and the expression of gB in genomic DNA (gDNA) or cDNA as: Lytic, Non-replicating or Latent. Five horses showed expression of LAT transcripts in blood samples. Four of those animals simultaneously tested positive in NPS for expression of gB in gDNA and cDNA, corresponding to the lytic viral state. Only one of the horses tested positive for LAT expression in the blood sample without expression of the gB gene in NPS, therefore corresponding to the latent viral state. In addition, only one horse tested positive for gB from gDNA detection in the blood sample without expression of LAT, corresponding to the non-replicative viral state. The authors suggested that categorization of distinct viral states might be regarded as simplistic, since "during an ongoing EHV-1 infection,

the viral state rarely satisfies the all or none rule, but rather progressively transitions from one state to the next" (Pusterla, Wilson et al. 2009). They also commented on the possibility that detection of gB DNA without expression of gB RNA could account for abortive EHV-1 infection or early reactivation from latency. If latency is defined as the presence in a cell of the viral genome, without detectable expression of viral genes (with the exception of the LAT transcripts) and without detectable virus production together with the ability to resume viral replication when exposed to a reactivating stimulus (Preston 2008), the detection of only one of those characteristics appears to be insufficient to determine latency.

Table 6.1 Determination of viral state corresponding with the molecular expression of the selected target genes in nucleic acids. Adapted from Pusterla 2009.

Target gene	Nucleic acid	Viral state		
		Lytic	Non-replicating	Latent
gB	*gDNA*	Yes	Yes	Yes
	cDNA	Yes	No	No
LAT	*cDNA*	No	No	Yes

As commented previously, Allen and colleagues also presented evidence that challenged the theory of PBLs as site of latency. In a study where samples from mandibular lymph nodes (MLN) and PBLs were collected *in vivo,* 18 out of 24 animals expected to be latently infected tested positive for EHV-1 DNA in MLN by a sequence-capture nested PCR. Those 24 horses had been infected with EHV-1 four to five years previous to this experiment. All the animals that tested positive in MLN tested negative in PBLs. The authors posed the question whether the detection of viral DNA in PBLs in previous investigations "represented a true latent state or active persistent infection by the virus" (Allen 2006).

After reviewing the published literature regarding EHV-1 latency and taking into account the results of my investigation where I was not able to detect latent EHV-1 in PBLs from horses presumed to be latently infected with EHV-1, I suggest that the concept of EHV-1 establishing latency in PBLs should be revised. The evidence suggests that future work involving EHV-1 sites of latency should focus on lymphoid tissue related to the respiratory tract.

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