

Characterizing and localizing EHV-1 chronic-persistent infection
in the natural host

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LIST OF ABBREVIATIONS

α -TIF	Alpha transducing factor	FAE	follicle-associated epithelium
ABC	avidin-biotin-complex	FAM	6-FAM-phosphoramidit
ADCC	antibody-dependent cell lysis	FeHV	Feline herpesvirus
B	bronchial lymph node	FFPE	formalin fixed paraffin embedded
BAC	bacterial artificial chromosome	g	gram
BCS	body condition score	GaHV	Gallid herpes virus
BHQ	black hole quencher	GALT	gut associated lymphatic tissue
BoHV	Bovine herpesvirus	gB	glycoprotein B
bp	basepair	gC	glycoprotein C
B2M	Equus caballus beta-2-microglobulin	gD	glycoprotein D
bw	body weight	gG	glycoprotein G
cDNA	complementary DNA	gH	glycoprotein H
CF	complement fixation	gK	glycoprotein K
CHV	Canine herpesvirus	gL	glycoprotein L
CNS	central nervous system	GCca	caudal cervical ganglion
CPE	cytopathic effect	GCcr	cranial cervical ganglion
CTL	cytotoxic T lymphocyte	GCI	ciliary ganglion
D	aspartate	GCoE	celiac ganglion
DD	D-Dimer	GM	mesenteric ganglion
DAB	diaminobenzidin	HEX	Hexachloro-Fluoresceine
DIVA	differentiating infected from vaccinated animals	HRP	horseradish peroxidase
DNA	Deoxyribonucleic acid	HSV	Herpes simplex virus
DRG	dorsal root ganglion	Hz	hertz
ds	double stranded	H&E	hematoxylin-eosin
E	early	ICTV	International Committee on Taxonomy of Viruses
ECE	equine coital exanthema	i.e.	id est
e.g.	example given	IE	immediate early
eGAPDH	equine glyceraldehyde-3-phosphate dehydrogenase	Ig	immunoglobulin
EHM	equid herpesvirus myeloencephalopathy	IHC	immunohistochemistry
EHV	Equid herpesvirus	ILTV	Infectious laryngotracheitis virus
EIA	equine infectious anemia	ISH	In-situ hybridization
ELISA	Enzyme-linked Immunosorbent Assays	IV	intravenous
		kbp	kilobase pair
		L	late

LAT	latency-associated transcript	SNP	single nucleotide polymorphism
LM	mesenteric lymph nodes	SPS	sympathetic/parasympathetic
LRT	lower respiratory tract	ss	single strand
LTT	large latency transcript	ST	sympathetic trunk
LR-RNA	latency-related RNA	TAMRA	Carboxy-Tetramethyl-Rhodamine
M	mandibular lymph node	TBS	Tris-buffered saline
MDV	Marek's disease virus	TG	trigeminal ganglion
mg	milligram	TRIS	Tris-(hydroxymethyl)-aminomethan
MHC	major histocompatibility complex	U _L	unique long
MHV	Murine herpesvirus	URT	upper respiratory tract
mi	micro	U _s	unique short
mL	milliliter	UV	ultraviolet
MLV	modified live virus	VN	virus neutralizing
MPS	mononuclear phagocyte system	VZV	Varicella zoster virus
mRNA	messenger Ribonucleic acid	WT	wild type
N	asparagine	μm	micrometer
nm	nanometer	μL	micrometer
nM	nanomolar		
NPS	nasopharyngeal secretions		
NSAID	nonsteroidal anti-inflammatory drug		
ORF	open reading frames		
oriLyt	lytic origins of replication		
P	pharyngeal roof		
PBL	peripheral blood leukocytes		
PBMC	peripheral blood mononuclear cells		
PCR	polymerase chain reaction		
PFU	plaque forming unit		
pi	post infection		
pmol	picomole		
PRV	Pseudorabies virus		
q	quantitative		
R	retropharyngeal lymph node		
RALT	respiratory tract associated lymph nodes		
R _i	internal repeat		
rpm	rounds per minute		
R _t	terminal repeat		
RT	reverse transcription		
S	spleen		
SHV	Suid herpesvirus		

I. INTRODUCTION

Herpesviruses belong to the order Herpesvirales and can be divided into three families (*Alloherpesviridae*, *Malacoherpesviridae* and *Herpesviridae*). The family *Herpesviridae* can be further subdivided into the subfamilies Alpha-, Beta- and Gammaherpesvirinae. Within the *Alphaherpesvirinae*, we distinguish the genera *Iltovirus*, *Mardivirus*, *Scutavirus*, *Simplexvirus* and *Varicellovirus* (KING et al., 2018). Primary herpesvirus infections occur at mucocutaneous junctions, mucosal or breached cutaneous surfaces often in the oronasopharyngeal or the urogenital tract. Herpesviruses spread through contact infection or through (short-distance) droplet infections. A hallmark characteristic of herpesviruses is their ability to cause a chronic-persistent, life-long infection in the host. Following a short-lived lytic infection, a latent infection commences, where the virus either retreats in sensory peripheral nerve cell bodies that are innervating the area of primary viral replication, or it infects immune cells of the mononuclear lineage. Herpesvirus latency is the dormant, silent stage of the chronic-persistent infection (BERNARD ROIZMAN, 2007).

Herpesviruses are large, double stranded DNA viruses with viral gene expression divided into three phases beginning with immediate early (IE) genes followed by early (E), and late (L) genes. Replication is a step-wise process downstream of genome transcription and translation, and it is the products of each of these steps that initiate the subsequent step (BERNARD ROIZMAN, 2007).

Herpesvirus latency is defined by a truncated genome transcription of IE genes and the absence of viral protein production (STEVENS, 1989; BERNARD ROIZMAN, 2007; HOGK et al., 2013). Well-defined circumstances and conditions are necessary to cause reactivation and recrudescence from latency, resulting once more in a lytic and productive infection in the host. As the location for latency in the body is histologically and physiologically connected to the original site of primary infection, recrudescence leads to active lytic infection at this site, and infectious virus can spread to other members of the population. As most herpesviruses are species-specific, latency is probably the most important mechanism to maintain their continuous presence in a finite population. Furthermore, a latent infection does not induce specific immunity and therefore,

future de novo infections are possible.

Equid herpesvirus type 1 (EHV-1), a member of the *Alphaherpesvirinae* is one of several herpesviruses that can be found in the horse. During lytic infection EHV-1 causes mild respiratory disease, and depending on the occurrence of a cell-associated viremia in peripheral blood mononuclear cells (PBMC), it can cause serious complications such as late-term abortions, equid herpesvirus myeloencephalopathy (EHM) and chorioretinopathies (GOEHRING et al., 2011; WILSTERMAN et al., 2011; HUSSEY et al., 2013; GOEHRING, 2015). Presumably, latency is established in any of the infected animals. Latent EHV-1 has been shown to persist in the trigeminal ganglion as well as in the mandibular and retropharyngeal lymph nodes (SLATER et al., 1994; CHESTERS et al., 1997; REED, 2004; PUSTERLA et al., 2012). Reactivation and recrudescence is thought to be infrequent; however, it can culminate in an outbreak after shedding of large amounts of virus into the environment. (SLATER, 2014).

Current vaccines do not protect completely from horizontal infection, and an eradication program to eliminate EHV-1 from horse populations is currently unrealistic. It is therefore prudent to deepen our knowledge on EHV-1 latency and its dynamics. If the establishment of virus latency during primary infection or reactivation from latency could be controlled, future outbreaks and their negative impact on the equine industry could be prevented.

As our long-term goal is either to prevent the establishment of latency or to prevent recrudescence, we will need to understand host and virus mechanisms that control this aspect of chronic-persistent infection. However, first we need to i) define locations of latency in the body of the horse, i.e. locations where active viral replication, lytic transcription and translation is absent, and ii) determine if strain differences have an impact on latency location or viral quantity in the horse.

For this, we collected a variety of neural parenchyma and lymphatic tissues of horses that were experimentally infected with either a wild-type strain of EHV-1 (Ab4) or one of two Ab4 mutant strain variants. Horses were euthanized 70 days post infection, and the collected tissues were evaluated by routine histology, quantitative (q)PCR, RT qPCR and (virus-specific) immunohistochemistry to assess for inflammation, (genomic) virus load, transcriptional activity, as well as viral translational activity in the various tissue samples.

II. LITERATURE REVIEW

1. Herpesviruses

1.1. Taxonomy

In 2009, the International Committee on Taxonomy of Viruses (ICTV) updated the herpesvirus classification due to recommendations of the *Herpesviridae Study Group*. The new order *Herpesvirales* consists of three families with more than 100 Herpesvirus species which have been isolated and classified and which are continuously updated by the ICTV (CARTER, 2013; KING et al., 2018). The three families consist of the former family *Herpesviridae*, which can be found in mammals, birds and reptiles, and two new families that were separated from the *Herpesviridae*: the *Alloherpesviridae*, infecting fish and amphibians and the *Malacoherpesviridae* infecting bivalve mollusk hosts (DAVISON et al., 2009; GROSE, 2012). Based on their biological properties, including which tissue or cell type is harboring latent virus and sequence similarity, the family *Herpesviridae* is divided into the subfamilies Alpha-, Beta- or Gammaherpesvirinae (EFSTATHIOU & PRESTON, 2005; DAVISON et al., 2009). The word Herpesvirus is derived from the word “*herpein*” (Greek = to creep) and alludes to the creeping spread of the rash induced by Herpes simplex virus in humans (MODROW, 2010). All known herpesviruses have in common, that once infection has occurred they remain in the host establishing life-long latency (EFSTATHIOU & PRESTON, 2005; OSTERRIEDER, 2011; CARTER, 2013). Most herpesvirus species are host-specific and most viruses affecting domestic animals belong to the *Alphaherpesvirinae*, genus *Varicellovirus* or *Simplexvirus* (DAVISON et al., 2009; OSTERRIEDER, 2011). Studies indicate, that the *Alphaherpesvirinae* have been in existence for more than 400 million years (GROSE, 2012). The *Alphaherpesvirinae* include viruses such as Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and Varicella zoster virus (VZV) in humans. Important members of the taxon *Alphaherpesvirinae* infecting animals are Marek’s disease virus (MDV, GaHV-2) and Infectious laryngotracheitis virus (ILTIV) in chickens, Pseudorabies virus (PRV) in pigs, Bovine herpesvirus types 1, 2 and 5 (BoHV-1, BoHV-2 and BoHV-5) in cattle, and Canine and Feline herpesviruses (CHV-1 and FeHV-1) in dogs and cats respectively

(OSTERRIEDER, 2011). In equids, nine herpesvirus species have been identified thus far (DAVISON et al., 2009; MA et al., 2013). Equid herpesvirus 1 (EHV-1), Equine herpesviruses 3 and 4 (EHV-3, EHV-4) as well as the Asinine herpesviruses 1 and 8 (formally named EHV-6 and EHV-8) and the Gazelle herpesvirus 1 (EHV-9) are members of the subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. EHV-2, EHV-5, Asinine herpesvirus 2 (EHV-7) and the recently identified Zebra herpesvirus 1 belong to the subfamily *Gammaherpesvirinae*. So far only *Alphaherpesvirinae* and *Gammaherpesvirinae* have been identified in the horse (EHLERS et al., 2008; DAVISON et al., 2009; MA et al., 2013; SLATER, 2014).

2. Alphaherpesvirinae

Alphaherpesvirinae have been found to cause similar disease processes within the different species they infect. These disease processes include respiratory (VZV, EHV-1, BoHV-1, PRV), neurological (HSV-1, HSV-2, VZV, EHV-1, PRV), as well as reproductive system pathology (EHV-1, BoHV-1, PRV), with the latter resulting in abortions. Neurotropism is another common feature of the *Alphaherpesvirinae* characterized by a lytic and latent state in nerve ganglia, with the ability to establish life-long latency (BLOOM, 2016). EHV-1 is notably as the latent virus also has been detected in the mandibular and retropharyngeal lymph nodes (SLATER et al., 1994; CHESTERS et al., 1997; REED, 2004; PUSTERLA et al., 2012).

Because research is mainly focused on human herpesviruses and notifiable, herpesvirus induced diseases with great economical impact on livestock animals, knowledge about EHV-1 infection is limited. A brief selection of important Alphaherpesvirus subfamily members, which share relevant characteristics with EHV-1, is summarized here.

2.1. Herpes simplex virus types 1 and 2

First contact with HSV-1 usually occurs in early childhood between ages of 6 and 18 months and results in oral infection. HSV-2 is sexually transmitted and results in genital infection (EFSTATHIOU & PRESTON, 2005; CARTER, 2013). Despite the classification of HSV-1 as a cause of oral infection and HSV-2 as a

cause of genital infections, there are increasing numbers of cases where HSV-1 infects the genitals and HSV-2 infects the face (CARTER, 2013).

After primary replication in the oral mucosa HSV-1 follows retrograde axonal transport to the neuron cell bodies in the trigeminal ganglia (TG) where it establishes latent infection (EFSTATHIOU & PRESTON, 2005). Reactivation can occur at any time, especially in immunocompromised hosts and strong, unfiltered UV-light is likely a common cause of HSV-1 recrudescence (ICHIHASHI et al., 2004). Furthermore, the degree of immunosuppression correlates positively with the likelihood of reactivation. When reactivated the virus starts replicating and is subsequently transported within the neuron to the initial site of infection. Lytic infection of the epithelial cells results in a cold sore. Sometimes serious complications such as encephalitis may occur, especially in severely immunocompromised hosts (CARTER, 2013).

The primary replication of HSV-2 takes place in the genital mucosa, subsequently following a similar retrograde axonal transport pathway but to the anatomically related sacral ganglia, where latency is established (EFSTATHIOU & PRESTON, 2005). In newborn babies infection with either HSV-1 or HSV-2 can result in serious disease (*Herpes neonatum*) (MODROW, 2010) with a mortality rate of about 54% (CARTER, 2013). When reactivated, both HSV-1 and HSV-2 can also be shed asymptotically (EFSTATHIOU & PRESTON, 2005).

2.2. Varicella zoster virus

Varicella zoster virus (VZV) has the smallest genome (125kbp) of the eight known human herpesviruses (M. DUMAS et al., 1980; DAVISON & SCOTT, 1986; GROSE, 2012). The virus is widespread and more than 95% of all humans are seropositive at 15 years of age. Infection normally occurs in childhood and causes chickenpox (MODROW, 2010). VZV is mainly transmitted horizontally and is the only human herpesvirus that is primarily transmitted by droplet aerosolization (GROSE, 2012). After aerogen transmission or direct contact with skin lesions, the virus replicates in the respiratory tract and the oropharynx causing varicella blisters typically seen in children, and then infects the PBMC. After viremia, the virus spreads to the mononuclear phagocyte system (MPS) and the endothelial cells. The rash on the skin is the result of inflammation moving from the endothelial cells of the capillaries to the epithelial cells of the skin.

During infection, the virus spreads via cell-to-cell contact with peripheral nerve endings. Latency is established in paravertebral dorsal root sensory ganglia (DRG). However, genomic DNA can also be found in the olfactory bulb and the *Corpus geniculatum* of the thalamus (MODROW, 2010). The VZV follows two main strategies: it first causes varicella blisters (chickenpox), then establishes latency, and finally may cause zoster (shingles) when reactivated from the DRG. Reactivation of VZV can be caused by immunosuppression, trauma to the spinal cord, psychological conditions and the use of various medications (MODROW, 2010). Normally VZV is a mild, non-life-threatening disease, but complications can occur in elderly or immunosuppressed people (DAVISON, 1991; MUELLER et al., 2008).

2.3. Bovine herpesvirus 1

In cattle, the notifiable Bovine herpesvirus 1 (BoHV-1) is the causative agent of the infectious bovine rhinotracheitis, infectious pustular vulvovaginitis and infectious balanoposthitis. Closely related to EHV-1 (OSTERRIEDER, 2011), BoHV-1 is worldwide a major pathogen in cattle which causes respiratory and genital disease.

Infection with BoHV-1 normally occurs through direct contact or inanimate vectors. The virus is spread through direct nose-to-nose contact or aerosolization over short distance. Genital infection occurs through virus contaminated semen or direct contact at mating (MUYLKENS et al., 2007). After infection, the virus first replicates in the mucosa of either the upper respiratory or the genital tract. This productive lytic infection causes cell death through apoptosis and necrosis, resulting in blisters. During viremia, BoHV-1 is transported to the secondary sites of infection, where clinical manifestation may cause late-term abortions in seronegative pregnant cows and fatal systemic infection in very young seronegative calves (NEWCOMER et al., 2017).

After replication at the primary sites of infection, the virus is retrogradely transported to the sensory ganglia of the head and the pelvis, the trigeminal and the sacral ganglion respectively, where latency is established (MUYLKENS et al., 2007). Although the ganglion cells are regarded as the main site of latency, BoHV-1 DNA could also be detected in tonsils, PBMC, lymph nodes and spleen even without detection of infectious virus (WINKLER et al., 2000; JONES et al.,

2011). Reactivation from latency can occur when animals are exposed to stressors (e.g. transportation, weaning) or after corticosteroid treatment (JONES et al., 2011). The virus is then transported to the site of primary infection through the axonal pathway, where it starts to replicate again. Infectious virus is then shed by symptomatic and/or asymptomatic animals to others nearby (MUYLKENS et al., 2007).

2.4. Pseudorabies virus

Suid herpesvirus type 1 (SHV-1), also known as Pseudorabies virus (PRV) or Aujeszky's disease virus is the causative agent of the economically important Aujeszky disease in swine (OSTERRIEDER, 2011). Transmission of the virus occurs directly through nose-to-nose contact or indirectly through contact with feces, secretions or fomites, included staff members. After infection, the virus replicates in the nasal and oral epithelial cells and the pharyngeal roof (tonsils). PRV is then spread to other organs via lymphatic and blood vessels resulting in a PBMC associated viremia (OSTERRIEDER, 2011). The virus can enter the central nervous system (CNS) either via retrograde transport through the TG or directly via the olfactory nerve through the olfactory neurons located in the nasal mucosa. Neurological, reproductive and respiratory signs are due to viral replication and the damage caused in related organs. The outcome of the disease highly depends on the age of the infected animal (VERPOEST et al., 2017). Immature development of the TG and CNS results in an inefficient suppression of viral replication, and therefore may explain the more severe neurological disease seen in 2-weeks old piglets (VERPOEST et al., 2017).

Similarly, to HSV-1 and BoHV-1 the primary site of PRV latency is the TG (CHEUNG, 1989; MAHJOUB et al., 2015). However, latent PRV genomes have been detected in other nervous tissues, such as the olfactory bulb and the brain stem. Tonsillar lymph nodes have also been reported a site of latency. Reactivation and shedding of the virus frequently occurs under stressful conditions, such as transportation, concomitant disease conditions or farrowing (POMERANZ et al., 2005). Carriers often do not show any signs of illness, but may silently shed the virus when it reactivates (OSTERRIEDER, 2011).

As PRV is known to have a broad host range, the infectious virus may also cause fatal encephalitis in many mammalian species, such as dog, cow and, although

rarely seen, horse (VAN DEN INGH et al., 1990; KIMMAN et al., 1991; CRAMER et al., 2011). Prevention of carry-over from wild boars and imported swine is of utmost importance and highlights the importance of the mechanism of latency, where a single infected animal that reactivates can infect others nearby and may lead to an outbreak.

To improve the knowledge on Alphaherpesvirus pathomechanisms, such as latency, PRV serves as a model organism to study the viral life cycle and the interactions with the host. PRV offers various advantages: it has a broad host range, it is easy to manipulate and still poses little hazard to laboratory personnel. Infection experiments can be performed in the natural host as well as in other model organisms, such as rats, mice and rabbits (POMERANZ et al., 2005). Furthermore, as neurological disease is more severe in neonates, both animals and humans, PRV infections in the natural host provides a suitable infection model to study neurological disease (VERPOEST et al., 2017).

2.5. Equid Alphaherpesviruses

The horse is the natural host to several herpesviruses, from which EHV-1, -3 and -4 belong to the subfamily *Alphaherpesvirinae*, genus *Varicellovirus*.

EHV-4 is an important Alphaherpesvirus affecting equids. It has a high degree of genetic and antigenic similarities with EHV-1 and, until 1981, both viruses were considered subtypes of a single species (SABINE et al., 1981; STUDDERT et al., 1981). Differentiation became possible in the 1990s, when one of the viral glycoproteins, gG, was shown to elicit type-specific antibodies (MA et al., 2013). Subsequently, assays for detection and differentiation between EHV-1 and -4 by polymerase chain reaction were developed (BORCHERS & SLATER, 1993; HU et al., 2014). Infections with EHV-4 cause rhinopneumonitis and occur through direct contact or through inanimate vectors. After primary replication of the virus in the upper respiratory tract (URT) mucosa, life-long latency is established within TG and lymphatic tissue (PATEL & HELDENS, 2005; SLATER, 2014). The virus has restricted tropism for epithelial and neuron cells (SLATER, 2014). In contrast to EHV-1, infections with EHV-4 are mainly associated with respiratory disease, and viremia is rarely seen (OSTERRIEDER, 2011; VANDEKERCKHOVE et al., 2011). However, recrudescence after stressors, such as transportation or hospitalization can lead to re-shedding and (re)infection

of other horses nearby.

EHV-3 is antigenically, genetically and pathogenically distinct from EHV-1/-4 and is the causative agent of equine coital exanthema (ECE), an acute venereal disease typically associated with formation of papules, vesicles, pustules and ulcers on the genital tract mucosa of mares and stallions. Tissue destruction is the result of a localized inflammatory response to the lytic infection (BARRANDEGUY et al., 2012). Nevertheless, infections can also be mild or subclinical. EHV-3 is primarily transmitted at mating but can also be spread indirectly through contaminated objects. As control of the disease mainly consists of the exclusion of infected animals from further breeding this disease has a significant impact on the equine industry worldwide (OSTERRIEDER, 2011; BARRANDEGUY & THIRY, 2012; TOISHI et al., 2017). Interestingly the exact location of EHV-3 latency is undetermined. The virus can reactivate spontaneously and experimental reactivation was observed after corticosteroid treatment (BARRANDEGUY et al., 2008).

The remaining equid Alphaherpesviruses are natural pathogens to donkeys (EHV-6 and -8) or are associated with wild equids, such as zebras (EHV-9). However, EHV-8 has been recently detected in association with abortion in mares (GARVEY et al., 2018). EHV-9 is the newest member of the *Alphaherpesvirinae*. It is a highly neurotropic herpesvirus and is closely related to the neuropathogenic EHV-1 (HARTLEY et al., 1999). It causes subclinical infections in the natural host, the Grevy's zebra and onager (*Equus hemionus*), but can cross the species barrier and cause encephalitis in wild animals such as the Thomson's gazelle, where it was first identified (FUKUSHI et al., 1997; SCHRENZEL et al., 2008). A recent report demonstrated that EHV-9 can also cause systemic infection in zebras (MOELLER et al., 2018). Though herpesviruses are known for species-specificity, EHV-1 and EHV-9 show the ability to infect multiple mammalian species, including Equidae, Rhinocerotidae and Bovidae (SCHRENZEL et al., 2008; ABDELGAWAD et al., 2016). EHV-9 showed tropism for nervous and respiratory tissues during experimental infections (DONOVAN et al., 2009) and establishes latency in the TG (GUEVARA et al., 2018).

2.6. Morphology

Besides similarities regarding the diseases caused and the ability to establish a

latent infection, all Alphaherpesviruses share similar morphology, that is different from all other viruses (DAVISON et al., 2009). Alphaherpesviruses are double stranded DNA (dsDNA) viruses and the size of these “large DNA viruses” varies from 180 to 250 nm in diameter (OSTERRIEDER, 2011). A linear DNA genome of 125 - 290 kbp is surrounded by an icosahedral capsid composed of 12 pentavalent and 150 hexavalent capsomeres (DAVISON, 2010; CARTER, 2013). The capsid is coated by a proteinaceous matrix, the tegument, which consists of more than 20 proteins of structural and functional importance, such as packaging of proteins for initiation of replication and virus transport (OSTERRIEDER, 2011). In turn, the tegument is covered by a lipid envelope (CAMPBELL, 2006) containing 16 different proteins, most of which are glycoproteins (DAVISON et al., 2009; DAVISON, 2010; CARTER, 2013). The envelope glycoproteins are each prefixed “g” (CARTER, 2013), bear antigen epitopes, and serve for viral cell entry pathways (OSTERRIEDER, 2011). Due to the enveloped structure, herpesviruses are vulnerable outside their host where they are rapidly inactivated by UV-light or mild detergents. Virus is spread through direct contact or inanimate vectors and short distance aerosols, but not through the air over long distances (OSTERRIEDER, 2011).

The genomic organization of all *Alphaherpesvirinae* has four general structural components (HAY, 2007) that consist of two unique regions (namely the unique long and unique short (U_L and U_S)), which are flanked by two inverted repeat regions (internal repeat (R_i) and terminal repeat (R_t)) (HAY, 2007; SLATER, 2014). The unique regions encode single copy genes, whereas the repeat regions might contain diploid genes necessary for cleavage and packaging of viral DNA (HAY, 2007). Replication of Alphaherpesviruses follows a highly regulated cascade of gene expression, giving rise to successively produce immediate-early (IE), early (E) and late (L) mRNA. Gene products from each phase have regulatory functions that enable up- and down-regulation of the subsequent phase. However, only the IE genes are capable of autoregulation (CARTER, 2013).

3. Equid herpesvirus 1

Infections with EHV-1 are probably the most important Alphaherpesvirus infections in the horse worldwide (OSTERRIEDER, 2011) and in contrast to

EHV-4, endothelial cells, lymphocytes and monocytes can also be infected (WILSTERMAN et al., 2011; SLATER, 2014). Thus, EHV-1 causes respiratory disease, but is much more feared because of devastating complications such as abortions and EHM (GOEHRING et al., 2006; GOEHRING, 2015). Therefore, EHV-1 has a high impact on the equine industry worldwide (SLATER, 2014). As outbreaks of infection and subsequent disease are single outbreaks, connected to a horse that returns from a (competitive) event, it is very likely that latent virus reactivates in a single horse, replicates and spreads horizontally leading to an outbreak. This again highlights the importance of EHV-1 latency and the need to improve our knowledge in this area.

3.1. The virus and its genomic organization

EHV-1 is an enveloped dsDNA virus with 150kbp size. The genome of EHV-1 is organized in the Alphaherpesvirus conformation: two unique regions, one long and one short, with the short unique region being flanked by two repeat regions, called the internal and the terminal repeat regions respectively (ELIZABETH A. R. TELFORD, 1992).

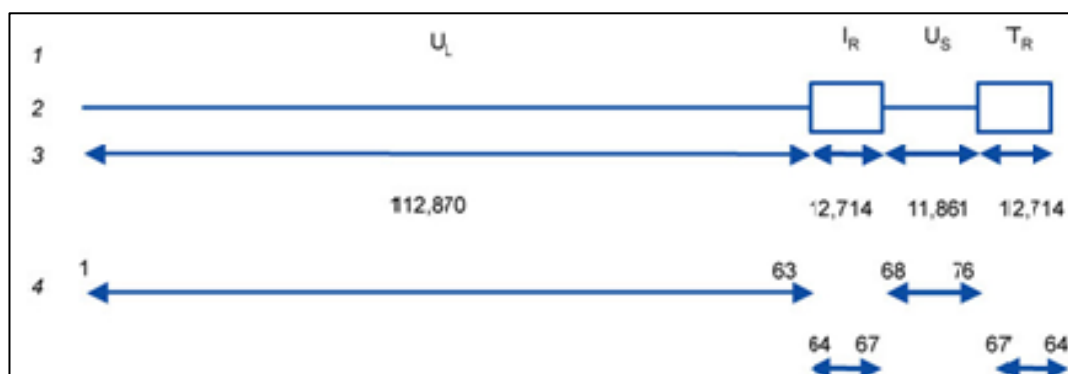


Figure 1: Organization of the EHV-1 genome. Note 1: two unique regions, one long (U_L) and one short (U_S), with the short U_S being flanked by two repeat regions, called the internal (I_R) and the terminal (T_R) repeat regions. **Note 2:** Schematic representation of the different genome regions. **Note 3:** Length of the different regions in base pairs. **Note 4:** Genes contained in the different regions: U_L contains gene 1 to 63, I_R contains genes 64 to 67, U_S contains genes 68 to 76 and T_R contains genes 67 to 64 (SLATER, 2014).

The linear dsDNA encodes 76 open reading frames (ORFs) (ELIZABETH A. R. TELFORD, 1992; SLATER, 2014). As four genes (gene 64-67) are located within the repeat regions, they are represented twice resulting in a total of 80 ORFs (MA

et al., 2013; SLATER, 2014). These ORFs include the sole immediate-early gene (IE), 55 early (E) and 20 late (L) genes (SLATER, 2014). Gene products have distinct functions: there are approximately 30 gene products that are associated with the virion (six for the capsid (PERDUE et al., 1974)), 12 that are associated with the tegument (MCLAUCHLAN & RIXON, 1992; PUREWAL et al., 1994), and 11 with glycoproteins. These glycoproteins have various functions, such as viral attachment for cell entry, fusion of infected cells and direct cell-to-cell spread. Five glycoproteins (gB, gD, gH, gK, gL) are required for replication, and therefore essential. Although glycoproteins are main viral immunogens, only three (gB, gC, gD) are considered immunodominant and are best recognized by the equine host. The remaining genes are associated with replication functions (SLATER, 2014). After the advent of complete genome sequencing in 1992 (ELIZABETH A. R. TELFORD, 1992), deeper studying of the pathogenesis of EHV-1 has been feasible; screening the whole genome for virulence factors became possible (SLATER, 2014).

It has been suggested, that circulating isolates in the field differ in their neuropathogenicity and the ability of causing abortions. Isolates with high virulence, such as Ab4, are more often associated with neurologic disease and abortions, than other isolates such as V592 (SLATER, 2014). Differences between the isolates were not necessarily associated with pathogenic potential. However, a single nucleotide polymorphism (SNP) in the viral polymerase (ORF 30), substituting G for A at position 2254 and resulting in an amino acid change from N (asparagine) to D (aspartate) at position 752 was significantly associated with nonneuropathogenic/neuropathogenic capacity, respectively (NUGENT et al., 2006; PERKINS et al., 2009). Studies, investigating recombinant viruses with different polymerase sequences, confirmed the causal relationship between this SNP in EHV-1 polymerase and neuropathogenicity in the horse (GOODMAN et al., 2007; VAN DE WALLE et al., 2009). However, neuropathogenicity seems to be multifactorial, with host response and virus genotype influencing the outcome of infection (WAGNER et al., 2011). Recent epidemiologic studies have revealed that EHM cases are not exclusively associated with the D752 variant, whereas higher association with prevalence of abortions was detected. More research is required to understand the genetic basis of the neuropathogenic phenotype in EHV-1 (SMITH et al., 2010; PRONOST et al., 2012).

3.2. Replication

Viruses do not have their own metabolism and thus need a living host cell to replicate. Viral replication can be generally divided into seven steps in which the mechanisms of the host cell are used. First step is attachment and adsorption, where the virus attaches to a functional receptor on the host cell. After cell entry, the virus gets uncoated, releasing its nucleic acids. Transcription, translation and viral genome replication processes make the fourth step. Following assembly, the virion exits the host cell through cell lysis or budding. Final post-translational modification of proteins in the progeny virus, complete the circle (TRUYEN, 2015).

These general steps of viral replication are common to all virus, regardless of the species. However, it is important to understand specific characteristics of the replication of each particular virus, to understand the pathogenesis of the disease it causes.

3.2.1. Lytic infection cycle

During productive lytic infection approximately 20 hours are needed for one replication cycle (SLATER, 2014), which starts with attachment of EHV-1 to the mucosal epithelial cells of the URT and involves binding to heparan sulfate first, followed by binding to the main receptor (CARTER, 2013). It has been suggested, that equine major histocompatibility complex (MHC) class I is a functional gD receptor that plays a pivotal role in EHV-1 entry into equine cells, and that efficient cell entry of EHV-1 (and -4) mainly depends on gD (SASAKI et al., 2011; AZAB, 2012). The virion envelope then fuses with the plasma membrane to enter the host cell (OSTERRIEDER, 2011; CARTER, 2013). This process is mediated by gB, gC, gD and presumably the gH/gL complex (NEUBAUER et al., 1997; OSTERRIEDER, 1999; CSELLNER et al., 2000). After cell entry, parts of the tegument proteins are released and transported to several sites within the cell, where they activate virus genes and downregulate host DNA, RNA and protein synthesis. Other tegument proteins remain associated with the nucleocapsid, which is rapidly transported along microtubules towards a nuclear pore (CARTER, 2013). This energy consuming process results in docking to a nuclear pore, where the capsid undergoes a transformational change that releases DNA into the nucleus (FRAMPTON et al., 2010; CARTER, 2013). Once in the nucleus, viral DNA forms a circular molecule and becomes associated with histones

(CARTER, 2013). Within the nucleus, viral DNA and viral proteins move to compartments where replication occurs. These ‘replication compartments’ can be seen under the electron and sometimes light microscope as intranuclear inclusions (ROY & WOLMAN, 1969). Genome replication starts with the expression of the immediate early protein (GRAY et al., 1987). Viral replication then follows a tightly regulated cascade of IE, E and L phases (CAUGHMAN et al., 1985; SLATER, 2014) (Figure 2). These three phases are controlled by six genes: the sole IE gene (ORF 64), four E genes (EICP 22, 27, 0 and TR2) and one L gene (ETIF) (SLATER, 2014). The 1487 amino acid IE protein is a regulatory protein (SMITH et al., 1992). It is expressed first and thus controls the following replication cascade. Through the capability of binding its own promotor, the IE protein can auto regulate itself and stop the replication cascade (SMITH et al., 1992). Hence, the IE protein functions as both: repressor and transactivator of the early and late gene promoters (KIM et al., 2012). Replication further requires activation of E genes (CARTER, 2013). Three (EICP 22, 27, TR2) of the E proteins co-act with the IE gene to transactivate E and L genes. In contrast, the EICP0 (ORF 63) downregulates the IE protein (KIM et al., 2003; ALBRECHT et al., 2005). Another protein, that downregulates the E genes and IE gene expressions is ETIF (KIM & O'CALLAGHAN, 2001). It is the product of L gene ORF 12 (LEWIS et al., 1997; KIM & O'CALLAGHAN, 2001) and is equivalent to the HSV-1 alpha transducing factor (α -TIF), which is important for cell-to-cell spread (CAMPBELL et al., 1984; VON EINEM et al., 2006; SLATER, 2014).

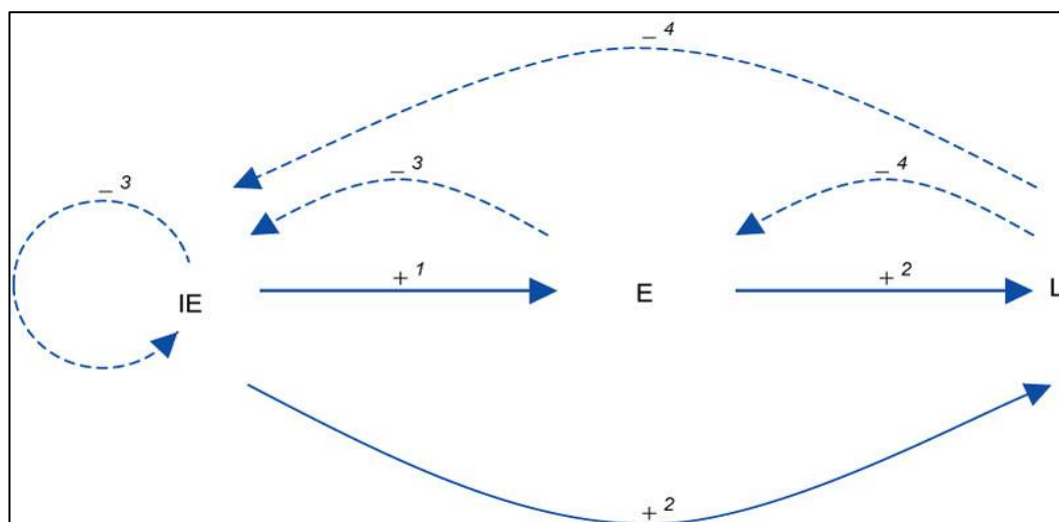


Figure 2: Replication cascade of EHV-1 during lytic infection. Gene expression follows a strictly regulated cascade of three phases: immediate early (IE), early (E) and late (L). Note 1: The IE transactivates promoters of

the E genes. Note 2: The IE and E genes transactivate L genes. Note 3: The E protein downregulates IE and the IE downregulates its own promoter. Note 4: The L proteins downregulate the expression of E genes and the IE gene (SLATER, 2014).

Furthermore, replication is dependent upon discrete sites that function as lytic origins of replication (*oriLyt*) (BOEHMER & NIMONKAR, 2003). Copies of origin binding protein bind at the *ori* sites and unwind the DNA. Double helix re-forming is prevented by a single stranded (ss) DNA-binding protein that binds to the ssDNA. A helicase acting complex of three proteins provides further unwinding and forming of a replication fork (CARTER, 2013). It is thought that the circular DNA is first amplified by Theta replication (bidirectional replication from *ori* with two replication forks) and then switches to Sigma replication (rolling circle replication). The exact mechanisms involved in this process are still unknown (BOEHMER & NIMONKAR, 2003). Replication products are long DNA molecules, so called concatemers, each of which consists of multiple copies of virus genomes (CARTER, 2013). Assembly of the virions starts with packaging of the newly synthesized DNA into the nucleocapsid which contains some tegument proteins. Entering the perinuclear space, the nucleocapsid is given a temporary envelope formed by the inner membrane of the nuclear envelope. This temporary envelope fuses with the outer membrane of the nuclear envelope and the nucleocapsid is released into the cytoplasm. Tegument proteins that were translated outside the nucleus are added. The virion envelope is now acquired from the Golgi complex and contains glycoproteins that were previously synthesized in the rough endoplasmic reticulum. The enveloped virion is transported in a vesicle to the plasma membrane where it is released from the cell. A lytic infection results in cell lysis (GRANZOW et al., 2001; CARTER, 2013).

3.2.2. Latent infection cycle

Latency is the dormant stage of the chronic-persistent infection and a hallmark of *Alphaherpesvirinae*. It plays a key role in the epidemiology, control and prevention of the disease. This section will give an overview of the latent infection cycle. Deeper insights of the current knowledge about the establishment of latency, expression of latency-associated transcripts (LAT), the prevalence and detection of EHV-1 latency, will be provided in section 4 'Latency'.

Commonly, latency location for *Alphaherpesvirinae* is the neural parenchyma, in particular sensory ganglia. After primary infection and replication, an Alphaherpesvirus enters sensible neuron endings and travels retrogradely towards the nerve nucleus, which is typically located in a sensory ganglion. As EHV-1 replicates in the epithelium of the nasal and pharyngeal mucosa, the TG has been identified as a latency location for this virus (SLATER et al., 1994; REED, 2004; PUSTERLA et al., 2012). However, and rather unusual for an Alphaherpesvirus, EHV-1 has also been detected in the mandibular and retropharyngeal lymph nodes (CHESTERS et al., 1997; REED, 2004; PUSTERLA et al., 2012) and in circulating CD8+ T-lymphocytes (CHESTERS et al., 1997; SLATER, 2014). Currently, all three tissues or cellular departments are considered primary latency sites.

For latent infection establishment, the steps until the virus enters into the nucleus are similar during both infection cycles. After attachment, the virus fuses with the plasma membrane and viral DNA is released into the nucleus. The exact mechanisms that control entry into lytic and latent infection cycles are not completely understood (SLATER, 2014). In nervous and lymphatic tissues, it appears that both cycles occur in parallel. However, during latency viral DNA is maintained in the nucleus and in contrast to the lytic infection, no viral replication takes place (CARTER, 2013). The virus is inactive and there is only limited transcription of the region antisense to the IE gene. From the human HSV-1, it is known that a latency-associated transcript (LAT) is continuously produced during the latent state (RAMAKRISHNAN et al., 1996; EFSTATHIOU & PRESTON, 2005). Presumably, EHV-1 has similar features that control latency, reactivation and suppression of transcriptional activity of IE gene transcription, which is a transactivator of the E gene promoter (SLATER, 2014). During latency, the viral genome persists in multiple copies in a continuous (circular) episomal form in the nucleus. Latently infected cells do not express viral antigens and are thus not detectable by the hosts immune system (i.e. they are non-immunogenic). After latency establishment, reactivation can occur secondary to a variety of stressors, including transport, illness and hospitalization (BURROWS & GOODRIDGE, 1984). The virus is then transported via anterograde axonal transport mechanisms towards the URT, where it replicates again. Virus is shed and may be transmitted to horses nearby (PUSTERLA et al., 2012). Thus, latently infected horses serve as

a natural reservoir (SLATER, 2014) and reactivation seems to be the major source of infective virus (PUSTERLA et al., 2012).

3.3. Pathogenesis

During EHV-1 infection, the virus enters the host through inhalation, ingestion or nose-to-nose contact. The virus then replicates in the URT mucosa where it causes cell death resulting in epithelial erosions necrosis, exudation and infiltration of phagocytic cells (REED, 2004; SLATER, 2014). After primary replication, the virus rapidly reaches the underlying lamina propia and is quickly spread to the regional respiratory associated lymph nodes, where it can typically be detected within 24-48 hours after infection. From these tissues, the virus enters the circulation and is disseminated to secondary sites during viremia, which is typically established 4 to 10 days following initial infection (HUSSEY, 2015). Vasculitis, thrombo-ischemia and secondary disease manifestation occur on days 9 to 13 after infection leading to EHM and/or abortion (PUSTERLA & HUSSEY, 2014; SLATER, 2014)

The ability to establish cell-associated viremia in PBMC is crucial to the pathogenicity of EHV-1 and a link between primary respiratory EHV-1 infection and secondary sites of disease manifestation as it allows the virus to be transported to other tissues (KYDD et al., 2012). Cell-associated viremia has been found most frequently within CD8+ T lymphocytes *in vivo*, followed by B-lymphocytes. However, viral DNA has also been detected in other subpopulations (CD4+ T lymphocytes and monocytes) (WILSTERMAN et al., 2011; SLATER, 2014). Following infection, EHV-1 infects endothelial cells and causes vascular lesions leading to thrombo-ischemia which may result in damage of the affected tissue (EDINGTON et al., 1986). The clinical thrombosis seen in horses with EHV-1 infection may be due to rapid stimulation of procoagulant activity in equine monocytes by EHV-1 (YEO et al., 2013). The activation of coagulation during EHV-1 infection has also been described (GOEHRING et al., 2013). In that study, EHV-1 viremia was associated with increases in D-dimers (DD), which are breakdown products of the coagulation cascade present after fibrinolysis (OLSON, 2015). Vascular endothelium infection occurs during viremia through direct contact with infected PBMC and cell-to-cell infection, rather than by viral egress from PBMC with an extracellular phase and subsequent endothelial cell infection (SMITH et al., 2002). One *in vitro* study demonstrated

EHV-1 infection of brain endothelial cells and endothelial cells from the carotid artery after cell-to-cell contact (GOEHRING et al., 2011). Free virus is rarely detected in the circulation and presumably would be neutralized by pre-existing antibodies in many cases (GOEHRING et al., 2011; SLATER, 2014).

Secondary disease manifestations of EHV-1 infections are EHM, EHV-1 abortions, neonatal foal death and chorioretinopathies. Horses that do not undergo viremia are unlikely to develop EHM or abortion (SOBOLL et al., 2010). In addition, the magnitude and duration of viremia is a critical determinant of whether secondary signs will develop or not. Genotypically neuropathogenic EHV-1 isolates are reported to induce longer and more severe viremia, which may explain the higher association with infections of the CNS and the uterus (GOODMAN et al., 2007; GOEHRING, 2015). Nevertheless, neuropathogenicity seems to be multifactorial and the appearance of EHM is dependent on both the host response and the virulence of the virus (SLATER, 2014). Vasculitis and local thrombosis lead to hypoxic degeneration and death of the nervous tissue, which causes myelopathy, encephalopathy or myeloencephalopathy depending on the region affected. Neurological signs associated with EHV-1 infection are therefore subsumed as equine herpesvirus myeloencephalopathy (EHM) (SLATER, 2014).

The pathogenesis of EHV-1 abortions includes the translocation of the virus to the endothelial cells of the uterus where it causes uterovascular lesions (SLATER, 2014). The pathogenesis of EHM and EHV-1 abortion at the vascular endothelium is thought to be similar (PUSTERLA & HUSSEY, 2014). In late pregnancy, the endothelial cells of the equine uterus seem more susceptible to EHV-1 than during early pregnancy (PUSTERLA & HUSSEY, 2014; SLATER, 2014). It has been suggested that abortions associated with EHV-1 during earlier pregnancy stages are caused by maternal stress or pyrexia (SLATER, 2014).

Another site of secondary infection with EHV-1 is the vasculature of the eye, inducing multifocal choroidal lesions in 50 to 90% of the horses experimentally infected with EHV-1 (HUSSEY et al., 2013). However, these ocular infections are subclinical and unlikely associated with loss of function (PUSTERLA & HUSSEY, 2014) and therefore of less economic importance. Nevertheless, it may serve as a surrogate model to study EHM pathogenesis, as the equine ocular fundus is anatomically and physiologically similar to the CNS (HUSSEY et al., 2013; PUSTERLA & HUSSEY, 2014).

3.4. Clinical signs

Infections with EHV-1 are associated with URT disease, abortions and neurologic disease. Affected horses typically show biphasic fever, anorexia, depression, tachypnea and dyspnea. Fever peaks occur 24 to 48 hours after infection and again on days 6 to 7. The first respiratory signs occur after an incubation period of up to 10 days. In contrast to equine influenza virus, coughing is not a major clinical sign of EHV-1 infection. Nasal discharge is present and changes rapidly from serous to mucous and mucopurulent, presumably due to secondary bacterial infection. Conjunctivitis and serous ocular discharge are common. There is a progressive lymphadenopathy, mainly of the mandibular, but also of the retropharyngeal lymph nodes (SLATER, 2014). Bloodwork changes include primary leukopenia followed by leukocytosis (GIBSON et al., 1992).

In horses previously exposed to EHV-1 clinical respiratory signs may be mild and of short duration or even absent. As a result, these animals may show no clinical signs of respiratory disease prior to abortion or recumbency (GOEHRING et al., 2005). Abortions typically occur in the last trimester of pregnancy and mares may abort while standing. Mares normally can conceive successfully shortly after abortion (SLATER, 2014). Neonatal deaths during delivery may occur if gestation reaches term, or neonatal foals that survive to birth can be affected and show weakness, lethargy, and profound respiratory distress (MURRAY et al., 1998). In this foals it is unclear whether infection occurs *in utero* or shortly after birth, but infected foals typically die within 2 to 3 days after birth (SLATER, 2014).

In horses suffering EHM, neurologic signs typically appear during the end of viremia, 6-10 days after infection and are often acute or peracute (REED, 2004). The viremic phase of EHV-1 infection is the critical phase, where virus is transported from the respiratory tract and the respiratory tract associated lymphatic tissues (RALT) to the CNS (GOEHRING et al., 2011). The presentation depends on the severity (number, size) and location of ischemic lesions in the spinal cord. Horses show different degrees and symmetry of ataxia, dysmetria, and weakness of the forelimbs and hindlimbs (GOEHRING, 2015). Debatably, caudal segments of the spinal cord and the lumbar intumescence are more intensely affected, resulting in cutaneous perineal and limb sensory but above all peripheral motor neuron deficits. In addition there is often bladder dysfunction, an upper or lower motor neuron bladder dysfunction resulting in

dysuria, atony, incontinence or urinary retention (SLATER, 2014). EHM shows with an acute onset and rapid progression during the first 24 to 48 hours, but then stabilizes (GOEHRING, 2015). Weeks to months are necessary for recovery, a return to full function is not guaranteed. For nonrecumbent horses, the prognosis is favorable, but once recumbent, the prognosis is guarded to poor (SLATER, 2014).

3.5. Immunity

The immune response to EHV-1 infection is very complex (SLATER, 2014). After replication in the mucosa, the virus enters blood vessels and lymphatics via endothelial cell infection (invasion phase) and is distributed throughout the horse in the viremic phase. Following lytic infection, the virus can evade the host immune system and establish a life-long persistent latent infection. Therefore, EHV-1 interacts closely with the equine immune system.

An effective immune response against EHV-1 requires a combination of mucosal and systemic humoral and cellular immunity (SLATER, 2014). Infection induces a transient protective immune response for 3 to 6 months (KYDD et al., 2006b). During this time, there is clinical protection and no viral shedding (SLATER, 2014). A strong humoral immunity with an initial short-lived (<3 months) immunoglobulin M (IgM) mediated response is followed by a long-lived (>12 months) IgG induction. Mucosal IgA is found only in small quantities. These antibodies are secreted onto mucosal surfaces for a short time (weeks), and the duration of this period does increase upon stimulation (KYDD et al., 2006b). Although a horse's EHV-1 antibody status provides information on past exposure to the virus, the strength of the humoral response does not seem to correlate with protection against disease (HENNINGER et al., 2007; DUNOWSKA, 2014a). Furthermore, it should be kept in mind that many cross-reactive epitopes exist between EHV-1 and EHV-4 (HARTLEY et al., 2005).

Infection also induces tissue (mucosal and lymphatic) and circulatory cellular immune responses mediated by CD8+ T-lymphocytes, which are associated with MHC I restricted cytotoxic T lymphocyte (CTL) activity (KYDD et al., 2006b). Thus, EHV-1-specific CTL can recognize and lyse virus-infected cells in a genetically restricted, antigen-specific manner (ALLEN et al., 1995). The virus becomes intracellular within hours of contact with host cells (KYDD et al., 1994a,

1994b), thereby evading the neutralizing effects of serum antibodies (KYDD et al., 2006b). There is a consensus between researchers that cellular and possibly mucosal immunity is more important for protection against EHV-1-induced disease than humoral responses (KYDD et al., 2012).

EHV-1 has evolved sophisticated mechanisms to escape elimination by the host's immune system (VAN DER MEULEN et al., 2006; MA et al., 2013). One such mechanism is the ability of EHV-1 to down-regulate cell surface MHC I on infected cells (MA et al., 2012). This downregulation of MHC I results in avoidance of natural killer cell-mediated lysis (DUNOWSKA, 2014b). The humoral immune evasion strategy includes interference with antibody-dependent cell lysis (ADCC), which makes infected cells insensitive to ADCC-mediated elimination (VAN DER MEULEN et al., 2003; VAN DER MEULEN et al., 2006). Further, establishment of latency is a key feature for not being eliminated by the host immune system (EDINGTON et al., 1994).

Vaccination remains a key strategy in protection against viral disease (ABBAS et al., 2014). The vaccines against EHV-1 must satisfy a difficult series of demands, including the induction of humoral and cellular immunity (SLATER, 2014). Thus far, modified live virus (MLV) and inactivated vaccines are available (MAYR A., 1968; BURROWS R., 1978; BURROWS et al., 1984; ALLEN & BRYANS, 1986; BURKI et al., 1990; BURKI et al., 1991; JESSETT D.M., 1998; MINKE et al., 2004), which are all capable of inducing some, but not all of the desired components. The MLV vaccines have an excellent safety record and can protect horses against clinical disease, however their efficacy in preventing viremia, abortion and neurological disease is unclear and has been only reported under experimental conditions (GOODMAN et al., 2006; KYDD et al., 2006b; BRESGEN et al., 2012; MA et al., 2013). Inactivated vaccines and MLV induce high titres of virus neutralizing (VN) antibodies, but there is little evidence, that significant cellular immunity is induced (SLATER, 2014). Thus vaccine development should be aimed at stimulating both CD8+ and CD4+ elements of cell mediated immunity, in particular CTLs, but at the same time maximise stimulation of mucosa and plasma VN antibodies (KYDD et al., 2006b). Therefore, new generation vaccines like subunit/vector vaccines, DNA vaccines and new generation MLV have been tested (MINKE et al., 2006; SOBOLL et al., 2006; ALLEN, 2008; SOBOLL et al., 2010; BANNAI et al., 2018). Despite all

these developed vaccine candidates, many are still unsatisfactory because of safety issues, lack of optimized target genes or poor antigenicity, and the ideal vaccine has yet to be found (SOBOLL et al., 2006; WAGNER et al., 2011; MA et al., 2013).

3.6. Diagnosis

Viral diagnostic methods can be generally divided into direct (antigen) and indirect (antibody) detection assays. The direct method includes virus isolation and cultivation in cell culture, electron microscope presentation and molecular biology detection assays (for antigen or viral nucleic acids). The indirect serological approach is based on the analysis of the host's immune response. The presence of specific antibodies can be detected by Enzyme-linked Immunosorbent Assays (ELISA), complement fixation (CF), specific CTL detection or other methods for serological investigation (FIELDS et al., 2013).

For EHV-1 detection, both direct and indirect methods are used. Diagnosis of EHV-1 infection can be further subdivided into ante mortem (for suspected acute infection) and post mortem (mainly for latent infection) diagnosis.

3.6.1. Ante mortem diagnosis of EHV-1 infection

Ante mortem diagnosis under outbreak conditions requires fast and reliable diagnostics for EHV-1, as it is important for further therapy and biosecurity measurements (GOEHRING et al., 2010). Accurate sample and case selection are crucial for further investigations (SLATER, 2014; BALASURIYA et al., 2015). Clinical signs are often very nonspecific and fever may be the only warning sign, which can be easily overlooked (POWELL, 1991; BALASURIYA et al., 2015). However, a presumptive diagnosis of EHM based on clinical signs should always be confirmed via laboratory testing on nasal swabs or venous blood samples (SLATER, 2014; GOEHRING, 2015).

Direct methods for EHV-1 diagnosis include demonstration of EHV-1 antigens by direct immunofluorescence test, virus isolation and detection of viral genomic DNA. Virus isolation remains the "gold standard" and involves the typical cytopathic effect (CPE) seen in cell culture (SLATER, 2014). However, this approach is time consuming requiring a minimum of 3-5 days (GOEHRING, 2015). In addition, both tests require the presence of replicating virus. Therefore,

qPCR is the method of choice and analysis of nasal swabs and blood samples is a fast and sensitive test for confirmation of EHV-1 infection (GOEHRING, 2015). Both conventional (gel-based) and real-time qPCR are available, which distinguish the gB sequence between EHV-1 and EHV-4 (SHARMA et al., 1992; LAWRENCE et al., 1994; HUSSEY et al., 2006). Tests are also available, which not only detect EHV-1, but also differentiate between EHV-1 isolates with D752 and N752 genetic markers (ALLEN, 2007; SMITH et al., 2012). However, these tests showed less sensitivity than the gB qPCR (PUSTERLA et al., 2009b).

Indirect evidence of infection can be investigated by serology. Serologic detection of EHV-1 infection can be achieved by demonstration of seroconversion or a four-fold increase in antibody titers between paired sera taken 3 weeks apart (BALASURIYA et al., 2015; GOEHRING, 2015). Different methods are available to determine EHV-1 serum antibody levels, of which CF and virus neutralization (VN) do not distinguish between EHV-1 and EHV-4, whereas glycoprotein G ELISA does (LANG et al., 2013; SLATER, 2014).

3.6.2. Post mortem diagnosis of EHV-1 infection

In some situations, post mortem diagnosis is required to confirm EHV-1 infection. This may be the case when multiple late term pregnancy losses occur, suggesting the occurrence of EHV-1 abortions. Appropriate testing of aborted fetuses is necessary to identify the causative agents and, in case of EHV-1 abortion, infective virus will be (most likely) detected (SLATER, 2014). However, post mortem diagnosis is mainly used for detection of latent EHV-1 infection, as the ante mortem confirmation of the presence of a latent infection remains challenging for various reasons (BALASURIYA et al., 2015): 1) Latently infected horses do not show any clinical signs; 2) No viral replication is present during latency and there is only a small number of viral genomes within the cells and some infected leukocytes; 3) Transcription is limited to the region antisense to the IE gene with only an EHV-1 LAT thought to be expressed during latent infection; and, 4) Serological investigation through antigen-detection assays is impossible, because major viral proteins (antigens) are absent.

Thus far, direct methods for post mortem analysis of latent infection mainly include assays to detect virus following in vitro reactivation and molecular tools for nucleic acid or viral protein detection. Demonstration of virus by co-

cultivation (an *in vitro* test for reactivation) is considered the “gold standard” because it unequivocally demonstrates the presence of reactivatable latent infection (WELCH et al., 1992; SMITH et al., 1998; SLATER, 2014). However, a negative result does not confirm the absence of a latent infection (BALASURIYA et al., 2015).

Molecular assays to demonstrate the presence of EHV-1 include PCR, *in-situ* hybridization (ISH) and immunohistochemistry (IHC). These are valuable for post mortem diagnosis and are used to detect viral DNA and viral proteins, respectively. For the diagnosis of latency, ISH may give insights about the exact localization of the viral DNA within fixed tissue samples. Following PCR detection of viral DNA, IHC distinguishes latent and lytic infectious cycles by the absence or presence of L gene proteins, respectively (EDINGTON et al., 1987; WHITWELL et al., 1992; SZEREDI et al., 2003b; SZEREDI et al., 2003a). For detection of latent infection, a nested PCR is available (BORCHERS & SLATER, 1993; EDINGTON et al., 1994). In addition, an ultrasensitive magnetic bead-based, sequence-capture nested PCR method was developed for detection of rare, low-abundance sequences below the detection threshold of conventional nested PCR (ALLEN, 2006; ALLEN et al., 2008). However, all of these techniques suffer from some disadvantages: virus isolation techniques are labor intensive and time consuming; IHC and ISH have limited sensitivity; and conventional PCR is not quantitative. Since 2006, a sensitive and quantitative test for EHV-1 is available (real-time qPCR), targeting glycoprotein B (L gene) (HUSSEY et al., 2006). However, demonstration of gDNA by qPCR is not sufficient, as it may be also present during reactivation or lytic infection (SLATER, 2014). Further sample processing is required, which aims to detect LAT or other IE gene mRNAs. LATs seem the only transcripts present during latency. Their detection would therefore confirm the presence of a latent infection. However, the detection of these is technically challenging, as LATs seem not to be universally expressed in all cells carrying latent virus. Thus, a functional marker for latency still needs to be identified (SLATER, 2014).

Hitherto, diagnosis of latent EHV-1 infection based on qPCR for DNA and reverse transcription (RT) qPCR for RNA detection includes three approaches. One approach is detection of viral DNA and an EHV-1 LAT within the same sample (CHESTERS et al., 1997; ABDELGAWAD et al., 2016). Another

approach is detection of EHV-1 gDNA and IE gene mRNA expression with simultaneous absence of L gene activity by RT qPCR (PUSTERLA et al., 2009b). In a third approach, latent EHV-1 infection is diagnosed by detection of viral DNA in the absence of detectable late structural protein genes mRNA (ALEMAN et al., 2012; PUSTERLA et al., 2012; SLATER, 2017). Samples required are typically venous blood samples for detection of latent EHV-1 in PBMC and various tissue samples, such as the TG or mandibular and retropharyngeal lymph nodes.

3.7. Treatment

After EHV-1 infection has been confirmed, appropriate treatment is required, which depends on the clinical presentation. Respiratory disease is generally mild and self-limiting and therefore does not require specific treatment (SLATER, 2014).

Abortions associated with EHV-1 occur without warning and mares typically do not receive special medications. Treatment of in-contact mares with antiviral medications does not reliably prevent abortion, however, a recent study demonstrated that following experimental infection with EHV-1, healthy foals were delivered, despite of the vicinity to aborted fetuses. (GARDINER et al., 2012).

Treatment of horses with EHM mainly consists of supportive care (LUNN et al., 2009) and is targeted towards reduction of inflammation associated with EHV-1 induced vasculitis (PUSTERLA et al., 2009b; PUSTERLA & HUSSEY, 2014; GOEHRING, 2015; GOEHRING et al., 2017).

3.8. Control during an outbreak

If despite all precautions an EHV-1 outbreak occurred, it is important to get the situation under control as quickly as possible to curtail the spread of infection within a premise and beyond. Therefore, a horse with acute neurological disease in combination with fever should always be considered as a potential EHM case and admission to hospital should be under strict isolation precautions (GOEHRING et al., 2010). In any case, as soon as disease is suspected on a premise, the horse should be immediately isolated, ideally with a separate airspace, and any in contact-horses should be monitored for signs of disease (GOEHRING et al., 2010). Literature agrees, that EHV-1 outbreak management

should be based on some fundamental strategies. These include: (1) recognition of a suspected case, (2) quarantine of the affected horse(s), (3) biosecurity measures including overalls, single-use protective gowns, dedicated boots, disposable gloves and hand sanitation between horses, (4) confirmation of the diagnosis via qPCR of nasal swabs, (5) monitoring of in-contact horses and the general population for clinical signs and fever (rectal temperature should be taken at least twice a day) (GOEHRING et al., 2010; SLATER, 2014; GOEHRING, 2015; GONZALEZ-MEDINA & NEWTON, 2015). It is recommended to lift a quarantine after three consecutive nasal swabs were tested negative plus an additional 14 days of isolation (GOEHRING et al., 2010). Until then horse movement should be stopped.

3.9. Prevention

Outbreaks may lead to high economic and emotional losses and disease prevention is crucial. Thus far, prevention of EHV-1 infection mainly consists of vaccination and strict biosecurity measures including the prevention of disease entry onto premises. However, due to the open character of training and boarding facilities, these measures are practically unfeasible (GOEHRING et al., 2006). Furthermore, the majority of horses are latently infected and do not show any clinical signs, but may reactivate after stress situations, such as transportation or introduction into a new herd. These silent shedders are of high risk for the spread of infection. If recrudescence of the virus could be controlled, new strong strategies in prevention of disease outbreaks would become feasible. Until then, the occurrence of an EHV-1 outbreak cannot be completely avoided but only reduced following appropriate measures, such as isolation.

Besides these measures, vaccination remains a key point in EHV-1 prevention, although there is currently no vaccine available that can reliably prevent EHM (GOEHRING, 2015). In addition, vaccination does not protect against the establishment of latency.

3.10. Epidemiology

After primary infection, the virus retreats to the TG (SLATER et al., 1994; REED, 2004; PUSTERLA et al., 2012) and the RALT (CHESTERS et al., 1997; REED, 2004; PUSTERLA et al., 2012), where it establishes a chronic-persistent, latent infection.

Although EHM is an uncommon manifestation of EHV-1, outbreaks have been reported consistently since 1966 (GOEHRING et al., 2006; MCFADDEN et al., 2016). Several circumstances may increase the risk of an EHM outbreak. These include season (fall, winter, and spring), age, breed, and stress factors such as transportation, exercise, mingling and crowding, and hospitalization (PUSTERLA et al., 2009a). It has been suggested that adult or adolescent (> 3 years of age) horses of tall breeds are more likely to develop EHM (GOEHRING, 2015). If these horses are transported to competitions, manifestation of EHM may ensue (GOEHRING, 2015). However, a number of studies has failed to detect recrudescence of EHV-1 under these conditions (YACTOR et al., 2006; CARR et al., 2011; SONIS & GOEHRING, 2013). The presence of the so called neuropathogenic isolate D752 (single nucleotide polymorphism (SNP)) in the DNA polymerase gene (CROWHURST et al., 1981; NUGENT et al., 2006) is discussed in several studies (STASIAK et al., 2017; BRYANT et al., 2018), and appears to be associated with higher incidence of EHM, possibly due to prolonged and/or higher levels of viremia (SLATER, 2014). However, in one fourth of horses affected by EHM the non-neuropathogenic variant N752 has been identified and experimentally, the D752 strain does not always lead to EHM (GOEHRING, 2015).

Based on the limited number of EHV-1 related disease outbreaks, reactivation and recrudescence out of latency has to be a rare event in the individual and latently infected horse. Taking this into consideration, deeper knowledge about the mechanisms involved in the establishment of EHV-1 latency and recrudescence are important to develop strategies for disease control and outbreak prevention.

4. Latency

Latency is the key feature of Alphaherpesviruses that allows the virus to establish a lifelong chronic-persistent infection in the host. It is defined as a type of infection without viral genome transcription (HOGK et al., 2013) except limited expression of a region antisense to the IE gene (EFSTATHIOU & PRESTON, 2005; SLATER, 2014). During latency, viral genome persists within tissue but no infectious virus, viral proteins or viral lytic transcripts can be detected (STEVENS, 1989; BLOOM, 2016). The virus is dormant but can be reactivated at

any time.

For HSV this classical definition of latency was overruled with the use of more sensitive techniques, which showed that latency is much more a dynamic state that requires looking not only at tissue, but also at cellular levels. Therefore, Bloom (2016) recently defined latency as *“the persistence of a viral genome within tissue where, at any given time, there is a population of cells that lack detectable infectious virus, viral proteins, or viral lytic transcripts that are dormant but have the capability of being reactivated”* (BLOOM, 2016). This suggests, that within the same tissue, different cells could harbor latent, lytic and/or reactivating virus at the same time.

Our knowledge on EHV-1 latency is extremely limited and whether this new definition is also applicable to EHV-1 has yet to be determined. The following sections will give more information on the current knowledge of Alphaherpesvirus latency in general and of EHV-1 in particular.

4.1. Establishment of latency

After cellular infection, the virus may either enter the lytic or the latent pathway (ALLEN, 2004). For EHV-1 the exact mechanisms controlling entry into lytic or latent infection and reactivation are not entirely known (SLATER, 2014). For HSV-1, the prototype of *Alphaherpesvirinae*, it has been suggested that the interaction and the amounts of incoming viral genome into neural cell type could influence the outcome. Furthermore, it has been shown, that productive lytic infection is not required for effective HSV-1 latency establishment (BLOOM, 2016).

In equids, EHV-1 latency is readily induced during primary respiratory epithelial infection where the virus penetrates the sensory nerve endings of the trigeminal nerve (cranial nerve V). From the nerve endings, the virus is assumed to be transported via retrograde axonal transport mechanisms towards the cell body of the neuron. All sensory cell bodies are combined in the TG on either side of the brain stem covering the trigeminal impression of the apex of the petrous part of the temporal bone (NICKEL, 2003). However as already mentioned, while EHV-1-induced state of latent infection has been shown in the TG, assumed latent virus has also been shown in circulating CD8⁺ T-lymphocytes (BAXI 1995; CHESTERS et al., 1997; SLATER, 2014) and in mandibular and retropharyngeal

lymph nodes (CHESTERS et al., 1997). In contrast, HSV-1 establishes latent infection only in nervous tissue, and specifically in populations of sensory neurons within the TG that are innervating the primary (epithelial) site of HSV-1 infection (BLOOM, 2016). However, latency is typically established only in a small population of the cells in the ganglion (RAMAKRISHNAN et al., 1996). It is assumed that the number of EHV-1 latently infected lymphocytes declines over time, as these cells are short-lived. However, the number of cells is increased during reactivation episodes or by exposure to new infections. In the long-lived neurons, latently infected cells are expected to be stable (SLATER, 2014).

During Alphaherpesvirus latency the viral genome is retained in the nucleus (CARTER, 2013) and persists in a circular form without integration into the host genome (GULATIY, 2015). The immediate early protein (IEP) encoded by the IE gene is associated with latency regulation. The expression of the IEP activates the E and L genes resulting in productive lytic infection. In contrast, inhibition of the IEP production downregulates the expression of E and L genes resulting in latency. This has been shown for EHV-1 (KYDD et al., 2006a; KYDD et al., 2006b). In turn, the gene product of ORF 12, the homolog to HSV-1 α -TIF (α trans-inducing factor) product is required for IE promotor activation (LEWIS et al., 1993). Another protein, which is supposedly necessary for the establishment of latency, is the EICP0, an early protein encoded by ORF 63 (KIM et al., 2003). EICP0 homologues have been found in HSV-1, BHV-1 and PRV (BOWLES et al., 1997). Latent virus can reactivate and result in symptomatic or asymptomatic virus shedding. EHV-1 is assumed to reactivate from the TG (SLATER et al., 1994) and from lymphocytes (SMITH et al., 1998).

For the equine industry, EHV-1 infections including the silent and non-silent shedders have a high impact, as reactivation in a single animal can have devastating outbreak consequences.

4.2. Latency associated transcripts

For HSV-1 it has been suggested that a LAT possesses regulatory functions balancing latency and recrudescence (KENT et al., 2003). There is evidence that LATs are transcribed from a DNA strand opposite to the viral immediate early IPC0 gene (RAMAKRISHNAN et al., 1996; KANG et al., 2006) and that they may down-regulate the expression of ICPO by an antisense mechanism.

Descriptions about the location of LAT within the genome of EHV-1 in previous publications is inconsistent. It has been claimed that the genomic EHV-1 LAT region is located either within the region of ORF 63 (HSV-1 ICP0 homologue) (BAXI 1995; ABDELGAWAD et al., 2016) or ORF 64 (CHESTERS et al., 1997; PUSTERLA et al., 2009b; SLATER, 2014). Potential regulatory functions during latency have also been suggested for the transcript of a gene located antisense to a region between ORF 64 and ORF 65 (HOLDEN et al., 1992; AHN et al., 2010).

In HSV-1, LAT has been studied in greater detail. Therefore, HSV-1 could serve as a model for EHV-1 LAT investigation. During latent infection of HSV-1, one single region of the genome remains transcriptionally active producing multiple nonpolyadenylated LATs. LATs are transcribed from the strand opposite to and overlapping the 3' end of the viral immediate early ICP0 gene in the inverted repeat region of the unique long region (RAMAKRISHNAN et al., 1996). The major form of LAT is an unusual stable 2.0 kbp intron (FARRELL et al., 1991) that is spliced from an 8.3 kbp primary transcript and persists as a lariat (loop shaped) (EFSTATHIOU & PRESTON, 2005; KANG et al., 2006). It has been proposed, that LAT promotes cell survival through an anti-apoptotic mechanism (PERNG et al., 2000; EFSTATHIOU & PRESTON, 2005) and inhibits viral transcription and productive lytic infection (JONES, 2013).

The presence of LAT is also well studied in BoHV-1. This Alphaherpesvirus establishes latent infection in TG and germinal centers of the pharyngeal tonsil. During latency a BoHV-1 latency-related RNA (LR-RNA) is expressed, which lies antisense to bovine ICP0 (bICP0) as it is known for HSV-1 ICP0. In contrast to all other Alphaherpesviruses, an additional gene ORF-E is abundantly transcribed during latent infection suggesting that both regulate the latency-reactivation cycle (JONES, 2003).

In PRV the TG is the primary site of latency (GUTEKUNST et al., 1980). It has been described that LATs of multiple sizes are transcribed from the opposite strand to the encoding EP0 (HSV-1 ICP0 homolog) and IE gene (IE180; HSV-1 ICP4 homologue) in a region overlapping the internal repeat region (CHEUNG, 1989; PRIOLA et al., 1990; CHEUNG, 1991; PRIOLA & STEVENS, 1991). The largest LAT is the 8.4-kb large latency transcript (*LLT*). A recent study has demonstrated that a 2.5-kb deletion that eliminates the expression of a cluster of nine microRNAs (miRNA) in the *LLT* transcript results in alteration of the host

response. That study, also demonstrated that neither miRNA expression nor high LLT expression levels are essential to establish latency in the TG (MAHJOUR et al., 2015).

VZV is the only Alphaherpesvirus, that does not appear to express LAT or any detectable miRNAs thus far (BLOOM, 2016).

In summary, there is general consensus that LATs are not essential for latency establishment, maintenance or reactivation (EFSTATHIOU & PRESTON, 2005).

For EHV-1, different locations of LAT genomic origin have been suggested. One study demonstrated a possible LAT being expressed from a region overlapping ORF 63 (HSV-1 ICP0 homologue) similar to HSV-1 using nested EHV-1 specific PCR in randomly selected naturally infected horses (BORCHERS). In that study, putative EHV-1 LATs were present only in TG (and in no other nervous or lymphatic tissues). However, not all EHV-1 DNA positive samples were also LAT positive. These findings suggest that the presence of a putative EHV-1 LAT is irregular and is consistent with HSV-1 infections, where not all cells positive for HSV-1 DNA express detectable levels of LAT (BLOOM, 2016). Baxi et al. also showed a putative EHV-1 LAT is transcribed from a region located antisense to ORF 63. In that study nervous tissues of experimentally infected ponies were analyzed by in situ hybridization (BAXI 1995). Results demonstrated that sequences overlapping ORF 63, but not ORF 64 were present at a low frequency in TG. More recently, Abdelgawad et al. demonstrated detection of a putative EHV-1 LAT also being transcribed from a region located antisense to ORF 63 in the sensory ganglia of zebras using a LAT specific primer during reverse transcription to cDNA. However, out of three samples positive for EHV gDNA using a pan herpesvirus nested PCR assay, transcriptional activity of a putative EHV-1 LAT could only be detected in one sample (ABDELGAWAD et al., 2016).

In contrast to these findings, putative EHV-1 LAT gene locations were also suggested to lie antisense to ORF 64. In one study, putative EHV-1 LAT were found in leukocytes but not in TG by random RT-PCR and southern blot hybridization in experimentally infected ponies (CHESTERS et al., 1997). In order to describe the location of a putative LAT, total RNA was extracted from peripheral blood leukocytes (PBL). Aliquots of the amplified cDNA were

hybridized with a radiolabeled, plasmid-purified EHV-1 DNA fragment spanning the region of the genome including ORF 63 and ORF 64. Part of the transcript lied in a 570-bp region within the internal repeat region, antisense to and overlapping the 3' end of the IE gene ORF 64 (HSV-1 IE-3 ICP4 homolog) (CHESTERS et al., 1997). In 2009, Pusterla et al. used an qPCR assay targeting to ORF 64 in order to determine the transcriptional activity of a putative EHV-1 LAT in blood samples and nasopharyngeal secretions (NPS) in horses following natural infection (PUSTERLA et al., 2009b). In that study, a four-year old Thoroughbred gelding index case with confirmed EHM and potentially exposed horses at a racetrack in California were sampled over a three-week period. In total 74 horses were sampled with three NPS and five blood samples positive for transcriptional activity of ORF 64 (IE gene) and negative for transcriptional activity of ORF 33 (L gene).

Holden et al. (HOLDEN et al., 1992) described another possible regulatory transcript being located between ORF 64 and ORF 65. The EHV-1 unique IR3 gene lies opposite to the IE gene, and the 5' end of the non-coding IR3 transcript harbors 117 nucleotides that are antisense to the 5' UTR of the IE mRNA (AHN et al., 2007). IR3 it is not essential for viral replication and establishment of lytic infection in mice. However, it has been suggested that the IR3 RNA plays a regulatory role by downregulating the IE gene expression in a mouse model (AHN et al., 2010)

Although various approaches have been made in the past to detect a putative EHV-1 LAT, there is still lack of evidence regarding the sequence and the location within the genome. It remains challenging to detect possible EHV-1 LAT due to sensitivity issues associated with molecular assays. Therefore, negative results for LAT could be due to LAT expression below detection level. Furthermore, specificity issues may generate false-positive results. As no published data is available regarding reproducible detection of possible EHV-1 LAT in consecutive studies using previously published assays and there is a lack of data confirming the EHV-1 LAT gene region by sequencing of corresponding PCR products, it remains questionable whether EHV-1 LAT or equivalent transcripts truly exist.

4.3. Prevalence and detection of EHV-1 latency

Infections with EHV-1 occur worldwide causing a serious impact to the equine industry. One study from 1994 (EDINGTON et al., 1994) reported 60% latent infection of EHV-1 and EHV-4 in 40 necropsied horses using co-cultivation in the United Kingdom. Using different PCR assays, a study in Brazil reported that the prevalence of latent infection was 88% of analyzed samples (PBL, pooled nervous and visceral tissues) from 116 horses (CARVALHO et al., 2000). Allen et al. (ALLEN et al., 2008) showed prevalence of 54% in 132 mandibular lymph nodes from thoroughbred broodmares, while Pusterla et al. (PUSTERLA et al., 2012) reported 25.7% in mandibular (and bronchial) lymph node samples analyzed post mortem in the USA in 2008 and 2012, respectively. In 2010, the EHV-1 prevalence was determined in TG and mandibular lymph nodes from 153 equids undergoing routine postmortem examination for various surgical and medical reason (PUSTERLA et al., 2010).

Twenty-one EHV-1 DNA positive (and L gene mRNA expression negative) TG and five mandibular lymph nodes were further differentiated whether so-called neurotropic (D variant) or non-neurotropic (N variant) EHV-1 strains can be detected simultaneously in TG and lymph nodes, respectively. Both EHV-1 variants were detected in twelve TG and three mandibular lymph nodes (PUSTERLA et al., 2010).

However, data about the prevalence of latent EHV-1 infections in the literature is poor and highly variable, suggesting that it is challenging to diagnose. Indeed prevalence of latency data may also vary because of regional/geographical differences.

The lack of clinical signs and the requirement for tissue samples, make ante mortem diagnosis of a latent infection nearly impossible. Furthermore, there is lack of antigen expression in infected cells (VAN DER MEULEN et al., 2003). Previous detection methods were based on co-cultivation and cell culture after reactivation (WELCH et al., 1992; BALASURIYA et al., 2015). With the advent of molecular techniques such as qPCR and RT-PCR, the diagnosis of latency is mainly reliant on the detection of viral DNA and RNA transcripts. Detection of a putative EHV-1 LAT remains technically challenging and is not commercially available. However, latency is said to be established in an animal when a sample is shown to be positive for viral genomic DNA using PCR and either i) negative

for L gene mRNA in RT-PCR (PUSTERLA et al., 2010; PUSTERLA et al., 2012; SLATER, 2017), ii) positive for IE gene mRNA and negative for L gene mRNA in RT-PCR (PUSTERLA et al., 2009b), or iii) positive for putative EHV-1 LAT and negative for L gene mRNA (ABDELGAWAD et al., 2016).

III. MATERIAL AND METHODS

1. Introduction to the study

Controlling latency is key EHV-1 outbreak prevention. Yet, our knowledge on this topic is extremely limited. To define locations of latency it is necessary to have horses with known infection history. Here we were able to obtain tissue samples from horses, previously tested negative for EHV-1, which were experimentally infected with EHV-1 and euthanized 70 days post infection, allowing presumably sufficient time for establishment of latency.

Therefore, aims of the study presented in this thesis verify established latency locations and identify putative novel locations in these horses. As three different EHV-1 strains, were used for infection, a wild type EHV-1 Ab4 and two deletion/insertion mutants, a comparison between three different strains was made.

2. Infection study

2.1. Animals

The study was evaluated and approved by the Michigan State University (MSU) Institutional Animal Care and Use committee. Detailed clinical information on this study has been previously published (HOLZ, 2017). In short: Samples from twenty-five (n=25) purpose-infected horses were included in this study. These were Western Stock yearling horses (range 12 – 19 month-of-age). Three separate infection studies were performed using different virus strains, in which individual groups of horses were infected a few months apart in the fall of 2014, and again in the spring and fall of 2015 at MSU, East Lansing, Michigan, USA.

Infection study 1, further referred to as *group 1*, included eight (n=8) horses that were infected in September 2014.

Infection study 2, further referred to as *group 2*, included nine (n=9) horses that were infected in May 2015.

Infection study 3, further referred to as *group 3*, included eight (n=8) horses that were infected in September 2015.

All three groups were bought from the same vendor, who purchased these horses in remote areas of North Dakota. Prior to shipping, all horses were tested negative for equine infectious anemia (EIA), dewormed and tested for previous exposure to EHV-1 and EHV-4 using serum neutralizations tests. Horses included in these experiments showed titers $< 1:4$ for EHV-1 and $< 1:40$ for EHV-4. Animals were of both sexes, equally distributed per group, and were group-housed in a closed barn with natural ventilation and adjustable sidewall openings. Animals were fed an ad libitum hay diet, twice-a-day pelleted concentrate, mineral supplements, and had 24 hours access to fresh water. Prior to infection, all horses were clinically healthy.

Each group was infected with a different EHV-1 strain (see section 2.2) via intrapharyngeal instillation of 5×10^7 PFU of the respective virus strain in 10 mL of saline on day 0. All horses were euthanized between day 70 and day 75 post infection. There was no uninfected control group.

2.2. EHV-1 Virus strains used for infection

Group 1 was infected with the neuropathogenic wild type (WT) EHV-1 strain Ab4. This strain was originally isolated from a quadriplegic mare and carries the aspartic acid amino acid (D) at location 752 (see section 3.1. The virus and its genomic organization) in ORF30 (CROWHURST et al., 1981; GOODMAN et al., 2007). Group 2 was infected with an EHV-1 mutant of Ab4, with an amino acid change at position 752 (from aspartate (D) to asparagine (N)) (GOODMAN et al., 2007) and group 3 was infected with another Ab4 mutant (TISCHER, 2006; AZAB, 2012), where gD of EHV-1 was replaced by EHV-4 gD as previously published (AZAB, 2012; HOLZ, 2017). During the experiments nasal virus shedding was followed via PCR for the different isolates. To confirm, that the horses within a group shed only the virus they were infected with, sequencing of the PCR products from nasal swabs collected at day 1, 2 or 3 pi was performed (HOLZ, 2017).

2.3. Clinical data

Detailed information can be found in the publication by Holz et al. (HOLZ, 2017). Clinical exams (including rectal temperature recording) and extensive sample collection were performed once daily for the first two weeks post infection, as well as various sample collections as determined by the main study.

2.4. Collection of postmortem tissues

Between day 70 and 75 post infection horses (2 or 3 horses per day) were euthanized on site. A jugular vein catheter was placed following a pre-medication with detomidine (Dormosedan®, Zoetis US, 0.012mg/kg bw IV) and butorphanol (Torbugesic®, Zoetis US, 0.025mg/kg bw IV). For euthanasia, an overdose of 100 mL (380mg/mL) of a pentobarbital solution (Socumb™, Henry Schein Animal Health, Dublin) per horse was injected via the catheter. Once death was confirmed, the body was transported to the adjacent (100m) Diagnostic Pathology Center necropsy floor, of the Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine at MSU, where an extensive necropsy exam started immediately. Once completed, a second and on occasion a third horse were euthanized and processed. Time between euthanasia and arrival at the unit was typically <10 minutes. Tissue collection was typically completed within 60-70 minutes. For the herein presented study, tissue samples were collected from already established sites of latent EHV-1 infection and from various other sites (see Table 1). The following samples were collected: trigeminal ganglia, mandibular and retropharyngeal lymph nodes. Additional sites were RALT, which included pharyngeal roof with primary lymphoid tissue and bronchial lymph nodes. To look into alternative sites of lymphoid tissue, mesenteric lymph nodes and spleen were collected. PBMC were collected on the day of euthanasia and were evaluated in a separate study. Regarding a possible further distribution of the virus within the horse, a number of additional ganglia with different functions (somatic sensory, parasympathetic and sympathetic) were collected.

Table 1: Tissue samples collected post mortem

lymphatic tissue		peripheral neural structures	
RALT	pharyngeal roof Lnn. mandibulares Lnn. retropharyngeales Lnn. bronchiales	somatic sensory ganglia	Trigeminal ganglion spinal cord dorsal root ganglion (mainly L1 - L6)
(PBMC)		parasympathetic ganglia	Ggl. ciliare

		sympathetic ganglia	<u>head location:</u> Ggl. cervicale craniale
			<u>thoracic location:</u> Ggl. cervicale caudale
GALT	<u>abdominal location:</u> Lnn. mesenteriales		<u>abdominal location:</u> Ggl. mesenteriale Ggl. coeliacum sympathetic trunc
spleen	spleen		

RALT=respiratory tract associated lymphatic tissue, GALT=gut associated lymphatic tissue;
PBMC=peripheral blood mononuclear cells

The entire left TG was available for this study. After dissecting part of the right TG for the study from Holz et al. (HOLZ, 2017), the remainder of the right TG tissue was returned and also available for this study. Most of the TG from group 1 and 3 were collected in (4%) phosphate-buffered formalin. The complete TG of group 2 were collected in RNALater™. All sympathetic/parasympathetic (SPS) ganglia (n=171), spinal cord dorsal root ganglia of L2-L4 and sympathetic trunk were immediately immersed in (4%) phosphate buffered formalin. Lymph node tissue (n=163) including RALT, mesenteric lymph node and spleen were immediately immersed in (4%) phosphate buffered formalin (n=61) or frozen in liquid nitrogen (n=102). All formalin fixed tissues as well as tissues in RNALater™ were trimmed after 24 hours and routinely embedded in paraffin. Therefore, samples were hydrated under running tap water for 30 minutes. Dehydration was performed using a standard 16 hours program with ascending alcohol series (Shandon Hypercenter XP, GMI Inc, Minnesota, USA). Final sample blocks were stored at room temperature. In 2 separate shipments tissue blocks or frozen tissue samples were shipped to the Ludwig-Maximilians-University (LMU) either at room temperature, or on dry ice.

3. Tissue preparation for analysis

At the LMU in Munich samples were further processed. Tissue cut sections were

prepared for histopathology and some for selective IHC. Cut sections were prepared for PCR assays following DNA/RNA-extraction.

3.1. Sample preparation

Further sample processing was performed in small batches, and according a specific routine. First TG and RALT samples of all horses were processed, followed by mesenteric lymph nodes and spleen. Finally, SPS ganglia and DRG were processed. For histological investigation, embedded tissue samples were serially sectioned for routine hematoxylin-eosin (H&E) staining and cut sections were collected for all PCR work. PCR-positive samples were further assessed using IHC.

For routine microscopy tissue blocks were sectioned at 4µm using a rotatory microtome (HM 315 Microtom, Thermo Scientific, Planegg, Germany). Samples were placed on an ice-plate to cool down the wax for efficient cutting. Tissue blocks were fixed on the block holder and the cutting depth was set at 4µm according to the manufacturer's instructions. Blades (Feather microtome blade R35, Engelbrecht Medizin- und Labortechnik GmbH, Edermünde, Germany) were wiped with RNase killer solution (Sigma-Aldrich, Darmstadt) and changed between each sample. Gloves, brushes and the work area were also wiped with RNase killer solution between samples. Samples were processed in batches for different types of tissues and on multiple days. Six serial sections were cut in total using the third cut for H&E staining. The other five sections were collected in a sterile Eppendorf tube, and stored on ice (Figure 3). Until further processing for quantitative real-time PCR these sections were kept at -80°C. Sections for staining were placed in a cold-water bath using a brush. The sections were transferred to a warm water bath (43°C) using microscope slides (STAR FROST® adhesive/white, Waldemar Knittel Glasbearbeitungs GmbH, Braunschweig, Germany) to allow the paraffin to stretch. Each section was then collected on a labeled microscope slide, placed in staining jars and dried overnight in a drying oven at 37°C.

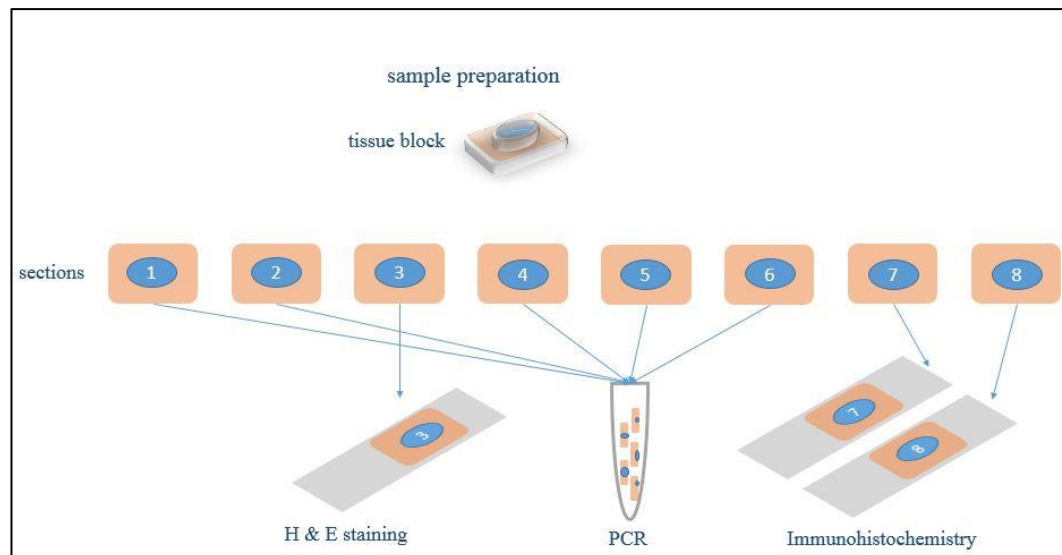


Figure 3: Sample preparation for histology and PCR: sections 1, 2, 4-6 were collected for PCR analysis following DNA/RNA extraction; section 3 was used for H & E staining; sections 7 and 8 were recuts from selectively chosen samples and were used for immunohistochemistry

3.2. H&E staining

For H&E staining, slides with paraffin sections were placed in a metal slide holder. Sections were deparaffinized and rehydrated as follows: 3 x 5 min in xylene, 2 x 5 min in 100% ethanol, 1 x 5 min in 96% ethanol, 1 x 5 min in 80% ethanol, 1 x 5 min in 70% ethanol, 1 x 5 min in 50% ethanol, 1 x 5 min in 30% ethanol and 1 x 5 min in distilled water. Excess liquids were drained and blotted before going from one reagent to the next. Nuclei of the sections were stained by placing slides for 10 min in Mayers haemalaun (commercially purchased, AppliChem GmbH, Darmstadt Germany). Slides were then placed under running tap water for 30 min. Excess water was blotted and slides were stained in eosin for 5 min. Eosin reagent was prepared as follows: 100ml of a stock solution (10g eosin yellowish (Sigma-Aldrich, Darmstadt) in 1000ml distilled water) mixed with 200ml of distilled water and 1ml 100% acetic acid. Sections were dehydrated in 1 x 5 min in distilled water, 1 x 5 min in 70% ethanol, 1 x 5 min in 80% ethanol, 1 x 5 min in 96% ethanol, 2 x 5 min in 96% ethanol, 3 x 5 min in 100% ethanol and 3 x 5 min in xylene. Slides were cover-slipped using xylene based Histokitt Nr. 1025/500 (Glaswarenfabrik Karl Hecht GmbH & Co KG, Sondheim v.d. Rhoen, Germany); a drop of Histokitt was placed on the slide using a glass rod and the coverslip was then angled to the slide and gently pressed to allow the Histokitt to spread beneath the coverslip covering all the tissue. Slides were dried

for 24 hours before further processing. H&E staining was done in the histology laboratory in the Department of Pathology, Veterinary Medicine Faculty, LMU Munich, Germany. All working steps were performed under a fume hood.

3.3. Histopathological examination and scoring

Neural tissue was specifically examined for the presence of ganglion cells, under the assumption that EHV-1 would be located within these cells. In cases in which no ganglion cells were detected sectioning and staining was repeated as described above, up to a total of three repetitions. Samples where cell bodies were not detected following the third sectioning and staining series were excluded from further investigation.

To determine cyto-histopathological changes these H&E stained FFPE samples were evaluated using a custom designed scoring system (Table 2). Ganglion cells, satellite cells, nerve fibers and inflammatory infiltration were evaluated and scored, according to the changes seen. For each group of characteristics (e.g. ganglion cells, satellite cells) the points were not summed, but the points of the most severe change were crucial for the total points. Points of all characteristics were summed. A maximum score of 12 points was possible, with 0 points indicating no changes, 1-4 points mild, 5-8 points moderate and 9-12 points severe changes.

Table 2: Evaluation of cyto-histopathological and infiltrative changes

ganglion cells				
ganglion cells	density	normal= 0	reduced= 1	increased= 1
	size	small= 1	normal= 0	large= 1
	neuronal cluster	none= 0	sporadic= 1	predominant= 2
	cytoplasm			
	Nissl substance	not homogenised = 0	coarse segregation= 1	chromatolysis =2
	cytoplasmic inclusions	absent= 0	present= 3 if present, type of inclusion:	
	nucleus with nucleolus			
	position	central= 0	paracentral= 1	
	vacuolization	absent= 0	present= 2	
	intranuclear inclusions	absent= 0	present= 3 if present, type of inclusion:	
satellite cells				
satellite cells	cell layers	single-row= 0	multiple= 2	
	hypertrophy & hyperplasia	absent= 0	present= 3	
	Nageotte´s bodies	absent= 0	occasional=1 (≤3)	multiple=2 (>3)
lymphocytic infiltration				
inflammation	position	perivascular	perineuronal	diffuse interstitial
	intensity	mild= 1	moderate= 2	severe= 3
	distribution	occasional= 1	multifocal= 2	diffuse= 3
	neuronophagia	absent= 0	present= 2	
nerve fibres				
nerve fibres	Myelin changes	absent= 0	present= 2 if present, type of changes:	
	Wallerian like degeneration	absent= 0	individual=1	multiple=2
	axonal spheroids	absent= 0	individual=1	multiple=2

Tissue preservation differed between fixation with formalin and impregnation with RNAlater®. To include these samples into our scoring system, we compared the two methods (Table 3).

Table 3: RNAlater® impregnation artefacts score system

grade	evaluation	cytoplasmic homogenization	nuclear vacuolization	neuronal shrinking
1	evaluation is possible in $\geq 75\%$ neurons, glial cells and blood vessels	partial homogenization $\leq 25\%$ cytoplasmic eosinophilia with minimal loss of nuclear details	core structure evaluation is possible $\geq 75\%$ of cells	$\leq 25\%$ shrunken
2	evaluation is possible in 50% neurons, glial cells and blood vessels	partial homogenization 50% cytoplasmic eosinophilia with partial loss of nuclear details	core structure evaluation is possible in 50% of the cells	$> 50\%$ shrunken
3	evaluation is possible in $\leq 25\%$ neurons, glial cells and blood vessels	$\geq 75\%$ cytoplasmic eosinophilia with complete loss of nuclear details	core structure evaluation is possible in $< 50\%$ of the cells	$\geq 75\%$ shrunken

4. Molecular biological characterization

4.1. DNA/RNA extraction

Formalin fixed (n=256) or RNAlater™ impregnated (n=13) and paraffin embedded tissue samples were stored at room temperature. For further analysis tissue blocks were cut into 4 μ m sections using a rotatory microtome (Microm HM 315 Microtome (Thermo Scientific, Planegg, Germany) as described in section 2.1 under sample preparation.

Total DNA and total RNA were extracted from 20 μ m (sections 1, 2, 4, 5, 6; see Figure 3) using a commercially available kit (AllPrep DNA/RNA FFPE Kit, Qiagen, Hilden, Germany) following to the manufacturer's instructions. Briefly: Paraffin was removed using deparaffinization solution, and then lysed with proteinase K digestion. Samples were cooled on ice and then centrifuged for 15 min at 20,000 x g (14000 rpm) to obtain RNA-containing supernatant and DNA-containing pellet. To extract total RNA, the supernatant was incubated for 15 min at 80°C then transferred to an RNeasy MinElute spin column, treated with DNase, and washed. RNA was eluted in 30 μ L RNase free water. To extract total DNA the

pellet was lysed with proteinase K digestion, then incubated for 2 hours at 90°C and transferred to QIAamp MinElute spin column. DNA was washed and eluted in 30µl Buffer ATE.

For DNA and RNA extraction from frozen tissues (n=108) a piece of tissue was separated with a microtome blade (Feather microtome blade R35) on ice in a 4°C cold room to avoid thawing. A maximum of 25mg of tissue for DNA extractions and 20mg of tissue for RNA extractions (maximum 10mg of spleen) were weighed on a high precision scale and further processed as follows:

Total DNA was extracted using the DNeasy Blood & tissue kit (Qiagen, Hilden, Germany) following to the manufacturer's instructions. In short, each sample was homogenized using the TissueLyser (30 Hz for 2 min) and then lysed with proteinase K digestion. To extract total DNA the homogenate was transferred to a DNeasy Mini spin column, washed and eluted in 200 µL Buffer AE.

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, including an additional on-column DNase digestion step according to Appendix D from the RNeasy Mini Kit handbook (Qiagen, Hilden, Germany). Briefly, each sample was homogenized using the TissueLyser (30 Hz for 2 min) and the homogenate transferred to a gDNA Eliminator spin column with an additional DNase treatment. Sample were then transferred to an RNeasy spin column, washed and eluted in 30µL RNase-free water.

Precautions were taken to avoid laboratory contamination during sample processing including disposable lab-ware, filtered pipette tips, separate facilities for RNA and DNA extractions. To avoid gDNA contamination of the RNA one hood was reserved for RNA extraction only. All equipment and gloves were sprayed with 70% alcohol, dried and then treated with RNase killer solution (Sigma-Aldrich, Darmstadt) before and during extraction. An extraction control was included in each extraction process to control precise working.

4.2. Quantitative real-time PCR

Unless stated otherwise, all real-time qPCR reactions for DNA and cDNA analysis were performed with the same thermal profile including an initial 95°C step for 2 min, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s (and hold 60°C for 60s). The qPCR was performed in a total reaction volume of 20µL using

1 x SensiFAST™ Probe Lo-ROX Kit (Bioline, Luckenwalde, Germany) and 5µl of the template. Amplification and detection were performed in strips of 8 PCR tubes and caps (BRAND®, Wertheim, Germany) using Stratagene Mx3000P cycler (Agilent Technologies, Waldbronn, Germany). All reactions included a non-template control (DNA/RNA-free water), an extraction control and a positive control for EHV-1 (EHV-1 gDNA extracted from RK infected cells) and for equine tissue (DNA extracted from equine liver). All primers and probes were purchased from Sigma-Aldrich, Darmstadt Germany.

4.2.1. Genomic EHV-1 qPCR

For detection of EHV-1 genomic DNA in the tissue samples a real-time qPCR targeting a region of the glycoprotein B gene within the open reading frame 33 (ORF 33) was performed as previously published (HUSSEY et al., 2006). The primers and probe sequences for glycoprotein B (gB) are as follows: gB forward 5'- CAT ACG TCC CTG TCC GAC AGA T -3', gB reverse 5'- GGT ACT CGG CCT TTG ACG AA -3'; gB probe 5'[FAM]- GGT ACT CGG CCT TTG ACG AA -[BHQ1]3'. Forward and reverse primers were added to a final concentration of 450nM each, and the probe to a final concentration of 100nM. Therefore, a primer-probe-mix (PPM) was prepared for qPCR containing 155 µL nuclease free water, 20 µL (10 pmol/µL) and 5 µL (10 pmol/µL) of each primer and probe, respectively. 2 µL PPM were added to each reaction. The primer-probe-mix was stored at -20.

To avoid cross-contamination precautions were taken using disposable lab-ware, powder free gloves, sterile filtered pipette tips of different size (0,5-10 µL, 2-20 µL, 2-200 µL, 50-1000 µL; BRAND®) and different facilities for preparing the master-mix and adding the samples.

4.2.2. B2M qPCR

For detection of equine host cells, *Equus caballus* beta-2-microglobulin (B2M) was used as a housekeeping gene. The sequences for primers and probe for equine B2M reference gene were previously described (ABDELGAWAD et al., 2016) and are as follows: equine B2M forward 5'- ATG GAA AGC CAA ATT TCC TG -3', equine B2M reverse 5'- ACC GGT CGA CTT TCA TCT TC - 3'; equine B2M probe modified with Hexachloro-6-carboxy-fluorescein and Black Hole Quencher at the 5' and 3'ends, respectively: 5'[HEX]-TGG GTT CCA TCC GCC

TGA GA –[BHQ1]3'. Forward and reverse primers were added to a final concentration of 600nM each, and the probe to a final concentration of 300nM. Therefore, a primer-probe-mix (PPM) was prepared for qPCR containing 150 μ L nuclease free water, 20 μ L (10 pmol/ μ L) and 10 μ L (10 pmol/ μ L) of each primer and probe, respectively. 3 μ L PPM was added to each reaction. The primer-probe-mix was stored at -20.

4.2.3. Standard curve

For final quantitation, absolute numbers of EHV-1 genomes were extrapolated to a standard curve generated with cloned EHV-1 oligonucleotides (with courtesy of W. Azab and N. Osterrieder (AZAB, 2012)). Previous efficiency testing was done with a log dilution series of the standard curve and a log dilution series of an EHV-1 positive control (EHV-1 gDNA extracted from RK infected cells). Therefore, 5 μ L of the EHV-1 fragment stock solution containing 10^7 copies/ μ L were diluted in 45 μ L TE-buffer, vigorously vortexed (Vortex Genie2) and briefly centrifuged to remove fluids from the lid. This procedure was repeated to create dilutions containing 10^6 , 10^5 , 10^4 , 10^3 , 10^2 and 10^1 copies/ μ L. EHV-1 positive control dilutions series was done by pipetting 5 μ L in 45 μ L TE-buffer. This step was repeated until getting six log dilutions steps. All pipetting steps were performed under the hood using disposable lab-ware, powder-free gloves, sterile 0,5 μ L Eppendorf tubes and filtered pipette-tips.

The number of cells was estimated using B2M as a housekeeping gene extrapolated to a standard curve generated with oligonucleotides specific to equine B2M (ABDELGAWAD et al., 2016). Therefore, 5 μ L of the EHV-1 fragment stock solution containing 10^7 copies/ μ L were diluted in 45 μ L TE-buffer as described above. Previous efficiency testing was done with a log dilution series of the standard curve and a log dilution series of a positive control (DNA extracted from equine liver).

DNA and mRNA quantification for the gB and cDNA transcripts, respectively, were compared with standard curves generated for each gene. Viral DNA and mRNA concentrations were expressed as copies per million cells, considering that each diploid eukaryotic cell contains two copies of beta-2-microglobulin (B2M) gene (ALLEN G.P., 2004; PUSTERLA et al., 2009b).

4.2.4. cDNA

Samples positive for EHV-1 gDNA were tested for the presence of viral transcripts using assays targeting to IE- and L- genes.

4.2.4.1. cDNA synthesis

For quantitative real-time PCR (qPCR) analysis, RNA extracted samples were reverse transcribed to complementary DNA (cDNA). Before reverse transcription, all extracted samples were tested to confirm the absence of genomic DNA by real-time qPCR using the housekeeping gene equine glyceraldehyde-3-phosphate dehydrogenase (eqGAPDH) as a marker. Primers and probe sequences were previously published (PUSTERLA et al., 2006) and are as follows: eqGAPDH forward 5'- GCC ATC ACT GCC ACC CAG -3', eqGAPDH reverse 5'- TGG CAG CAC CAG TAG AAG CA -3'; probe 5'[6FAM]- AGG GGC TGC CCA GAA CAT CAT CC - [TAMRA]3'.

Each PCR reaction was performed in a total reaction volume of 20µL, containing 2µL of each primer in a final concentration of 1000nM each, 1µL probe in a final concentration of 500nM, 10 µL 1 x SensiFAST™ Probe Lo-ROX Kit (Bioline, Luckenwalde, Germany), 4µL nuclease free water and 1µL of the template. Amplification and detection were performed using Stratagene Mx3000P cyclers (Agilent Technologies, Waldbronn, Germany) with the following thermal profile: initial 95°C step for 2 min, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s (and hold 60°C for 60s). Samples positive for genomic DNA were treated with the RQ1 RNase-free DNase Kit (Promega) according to the manufacturer's instructions. Briefly: 1-8 µL of RNA sample were added to 1 µL RQ1 RNase-Free DNase 10X Reaction Buffer, 1 unit RQ1 RNase-Free DNase per µg RNA and nuclease-free water to a final volume of 10 µL and incubated at 37°C for 30 min. To terminate reactions 1µL of RQ1 DNase Stop Solution was added, followed by a final incubation step at 65°C for 10 minutes to inactivate the DNase. Samples were re-tested for the presence of gDNA. Only negative samples were used for cDNA synthesis. Up to 5µg of RNA was reverse transcribed using the Quantinova Reverse Transcription Kit (Qiagen, Hilden, Germany) with random primers following manufacturer's instructions. To assess if the reverse transcription was successful, the QuantiNova Internal Control RNA (QN IC RNA) was included in each reaction according to manufacturer's recommendations. Briefly: 7µL of gDNA free RNA were incubated with reverse transcription master-mix containing

2 μ L gDNA Removal Mix, 1 μ L Internal control RNA, 1 μ L Reverse Transcription enzyme, 4 μ L reverse Transcription Mix and 5 μ L RNase free water at 25°C for 3 min. This was followed by incubation at 45°C for 10 min and at 85°C for 5 min to inactivate Reverse Transcriptase Enzyme, before placing on ice. The cDNA was adjusted to 100 μ L with RNase/DNase-free water and cDNA samples were stored at -20°C until further analysis. All incubations were performed in sterile 0,5 ml Eppendorf tubes in Eppendorf Thermomixer R Mixer, 0.5ml Block (Sigma-Aldrich, Darmstadt).

4.2.4.2. Internal control

To report instrument or chemistry failures, errors in assay set up or inhibitors QN IC RNA was added during conversion as earlier described. All samples were tested in a real-time qPCR using QuantiNova Probe RT-PCR kit (Qiagen, Hilden, Germany) to monitor successful cDNA synthesis according to manufacturer's instructions. Signal detection was performed at filter HEX of the thermal cycler. A no template control was included in each run.

4.2.4.3. Immediate early gene activity

For detection of IE gene transcripts, immediate early gene activity was monitored. To detect transcripts located within ORF 64 primers and probe were used as previously published (PUSTERLA et al., 2009b). The primers and probe sequences are as follows: primer ORF64 EHV-1 forward 5'- GGG TGC TGG AGG TGA GGA C -3', primer ORF64 EHV-1 reverse 5'- GCG ATC AGC CAG TAC CAC ATC -3'; ORF64 EHV-1 probe [6FAM] – GGC TGA GG – [TAMRA]. The probe contained in this protocol was purchased from Roche (Roche® universal probe #30). Forward and reverse primers were added to a final concentration of 800nM each, the probe to a final concentration of 400nM. Therefore a PPM was prepared for qPCR containing 13,2 μ L nuclease free water, 2,4 μ L (10 pmol/ μ L) and 12 μ L (10 pmol/ μ L) of each primer and probe respectively. 2 μ L PPM were added to each reaction. The primer-probe-mix was stored at -20°C. The amplified 113bp long product was detected using real-time qPCR.

4.2.4.4. Late gene activity

For detection of L gene transcripts in the cDNA of tissue samples a real-time qPCR targeting a region of the glycoprotein B gene (ORF 33) was performed as

previously published (HUSSEY et al., 2006). The primers and probe sequences for glycoprotein B (gB) are as follows: gB forward 5'- CAT ACG TCC CTG TCC GAC AGA T -3', gB reverse 5'- GGT ACT CGG CCT TTG ACG AA -3'; gB probe 5'[FAM]- GGT ACT CGG CCT TTG ACG AA -[BHQ1]3'. Forward and reverse primers were added to a final concentration of 450nM each, probe to a final concentration of 100nM. Therefore, a primer-probe-mix (PPM) was prepared for qPCR containing 155 µL nuclease free water, 20 µL (10 pmol/µL) and 5 µL (10 pmol/µL) of each primer and probe, respectively. 2 µL PPM were added to each reaction. The primer-probe-mix was stored at -20.

Table 4: Primers and probes

eGAPDH	
egapdh (F)	5'- GCCATCACTGCCACCCAG-3'
egapdh (R)	5'- TGGCAGCACCAGTAGAAGCA-3'
egapdh (probe)	5'[6FAM]- AGGGGCTGCCAGAACATCATCC - [TAMRA]3'
B2M	
B2M (F)	5'-ATGGAAAGCCAAATTCCTG-3'
B2M (R)	5'-ACCGGTCGACTTTCATCTTC-3'
B2M (probe)	5'[HEX]-TGGGTTCCATCCGCCTGAGA -[BHQ1]3'
gB (L gene)	
gB (F)	5'- CATACTCCCTGTCCGACAGAT -3'
gB (R)	5'- GGTACTCGGCCTTTGACGAA -3'
gB (probe)	5'[FAM]- GGTACTCGGCCTTTGACGAA -[BHQ1]3'
ORF 64 (IE gene)	
ORF 64 (F)	5'- GGGTGCTGGAGGTGAGGAC -3'
ORF 64 (R)	5'- GCGATCAGCCAGTACCACATC -3'
ORF 64 (probe)	[6FAM] - GGCTGAGG - [TAMRA]

4.3. Sequencing

To confirm the qPCR results, selected samples positive for IE gene transcripts (EHV-1 ORF64) were submitted for sequencing: The real-time qPCR product was amplified with a Q5® High-Fidelity DNA Polymerase (Roche, Grenzach-Wyhlen,

Germany) in a conventional nested PCR. The protocol included an initial 98°C step for polymerase activation, followed by 30 cycles of 95°C for 10 s and 64°C for 60 s and a final extension step at 72°C for 5 min. Amplified PCR products were separated by gel electrophoresis in 2 % agarose gel stained with GelRed nucleic acid stain (Phenix Research Products, Chandler, NC USA). Gel electrophoresis was performed at 50V for 85min with 13µl of Quick-Load® 2-Log DNA Ladder (BioLabs, Frankfurt a. M., Germany) in a dilution of 1:10 (0.1-10.0 kb), 10 µl gel loading dye, blue (x6) (BioLabs, Frankfurt a. M., Germany) and 12 µl of each sample. Bands at the required size were cut under a UV-lamp and cleaned using Wizard SV gel and PCR clean-up system (Promega, Mannheim, Germany) according to manufacturer's instructions. Samples were inserted in E.coli using One Shot™ TOP10 Chemically Competent E. coli (Invitrogen) and cloned using TOPO™ TA Cloning™ Kit for Sequencing (Invitrogen) according to the manufacturer's instructions. Five clones of each sample were picked and cleaned using PureYield™ Plasmid Miniprep system (Promega, Mannheim, Germany) following the manufacturer's instructions. Plasmid clones were diluted a thousandfold and additional ten-fold serial dilutions were created to test the clones for the presence of EHV-1 IE gene using real-time qPCR for ORF64. PCR product concentration was determined by mass spectrophotometry using the NanoDrop® Spectrophotometer ND-1000 (peqlab Biotechnologie GmbH, Erlangen, Germany). A conventional PCR with plasmid DNA and additional separation in a 2 % agarose gel at 50V for 85min was performed for quality control. Selected clones were prepared for sequencing using the sequencing service of Eurofins Munich, Germany.

4.4. Immunohistochemistry

PCR (genomic) positive tissue sections were re-cut and subjected to IHC using EHV-1/ERV polyclonal antiserum to control for lytic virus replication. EHV-1 gDNA PCR positive (Ct < 13) lung tissue from an EHV-1 aborted fetus was used as the positive control. IHC was carried out on samples positive for EHV-1 genomic DNA. Sections were processed according to the following EHV-1 IHC protocol. Paraffin sections were cut as previously described (section 3.1) and dried overnight at 37°C. After dewaxing for 20 minutes in xylene, hydrating in descending ethanol series and rinsing in distilled water, samples were transferred into citric acid – sodium citrate buffer (0.1M, pH 6.0). Therefore, a stock solution

was prepared diluting 21.01g citric acid (solution A) and 29.51g sodium citrate (solution B) in 1000 mL distilled water. For the working solution, 9mL of solution A and 41mL of solution B were mixed with 450mL distilled water. Samples were incubated in the microwave at 700W for 2 x 10 minutes and then left for another 20 minutes to cool down. After rinsing with distilled water, samples were blocked with 1% hydrogen peroxide (H₂O₂) for 15 minutes at room temperature and then rinsed with Tris-buffered saline (TBS) (pH 7.6). Therefore, a stock solution of TBS was prepared by diluting 121.0g Tris-(hydroxymethyl)-aminomethan (TRIS) in 1000mL distilled water. The working solution consists of TBS in a 1:10 dilution. Slides were incubated with rabbit antiserum diluted 1:10 in TBS for 30 minutes, directly followed by incubation with polyclonal caprine EHV-1/ERV antiserum (VMRD, Pullman, USA) diluted 1:1600 in TBS for 1 hour at room temperature without washing. After incubation, slides were rinsed with TBS and incubated with the secondary rabbit anti goat biotinylated antibody (Vector Laboratories LTD, Burlingame, USA) at room temperature for 50 minutes. To increase target amplification slides were incubated with avidin-biotin-complex (ABC) (Vector Laboratories LTD, Burlingame, USA) diluted 1:100 in TBS for 30 minutes at room temperature and were rinsed with TBS before and afterwards. For detection, diaminobenzidin (DAB) horseradish peroxidase (HRP) (Vector Laboratories LTD, Burlingame, USA) substrate was applied on the slides and incubated for 1 minute. Samples were rinsed with running tap water for 5 minutes to stop detection and subsequently counterstained with filtered Mayers haemalaun (commercially purchased, AppliChem GmbH, Darmstadt Germany) terminating with another wash with tap water for 5 minutes. Samples were dehydrated in an ascending ethanol series ending in xylene. A cover-slip was applied using xylene based Histokitt Nr. 1025/500 (Glaswarenfabrik Karl Hecht GmbH & Co KG, Sondheim v.d. Rhoen, Germany).

5. Statistical Analysis

Statistical analysis was performed using IBM-SPSS-Statistics 24.0 software (IBM Deutschland GmbH, Ehningen, Germany). Cross tabulation and *U* tests for group ranked data were used for the following comparisons: 1) *tissue type (different sample site) regardless of the group (virus strain)*: significance of number of

EHV-1 gDNA-positive samples and viral loads of a specific site (tissue type) (among all tissues and among nervous tissue); 2) *tissue type (different sample site) among groups (different virus strain)*: significance of number of EHV-1 gDNA-positive samples of a specific site (tissue type) and viral loads among groups (significance of different virus types); 3) *tissue type (different sample site) within each group (same virus type)*: significance of number of EHV-1 gDNA-positive samples and viral loads of a specific site (tissue type). Differences between results for paired tissue samples from both body sides were compared with a Wilcoxon signed-rank test. Mann-Whitney U test was used to analyze the correlation between cell number (determined using B2M and ganglion cells) and qPCR results. Correlations between qPCR results and severity of histopathological changes within the nervous tissue samples were determined using cross tabulation and chi-squared test. Within sympathetic/parasympathetic ganglia, the presence of lymphoid follicles within the sample and the correlation to the qPCR result was determined using cross tabulation and chi-squared test. In all statistical analysis $p < 0.01$ was considered significant.

IV. RESULTS

For this study, we aimed to collect different tissues to be examined for EHV-1 presence 70 days post infection. Specifically these tissues were RALT, abdominal lymphatic tissue (mesenteric lymph node and spleen), as well as somatic sensory TG, spinal cord DRG and various sympathetic/parasympathetic (SPS) ganglia. A total of 377 samples were collected. Unfortunately, upon on analysis some of the collected material did not contain the cell population of interest. In addition, two of eight horses in group 1 were euthanized due to neurological complications on day 10 post infection and could not be included in this study.

From RALT 87.0% (120/138) of the aimed samples were collected. Out of these 17.5% (21/120) were in group 1, 44.2% (53/120) were in group 2 and 38.3% (45/120) were in group 3. From the abdominal lymphatic tissue, 43 samples (93.5%; 43/46) were available, from which 12 (27.9%; 12/43) were in group 1, 16 (37.2%; 16/43) in group 2 and 15 (34.9%; 15/43) in group 3. From TG 93.5% of the samples were present, from which 25.6% (11/43) were in group 1, 39.5% (17/43) in group 2 and 34.9% (15/43) in group 3. Eighty-one percent (149/184) of the SPS samples could be collected (21.5%; 32/149 in group 1, 38.2%; 57/149 in group 2, 40.3%; 60/149 in group 3). From the DRG samples, 95.7% were available, with 27.2% (6/22) in group 1 and 36.4% (8/22) in groups 2 and 3, respectively.

From the available 377 lymphatic and neural tissue samples, 57 (15.1%) of the neural tissue samples could not be analyzed because of the absence of neuron cell bodies within the SPS ganglia and DRG samples.

Altogether, 320 samples were analyzed for the presence of EHV-1 gDNA and IE- and L gene mRNA expressions using real-time qPCR. Samples were also scored for histopathological changes, and EHV-specific IHC staining was applied to all genome positive TG and lymphatic tissue samples and selectively (three samples with the lowest Ct from each type of tissue) to genome positive SPS ganglia and DRG samples. Correlations between qPCR and histopathological results were determined. Results were compared between groups, to find out, whether there are differences among the three EHV-1 isolates.

1. Molecular biological characterization

1.1. Detection of viral genomic DNA using PCR

Viral loads (copies per 1×10^6 cells (estimated)) and the distribution of EHV-1 gDNA within the nervous and lymphatic tissues of each horse in each group are shown in Table 5. In the qPCR, 28.75% (92/320) were positive ($Ct < 40.0$) for EHV-1 gDNA.

A higher cell number did not affect the viral load, as no correlation was found between the cell number (estimated by B2M and counted ganglion cells) and the viral load ($p=0.05$ and $p>0.5$, respectively). If paired tissue samples were available for analysis, differences between sides were not detected ($p=0.387$).

1.1.1. Lymphatic tissue

1.1.1.1. Respiratory tract associated lymphatic tissue

A total of 120 RALT samples were analyzed, out of these 17.5% (21/120) were found positive for EHV-1 gDNA. Seven out of 40 (17.5%) mandibular lymph node samples and five out of 40 (12.5%) retropharyngeal lymph nodes were EHV-1 gDNA positive. Despite equal number of samples, 30.0% (6/20) of the tonsil samples were tested EHV-1 gDNA positive, while only three out of 20 bronchial lymph nodes (15.0%) were positive. These three samples came from group 3. Highest frequency of positive RALT samples ($n=21$) were within group 3: 13/21, 61.9%; followed by group 1 (6/21, 28.6%) and group 2 (2/21, 9.5%). Nevertheless, high viral loads ($\geq 1 \times 10^3$ copies/ 1×10^6 cells) were only found in group 1 (1/6 = 16.7%) and group 2 (2/2 = 100%). All RALT samples of group 3 had viral loads $< 1 \times 10^3$ copies/ 1×10^6 cells (Table 5).

1.1.1.2. Abdominal lymphatic tissue

Analyzed abdominal lymphatic tissue included spleen and mesenteric lymph nodes. A total of 43 samples were analyzed, where 32.6% (14/43) were positive for EHV-1 gDNA. Nine out of 22 spleen samples (40.9%) and five out of 21 (23.8%) mesenteric lymph nodes were positive. The largest number of positive samples were found in group 1 (spleen: 66.7%, 4/6; mesenteric lymph node: 50%, 3/6) followed by group 3 (spleen: 50%, 4/8; mesenteric lymph node: 28.6%, 2/7). In group 2 only one sample (spleen: 12.5%, 1/8) was positive.

Similar to the RALT samples, viral loads $\geq 1 \times 10^3$ copies/ 1×10^6 cells were found in group 1 (4/7; 57.1%) and 2 (1/1; 100%), while in group 3 all abdominal lymphatic tissue samples had viral loads $< 1 \times 10^3$ copies/ 1×10^6 cells (Table 5).

Table 5: Distribution of EHV-1 gDNA within lymphatic and nervous tissues

Distribution of EHV-1 genomic DNA within lymphatic and nervous tissues [EHV-1 genomic copies/ 1.0×10^6 cells*]		lymphatic tissue												nervous tissue											
		respiratory tract associated lymphatic tissue						abdominal lymphatic tissue		trigeminal ganglion				additional ganglia											
group	horse	P	M	I	r	R	I	r	B	S	LM	TG	I	r	GCI	I	r	GCr	I	r	GCca	GM	GCoe	ST	DRG
1	H1	█																							
	H2	█																							
	H3																								
	H4																								
	H5																								
	H6																								
2	H7																								
	H8																								
	H9																								
	H10																								
	H11																								
	H12																								
3	H13																								
	H14																								
	H15																								
	H16																								
	H17																								
	H23																								

*determined using B2M housekeeping gene as previously described (see section material & methods)

█ $\geq 1.0 \times 10^3$ copies/ 1.0×10^6 cells

▨ $< 1.0 \times 10^3$ copies/ 1.0×10^6 cells

□ 0 copies/ 1.0×10^6 cells

▩ no ganglion cells

▫ sample not available

GM	mesenteric ganglion	I	left
GCoe	celiac ganglion	r	right
ST	sympathetic trunk	S	spleen
DRG	dorsal root ganglion	LM	mesenteric lymph node
P	pharyngeal root		
B	bronchial lymph node		
TG	trigeminal ganglion		
Gci	ciliary ganglion		
GCr	cervical cranial ganglion		
GCca	caudothoracic (stellate) ganglion		
M	mandibular lymph node		
R	retropharyngeal lymph node		

1.1.2. Nervous tissue

1.1.2.1. Trigeminal ganglia

A total of 43 trigeminal ganglia were analyzed including one paired sample of both body sides. Eleven out of 43 (25.6%) were EHV-1 gDNA positive (group 1:

36.4%, 4/11; group 2: 29.4%, 5/17; group 3: 13.3%, 2/15). From these 11 positive samples, 36.4% (4/11) came from group 1; 45.4% (5/11) from group 2 and 18.2% (2/11) from group 3. Eight out of eleven (72.7%) samples showed low viral loads ($<1 \times 10^3$ copies/ 1×10^6 cells). Three samples showed high viral loads ($\geq 1 \times 10^3$ copies/ 1×10^6 cells) but none of these were in group 1 (Table 5).

1.1.2.2. Sympathetic/Parasympathetic ganglia

SPS ganglia (n=149) were also analyzed for the presence of EHV-1 gDNA. Ganglion cells could not be found in 28.2% (42/149) of the samples and were not used for analysis. Of the remaining samples, 36.5% (39/107) were positive for EHV-1 gDNA. Out of these, 18.0% (7/39), 51.3% (20/39) and 30.7% (12/39) were positive within group 1, 2 and 3, respectively.

Three out of 22 (13.6%) ciliary ganglion samples were positive for EHV-1 gDNA. All three positive samples were within group 2.

In 12/27 (44.4%) cranial cervical ganglion samples EHV-1 gDNA was detected, seven samples came from group 2 (7/12; 58.3%) and five out of group 3 (5/12; 41.7%).

For caudal cervical ganglion samples, EHV-1 gDNA was detected in 3 out of 6 (50%) samples; positive samples were evenly distributed among the three groups.

Thirty five percent (7/20) of cranial mesenteric ganglion samples were EHV-1 gDNA positive. Out of these, three samples (42.9%) came each from groups 1 and 2, and one sample (14.2%) came from group 3.

For celiac ganglion samples, in 4/13 samples (30.8%) EHV-1 gDNA was detected with two samples from group 2 and the other two from group 3.

Ten out of 19 (52.6%) sympathetic trunk samples were positive for EHV-1 gDNA, out of these 30% (3/10), 40.0% (4/10) and 30% (3/10) came from groups 1, 2 and 3, respectively.

From the positive samples, 23.1% (9/39) showed high viral loads ($\geq 1 \times 10^3$ copies/ 1×10^6 cells) with the samples equally distributed among the groups (Table 5).

1.1.2.3. Dorsal root ganglia

Twenty-two DRG samples were available for EHV-1 gDNA detection. Neuron

cell bodies were not present in 15 samples (68.2%) and were therefore not analyzed. All of the remaining seven samples (7/7) were EHV-1 gDNA positive. Positive samples were distributed as follows: 1/7 group 1 (14.3%); 2/7 group 2 (28.6%); 4/7 group 3 (57.1%). High viral loads ($\geq 1 \times 10^3$ copies/ 1×10^6 cells) were detected in six out of seven (85.7%) samples (Table 5).

1.2. Detection of viral RNA using reverse transcription PCR

All RNA samples converted to cDNA included an internal control to confirm correct transcription. Only samples positive for the internal control were further processed. To determine whether infection was latent or lytic all samples were analyzed for mRNA expression of IE and L genes.

Trigeminal ganglia, RALT and abdominal lymphatic tissues were systematically analyzed using qPCR targeting for IE gene ORF 64 as previously published (PUSTERLA, 2009). Although qPCR results showed expression of ORF 64 throughout tissue samples, sequencing of some of these PCR products did not show any matches to EHV-1. Therefore, qPCR results for IE gene ORF 64 were considered false positive results.

All 320 tissue samples were analyzed for L gene expression using real-time qPCR targeting to ORF 33 (HUSSEY et al., 2006) and were consistently negative.

1.3. Immunohistochemistry

Samples positive for EHV-1 gDNA and negative for L gene RNA were additionally analyzed for L gene protein expression of lytic infection using an IHC assay. All samples showed background signals and iron pigments in mononuclear cells as well as artefacts at the border zones. One lymphatic tissue sample showed questionable weak positive signals within few lymphocytes. All results were verified by an expert pathologist. All other EHV-1 gDNA positive but cDNA L gene negative samples of RALT, abdominal lymphatic tissue and neural parenchyma were tested also negative for L gene transcriptional activity in the IHC.

2. Pathological findings

2.1. Macroscopic examination

Macroscopic examinations were performed at the MSU and results were as follows (L.S. GOEHRING, personal communication): All animals were in a good body condition (average 5/9). Some macroscopic findings included subarachnoid spongiform haemorrhage between C1 and C3 and enlarged mandibular, retropharyngeal and bronchial lymph nodes. Subarachnoid bleeding was associated with centesis of CNS between C1 and C2 on day 5 and on day 11 post infection. With exception of lymphadenopathy of many of RALT tissue samples, none of the samples showed macroscopic abnormalities.

2.2. Microscopic examination

Paraffin embedded nervous tissue samples (n = 151) underwent microscopic evaluation and were graded based on criteria of inflammation, degeneration and leukocyte/lymphocyte infiltration. Most of the TG of group 1 and 3 were fixed in formalin prior to embedding, while most of group 2 TG were collected in RNAlater® prior to embedding. As the sample quality of H&E stained tissue cut sections had changed dramatically between groups 1 & 3 and group 2, a brief comparative study in TG was conducted. Therefore, TG from three EHV-1 (PCR) negative horses were collected, divided into equal portions and stored in either formalin or RNAlater® (Annex Table 20). While dramatic differences in cut section quality was noticed between the two fixation methods, inflammation/degeneration of samples could still be appreciated in any of the samples.

No inflammatory/degenerative changes were detected in 37/151 (24.6%) of the samples. Mild grade 1-changes were noticed in 105/151 (69.5%) and moderate (grade 2) changes in 9/151 (6.0%) of the samples. All samples with moderate changes were found in SPS ganglia and DRG, while TG scored ≤ 1 . Cyto-histopathological changes mainly consisted of Nageotte's bodies presence (as a sign of neural degeneration) and lymphocytic infiltrations.

As we were currently unable to assign gDNA to cell type of EHV-1 positive cells, neural vs. lymphocytic/monocytic infiltration, we tested for correlation between high histopathological score and the corresponding low Ct qPCR results.

However, correlation coefficient showed no evidence for this correlation with $p>0.05$.

3. Comparative analysis between EHV-1 strains

3.1.1. Comparison among tissue types

When all EHV-1 gDNA containing locations were compared, regardless of group assignment, a highest genomic load of EHV-1 gDNA was found ($p<0.001$) in DRG. Likewise, a significant higher affinity to DRG could be detected compared to the other SPS ganglia ($p<0.05$). The affinity of the virus to a certain tissue type was defined by the frequency and the viral genomic load found in that tissue sample.

In groups 1 and 2 viral loads were high ($\geq 1 \times 10^3$ copies/ 1×10^6 cells) within DRG (except one sample in group 2 with low viral loads), but low ($< 1 \times 10^3$ copies/ 1×10^6 cells) within TG (except one sample in group 2 with high viral loads). Viral loads were high for both, the DRG and the TG, in group 3 (Table 5).

3.1.2. Comparison among tissue types and groups

To further determine if these tissue affinities differ between groups, the localizations and groups were compared. The affinity to RALT was significantly higher in groups 1 and 3 compared to group 2 ($p<0.005$). No difference was detected between groups 1 and 3 ($p>0.05$). For abdominal lymphatic tissue and TG the affinity was higher in group 1 compared to group 2 ($p<0.005$ and $p<0.01$, respectively). No differences were detected for the other localizations between groups ($p>0.05$). With regards to EHV-1 gDNA containing localizations within nervous tissues a significantly higher affinity to the SPS ganglia could be detected in groups 1 and 2 compared to group 3 ($p<0.001$). Differences between groups within TG and DRG were not detected ($p>0.05$).

3.1.3. Comparison among tissue types within each group

To determine the affinity of each EHV-1 strain to a certain type of tissue, the distribution of EHV-1 gDNA was compared within each group. In groups 1 and 3 an even distribution of the positive samples between the nervous (50.0% in group 1; 45.7% in group 3) and lymphatic (50% in group 1; 54.3% in group 3) tissues

was detected. In group 1, differences between tissue types were not significant ($p>0.05$). In group 3, a high affinity to DRG was seen and a moderate affinity to all other tissues ($p<0.001$ and $p<0.005$, respectively). In group 2, higher affinity was found in the nervous tissue (90.6%; 29/32) when compared to the lymphatic tissue (9.4%; 3/32) ($p<0.005$). In addition, in this group there was a high virus affinity to the DRG and mild to moderate affinity to TG and SPS ganglia ($p<0.005$).

No evidence for a regular and systematic distribution pattern of EHV-1 gDNA within tissue types could be detected ($p>0.05$) (Annex tables 6 - 17).

V. DISCUSSION

EHV-1 gDNA was detected in already previously described and well-documented locations of putative latency in the horse; i.e. the TG and in the mandibular or retropharyngeal lymph nodes. Furthermore, genomic EHV-1 DNA was also detected in various other lymphatic tissues, as well as in previously undocumented nervous tissues of horses at day 70 post experimental EHV-1 infection.

In the present study, EHV-1 gDNA was detected in many but not all of the TG, the mandibular and retropharyngeal lymph nodes, in sections of the pharyngeal roof containing primary lymphoid follicles, in bronchial lymph nodes, as well as in mesenteric lymph node and the spleen. Moreover, EHV-1 gDNA was repeatedly detected in spinal cord DRG and in SPS ganglia.

The TG as well as the mandibular and retropharyngeal lymph nodes are considered established sites of EHV-1 latency (SLATER et al., 1994; PUSTERLA et al., 2012; SLATER, 2014). Therefore, our findings are consistent with previous publications (SLATER et al., 1994; PUSTERLA et al., 2012; SLATER, 2014).

For members of the *Alphaherpesvirinae*, such as VZV, HSV-1 and HSV-2 the typical latency location is nervous tissue. These locations are connected via dermatomes to the primary site of infection. Moreover, HSV-1 establishes latency presumably only in nervous tissue (BLOOM, 2016). The detection of EHV-1 gDNA in various structures of the RALT, mesenteric lymph nodes, spleen as well as in additional neural parenchyma is therefore extremely interesting. It would be expected that positive tissue is regional lymphatic tissue associated with the respiratory tract. However, the frequent identification of EHV-1 gDNA in the mesenteric lymph nodes and the spleen, while PBMC were consistently negative, is a novel finding, which requires further investigation. Samples were preserved on day 70 pi, which was expected to be enough time to establish a non-lytic, latent EHV-1 infection and is consistent with previously published investigations of latent PRV (BROCKMEIER et al., 1993). However, whether it is true latency measured in the herein presented samples remains a central question and will be discussed in detail further down the line.

EHV-1 gDNA in the present study was detected regularly in the equine pharyngeal roof. Horses do not have discrete masses of lymphoid tonsillar tissues.

However, they do possess follicle-associated epithelium (FAE) overlying primary lymphoid tissue located within the walls and the roof of the pharynx (KUMAR & TIMONEY, 2001, 2005). This FAE is composed of M cells, which are specialized in the uptake and transcytosis of macromolecules and microorganisms (HATHAWAY LJ, 2000). Following transcytosis, a virus could reach the primary follicles and be subsequently transported to the (secondary) mandibular and retropharyngeal lymph nodes. Therefore, the detection of virus in the pharyngeal roof as identified in this study was not unexpected. To the authors' best knowledge, this is the first report of EHV-1 gDNA in this location by day 70 pi. However, it might be explained by the fact that these tissues simply have not been judiciously tested in the past and suggests that these should be included in future investigations. In addition, our findings are consistent with other members of the *Alphaherpesvirinae*, being detected in pharyngeal lymphatic tissue, the tonsil, of the host species. Winkler et al. (WINKLER et al., 2000) described the consistent detection of latent BoHV-1 in tonsils of latently infected calves, suggesting that the tonsil is a site for viral persistence or latency in cattle. In another study, BoHV-1 was detected in the tonsils of experimentally infected calves using BoHV-1 wild type and one strain mutant variant (PEREZ et al., 2005). Furthermore, PRV DNA has been detected by PCR at low frequencies in the tonsils (and at high frequencies in the TG) of latent infected pigs 70 days after experimental infection (BROCKMEIER et al., 1993). In that study pigs were believed to be latent infected 70 days after oronasally exposure to virulent PRV. In addition, Romero et al. (ROMERO et al., 2003) detected PRV DNA (PCR) in tonsils (as well as in TG and Sacral ganglia) from naturally infected (seropositive) feral pigs that were trapped and kept in isolation for 2-3 years. Subsequent inoculation of tissue suspensions in Vero cell cultures did not yield virus and it was suggested that the detected DNA corresponded to latent virus (ROMERO et al., 2003). Hence, a latent PRV infection was defined as the presence of viral DNA in combination with a negative viral isolation in a cell culture. Considering these previous observations and the results of this study, it is plausible that the pharyngeal roof could also be a site for EHV-1 latency in equids.

EHV-1 gDNA was also identified in bronchial lymph nodes. As the bronchial lymph node is component of the RALT, the virus may be transported to this site via identical mechanisms as those involved in viral transport to the mandibular

and retropharyngeal lymph nodes. Alternatively, replicating virus may reach the lower respiratory tract (LRT), which drains into the bronchial lymph nodes. And although EHV-1 primarily infects the URT, infection of the LRT may result from dissemination through airway surfaces or via blood vessels during a cell-associated viremia (CRABB & STUDDERT, 1995). Therefore, the virus may be transported to the bronchial lymph node following possible replication in the LRT. In addition, it is plausible that the bronchial lymph nodes are reached by the virus via antigen presenting cells (APCs). EHV-1 has been demonstrated to use APCs, especially monocytes and dendritic cells, for transportation from the apical side of the mucosal epithelium into the lymphatics and blood vessels (BAGHI, 2014). Therefore, the finding of EHV-1 gDNA in the bronchial lymph nodes of horses in the present study is not completely unexpected. Furthermore, it is in agreement with findings by Pusterla et al. (2012) who demonstrated that 8.6% (6/70) of Thoroughbred race horses that were necropsied following euthanasia due to musculoskeletal injuries, were positive for EHV-1 gDNA in the bronchial lymph node (PUSTERLA et al., 2012).

Somewhat surprisingly, EHV-1 gDNA was also identified in spleen and mesenteric lymph node samples collected 70 days pi. To the author's knowledge, this has not been previously reported. However, the spleen has been described as a site of BoHV-1 latency in experimentally infected cattle (MWEENE et al., 1996). Hitherto, BoHV-1 was the only Alphaherpesvirus species in which splenic latency had been documented. In contrast to many *Alphaherpesvirinae*, the lymphatic tissue has been commonly identified as a latency site for *Gammaherpesvirinae*, where splenic latency is established preferentially in splenic germinal centers and memory B cells (FLAÑO et al., 2002). For the murine Gammaherpesvirus 68 (MHV-68), which is used as an experimental in vivo model of Gammaherpesvirus infections in the host, the spleen is a known site of latency. Also well documented is the finding that immunocompetent lymphocytes reach the spleen via blood vessels and proliferate locally (BUTCHER & PICKER, 1996). During viremia, EHV-1 gDNA has been identified in CD8⁺ T-lymphocytes, B-lymphocytes, monocytes, and less likely in CD4⁺ T-lymphocytes of horses experimentally infected with EHV-1 (WILSTERMAN et al., 2011). Therefore in horses, during a productive lytic infection and viremia, the virus may reach the spleen and could possibly be retained in splenic germinal centers during potential latent infection.

Alternatively, the detection of EHV-1 gDNA in splenic tissue may be due to its presence in circulating latently infected lymphocytes. Lymphocytes home repeatedly to secondary lymphoid organs, including the spleen and lymph nodes, reside in these organs transiently, and return to the circulation (ABBAS et al., 2014). Through this recirculation, EHV-1 infected lymphocytes move continuously through the blood and lymphatic vessels, as well as through secondary lymphatic tissues.

In this study, all but one horse with low viral loads that were EHV-1 gDNA positive in the mesenteric lymph nodes were also positive in various other respiratory associated and/or abdominal lymphatic tissues. This suggests a possible role of (re)circulating infected lymphocytes in the detection of EHV-1 gDNA in the spleen and the mesenteric lymph nodes. However, whether the viral DNA identified in the spleen and mesenteric lymph nodes represent a potentially true localization for EHV-1 latency or if these results are a consequence of recirculation of lymphocytes remains to be determined. It looks like viral DNA has settled in a variety of lymphatics while circulating PBMC were all negative. Still, the mechanisms of possible latency establishment at these sites are not well understood; therefore, further investigations would be required.

In this study EHV-1 gDNA was found in TG, considered an established site of latency. While easy to understand, as primary infection allows retrograde axonal transport to the TG, as sensible nerve fibers are closely associated with respiratory tract mucosa, another finding not previously reported was the presence of EHV-1 gDNA in various ganglia throughout the equine body. The unique tissue sample collection of our study allowed us to describe novel and putative EHV-1 latency sites in nervous tissues. More precisely, EHV-1 gDNA was found in somatic sensory (TG, spinal cord DRG), parasympathetic (ciliary ganglion (GCi)) and sympathetic ganglia (cranial cervical (GCcr), caudal cervical (GCca), mesenteric (GM), celiac (GCoe) and sympathetic trunk (ST) ganglia). However, it remains unclear whether the EHV-1 gDNA is present in the ganglion cell (neuron), in the support cell (e.g. satellite cells), or in infiltrative lymphocytes/monocytes. The presence of EHV-1 gDNA in the GCi and GCcr could be due to the close proximity of primary replication and the vicinity to the guttural pouch epithelium (GCi). Virus may establish latency in these sites using retrograde axonal transport, similar to the TG pathway. These findings are consistent with a recent publication,

where FeHV-1 was detected in various nervous tissues of experimentally infected cats including GCi, GCcr and pterygopalatine ganglion (TOWNSEND et al., 2013). Furthermore, latent VZV has also been demonstrated in the GCcr of human specimens (GILDEN et al., 1983; HYMAN et al., 1983). EHV-1, VZV and FeHV-1 belong to the genus *Varicellovirus* and there may be commonalities regarding the sites where latency is established in the respective natural host. In addition, it has also been previously demonstrated that HSV-1 can establish latency in the GCi (BUSTOS & ATHERTON, 2002), and HSV-1 is regarded as a model in studying EHV-1 latency. However, whether similarities in latency establishment in the GCi between HSV-1 and EHV-1 exist, requires further investigation.

EHV-1 gDNA could herein be detected in the equine GCca, GM, GCoe, ST and DRG. As these sites are not in the vicinity to the primary site of the infection, it is not clear how the virus reached these ganglia to establish a putative latent infection. VZV typically establishes a latent infection in the somatic sensory DRG after cell-associated viremia (KENNEDY et al., 1999; MODROW, 2010). However, VZV and HSV-1 were also detected in the autonomic nervous system in the GCoe (GILDEN et al., 2001). Herpesviruses must travel selectively in a retrograde direction from the periphery to the neuron cell body for latency establishment and travel in an anterograde direction in the same axons to innervated tissues when reactivated (LAVAIL et al., 2007; ANTINONE & SMITH, 2010). Anterograde transport to synapses connected with other neurons may result in virus transmission to higher-order neural sites (NEGATSCH et al., 2010). As this anterograde transport is present during reactivation, direct crossing of nerves via synapse saltation seems very unlikely for latency establishment. Therefore, possible explanations for how EHV-1 establishes latency within these SPS ganglia and DRG could include either direct neuro-neural contact or viremia. Although, ischemic necrosis of the supporting vessels of the neural parenchyma caused by viremia leads to increased permeability, EHV-1 egress from the CNS to sites with close contact seems unlikely. However, viremia is crucial to EHV-1 pathogenesis and may play a role in viral dispersion and transmission to these neural sites. Viral DNA has been detected in all PBMC subpopulations of experimentally infected horses during acute infection and cell-associated viremia leads to the spread of virus in the horse's body by cell-to-cell contact

(GOEHRING et al., 2011; WILSTERMAN et al., 2011). Viral DNA could also be present in the endothelial cells of arterioles or venules within or in vicinity to the ganglia or within perivascular mononuclear cuffing. Both findings have been previously described during lytic infection of the uterine tissue and the spinal cord (SMITH & BORCHERS, 2001; STIERSTORFER et al., 2002). If this is also occurring during latency, it would suggest that EHV-1 is present in vascular endothelial cells and therefore, 'accidentally' present within the nervous tissue samples.

In addition, inflammation could have transported virus-positive lymphocytes in the vicinity of the nervous tissue of interest through lymphocytic/monocytic influx, resulting in a false-positive 'nervous tissue result'. Inflammation following EHV-1 infection results in higher circulation and increased chemotaxis of PBMC. As EHV-1 latency is known to be established in CD8⁺ T-lymphocytes, higher lymphocytic infiltrations in nervous tissue samples could influence PCR outcome. To test the hypothesis of whether a high inflammation/degeneration grade resulted in high positivity of these nervous tissue samples, a purpose-designed scoring system was created. This score was used to semi-quantitatively determine the cyto-histopathological changes associated with inflammation, infiltration and degeneration of neuron cell bodies and nerve fibers. Results were graded from 0 (=no changes), 1 (=mild), 2 (=moderate) to 3 (=severe) changes. The cyto-histopathological evaluation of the nervous tissue samples showed no, or only mild/moderate changes in EHV-1 gDNA positive samples. The changes detected were mainly lymphocytic/monocytic infiltrations. In addition, the presence of lymphoid follicles was confirmed in some nervous tissue samples (except TG). The presence of lymphocyte infiltration and lymphoid follicles in the SPS ganglia and DRG was initially suspected to cause false positive qPCR results. However, this assumption could not be confirmed, as several samples were EHV-1 gDNA positive without containing lymph nodes or lymphocytic infiltrations and many others were qPCR negative while containing these structures. Furthermore, a correlation between the presence of lymphoid follicles and/or inflammation with the results of qPCR was not detected. Also no correlation could be identified between cyto-histopathological changes and the qPCR results. As no changes were present in a large number of samples (i.e. no inflammation) which were positive for gDNA while negative in the IHC analysis, it was suspected that these

nervous tissues may be a true site of EHV-1 latency. However, thus far, how the virus is transported to these nervous tissues remains unknown and further studies are required to determine the viral pathways to these latency sites. In addition, for definitive confirmation of whether these ganglia are true sites of EHV-1 latency, further analysis using in-situ hybridization would be necessary to localize the virus within these samples and, based on the found localization of viral DNA, to further characterize the cell type.

Though carry-over of viral DNA during sample preparation via lymphocytic/monocytic infiltrates into nervous tissue cut-sections must be acknowledged as a plausible cause of false-positive results in the analyzed nervous tissue samples, this was deemed highly unlikely because all precautions were taken to prevent any foreseen type of contamination. Moreover, samples were cut in batches subdivided in tissue types and on different days. During DNA extraction, controls were also included (extraction control) to avoid laboratory and/or cross contaminations among samples. Furthermore, during qPCR processing, negative and positive controls were included in each run, and results were only considered as valid, if the negative controls remained negative and the positives controls were positive.

Another possible limitation of this study was previous exposure to EHV-1 in the subjects enrolled. While it is not possible to completely ascertain whether these horses truly never had previous contact with EHV-1, all enrolled horses were young and seronegative, and came from a region (North Dakota) where EHV-1 infection is deemed unlikely. The horses were therefore assumed to be EHV-1 naive at the beginning of the experimental infections.

One of the main objectives of this study relied on establishing whether EHV-1 gDNA presence in the samples was due to lytic or latent infection. Unfortunately, the qPCR for identification of an IE gene mRNA product rendered questionable results. Therefore, putative EHV-1 latency for the interpretation of test results was defined as the sample being positive for EHV-1 specific gDNA in combination with negative EHV-1/4 specific IHC and negative L gene mRNA expression in the RT qPCR. A similar definition based only on the combination of the PCR tests has been previously used by Pusterla et al (PUSTERLA et al., 2010; PUSTERLA et al., 2012) and Slater et al (personal communication 2017).

The requirement of a negative IHC result in this study adds strength to the indirect method of latency identification. Despite herpesvirus latency being considered a dynamic process, EHV-1 latency was reported to be established by day 21 pi (SLATER, 2014). Horses in this study were euthanized 70 days pi, which was deemed sufficient for latency to be established. Latency is considered the dormant stage, with no viral replication and transcriptional activity limited to a region antisense to the IE gene producing a latency-associated transcript (LAT) (EFSTATHIOU & PRESTON, 2005; SLATER, 2014). The presence of a possible EHV-1 LAT was previously described (BAXI 1995; CHESTERS et al., 1997; PUSTERLA, 2009; SLATER, 2014; ABDELGAWAD et al., 2016). Pusterla et al. (PUSTERLA et al., 2009b) defined latent infected tissue when qPCR for gDNA was positive, with simultaneous presence of IE gene activity (mRNA, RT PCR positive) and absence of L gene activity (mRNA, RT PCR negative). In the present study, L gene activity was not detected in any of the samples. Initial IE transcript detection methodology was rejected once the PCR products were sequenced and deemed false-positive.

Despite the failure to detect simultaneous presence of IE gene in suspected EHV-1 latently infected tissues in this study, there is still a general consensus that LATs are not essential for latency establishment, maintenance or reactivation in all herpesviruses (EFSTATHIOU & PRESTON, 2005). In addition, the presence of a LAT is thought to be irregular, with not all cells positive for gDNA also expressing detectable LATs (BORCHERS; BLOOM, 2016). Moreover, there are Alphaherpesvirus species such as VZV that seem not to express LATs during latency (BLOOM, 2016). For EHV-1 LAT or equivalent IE transcripts, reports in the literature are inconsistent about the location within the virus genome. It has been claimed that EHV-1 LAT is either transcribed from a region located antisense to the E gene (ORF 63; HSV-1 ICP0 homologue) (BAXI 1995; ABDELGAWAD et al., 2016), or the IE gene (ORF 64) (CHESTERS et al., 1997; PUSTERLA et al., 2009b; SLATER, 2014). Potential regulatory functions during latency have been also suggested for the transcript of a gene located antisense to a region between ORF 64 and ORF 65 (HOLDEN et al., 1992; AHN et al., 2010). Although out of scope of this study, we additionally tested various strong positive EHV-1 gDNA samples for transcription of a region antisense to the E gene by specifically transcribing RNA to cDNA using the methodology published by

Abdelgawad (ABDELGAWAD et al., 2016). Samples were also tested for the presence of the transcript published by Holden (HOLDEN et al., 1992; AHN et al., 2010). Results for both assays were invariably negative (unpublished data). It therefore remains to be determined whether EHV-1 LAT or an equivalent transcript exist, or if different EHV-1 LATs may be present and what their location within EHV-1 genome is. Further studies are required to be able to assess the expression of potential EHV-1 LATs, latency associated miRNAs or equivalent transcripts expressed during latency, as well as IE gene mRNA production during latency in horses. As it is expected that the copy number of IE RNA present during latency is extremely low, the use of novel techniques such as next generations sequencing (NGS) potentially could help identify specific EHV-1 sequences expressed during latency.

To differentiate between lytic and latent infection in the present study, qPCR gDNA positive tissue samples were screened for translational protein expression using an established EHV-1/4 specific IHC assay. During latency, viral protein expression should be absent. Therefore, IHC should only be positive in gDNA positive tissue sections during lytic infection. All samples except one were tested negative in IHC for translational protein expression. One left mandibular lymph node sample showed questionable weak positive signals within few lymphocytes with the corresponding right side mandibular lymph node tested negative in the IHC. As these signals were very rare, present only in one sample and PCR results showed left/right discrepancies, it was suspected that these signals were false positive. Although, IHC is reported to be a reliable tool for post mortem identification of EHV-1 in FFPE tissues (RIMSTAD & EVENSEN, 1993; SZEREDI et al., 2003b; HUSSEY et al., 2006), the sensitivity of the IHC assay used in this study might have been too low to detect viral protein expression in the analyzed samples. For a separate study, testicular samples were tested for the presence of EHV-1 infection. EHV-1 crosses the blood-testis barrier. As barrier damage is key to EHM and abortions, infections of the testes may serve as a model to investigate the detection of virus and tissue pathologies. In addition, intact male horses have been shown to shed infectious virus in their semen following infection. Shedding was noticed for up to 3 and 4 weeks respectively, following natural and experimental infections (TEARLE et al., 1996; WALTER et al., 2012). Co-workers of this study confirmed EHV-1 gDNA in the majority of

testicles of intact male horses by day 70 pi (C. HOLZ & G. SOBOLL-HUSSEY 2018; publication in progress). Furthermore, EHV-1/-4 specific IHC was positive in EHV-1 gDNA positive testicular samples. Available testes samples were retested in our laboratory and results were similar to those observed by our colleagues in North America. However, L gene transcriptional activity was consistently undetectable. We concluded that mRNA detection may have been insufficient probably due to i) low RNA transcriptional activity in the testes prior to preservation, ii) RNA degradation during preservation and storage, and/or iii) due to loss/destruction of RNA during extraction/reverse transcription. Though low sensitivity of the L gene RT PCR assay may explain the occurrence of these false negative results, previous efficiency testing with a log dilution series of the positive control showed a good sensitivity. In addition, our positive control for IHC – an aborted equine fetal lung – was positive for gDNA and weak positive for L gene mRNA while strong positive in the IHC.

Altogether, we cannot be absolutely certain in what form these tissues are EHV-1 infected. However, the presence of EHV-1 gDNA with simultaneous absence of L gene activity in combination with the absence of translational activity as demonstrated by IHC provide strong evidence that in these horses an arrested, potentially latent form is present in both nervous and lymphatic tissues by day 70 pi.

Comparison between groups was used to determine the presence of tissue preferences among EHV-1 Ab4 WT and the two strain mutant variants. Clear differences in the distribution of EHV-1 gDNA could be identified between the groups and therefore between the EHV-1 isolates. Group 2 (EHV-1 Ab4 N752) was significantly different from group 1 (Ab4 WT) and group 3 (Ab4 gD4). Infections with this EHV-1 Ab4 N752 mutant reportedly had most severe respiratory clinical signs after infection (HOLZ, 2017) but viral DNA was found only in less than 10% of the analyzed RALT samples. In a study by Quintana et al., the equine airway epithelium was characterized immunologically *in vitro*; it was suggested that early events in the respiratory tract may shape downstream responses and clinical outcome (QUINTANA et al., 2011; SOBOLL HUSSEY et al., 2014). In addition, local virus-specific mucosal immunity is believed to represent a first line of defense against EHV-1 infection and may impede the establishment of viremia (BREATHNACH et al., 2006). In the present study,

horses infected with EHV-1 Ab4 N752 did not show secondary fevers and EHV-1 Ab4 N752 gDNA was detected in various nervous tissues, suggesting that a rapid latency establishment occurred at these sites. However, the exact mechanisms of latency establishment and identification of cell type carrying EHV-1 genome after infection need to be further investigated.

Horses infected with EHV-1 Ab4 WT showed significantly higher viremia with classical bi-phasic fever than the other groups. A fraction (n=3) developed clinical signs of EHM although some transient. None of the horses in the other groups showed signs of EHM (HOLZ, 2017). Despite the absence of severe clinical signs in horses infected with EHV-1 Ab4 gD4, and the possible role of gD4 in disease attenuation, no significant differences in viral genome distribution were identified between those two isolates. In both groups, presumed latent virus could be detected in nervous and lymphatic tissues throughout the horses' body.

Conclusion:

The presence of EHV-1 gDNA was confirmed in multiple locations in horses infected with three different EHV-1 isolates (Ab4 WT and two mutants) that have never been described before. Consistent with previous publications, possible EHV-1 latency was defined as samples that were positive for EHV-1 gDNA in qPCR with simultaneous absence of L gene transcriptional and translational activity using RT qPCR and IHC, respectively. Using this definition, we suspect that in this study EHV-1 established a latency-like, arrested, non-lytic stage of infection in vast parts of nervous and lymphatic tissue in horses euthanized 70 days pi. The findings presented herein indicate that EHV-1 may become arrested in many different tissues at the same time. However, the exact mechanism involved in transmission of (arrested) EHV-1 to these tissues and the establishment of a presumed latency in the horse 70 days pi could not be determined. This highlights the complexity of EHV-1 latency and suggests that selectively examining tissues close to primary sites of replication is inappropriate for latency diagnosis. In contrast, the known sites of latency (TG, mandibular and retropharyngeal lymph nodes) do not seem to be the only "guardian" sites of infection and even if negative, other lymphatic and nervous tissues still may contain latent EHV-1. Further investigations are required to get more insights about how the virus is transported to these new sites, what cell type conclusively harbors virus and whether all of these are true sites of EHV-1 latency

establishment. The tissue tropism of different EHV-1 isolates for latency establishment also requires further investigation. As this is a moment-in-time investigation 70 days pi, it would be of great interest to assess the development of the EHV-1 latency state in horses over a longer period of time, and to compare these findings with the results of the present study. However, these kind of experimental infection studies may be difficult to perform due to enormous personal and financial efforts. One would need samples of horses kept together longer after infection or a random horse population. Last, but not least, while changes in the pathogenicity of Ab4 WT have been made to get the two other infection groups, one have to realize that Ab4 belongs to an EHV-1 subfamily that is different from other circulating EHV-1 WT strains (NUGENT et al., 2006; GOODMAN et al., 2007).

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VI. SUMMARY

Equid herpesvirus 1 (EHV-1) is a member of the *Alphaherpesvirinae*. EHV-1 is species-specific, and a worldwide cause of respiratory disease, abortions and neurological disease in horses. *Alphaherpesvirinae* have the ability to cause a chronic-persistent latent (dormant) infection following an acute infection. *Alphaherpesvirinae* can reactivate out of dormancy and (re)infect other herd members, which is crucial for this group of viruses to maintain presence in the horse population. Little is known on EHV-1 latency, while understanding mechanisms of reactivation and recrudescence would be crucial for prevention in the future. EHV-1 latency locations in the horse have been detected in the mandibular and retropharyngeal lymph nodes; in PBMC, and in TG using genomic and transcriptional PCR-based assays as well as virus-specific immunohistochemistry (IHC). The short-term goal of this study was to define locations of chronic-persistent, latent infection in the horse, and to determine if different strains have impact on latency location.

Nervous somatic sensory (trigeminal and spinal cord dorsal root) ganglia; a variety of sympathetic/parasympathetic ganglia, and lymphatic tissues of respiratory tract origin as well as abdominal origin (mesenteric lymph nodes and spleen) were analyzed in three groups of horses experimentally infected with either a wild-type EHV-1 Ab4 strain, or a mutant strain Ab4 variant (Ab4 N752 or Ab4 gD4). Horses were euthanized 70 days pi and tissue samples were collected. Samples were analyzed for genomic EHV-1 presence, transcriptional and (in part) for translational activity using published qPCR protocols for viral DNA IE gene and L gene mRNA transcripts detection. IHC was carried out on the majority of samples positive for gDNA to determine the presence of translational activity. Histopathological evaluation on H&E stained tissue samples was also performed. As three different viruses were used, results were compared between groups to determine strain differences. Latent virus presence was defined when there was tissue evidence of viral genomic DNA in the absence of L gene transcriptional activity combined with a negative IHC result.

Using this definition of latency, the previously defined locations of EHV-1 latency were confirmed in the majority of horses. Surprisingly, at day 70 pi,

numerous alternative locations were found in nervous and lymphatic tissue (evidence of genomic presence) while transcriptional and translational activity in none of the samples could be detected.

Cyto-histopathological changes of degeneration and inflammation in the nervous tissue were variable and consisted more commonly of lymphocytic/monocytic infiltrates. A possible carry-over of viral DNA via lymphocytic/monocytic infiltrates into nervous tissue cut-sections was addressed, as a DNA positive nervous tissue result was not associated with lymphocytic/monocytic infiltrates ($p>0.05$). Among groups, in the group of EHV-1 Ab4 N752 infected animals, we found very little evidence of virus in the lymphatic tissue ($p>0.005$) when compared to the other two groups.

The results of this study provide evidence of already known and alternative sites of EHV-1 genomic presence in horses 70 days post infection. Based on the absence of transcriptional and translational activity in EHV-1 genome positive samples this is likely a chronic-persistent, latent infection. In addition, EHV-1 strain differences may be responsible for tissue preferences during chronic-persistent infection.

VII. ZUSAMMENFASSUNG

Das Equide herpesvirus 1 (EHV-1) ist spezies-spezifisch und gehört zu Subfamilie der *Alphaherpesvirinae*. Infektionen mit EHV-1 verursachen bei Pferden weltweit respiratorische Symptome, Aborte und neurologische Störungen. Ein charakteristisches Merkmal für alle Vertreter der *Alphaherpesvirinae* ist die Fähigkeit nach einer akuten, eine chronisch-persistierende latente (schlummernde) Infektion im Wirt einzustellen. Dieser Zustand der Latenz kann jederzeit durch eine Reaktivierung des Virus unterbrochen werden, was durch erneute Virusausscheidung zur (Re-)Infektion benachbarter Tiere führt und somit ausschlaggebend für den Erhalt des Virus innerhalb der Pferdepopulation ist. Das Wissen um die EHV-1 Latenz ist limitiert. Um jedoch langfristig mögliche EHV-1 Ausbrüche verhindern zu können, ist ein besseres Verständnis der Mechanismen notwendig, die die Latenz und Virus-Reaktivierung beeinflussen. Latentes EHV-1 konnte im Pferd bisher in den mandibularen und retropharyngealen Lymphknoten, sowie in PBMC und den Ganglia trigeminale mittels qPCR und RT qPCR sowie virusspezifischer Immunhistochemie (IHC) nachgewiesen werden. Das Ziel dieser Studie war es, mögliche Lokalisationen der chronisch-persistierenden latenten EHV-1 Infektion im Pferd zu bestimmen sowie festzustellen ob es Unterschiede bezüglich der Präferenz in den Lokalisationen zwischen den verschiedenen Isolaten gibt.

Hierfür wurde neurales (Ganglion trigeminale, sympathische/parasympathische Ganglien und Spinalganglien) und lymphatisches (Respirationstrakt-assoziiertes und abdominales) Gewebe von Pferden analysiert, welche experimentell mit EHV-1 Ab4 WT oder zwei Virusmutanten (Ab4 N752 oder Ab4 gD4) infiziert und 70 Tage nach Infektion euthanasiert wurden. Das entnommene Gewebe wurde mittels qPCR für genomische virale DNA und mittels RT qPCR für die mRNA Expression der sog. „sehr frühen“ (IE) und späten (L) Gene analysiert um chronisch-persistierendes, latentes EHV-1 in den Proben zu bestimmen. DNA positive Proben wurden weiterhin mittels IHC auf translationale Aktivität getestet. Zusätzlich wurden H&E Schnitte der Proben zytopathologisch untersucht. Bei allen Untersuchungen wurden gruppenspezifische und somit virusspezifische Unterschiede mitbeurteilt. Virus wurde als latent definiert, wenn die Probe positiv für genomische virale DNA war bei gleichzeitiger Abwesenheit der

transkriptionellen Aktivität des L Gens und einem negativen Ergebnis in der IHC.

Anhand dieser Definition konnte im Großteil der Pferde latentes EHV-1 in den bereits zuvor beschriebenen Latenz-Lokalisationen nachweisen. Überraschenderweise, konnte in der vorliegenden Studie am Tag 70 nach Infektion latentes EHV-1 regelmäßig auch in allen zusätzlichen neuronalen und lymphatischen Geweben nachgewiesen werden.

Die zytohistopathologischen degenerativen und entzündlichen Veränderungen im neuronalen Gewebe variierten und bestanden hauptsächlich aus lymphozytären/monozytären Infiltraten. Eine mögliche Übertragung viraler DNA in die neuronalen Gewebsproben durch die lymphozytären/monozytären Infiltrate konnte nicht bestätigt werden, da ein positives DNA Ergebnis des neuronalen Gewebes nicht mit dem Vorhandensein von lymphozytären/monozytären Infiltraten in Verbindung gebracht werden konnte ($p>0.05$). Im Gruppenvergleich waren in Gruppe zwei (EHV-1 Ab4 N752) kaum Hinweise auf Virus in den lymphatischen Geweben ($p>0.005$) im Vergleich zu den anderen Gruppen.

Die Ergebnisse dieser Studie bestätigen das Vorhandensein von EHV-1 in bereits zuvor beschriebenen sowie neuen alternativen Lokalisationen im Pferd am Tag 70 nach Infektion. Basierend auf der transkriptionellen und translationalen Aktivität in EHV-1 positiven Proben handelt es sich hierbei mit großer Wahrscheinlichkeit um eine chronisch-persistierende, latente Infektion. Darüber hinaus scheinen die Unterschiede in den EHV-1 Isolaten für die verschiedenen Gewebspräferenzen während einer chronisch-persistierenden Infektion verantwortlich zu sein.

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IX. ANNEX

Table 6: Distribution of EHV-1 gDNA within localizations

		tissue				
		negative	positive*	positive**	total	
localizations	RALT	amount	99	18	3	120
		within localization	82.5%	15.0%	2.5%	100.0%
	ALT	amount	29	9	5	43
		within localization	67.4%	20.9%	11.6%	100.0%
	TG	amount	32	8	3	43
		within localization	74.4%	18.6%	7.0%	100.0%
	SPS	amount	68	30	9	107
		within localization	63.6%	28.0%	8.4%	100.0%
	DRG	amount	0	1	6	7
		within localization	0.0%	14.3%	85.7%	100.0%
	total	amount	228	66	26	320
		within localization	71.3%	20.6%	8.1%	100.0%

RALT= respiratory tract associated lymphatic tissue; ALT=abdominal lymphoid tissue; TG=trigeminal ganglion; SPS= sympathetic/parasympathetic ganglia; DRG=dorsal root ganglion; *low viral loads ($< 1 \times 10^3$ copies/ 1×10^6 cells); **high viral loads ($\geq 1 \times 10^3$ copies/ 1×10^6 cells)

Table 7: EHV-1 gDNA distributions within RALT per group

		RALT			
		negative	positive*	positive**	total
group	1	15	5	1	21
	2	51	0	2	53
	3	33	13	0	46
total		99	18	3	120

RALT= respiratory tract associated lymphatic tissue; *low viral loads ($< 1 \times 10^3$ copies/ 1×10^6 cells); **high viral loads ($\geq 1 \times 10^3$ copies/ 1×10^6 cells)

Table 8: EHV-1 gDNA distributions within ALT per group

		ALT			
		negative	positive*	positive**	total
group	1	5	3	4	12
	2	15	0	1	53
	3	9	6	0	15
total		29	9	5	43

ALT=abdominal lymphatic tissue; *low viral loads ($< 1 \times 10^3$ copies/ 1×10^6 cells); **high viral loads ($\geq 1 \times 10^3$ copies/ 1×10^6 cells)

Table 9: EHV-1 gDNA distributions within TG per group

		TG			
		negative	positive*	positive**	total
group	1	7	4	0	11
	2	12	4	1	17
	3	13	0	2	15
total		32	8	3	43

TG=trigeminal ganglion; *low viral loads ($< 1 \times 10^3$ copies/ 1×10^6 cells); **high viral loads ($\geq 1 \times 10^3$ copies/ 1×10^6 cells)

Table 10: EHV-1 gDNA distributions within SPS per group

		SPS			
		negative	positive*	positive**	total
group	1	9	4	3	16
	2	26	17	3	46
	3	33	9	3	45
total		68	30	9	107

SPS=sympathetic/parasympathetic ganglia; *low viral loads ($< 1 \times 10^3$ copies/ 1×10^6 cells); **high viral loads ($\geq 1 \times 10^3$ copies/ 1×10^6 cells)

Table 11: EHV-1 gDNA distributions within DRG per group

		DRG			
		negative	positive*	positive**	total
group	1	0	0	1	1
	2	0	1	3	4
	3	0	0	2	2
total		0	1	6	7

DRG=dorsal root ganglion; *low viral loads ($< 1 \times 10^3$ copies/ 1×10^6 cells); **high viral loads ($\geq 1 \times 10^3$ copies/ 1×10^6 cells)

Table 12: EHV-1 gDNA distributions within tissues in group 1 (Ab4 WT)

		tissue			
		negative	positive*	positive**	total
RALT	amount	15	5	1	21
	within localization	71.4%	23.8%	4.8%	100.0%
ALT	amount	5	3	4	12
	within localization	41.7%	25.0%	33.3%	100.0%
TG	amount	7	4	0	11
	within localization	63.6%	36.4%	0.0%	100.0%
SPS	amount	9	4	3	16
	within localization	56.3%	25.0%	18.8%	100.0%
localizations DRG	amount	0	0	1	1
	within localization	0.0%	0.0%	100.0%	100.0%
total	amount	36	16	9	61
	within localization	59.0%	26.2%	14.8%	100.0%

RALT= respiratory tract associated lymphatic tissue; ALT=abdominal lymphoid tissue; TG=trigeminal ganglion; SPS= sympathetic/parasympathetic ganglia; DRG=dorsal root ganglion; *low viral loads ($< 1 \times 10^3$ copies/ 1×10^6 cells); **high viral loads ($\geq 1 \times 10^3$ copies/ 1×10^6 cells)

Table 13: EHV-1 gDNA distributions within tissues in group 2 (Ab4 N752)

		tissue				
		negative	positive*	positive**	total	
localizations	RALT	amount	51	0	2	53
		within				
		localization	96.2%	0.0%	3.8%	100.0%
	ALT	amount	15	0	1	16
		within				
		localization	93.8%	0.0%	6.3%	100.0%
	TG	amount	12	4	1	17
		within				
		localization	70.6%	23.5%	5.9%	100.0%
	SPS	amount	26	17	3	46
		within				
		localization	56.6%	37.0%	6.5%	100.0%
	DRG	amount	0	1	3	4
		within				
	localization	0.0%	25.0%	75.0%	100.0%	
total	amount	104	22	10	136	
	within					
	localization	76.6%	16.2%	7.4%	100.0%	

RALT= respiratory associated lymphoid tissue; ALT=abdominal lymphoid tissue; TG=trigeminal ganglion; SPS= sympathetic/parasympathetic ganglia; DRG=dorsal root ganglion; *low viral loads ($< 1 \times 10^3$ copies/ 1×10^6 cells); **high viral loads ($\geq 1 \times 10^3$ copies/ 1×10^6 cells)

Table 14: EHV-1 gDNA distributions within tissues in group 3 (Ab4 gD4)

		tissue				
		negative	positive*	positive**	total	
localizations	RALT	amount	33	13	0	46
		within localization	71.7%	28.3%	0.0%	100.0%
	ALT	amount	9	6	0	15
		within localization	60.0%	40.0%	0.0%	100.0%
	TG	amount	13	0	2	15
		within localization	86.7%	0.0%	13.3%	100.0%
	SPS	amount	33	9	3	45
		within localization	73.3%	20.0%	6.7%	100.0%
	DRG	amount	0	0	2	2
		within localization	0.0%	0.0%	100.0%	100.0%
	total	amount	88	28	7	123
		within localization	71.5%	22.8%	5.7%	100.0%

RALT= respiratory associated lymphoid tissue; ALT=abdominal lymphoid tissue; TG=trigeminal ganglion; SPS=sympathetic/parasympathetic ganglia; DRG=dorsal root ganglion; *low viral loads (< 1x10³ copies/1x10⁶ cells); **high viral loads (≥ 1x10³ copies/1x10⁶ cells)

Table 15: EHV-1 gDNA distributions within SPS ganglia per group

		Sympathetic/Parasympathetic ganglia				
		negative	positive*	positive**	total	
group	1	amount	9	4	3	16
		within localization	56.3%	25.0%	18.7%	100.0%
	2	amount	26	17	3	46
		within localization	56.5%	37.0%	6.5%	100.0%
	3	amount	33	9	3	45
		within localization	73.3%	20.0%	6.7%	100.0%
total		amount	68	30	9	107
		within localization	63.6%	28.0%	8.4%	100.0%

*low viral loads (< 1x10³ copies/1x10⁶ cells); **high viral loads (≥ 11x10³ copies/1x10⁶ cells)

Table 16: Correlation between the nervous tissues

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
(1) TG								
(2) GCi	<i>p</i> >0.05							
(3) GCcr	<i>p</i> >0.05	<i>p</i> >0.05						
(4) GCca	<i>p</i> >0.05	<i>p</i> >0.05	<i>p</i> >0.05					
(5) GM	<i>p</i> >0.05	<i>p</i> >0.05	<i>p</i> >0.05	<i>p</i> >0.05				
(6) GCoe	<i>p</i> >0.05	<i>p</i> >0.05	<i>p</i> >0.05	<i>p</i> >0.05	<i>p</i> >0.05			
(7) ST	<i>p</i><0.05	<i>p</i> >0.05	<i>p</i> >0.05	<i>p</i> >0.05	<i>p</i> >0.05	<i>p</i> >0.05		
(8) DRG	<i>p</i><0.001	<i>p</i><0.005	<i>p</i><0.005	<i>p</i><0.05	<i>p</i><0.001	<i>p</i><0.001	<i>p</i><0.05	

TG=trigeminal ganglion; GCi= ciliary ganglion; GCcr= cranial cervical ganglion; GCca= caudal cervical ganglion; GM= mesenteric ganglion; GCoe= celiac ganglion; ST= sympathetic trunk; DRG= dorsal root ganglion

Table 17: Samples positive for EHV-1 gDNA within nervous and lymphatic tissues

	lymphatic tissue	nervous tissue	total
group 1	13	12	25
group 2	3	29	32
group 3	19	16	35
total	35	57	92

Comparative study to determine fixation and impregnation artefacts in TG

Evaluation of fixation (formalin) vs. impregnation (RNAlater™) artefacts was performed on TG from equine carcasses submitted to the LMU pathology service. Samples were collected and further prepared as follows: TGs were extracted from the opened equine cranial. Each TG was cut lengthways and then into 2 pieces. From each side one piece of the TG was immersed into 4 % buffered formaldehyde solution in a proportion of 1:10. The other piece was immersed into RNAlater™ in a proportion of 1:10 respectively (Figure 4). After 24 and 48 hours, respectively, the TGs were transferred to 50% ethanol as an intermedium before further preparation. Samples were hydrated under running tap water for 30 minutes. Dehydration was performed using the standard 16 hours program (Shandon Hypercenter XP) (Table 18). After dehydration, samples were embedded in wax and sectioned for H&E staining.

Table 18: Standard dehydration protocol (Shandon Hypercenter XP)

Step	Reagent / Conc. %	Temp.	Immersion
01	Alcohol /70	35	01:20:00
02	Alcohol /70	35	01:20:00
03	Alcohol /80	35	01:20:00
04	Alcohol /96	35	01:20:00
05	Alcohol /96	35	01:20:00
06	Alcohol /100	35	01:20:00
07	Alcohol /100	35	01:20:00
08	Alcohol /100	35	01:20:00
09	Xylol	35	01:20:00
10	Xylol	35	01:20:00
11	Paraffin	62	01:20:00
12	Paraffin	62	01:20:00

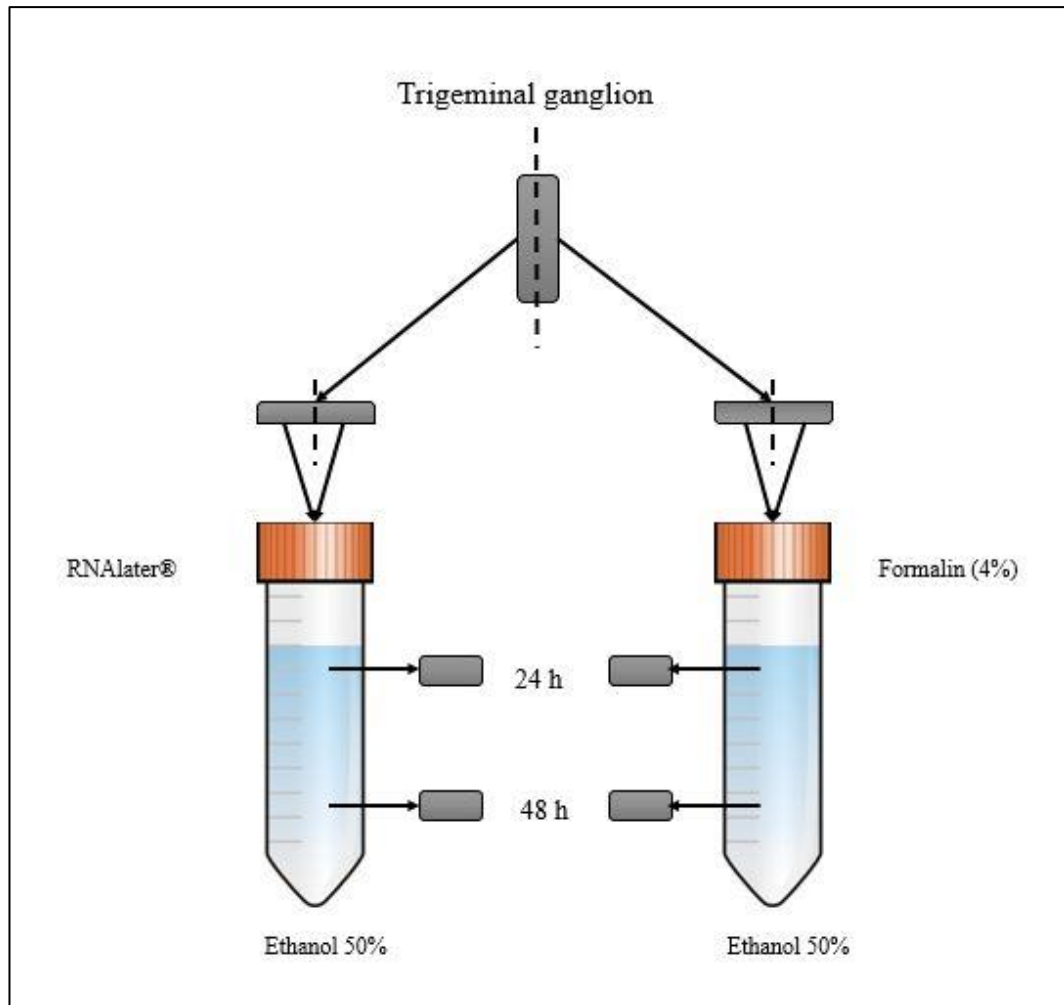


Figure 4: Sample preparation for evaluation of fixation artefacts

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