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Frequency of latent equine herpesvirus 1 (EHV-1) in New Zealand horses

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Abstract

AIM: To estimate the frequency of infection with equine herpesvirus 1 among a selected population of horses from the central North Island of New Zealand, including determination of the open reading frame (ORF) 30 N/D752 genotype.

METHODS: Fresh heads were collected from horses euthanised for unrelated reasons between March and November 2015. Small pieces of retropharyngeal lymph nodes (RLN) and submandibular lymph nodes (SLN) were dissected from the heads and transported to the laboratory in RNA later solution. DNA extracted from these tissues was subjected to enrichment for EHV-1 sequences by hybridisation with biotin-labelled EHV-1 specific probe, followed by recovery of EHV-1 sequences on streptavidin-coated magnetic beads. The enriched samples were tested for the presence of EHV-1 using nested PCR. The EHV-1 amplicons were sequenced to determine the ORF30 genotype of the virus.

RESULTS: Overall, EHV-1 DNA was detected in RLN samples from 6/63 (9.5%) horses. Of those, three were also positive for EHV-1 DNA in SLN samples. There was no association between EHV-1 positivity and age, sex, or breed of the animals sampled. All EHV-1 positive horses harboured ORF30 N752 genotype. The D752 genotype, which has been linked to increased neurovirulence, has not been detected in any of the samples.

CONCLUSION: Equine herpesvirus 1 continues to circulate among horses in New Zealand. The RLN appear to be the sample of choice for detection of EHV-1 DNA in a recently euthanised horse. The frequency of latent EHV-1 infection among sampled horses may have been higher than detected, as some of latently infected horses may have harboured EHV-1 DNA at the levels beyond the sensitivity limit of the assay or at anatomical sites not sampled in the study. Lack of detection of EHV-1 with ORF30 D752 genotype, together with detection of only one horse positive for that genotype in the previous South Island based study (Dunowska et al., 2015) suggest that infection with this genotype is not common in New Zealand.

CLINICAL RELEVANCE: If live animals are tested using SLN biopsy it should be kept in mind that negative results do not rule-out the presence of latent EHV-1 at other sites inaccessible for testing. While EHV-1 with ORF30 D752 genotype was not detected in this study, the importance of this genotype should not be over-interpreted because the markers for EHV-1 neurovirulence are most likely more complex than this single amino acid substitution. Viruses with either genotype have been recovered from equine

herpesvirus encephalopathy cases worldwide. The data presented provided baseline information on the frequency of EHV-1 infection among horses in New Zealand. These can provide useful information during any future outbreaks of EHV-1 associated diseases and for the development of control measures to minimise the impact of such viral disease for horses and their owners.

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Publication

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Abbreviations

A	adenine
Ab	antibody
ADCC	antibody dependent cell cytotoxicity
AHL	Animal health laboratory
bp	base pair
CFT	complement fixation test
CMI	cell mediated immunity
CNS	central nervous system
Cq	quantification cycle
CTAB	cetyltrimethyl ammonium bromide
CTL	cytotoxic T-lymphocytes
D752	aspartic acid at amino acid position 752
DNA	deoxyribonucleic acid
ECE	equine coital exanthema
EHV	Equine herpesvirus
EHM	Equine herpes myeloencephalopathy
ELISA	enzyme link immunosorbent assay
ER	endoplasmic reticulum
FFPE	formalin fixed paraffin embedded
G	guanine
gB	glycoprotein B
gC	glycoprotein C
gD	glycoprotein D
gG	glycoprotein G
H&E	haematoxylin and eosin
HSV	herpes simplex virus
ICTV	International Committee on Taxonomy of Viruses
IE	immediate early
Ig	immunoglobulin
iiPCR	insulated isothermal polymerase chain reaction
IP	immunoperoxidase
IR	inverted repeat
kbp	kilo base pair
LAT	latency associated transcript

LN	lymph node
MHC	major histocompatibility complex
MPI	Ministry for Primary Industries
mRNA	messenger RNA
N752	Asparagine at amino acid position 752
NK	natural killer
NTC	no template control
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PC	positive control
PCR	polymerase chain reaction
ppm	parts per million
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RLN	retropharyngeal lymph nodes
SLN	submandibular lymph nodes
SoVS	School of Veterinary Science
TAP	transporters associated with antigen processing
Tc	cytotoxic T lymphocytes
Th	T helper cells
TR	terminal repeat
VI	virus isolation
VNT	virus neutralisation test
UL	long unique
US	short unique

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Figure 1. Long unique (UL) and short unique (US) regions of EHV-1 genome (ORF1 to ORF 76). Not shown in this figure are the very short small inverted (IR_L) and terminal (TR_L) repeats of 32 bp each, flanking the UL region. 3

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1 Review of literature

Equine herpesvirus 1 (EHV-1) is a highly prevalent viral pathogen of horses worldwide (Pusterla et al., 2010a). Although not every EHV-1 infection leads to clinical disease, it can result in clinical signs that include respiratory disease, abortion in mares, perinatal death and myeloencephalopathy (Dunowska, 2014a; Pusterla et al., 2016). The disease can result in high economic cost, usually due to direct expense in implementing interventions and controls; interruptions of mating activities at stud farms or when it interferes with racetrack operations (Damiani et al., 2014). An outbreak of equine herpes myeloencephalopathy (EHM), a neurological disease form of EHV-1, occurred for the first time in New Zealand in 2014 (McFadden et al., 2016). A virus with open reading frame (ORF) 30 D752 genotype was detected from several horses affected by neurological disease during this outbreak. The EHV-1 D752 genotype has been shown to be associated ($P < 0.0001$ by Fisher's exact test) with neuropathogenic capacity (Nugent et al., 2006). Currently, although EHV-1 is endemic in New Zealand (Jolly et al., 1986), the origin of the EHM outbreak and prevalence of the EHV-1 D752 genotype is unknown. To understand EHV-1 and its associated diseases, the existing knowledge on EHV-1 infection, pathobiology and epidemiology is presented.

1.1 *Classification and nomenclature of equine herpesviruses*

The order *Herpesvirales* includes the family *Herpesviridae* which is divided into 3 subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* (Davison et al., 2009). Subfamilies are further divided into different genera. Historically, a virion's structure was the primary means of identifying members of the herpesvirus family while antigenic and biological property relatedness were used to group them into subfamilies (Davison, 2002). Biological properties include composition, size and arrangement of genome; tissue tropism; and pathogenicity (Davison, 2002; Roizman, 2013). Such traditional basis for classifying herpesviruses however was superseded by the increasing availability of genomic sequences and molecular data (Davison, 2002).

An online report published by the International Committee on Taxonomy of Viruses (ICTV) recently adopted a proposed change in herpesvirus species nomenclature to include the subfamily designation (ICTV, 2017). According to ICTV, the subfamily prefix addition to the "herpesvirus" word would easily provide reference to which subfamily a virus species belongs. For example, the species name of EHV-1 was renamed as "*Equid alphaherpesvirus 1*", equine herpesvirus type 4 (EHV-4) is now "*Equid alphaherpesvirus 4*", equine herpesvirus type 2 (EHV-2) is "*Equid gammaherpesvirus 2*" and so on.

Equine herpesvirus 1 is a member of the genus *Varicellovirus*, subfamily *Alphaherpesvirinae* (Davison et al., 2009). Two additional equid herpesviruses, equine herpesvirus type 3 (EHV-3) and equine herpesvirus type 4 (EHV-4), also belong to this genus. Prior to 1980s, EHV-1 and EHV-4 had been treated as abortifacient and respiratory subtypes of EHV-1 or equine rhinopneumonitis virus (Patel et al., 2005). While these two subtypes demonstrated a high degree of antigenic similarity, there were differences observed in cross neutralisation studies (Horner, 1981) and restriction endonuclease DNA pattern analysis (Studdert et al., 1981). Eventually, such variation in the DNA fragment fingerprint became the basis for the designation of the two subtypes as two separate viruses, EHV-1 and EHV-4 (Studdert et al., 1981). EHV-1 was more often associated with abortion hence the name "equine abortion virus" Hutton et al. (1977), while EHV-4 isolates were restricted mostly to respiratory infections thus retaining the common name "equine rhinopneumonitis virus" (Studdert et al., 1984). The remaining equine herpesvirus, EHV-3, is the causal agent of equine coital exanthema in mares and stallion (Barrandeguy et al., 2010).

The two other equine herpesviruses (EHV-2 and EHV-5) are members of the genus *Percavirus*, subfamily *Gammaherpesvirinae* (Davison et al., 2009). These slow growing gammaherpesviruses are ubiquitous in the horse population worldwide. The initial isolates of EHV-5 were obtained in Australia from horses with upper respiratory tract disease (Turner et al., 1970; Wilks et al., 1974). These isolates were preliminarily identified as EHV-2 until genetic study demonstrated them as distinct members of *Gammaherpesvirinae* subfamily (Browning et al., 1987; Sharp et al., 2007).

Equid herpesviruses that infect donkeys, zebra and asses include the following: EHV-6, EHV-7 and EHV-8 (Bell et al., 2008; Browning et al., 1988). EHV-6 (asinine herpesvirus 1) and EHV-8 (asinine herpesvirus 3) are classified within the genus *Varicellovirus,* while EHV-7 (asinine herpesvirus 2) is an unassigned member of the *Gammaherpesvirinae subfamily* (Davison et al., 2009). An additional equid alphaherpesvirus, EHV-9 (Gazelle herpesvirus-1) was originally isolated from gazelle and has been reported to infect other hosts including dogs, cats and polar bear (Fukushi et al., 1997; Fukushi et al., 2012).

1.2 *General characteristics*

1.2.1 Virion structure

The main structures of a herpesvirus virion include a core, capsid, tegument and an envelope. The core, which includes a linear double stranded deoxyribonucleic acid (DNA), is enclosed by an icosahedral capsid containing 12 pentameric and 150 hexameric capsomers (Davison, 2002; Davison et al., 2009). Surrounding the capsid is an amorphous tegument containing viral proteins, while the outer host-derived lipid envelope is embedded with viral glycoproteins. Variability in the tegument thickness

and envelope dimension influence the herpesvirus particle size that could range from 200 to 250 nm in diameter (Davison, 2002; Roizman, 2013).

1.2.2 Genome structure

The genomes of herpesviruses range from 125 to 290 kilo base pairs (kbp) (Davison, 2002; Davison et al., 2009). The reference EHV-1, Ab4 strain, is 150,223 base pairs (bp) in size (Telford et al., 1992). The genome of EHV-1 (Figure 1) is divided into a 112,870 bp unique long region (UL) and 11,861 bp unique short region (US) (Telford et al., 1992). The US region is further flanked by short inverted repeat (IR_S) and short terminal repeat sequences (TR_s) of 12,714 bp each. The UL region is flanked by a small inverted (IR_L) and terminal (TR_L) repeat of 32 bp each (Telford et al., 1992). The linear double-stranded EHV-1 DNA genome with G + C content of around 56 to 57 % codes for 76 genes (Pusterla et al., 2014; Telford et al., 1992). Previous hybridisation studies demonstrated the colinear arrangement of related regions of EHV-1 and human herpes simplex viruses (Allen et al., 1988; Davison et al., 1983; Whalley et al., 1989). EHV-1 also shares a common gene sequence arrangement with EHV-4, pseudorabies virus, and Varicella Zoster virus (Davison, 2002; Purewal et al., 1994). Unlike its close relative, EHV-4, which exhibits extensive recombination, EHV-1 is a genetically stable virus (Vaz et al., 2016). High throughput sequencing showed very little or no recombination in the genomes of 11 international EHV-1 isolates studied (Vaz et al., 2016). These findings agreed with the earlier restriction endonuclease profiling of EHV-1 where no considerable differences existed among virus isolates (Kirisawa et al., 1993b; McCann et al., 1995).

Figure 1. Long unique (UL) and short unique (US) regions of EHV-1 genome (ORF1 to ORF 76). Not shown in this figure are the very short small inverted (IR_L) and terminal (TR_L) repeats of 32 bp each, flanking the UL region.

1.2.3 Herpesvirus replication, transcription and translation

The herpesvirus virion attaches to host cellular receptor with the envelope glycoprotein spikes. After attachment, the viral envelope fuses to the cell plasma membrane in a pH-dependent manner. The virion is then taken in and disassembled as the nucleocapsid enters the cytoplasm. The viral DNA-protein complex is released from the capsid and moves towards the cell nucleus (Roizman, 2013). A virion tegument protein (VP16) enters the nucleus and is a vital part of the transcription factor complex recognized by the host RNA polymerase. Once the linear genome is in the nucleus, the viral DNA is transcribed successively into 3 classes such as α (immediate early, IE), β (early) and γ (late) mRNA (messenger RNA) during lytic infection (Gray et al., 1987). The viral genome is transcribed in a cascade

manner with the help of the host cellular RNA polymerase. The virus uses host mRNA modification enzymes. The α mRNA are transcribed in the nucleus then transferred to the cytoplasm and translated into α (IE) proteins. The α proteins move to the nucleus where they allow β promoters to be used by the host RNA polymerase for the transcription to β mRNA, that are translated into β (early) proteins, which regulate the transcription of more α mRNA. This initiates viral DNA replication using the viral α and β proteins, and the host-cell proteins. The γ mRNA are then translated into γ (late) proteins. During the replication cycle, more than 70 virus-encoded proteins are created. The α and β proteins are virus coded enzymes such as thymidine kinase and DNA polymerase that play roles in viral replication. The γ proteins mostly are viral structural proteins such as tegument, capsid and envelope glycoproteins. The transcription of viral DNA happens in the nucleus, which is followed by the translation of mRNA into proteins in the cytoplasm. Such proteins may either remain in the cytoplasm, moved to the nucleus, or be inserted into cellular membranes to become a part of the viral envelope. Complete segments of viral DNA are bundled to form nucleocapsids (Davison et al., 2009; Roizman, 2013). Capsid proteins assemble in the nucleus. The capsids obtain a primary envelope by budding through the inner nuclear membrane containing viral membrane proteins such as tegument. The virus loses the envelope as it attaches to the outer nuclear membrane. The nucleocapsid travels to the cytoplasm then buds into Golgi-apparatus where it acquires a secondary envelope with more mature tegument (Granzow et al., 1997). The virus is released from the cell by exocytosis.

1.2.4 Latency

Latency, an important biological property, enables herpesviruses to strategically evade the immune system and allows them to stay indefinitely in their natural host (Field et al., 2006). During latency, viral gene expression is limited, preventing the production of infectious virions. Also, viral replication is not active hence the virus becomes dormant in certain cells (Nicoll et al., 2012). Members of the *Alphaherpesvirinae* subfamily establish latency primarily in nervous tissues (Rock, 1993), members of *Betaherpesvirinae* in secretory glands and other tissues, while viruses from *Gammaherpesvirinae* "hide" in lymphocytes (Davison et al., 2009; Roizman, 2013). Several reviews have been published regarding latent infection of herpes simplex virus 1 (HSV-1) using animal models (Nicoll et al., 2012; Roizman et al., 2013), however the exact cellular mechanisms of latency establishment, maintenance and reactivation for the majority of herpesviruses are still unclear (Roizman et al., 2013).

Trigeminal ganglia (Baxi et al., 1995), lymphoid tissues draining the respiratory tract (Allen et al., 2008; Dunowska et al., 2015; Edington et al., 1994; Welch et al., 1992) and peripheral blood leucocytes (Chesters et al., 1997; Welch et al., 1992) serve as latency sites of EHV-1. In one study, a latency associated transcript (LAT) was identified in peripheral blood leucocytes of a horse as IE gene 64 (Chesters et al., 1997). Immediate early gene 64 of EHV-1 is a homologue of herpes simplex 1 gene IE-3 and was hypothesised to be the same with IE LAT described in other members of *Alphaherpesvirinae*. Latency in

blood leucocytes was supported by the in vitro re-activation of EHV-1 following stimulation of CD5 and CD8 leucocytes indirectly with chorionic gonadotrophin and interleukin 2 (Smith et al., 1998). The EHV-1 LAT found in trigeminal ganglia however was different and was derived from ORF63, a latency promoter binding factor with unknown function (Baxi et al., 1996), suggesting that the mechanism of latency may be different in different cell types. The importance of this finding is currently unknown.

Re-activation from EHV-1 latent infection, although not always, was possible through administration of corticosteroid (Edington et al., 1985; Gibson et al., 1992; Pusterla et al., 2010a).

1.3 *Disease outcomes*

1.3.1 Equine herpesvirus 1

The reported incubation period for EHV-1 infection varies, although in most cases ranges between 3 and 8 days. Such variation may be due to pathogenic potential of the virus, immune status of the host or other external factors. On one study, clinical signs of mild upper respiratory tract infection were observed between one and five days after experimental EHV-1 infection (Gibson et al., 1992). However, incubation period tends to be longer (6 to 10 days) following exposure to diseased animals in the field (Crabb et al., 1995; Slater et al., 2006). One possible explanation for this discrepancy is that the observable clinical signs of disease in the field are due to secondary bacterial infection, and mild or subclinical disease due to primary EHV-1 infection may be missed by the owners.

Disease associated with EHV-1 infection in horses has three presentations with varying degree of severity. The clinical outcomes following EHV-1 infection include mild respiratory disease, sporadic abortion or 'abortion storm', perinatal death, and in more serious cases, neurologic syndrome (Pusterla et al., 2016; Smith et al., 2001; Walter et al., 2013). Although severe respiratory disease with fatal outcome has been occasionally linked to field EHV-1 infections of young animals, most EHV-1 infections are subclinical (Gibson et al., 1992). Clinical signs observed after experimental infection with EHV-1 include malaise, pharyngitis, inappetence, serous nasal discharge, sporadic coughing (Heldens et al., 2001; Pusterla et al., 2016; Sutton et al., 1998); neutropenia and lymphopenia, and/or submandibular or retropharyngeal lymphadenopathy (McCulloch et al., 1993). Secondary bacterial infections are common in field infections and manifest as mucopurulent nasal discharge and pulmonary disease (McCulloch et al., 1993; Pusterla et al., 2016; Whitwell et al., 1992a). Infection with EHV-1 is often mild or inapparent in horses previously exposed to the virus. Equine herpesvirus 1 infected horse develops biphasic fever. The first episode of fever typically occurs within 24–36 hours of infection and is likely due to viral replication and tissue destruction within the upper respiratory tract (Goehring et al., 2013). The second rise in temperature usually coincides with cell-associated viremia (Henninger et al., 2007; Van Maanen et al., 2001). In experimentally infected naïve SPF foal, fever was observed to be short-lived (Sutton et al., 1998).

EHV-1 infection can also result in chorioretinopathy initiating permanent lesions of the retina. Following experimental infection of EHV-1, the frequency of ocular lesions was 50 to 90% (Hussey et al., 2013).

1.3.1.1 Abortion

Herpesvirus-induced abortion in mares occurs without preliminary clinical signs. In an outbreak of abortion storm on a Thoroughbred stud farm in New South Wales, 33 out of 44 resident pregnant mares aborted during the last trimester of gestation (Carrigan et al., 1991). Another six mares, presumed to be in the 1st trimester of pregnancy, did not give birth indicating that embryonic loss might have occurred. One mare foaled, but the foal died within 36 hours after birth. Others also reported that the majority of EHV-1 induced abortions occurred between 8 and 11 months of gestation, about 2 to 12 weeks post infection, and only a few cases occurred earlier in the gestation period (Humelt et al., 2016; Laugier et al., 2011). Aborted foetuses were normal with no evidence of autolysis. There was no evidence of post EHV-1 abortion complications in mares and the infection had no effect on subsequent pregnancies.

Mares in late gestation that were exposed to EHV-1 may not abort but may give birth to live foals with viral pneumonitis. These foals are prone to secondary bacterial infections and typically do not survive for long (Edington et al., 1991).

1.3.1.2 Equine herpesvirus myeloencephalopathy

Clinical signs seen in cases of EHM include urinary incontinence, incoordination, recumbency, lethargy, depression anorexia and conjunctivitis (Goehring et al., 2013). Because EHV-1 most commonly affects the spinal cord, the severity of disease ranges from asymmetrical ataxia with hind limb weakness to complete paralysis (Henninger et al., 2007). Affected horses may lose skin sensation in the perineal and inguinal areas. Neurological deficits typically occur around or after the second rise in temperature, which may be after cessation of virus shedding in nasal secretions and viremia (Henninger et al., 2007; Walter et al., 2013), making laboratory confirmation of sporadic EHM cases challenging. Many affected horses recover with appropriate supportive care, although neurological deficits may persist for weeks to months in some cases (Henninger et al., 2007; McFadden et al., 2016). Rarely, the paralysis may progress to quadriplegia and death. Prognosis depends on the severity of signs, the length of time the horse is recumbent, and the level of supportive care provided to the horse (Van Maanen et al., 2001) .

1.3.2 Other equid herpesviruses

Equine herpesvirus 3 is the causal agent of equine coital exanthema (ECE), a disease characterized by formation of painful papules or pox-like lesions, vesicles, pustules and ulcers on the external genitalia of mares and stallions (Barrandeguy et al., 2010). This virus is highly contagious but non-invasive and causes relatively benign venereal disease (Allen et al., 2004; Barrandeguy et al., 2012a; Barrandeguy et al., 2012b). The economic impact of ECE is mainly due to temporary disruption of reproductive activities between stallions and mares (Barrandeguy et al., 2012a).

Equine herpesvirus 4 is a primary pathogen of the respiratory tract of young horses from weanling to 2 years of age (Gilkerson et al., 1994). As with EHV-1, many EHV-4 infections are subclinical (Gilkerson et al., 1999a). Disease associated with EHV-4 infection is characterized by rhinopharyngitis and tracheobronchitis, similar to the respiratory form of EHV-1 (Patel et al., 2005).

Equine herpesvirus 2 and EHV-5 infections are linked to mild forms of respiratory disease in horses manifested by nasal discharge, coughing, fever, pharyngitis, keratoconjunctivitis and lymphadenopathy (Dunowska et al., 2002b; Kershaw et al., 2001; Pusterla et al., 2013). Horses affected by respiratory disease are often infected with more than one virus, including equine α and γ herpesviruses (Dunowska et al., 2002b; Pusterla et al., 2013). Apart from respiratory cases, EHV-2 was also detected using polymerase chain reaction (PCR) assay in a horse with granulomatous dermatitis (Sledge et al., 2006). A recently described form of interstitial pneumonia, called equine multinodular pulmonary fibrosis (EMPF), has been linked to EHV-5 (Williams et al., 2007) . Cases of horses with EMPF have well demarcated nodular areas of pulmonary interstitial fibrosis, inflammatory cell infiltration and type II pneumocyte hyperplasia, although these myriad of clinico-pathological signs are difficult to differentiate from other causes of interstitial pneumonia (Wilkins, 2008). In addition to EMPF, EHV-5 was also demonstrated in horses with multicentric lymphoma (Vander Werf et al., 2014).

1.4 *Pathogenesis of EHV-1 infection*

1.4.1 Respiratory invasion and viremia

The nasal mucosa is the primary replication site following inhalation of EHV-1 (Figure 2). Infection occurs via direct contact with shedding animals, infectious respiratory droplets or fomites (Edington et al., 1986; Edington et al., 1991; Kydd et al., 1994). After inhalation, the virus enters the host cell by direct fusion with the cell membrane, a process that is believe to be mediated by the viral glycoproteins B, C and D (Azab et al., 2012; Csellner et al., 2000; Osterrieder, 1999; Wellington et al., 1996). Equine herpesvirus 1 then multiplies in the epithelium of the respiratory tract including pharynx, soft palate, tracheal epithelial cells, turbinates and lungs (Gryspeerdt et al., 2010; Kydd et al., 1994). The virus can be isolated

from these sites around 12-48 hours post experimental intranasal infection (Edington et al., 1991; Kydd et al., 1994). Also, immunofluorescent EHV-1 was observed in the endothelial cells of the rete arteriosus in the nasal mucosa four days after experimental nasal inoculation of mares (Edington et al., 1986). Gryspeerdt et al. (2010) suggested that mucosal dendritic cells attracted to the site of infection become infected with EHV-1. The virus is transported within dendritic cells across the basement membrane through the connective tissues and gains access to the vascular and lymphatic system in draining lymph nodes (Gryspeerdt et al., 2010).

Viremia occurs through infection of the peripheral blood mononuclear cells (PBMC), particularly monocytic cells and some T lymphocytes within 48 hours up to 7-14 days post experimental inoculation (Edington et al., 1985; Gryspeerdt et al., 2010; Hussey et al., 2006). The reported duration of viremia varied among experimentally infected foals (4 to 5 days) (Edington et al., 1985); ponies (4 to 10 days) (Hussey et al., 2006) and mares (3 to 18 days) (Smith et al., 1992), although such variation may be attributed to different detection techniques used. The pattern of viremia following EHV-1 reactivation by corticosteroid injection appears to be similar to that described above for the primary infection (Pusterla et al., 2010a). A consequence of cell associated viremia is the distribution of EHV-1 into different tissues including the vascular endothelium of the uterus or nervous tissues where it may lead to abortion or EHM, respectively. Secondary virus replication may also occur in leukocytes of the submandibular and retropharyngeal lymph nodes where eventually the virus establishes latency (Kydd et al., 1994; Patel et al., 1982).

1.4.2 Reproductive tissue and foetal localisation

Cell associated viremia may result in translocation of EHV-1 through the utero-placental barrier. Virus infection of the vascular endothelial cells of pregnant mare's uterus can induce vasculitis, thrombosis, ischemia and infarction of the microcotyledons (Smith et al., 1992). Infarcted areas facilitate the leakage of the virus from the vessels into surrounding tissues, and promote transplacental spread leading to infection of the foetus and subsequent abortion or perinatal death of the foal (Edington et al., 1991; Smith et al., 2001). Equine herpesvirus 1 reactivation within the pregnant uterus in late gestation may explain sporadic EHV-1 abortions in closed breeding herds (Smith et al., 1992; Smith et al., 1996). Subsequent horizontal spread of the virus may lead to abortion storms (Carrigan et al., 1991). Association between early embryonic death and EHV-1 infection of uterine endothelium has been speculated although it has not been well examined (Carrigan et al., 1991).

1.4.3 Myeloencephalopathy

Viral infection of the vascular endothelial cells surrounding the nervous tissues appears to be responsible for the development of EHM. Resultant vasculitis and thrombo-ischemia following lytic viral

replication causes lesions such as myelopathy or encephalopathy, depending on the affected areas of the central nervous system (CNS). Such pathological lesions are responsible for clinical manifestations of EHM including ataxia, paresis and other nervous system disorders (Allen, 2008). Lesions in the spinal cord cause neurologic deficits predominantly affecting the hind limbs with many cases displaying tail paralysis or weakness, rectal paralysis and bladder paralysis leading to urinary incontinence. However, despite evidence of EHV-1 replication in horse brain derived endothelial cells in-vitro (Goehring et al., 2011; Hasebe et al., 2006), lytic viral replication has not been observed in the nervous tissues of horses (Edington et al., 1986; Patel et al., 1982; Whitwell et al., 1992a). This is in contrast to the mouse model-experiment where EHV-1 exhibited a dual affinity of neurotropism in the brain and endotheliotropism in visceral organs (Gosztonyi et al., 2009). Regardless, it was suggested that some EHV-1 isolates have greater affinity and virulence for the endothelial cells of the blood vessel than other isolates (Smith et al., 2010). In recent years, EHM has been associated with EHV-1 ORF30 D752 genotype, containing a single amino acid mutation in the DNA polymerase region compared to ORF30 N752 isolates. The ORF30 D/N752 viruses will be referred to as N/D752. The D752 virus was found to reproduce more efficiently in horses than the N752 virus, causing higher levels of cell-associated viremia (Goodman et al., 2007; Van de Walle et al., 2009). In a study by Goodman et al. (2007), the D752 genotype infected ponies had viral titre that were more than 100-fold (p=0.03) higher than those observed following the N752 infection as determined by quantitative PCR (qPCR) of the PBMCs.

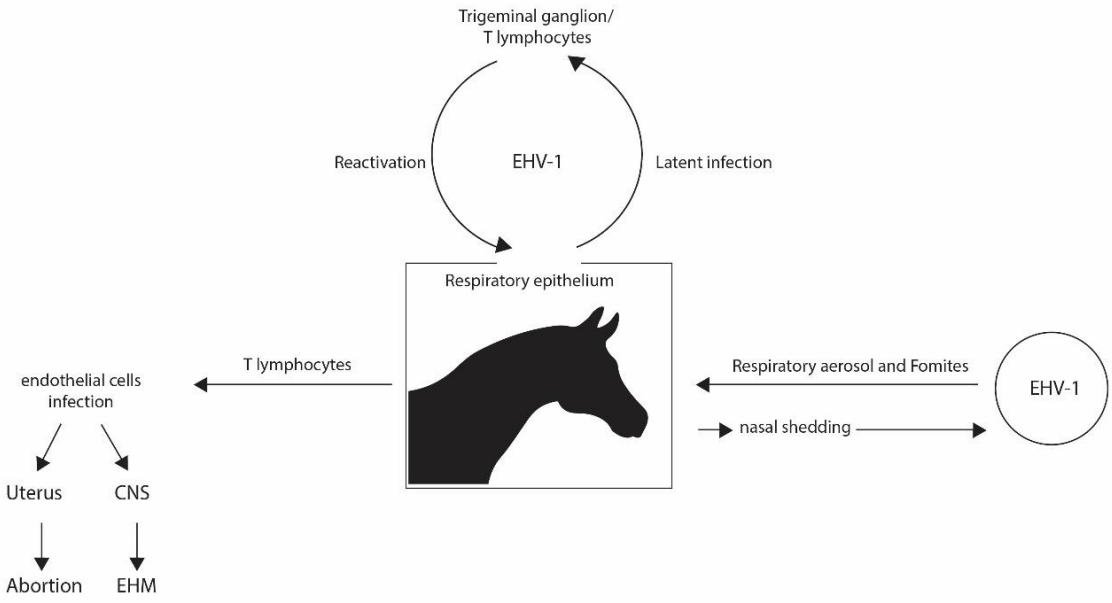

Figure 2. Pathogenesis of equine herpesvirus 1.

1.5 *Immunity and immune evasion following EHV-1 infection*

Infection with EHV-1 or vaccination stimulates both humoral and cell mediated immune (CMI) responses in horses (Goodman et al., 2012). However, acquired immunity to EHV-1 is short-lived and re-infection may occur within 3-6 months (Kydd et al., 2006). Virus neutralising (VN) antibody (Ab) interacts mainly with the glycoprotein (g) B and gC and persist up to a year post infection (Allen et al., 1992). In one study, the levels of VN Ab correlated with reduced nasal viral shedding, however the establishment of viremia was not affected (Goodman et al., 2006). Circulating neutralising Ab can neutralise cell free virions, however, EHV-1 is usually cell associated (Kydd et al., 1994), and thus inaccessible to direct neutralisation. Elimination of infected cells can occur via Ab dependent cell cytotoxicity (ADCC) and complement mediated cell lysis (Ma et al., 2013). The mechanism of ADCC involves the formation of complexes between the virus specific Ab and viral envelope proteins that are present in the plasma membrane (Tizard, 2013). The protruding Fc domain of the antigen-bound Ab is recognised by the Fc receptors expressed by natural killer (NK) cells and other phagocytic cells. Such binding then initiates the release of perforin and proteases that lead to lysis of the infected cell. In addition, the Ab-viral antigen complexes on the cell surface can also activate the complement cascade that may eventually lead to the formation of membrane attack complex composed of complement proteins on the surface of virus infected cells, leading to cell membrane disruption and cell lysis (Tizard, 2013).

Presence of EHV-1 specific mucosal antibodies, Immunoglobulin (Ig) A, was shown in nasal secretions after experimental intranasal infection of horses with EHV-1 (strain A183) (Breathnach et al., 2001). Although the authors recognised the importance of mucosal defence in reducing viral shedding, virus-specific IgA were only detected in infected, and not in vaccinated horses.

Cytotoxic T-lymphocytes (CTL) are important players in carrying out CMI response, which is believed to be important in protection from and clearance of EHV-1 infection (Kydd et al., 2006). This was indicated by the proliferation of lymphocytes of the $CD8^+$ type in the blood and lung after experimental EHV-1 infection (Kydd et al., 1996; Lunn et al., 1991). High levels of CTL against EHV-1 in the peripheral blood were linked to protection from abortion, while horses with low levels of specific CTL at pre-infection had higher risk of aborting following experimental challenge with EHV-1 (Kydd et al., 2003). The virus specific CTL activity is highly dependent on the efficient identification of virus derived peptides presented within major histocompatibility complex (MHC) class I proteins on the cell surface (Tizard, 2013). Cell mediated immunity is initiated by the activation of T helper 1 (Th1) lymphocytes through the presentation of endogenous peptides from protein products of viral transcription in conjunction with MHC I molecules. Activated Th1 lymphocytes stimulate the proliferation of cytotoxic lymphocytes (Tc cell) that destroy virus infected cells with antigen-MHC I complex molecules in their surface by inducing apoptosis or programmed cell death.

Equine herpesvirus 1, along with most other herpesviruses adopted the "infect and persist" strategy to ensure continued presence in the host for indefinite period (Peterhans et al., 2010). The way to accomplish this goal is to develop latency after primary infection, with occasional re-activation that allows horizontal spread of the infectious virus to susceptible horses. Establishment of latency is facilitated by several viral proteins that interact with the immune system and allow the virus-infected cells to escape elimination by innate and adaptive immune responses (van der Meulen et al., 2006). Well characterised EHV-1 evasion strategies include interference with ADCC and disruption of the $CD8^+$ CTL mediated infected cell lysis. The inefficiency of VN Ab to clear the infection is somewhat counter-balanced by binding of virus specific antibodies to viral antigens presented within the cell membrane to facilitate the cell lysis function of NK cells, macrophages, CTL and the complement system. However, in vivo and in vitro studies showed that EHV-1 envelope proteins are not expressed on the cell surface of the majority of infected PBMCs, thus making recognition of these cells by virus specific Ab difficult (Ma et al., 2013; van der Meulen et al., 2006). Without the formation of antibody and viral antigen complexes on the cell surface, elimination of virus infected cells via ADCC cannot occur (van der Meulen et al., 2006). In addition, EHV-1 gC has been shown to have the ability to sequester the C3 complement component in in-vitro study (Huemer et al., 1995). The absence of C3 disables the complement cascade of cell mediated lysis (Huemer et al., 1995).

The activation of CTL cytotoxic function necessitates the presentation of viral antigens by MHC class I in all nucleated cells (Harty et al., 2000). Viral antigens are peptides derived either from newly endocytosed virus or newly synthesised viral protein (Uebel et al., 1999). The transporters associated with antigen processing (TAP) molecules then help to translocate viral peptides into the endoplasmic reticulum (ER), where they are fused with MHC class I molecules (Abele et al., 2004). After the formation of the MHC class I-antigen complex, the molecule is transported and presented on the cell surface for recognition and lytic action of CTL (Spiliotis et al., 2000). To get away from the lytic activities of CTL, EHV-1 restricts the MHC class I-antigen presentation by down regulating the action of TAP using UL49.5 viral protein (Koppers-Lalic et al., 2005). Koppers-Lalic et al. (2005) also reported the activity of UL49.5 orthologues in bovine herpesvirus 1, pseudorabies and EHV- 4 in inhibiting the TAP function.

The interference of Equine herpesvirus 1 with cytokines responses to suppress lymphocyte activation has also been reported and reviewed extensively by Ma et al. (2013) and van der Meulen et al. (2006).

1.6 *Epidemiology*

1.6.1 Virus transmission

Infection with EHV-1 is acquired through direct or indirect contact with infected horses (Foote et al., 2003; Pusterla et al., 2009b). The primary portal of entry is the upper respiratory tract by inhalation of the virus. Infectious virions usually come from expelled respiratory droplets, and in cases of EHV-1 associated abortion, dead foetus, placenta and secretions from the reproductive tract are good virus sources (Gerst et al., 2003; Stasiak et al., 2015; Szeredi et al., 2003). Indirect mode of viral spread can be through contaminated fomites such as clothing, footwear, farm tools and equipment (Goehring et al., 2010). From the nasopharynx of a horse with respiratory disease, infectious EHV-1 is shed into the environment (Burgess et al., 2012; Hussey et al., 2006). Shedding may commence as early as one day after experimental challenge (Gardiner et al., 2012) and majority of infected horses stop virus shedding within 1 to 2 weeks post-infection, with some showing intermediate shedding up to 21 days post infection (Perkins et al., 2008). In one report of naturally occurring outbreak of EHM, EHV-1 DNA was detected by qPCR in nasal secretions at a minimum of 9 days after the start of neurologic signs (Burgess et al., 2012). The reported duration of EHV-1 nasal shedding after recrudescence of latent infection using corticosteroids was similar to that reported during field outbreaks: between 2 and 7 days (Pusterla et al., 2010a) and 1 and 12 days (Edington et al., 1985). Overall, differences in the shedding pattern are likely to be attributed to the level of immunity of an infected horse and the characteristics of the involved EHV-1 isolate (Dunowska, 2014b).

The presence of EHV-1 DNA in horse semen with no clinical signs also suggested a possibility of a reproductive route of viral spread (Hebia-Fellah et al., 2009). In a farm with reported neurological and abortion cases, semen from an asymptomatic stallion was coincidentally positive for EHV-1 D752 genotype (Fritsche et al., 2011). Virus DNA in horse semen can be detected for up to three weeks in both natural infection, as shown in an acute outbreak (Walter et al., 2012) and in experimentally infected horses (Tearle et al., 1996). The importance of this putative venereal route of transmission needs further investigation (Carvalho et al., 2000; Hebia-Fellah et al., 2009). Nevertheless, EHV-1 was found to have no effect on the fertility of stallions (Hebia-Fellah et al., 2009).

Maintenance of EHV-1 infectivity in the environment depends on factors associated with the integrity of the viral envelope. In general, EHV-1 is a fragile virus, that is susceptible to inactivation by heat and common disinfectants. However, EHV-1 could probably survive longer (up to three weeks) at favourable environmental conditions, such as in high humidity, low ambient temperature (4°C to 20°C), high salinity (3,000 to 35,000 ppm), and very turbid or high sediment concentration (Dayaram et al., 2017). The survivability of EHV-1 at various conditions merits for additional examination because if it is proven, it could be an important risk pathway for EHV-1 transmission.

1.6.2 Maintenance of EHV-1 in horse population

In closed herds where the virus is endemic, mares and foals were considered the primary source of circulating EHV-1, with or without evidence of associated clinical signs of infection (Gilkerson et al., 1999a; Gilkerson et al., 1998). Reactivation of latent virus in this setting plays an important role in the maintenance of EHV-1 in the herd. An initial "mare-to-foal" spread can occur immediately after birth (Gilkerson et al., 1999a), followed by a lateral "foal to foal" spread (Gilkerson et al., 1998). In a cross sectional serological survey conducted on a large stud farm, mares were approximately thrice (Odds ratio = 2.78, 95% confidence interval 1.63-4.73) more likely to have antibody against EHV-1 than foals (Gilkerson et al., 1999a). Active EHV-1 infection in mare was believed to be due to recrudescence of the virus as a consequence of stress caused by pregnancy or parturition (Gilkerson et al., 1999a). The same authors reported that mares with EHV-1 antibodies were more likely to have EHV-1 antibody positive foal (Odds ratio = 3.98, 95% CI 1.58-10.09) than EHV-1 antibody negative mares. These data suggest that mare-to-foal transmission is common in farms where virus is endemic. In an unvaccinated population, an increasing level of EHV-1 antibodies in foals aged from few hours to 90 days provided evidence of virus circulation among this population of horses (Gilkerson et al., 1997). In contrast to protective maternal antibodies that decline overtime as the foal becomes older, an increasing titre was suggestive of active infection. It was presumed that transmission of EHV-1 to foals was from their dams, since foals less than 30 days old did not have much contact with other horses outside of their paddock before weaning (Foote et al., 2004; Gilkerson et al., 1999a). Mare-to-foal transmission was also possible even in vaccinated population (Marenzoni et al., 2008).

A "foal to foal" transmission ensued when foals co-mingled or were grouped together before or after weaning (Gilkerson et al., 1999b). In a parallel longitudinal study of 200 foals on two stud farms in the Hunter Valley, serological evidence of EHV-1 infection among foals was reported before and after weaning (Gilkerson et al., 1998). This foal-to-foal spread was demonstrated by the seroconversion of foals within groups which was detected by a type specific enzyme link immunosorbent assay (ELISA). The seroconversion to EHV-1 of seronegative foals was believed to be due to mixing with EHV-1 antibody-positive foals, that were possibly shedding the virus around weaning. As foals recover from the initial infection and become latently infected, fillies that eventually enter the broodmare population can be a source of infectious virus following recrudescence at the time of pregnancy stress (Foote et al., 2006). Using an EHV-1 type specific blocking ELISA, Donald (1998) also described an increasing pattern of seroconversion from younger to older animals beginning at 6 to 12 months old (29% of 256 horses) and 13-14 months old (48% of 143 horses). These findings were supported by a comparative frequency of seropositive Thoroughbred yearlings (56% of 21 horses) as detected by similar blocking ELISA and appeared to be common in horses with respiratory disease (67% of 45 horses) (Dunowska et al., 2002a; Dunowska, 2014b). Such virus circulation had been observed even within vaccinated groups of horses (Foote et al., 2006).

In naïve herds, the introduction of EHV-1 to a population of pregnant mares may lead to abortion(s) in breeding herds (Carrigan et al., 1991) and epizootic respiratory disease with or without neurologic involvement (Friday et al., 2000; Matsumura et al., 1994) in non-pregnant horses. After establishing infection, EHV-1 can undergo latency and follow the pattern of virus circulation in endemic herd with or without manifestation of clinical disease.

1.6.3 Reactivation from latency

Establishment of and reactivation from latency are important to the epidemiology of EHV-1. Latent viral genome is maintained in the trigeminal ganglia or in lymphoid tissues, without expression of structural genes and without production of infectious viruses (Allen, 2006; Chesters et al., 1997; Slater et al., 1994). Latently infected horses become infectious following EHV-1 recrudescence in the respiratory tract, and can be a source of infectious virus to other susceptible animals (Dunowska, 2014b; Edington et al., 1985; Gibson et al., 1992). Evidence of reactivation was shown by the presence of glycoprotein B (gB) mRNA sequences, an expressed marker of a replicating EHV-1, in the nasal secretions and blood using a qPCR analysis (Pusterla et al., 2010a).

Independent to EHV-1 recrudescence in the respiratory tract, local reactivation of the virus within the blood vessels of the pregnant uterus or the CNS may also occur, resulting to sporadic cases of abortion and neurological disease in a closed group of horses (Crowhurst et al., 1981; Greenwood et al., 1980; McFadden et al., 2016). Not all virus reactivation, however, will result in disease or transmission of the virus. In an experiment conducted by Pusterla et al. (2010a), only one out of four horses with reactivated EHV-1 developed fever for three days. In the same study, direct transmission of EHV-1 from a horse with reactivated virus to susceptible sentinel horses was not seen possibly due to the short duration of observed shedding and the low level of virus in secretions (Pusterla et al., 2010a).

Recrudescence of EHV-1 was demonstrated following experimental administration of corticosteroid (Edington et al., 1985; Slater et al., 1994). Stressful conditions such as long distance transport, sales or competitions, unsettled social structure, weaning, castration, or strenuous exercise were generally regarded to induce EHV-1 recrudescence (Dunowska, 2014b). Immunocompromised animals such as pregnant mares or horses with underlying disease are also susceptible to recrudescence (Gilkerson et al., 1999a; Pusterla et al., 2009a). An example of virus reactivation due to history of long distance travel was shown when 8 out of 302 horses shed EHV-1 from their nasal secretions (Pusterla et al., 2009a). However, results of some studies challenged this assumption, as active EHV-1 infection was not detected in any of the 124 hospitalised critically ill horses (Carr et al., 2011) and nasal shedding of the virus was detected in only a very small proportion of horses after multi-day international transport (Smith et al., 2018) . Altogether, while reactivation from latency is of key importance in the epidemiology of EHV-1, the exact mechanisms of recrudescence, including its environmental triggers, are poorly understood.

1.6.4 Geographic distribution

Outbreaks of diseases associated with EHV-1 have been reported in various countries suggesting a worldwide distribution of the virus (Anagha et al., 2017; Burgess et al., 2012; Carrigan et al., 1991; Damiani et al., 2014; Goehring et al., 2010; Gryspeerdt et al., 2011; Henninger et al., 2007; Matsumura et al., 1994; McFadden et al., 2016; Negussie et al., 2017; Pronost et al., 2012; Pusterla et al., 2012a; Stasiak et al., 2017; Studdert et al., 2003; Yilmaz et al., 2012). This is in agreement with a 1960 study where antibodies against EHV-1 were detected by complement fixation test (CFT) and virus neutralisation test (VNT) in sera from horses affected with rhinopneumonitis (Matumoto et al., 1965). Although during that time, distinction between EHV-1 and EHV-4 was not yet established. Country specific seroprevalence data of EHV-1 infection is often not available in the current literature. This may be due to limited availability of serological tests that discriminate between EHV-1 and EHV-4 (Hartley et al., 2005) and the increasingly common incorporation of EHV-1 vaccination to the health program of various horse populations (Dunowska, 2014a). In a structured serological survey conducted in an unvaccinated population of Thoroughbreds in New Zealand, 70% of >24 months old horses had antibody to EHV-1 and were presumed to be latently infected (Donald, 1998). In contrast, before the widespread adoption of vaccination in 1997, the reported seroprevalence of EHV-1 in Australian Thoroughbreds was relatively lower than in New Zealand (Dunowska, 2014b). In the 1993 (Crabb et al.) work, the frequency of healthy Thoroughbreds with EHV-1 Ab was low (10%) in randomly chosen archived sera (75) collected between 1967-1974 and 28% of 97 racing Thoroughbreds (Crabb and Studdert's 1995 publication as cited by Dunowska et al., 2014b). Similarly, 26% (229) of mares and 11% of foals in an Australian stud farm was found to have EHV-1 specific antibodies in 1995 (Gilkerson et al., 1999a). However, such difference that was pointed out could be due to variation in testing methods (Dunowska, 2014b).

A common view is that the detection of latent infection suggests the widespread distribution of EHV-1 in horse population thus several investigators tried to describe the prevalence of latent EHV-1 using either co-cultivation of the virus or molecular detection techniques on latently infected cells. In the United Kingdom, 50% of 40 randomly selected healthy horses slaughtered in an abattoir were found to harbour EHV-1 using co-cultivation and PCR analysis of mostly bronchial lymph nodes draining the respiratory tract (Edington et al., 1994). A similar proportion of latent EHV-1 (54%) was found in submandibular lymph nodes of 132 Thoroughbreds necropsied at the University of Kentucky, using a protocol that included enrichment for EHV-1 sequences prior to PCR testing (Allen et al., 2008). However, a relatively lower prevalence of latent infection (26%) in lymphoid tissues and trigeminal ganglia was reported among 70 Thoroughbred horses in California (Pusterla et al., 2012b) and 32% of 52 New Zealand horses were positive for EHV-1 DNA in either retropharyngeal lymph nodes or trigeminal ganglia (Dunowska et al., 2015).

1.6.5 Emergence of a variant associated with neuro-pathogenicity

The increasing number of myeloencephalopathy cases associated with EHV-1 have been reported in the United States of America and Europe for the past five years (Burgess et al., 2012; Pronost et al., 2012; Pusterla et al., 2014). A single amino acid point mutation in the highly conserved region of open reading frame (ORF) 30 coding for DNA polymerase was reported to be associated with neuro-pathogenic capacity of EHV-1 (Nugent et al., 2006). This important genetic marker was discovered when nucleotide variation of EHV-1 Ab4 sequences, a genotype associated with neuropathogenicity, was compared to a relatively non-virulent EHV-1 V592. According to Nugent et al. (2006), 78 (95%) isolates from outbreaks without neurological disease encoded the nucleotide A at 2254 position (amino acid N752), while 42 (86%) isolates obtained from cases of EHM had the nucleotide G at position 2254 (amino acid D752) of ORF30. This suggested that the shift from asparagine to aspartic acid (N752 to D752) in the DNA polymerase region may have increased the neuro-pathogenic potential of EHV-1.

Goodman et al. (2007) demonstrated the increased ability of a D752 genotype to induce viremia, central nervous system inflammation and ataxia in experimentally infected horses when compared to N752 mutant virus. The N752 virus in this experiment was engineered from the D752 virus Ab4, resulting in the loss of neuropathogenicity of this virus. In a similar reverse genetics experiment (Van de Walle et al., 2009), a D752 mutant, created from a non-neurovirulent EHV-1, caused neurologic disease in 2 out 6 infected horses, and demonstrated high level of viremia in PBMC. Although both studies (Goodman et al., 2007; Van de Walle et al., 2009) supported the importance of D/N752 mutation for the neuropathogenic potential of the virus, the mechanism for EHV-1 neuro-virulence is still unclear. The virus with D752 genotype was also isolated from cases of abortion without neurological signs (Marenzoni et al., 2013; Smith et al., 2010). Equally, in several instances of neurologic cases, only the virus with N752 genotype was detected. Most likely, aside from viral genotype, other host (sex, breed and age) and environmental risk factors have played roles in the development of EHM (Goehring et al., 2006; Goehring et al., 2010; Henninger et al., 2007; Pronost et al., 2010)

1.7 *Laboratory Diagnosis*

Presumptive diagnosis of EHV-1 infection in horses is based on history and presenting clinical signs. While this can be confirmed by an array of definitive diagnostic tests, the selection of the right samples for the relevant assay and the timing of collection based on manifested clinical signs is an important aspect of laboratory diagnosis (Balasuriya et al., 2015). Commonly employed diagnostic methods are agent identification by virus isolation (Carrigan et al., 1991), nucleic acid detection by PCR (Diallo et al., 2007) and demonstration of increase in antibodies against EHV-1 through an EHV-1 type-specific ELISA (Hartley et al., 2005). Other test methods include histopathology, electron microscopy;

immunohistochemistry, indirect immunofluorescence test; and other serological assays such as complement fixation test, serum neutralisation test and agar gel immunodiffusion assay. The ideal diagnostic test is fast and sensitive which is critical for the immediate treatment of an affected horse. Albeit each individual method has its own limitations, selection of the most appropriate test is also dependent on the complexity and capability of the local diagnostic laboratory.

1.7.1 Virus isolation

Virus isolation (VI), culture and identification of EHV-1 from nasal or nasopharyngeal swabs and whole blood samples, was traditionally regarded as the 'gold standard test' for EHV-1 diagnosis. However, VI is slow and relatively less sensitive (Edington et al., 1991) compared to qPCR especially when confronted with either a limited amount or a poor quality of clinical specimen submitted. It takes at least 2-3 days for cytopathic effect (CPE) to be visible and the virus is identified. The presence of replicating virus in the nasal mucosa and the upper respiratory tract is usually short lived (<5 days post infection). Thus, to increase the chance of isolating the virus, nasal swab or nasopharyngeal swab samples (McBrearty et al., 2013) should be collected during the acute early febrile phase of the disease. Fever was observed to have a direct correlation with EHV-1 load in the nasal secretions during this time (Balasuriya et al., 2015) and usually the virus disappears from the blood after the onset of neurological signs (Pusterla et al., 2014). Swabs soaked in virus transport media and blood in EDTA tubes should be shipped in chilled condition to the laboratory for inoculation to permissive continuous cell lines (rabbit kidney-13, baby hamster kidney-21, Madin-Darby bovine kidney or equine kidney or lung cells) (Balasuriya et al., 2015). Other samples where VI can be attempted to are from aborted foetus tissues (lung, liver, spleen), placenta, buffy coat cells and nerve tissues or cerebrospinal fluid from EHM cases if available. Although VI is important to retrospective molecular characterisation of the virus, VI is not a quick test and the use of rapid molecular PCR testing to support diagnosis during outbreak situation is recommended (Balasuriya et al., 2015).

1.7.2 Detection of virus nucleic acid by PCR

With the advent of validated molecular diagnostic tools such as PCR ("OIE Terrestrial manual ", 2017), these tests have largely replaced VI for the diagnosis of EHV-1 infection in the laboratory. This is primarily due to the high sensitivity and specificity of PCR, fast turn-around time and a relatively low resource requirement. Polymerase chain reaction results can be available within hours. This technique relies on repeated heating and cooling cycles to amplify a specific fragment of the virus genomic DNA if it was present in the samples. Like VI, routine samples for PCR detection are nasal swabs, nasopharyngeal swabs and uncoagulated blood. Polymerase chain reaction can also be used to detect EHV-1 DNA in paraffin-embedded tissues or in cell culture lysates (Gerst et al., 2003; Perkins et al., 2009; Studdert et al., 2003). Common EHV-1 genes that are targeted in PCR-based assays are gB, gD, and DNA polymerase. Early protocols for conventional PCR were described (Borchers et al., 1993; Kirisawa et al., 1993a) while

an example of qPCR to illustrate viral shedding and viremia was reported by Hussey et al. (2006). Recently, analyses of ORF 30 sequence using various PCR approaches were described to discriminate between D752 and N752 variants (Allen, 2006; Nugent et al., 2006; Pusterla et al., 2009b). A multiplex qPCR technique to differentiate EHV-1 from EHV-4 in a single reaction was also described by Diallo et al. (2007).

Other methods that have been developed for latency detection are sequence capture qPCR (Allen et al., 2008) and qPCR technique for expression of late structural genes (Pusterla et al., 2012b). Latency is confirmed when tissue samples are PCR positive for the late structural gB gene in the absence of detectable gB gene mRNA (Pusterla et al., 2012b). An insulated isothermal PCR (iiPCR) method, for point-of-need detection of EHV-1 targeting the ORF30 gene to detect both D752 and N752 viruses, was recently used in archived nasal swabs, buffy coats and tissues from abortion materials (Balasuriya et al., 2017). The sensitivity (100%) and specificity (90.20%) of iiPCR was reported to be comparable to a reference qPCR with the two methods having a 95.19% (CI 95%: 90.48-99.90%) agreement and kappa value of 0.90 (Balasuriya et al., 2017).

1.7.3 Serology

A four-fold increase in serum antibody titre between acute and convalescent samples of clinically affected horses indicates recent EHV-1 infection and is suggestive of EHV-1 induced disease (Friday et al., 2000; Hartley et al., 2005; Van Maanen et al., 2001; Walter et al., 2013). However, traditional serological surveys have always been confounded by the broad antigenic cross-reactivity between EHV-1 and EHV-4 especially when using virus neutralization or complement fixation (Hartley et al., 2005). A type-specific antibody ELISA using *E.coli* expressed fusion proteins containing variable regions of glycoprotein G (gG) was developed in the early 1990's (Crabb et al., 1995; Crabb et al., 1992). It was found that type-specific epitopes at the carboxyl-terminal of the amino acid sequence of gG of both EHV-1 and EHV-4 stimulate a type-specific humoral immune response which enabled the differentiation of EHV-1 from EHV-4-specific antibodies (Crabb et al., 1995). However, the use of type-specific antibody ELISA to classify animals that are latently infected with EHV-1 or EHV-4 may not be as reliable as detection of actively infected horses (Dunowska et al., 2015). This test has been useful in investigations of EHV-1 infection and management of EHV-1 disease outbreaks (Dunowska et al., 2015; Studdert et al., 2003). The limitations of serologic testing in the diagnostic confirmation of EHV-1 infection in a horse should not hold the diagnostician from using such assays. Testing of paired serum samples from in-contact horses is encouraged to determine the circulation of the virus within group of horses and to provide support for EHV-1 involvement in cases of outbreaks of abortions or neurological disease.

1.7.4 Histopathology and immunohistochemistry

Pathological changes such as presence of characteristic Cowdry type A intranuclear eosinophilic inclusion bodies in liver cells and respiratory tract epithelium of aborted foetuses or foals, together with vasculitis and thrombosis in small blood vessels of the spinal cord can be demonstrated in tissue samples taken from horses infected with EHV-1 (Murray et al., 1998). This involves fixing of tissues in formalin, embedding in paraffin wax, staining with haematoxylin and eosin (H & E) and observation under light microscope (Murray et al., 1998; Schultheiss et al., 1993).

Immunoperoxidase (IP), an immunohistochemical staining method, has been used in several EHV-1 studies (Kydd et al., 1994; Whitwell et al., 1992b). An IP test can be used to determine the cell type and intracellular location of an antigen (protein or glycoprotein) and can also be performed on virus inoculated cell cultures. This method relies on the use of antigen-specific antibodies that are either conjugated to a peroxidase enzyme or detected by secondary peroxidase conjugates. The peroxidase enzyme catalyses a chemical reaction to produce a colored product after addition of a colorless substrate. Hence, the test result can be interpreted based on a distinct colour or pigmentation reaction. Several researchers (Gerst et al., 2003; Schultheiss et al., 1993; Whitwell et al., 1992b) used IP test to detect EHV-1 in formalin fixed, paraffin embedded (FFPE) tissue samples from aborted equine foetuses and placental tissues. In addition, an IP method was utilised to detect viral antigens in FFPE sections with the use of a polyclonal rabbit anti-EHV-1 antibody. The IP test method is also suitable for testing FFPE archival samples.

1.8 *Prevention and control*

Eradication of EHV-1 in equine premises is difficult and unlikely to be successful. A primary reason is that the virus establishes life-long latency after primary infection. Although the introduction of an EHV-1 infected horse can start EHV-1 related disease outbreak, the presence of latently infected animals is the main risk factor for closed herds. When stressed, some horses may reactivate EHV-1 which may then be transmitted to other susceptible in-contact horses. The resulting infection could be subclinical or accompanied by clinical disease of various severity. Thus, the goals of preventing disease to occur in this kind of situation are: firstly, to minimise the level of stress to limit the reactivation of any latent EHV-1; secondly, to reduce the exposure of susceptible horses to potential shedders; thirdly, to increase individual immunity by vaccination; and lastly, to treat and isolate animals with clinical infection away from the healthy group.

One way of reducing stress is to segregate horses into smaller groups, preferably by age or by type of management. This not only minimises stress that could be brought by overstocking but also lessen the exposure of susceptible horses to other horses that might be shedding the virus. Newly arrived horses should undergo at least three weeks of quarantine away from a group of healthy but susceptible members

of the population, before allowing them to join the group. Another important thing to establish is to prevent the transfer of potentially contaminated fomites such as farm tools or cleaning materials from one group to another. Handling of animals should start from groups of healthy or high-risk horses such as pregnant mares, towards the direction of potential shedders such as recently weaned foals, yearlings or sick animals. Vaccination strategy is also an important management tool as it may lessen the impact of clinical disease. It does not, however, prevent virus shedding nor does it provide protection to young animals less than one month of age in the presence of maternal antibodies (Gilkerson et al., 1999b).

1.9 *Equine herpesviruses in New Zealand*

Serological evidence of the presence of EHV-1 in New Zealand was reported in the early 1960s where antibodies against EHV-1 were detected by CFT and VNT in sera from horses affected by rhinopneumonitis (Matumoto et al., 1965). Horner et al. (1976) reported the first isolation of equine herpesvirus that resembled EHV-1 from foals with respiratory disease in New Zealand. Two virus isolates were believed to be EHV-1 because of a relatively fast CPE development. These EHV-1 like isolates however did not react with the hyperimmune sera prepared against a reference strain of EHV-1 in neutralisation test. During that time, distinction of EHV-1 from EHV-4 was not yet established and possibly the hyperimmune sera used may have been anti-EHV-4 rather than anti-EHV-1. Alternatively, the viruses isolated may have been equine γ herpesviruses. Subsequently, Hutton et al. (1977) successfully isolated and identified EHV-1 from aborted foetuses from a South Island property. Horner (1981) later described that the EHV-1 isolated from respiratory and abortion cases were different based on cross neutralisation studies performed. Such antigenic differences supported the existence of two EHV-1 subtypes (Studdert et al., 1981), which afterwards were segregated into two distinct viruses, EHV-1 and EHV-4. Since then, serological studies (Dunowska et al., 2002b; Dunowska et al., 2015; Jolly et al., 1986) and isolation of EHV-1 substantiated that equine herpesvirus circulates among New Zealand horses and may have contributed to unreported occurrences of clinical diseases (Dunowska et al., 2002a; McBrearty et al., 2013)

The first laboratory confirmed outbreak of equine herpesvirus myeloencephalopathy (EHM) occurred in New Zealand in 2014 in the Waikato region (McFadden et al., 2016). The affected stud farm comprised about 180 mares and foals in one management unit and 80 yearlings in a separate unit. Mares had been vaccinated against EHV-1 (Pneumequine, Merial) a year prior to the outbreak. The index case, a mare, was reported to have urinary scalding behind the hindlimbs, presumably due to urinary incontinence, for some time before being recognized as neurologically impaired. The mare was not noted as febrile at the time of the commencement of the outbreak investigation, although it may have occurred and was undetected during the early part of the disease process. Over a period of the following 33 days, additional 14 mares exhibit neurologic signs. The clinical signs observed included hindlimb paresis, ataxia, head tilt, and facial paresis. Seven horses became recumbent and were euthanised. The remaining eight

recovered. EHV-1 DNA was detected in 12 out of 15 cases by qPCR of nasal swab and blood samples. In addition, blood and nasal swabs were also collected from in-contact healthy mares in the high-risk area at the farm and 20/21 (95%) were EHV-1 positive by PCR. Sequencing of the EHV-1 DNA polymerase gene revealed the presence of both D752 and N752 variants of EHV-1 on the farm. The origin of the outbreak was not determined, although it was thought to be a reactivation of a latent infection in one of the resident horses, with subsequent horizontal spread. Around 7 months after the EHM outbreak, two mares aborted, and EHV-1 with D752 genotype was recovered from placenta and aborted foetal tissues (lung spleen, heart, kidney and liver). This is in agreement with other findings where EHV-1 with D752 genotype infection was not accompanied by any neurologic deficits (Nugent et al., 2006; Perkins et al., 2009). Interestingly, one sample collected from trigeminal ganglia from a horse following routine slaughter at the processing plant in the South Island two years prior to this 2014 outbreak was also positive for EHV-1 ORF30 D752 variant (Dunowska et al., 2015).

1.10 *Aim of the Study*

The aim of the current study was to estimate the frequency of latent EHV-1 infection among a selected population of New Zealand horses from the central North Island. As the study was conducted after the 2014 EHM outbreak in the Waikato region of the North Island, the second aim of the study was to determine the frequency of detection of the ORF30 D752 genotype, as well as any host-factors that may be associated with infection with this genotype. The data reported complement results of a similar study conducted in the South Island of New Zealand in 2012 (Dunowska et al., 2015).

2 Methodology

2.1 *Study design*

A cross sectional study to detect EHV-1 infection was carried out using samples collected between March and November 2015. The target population was horses from the upper and central North Island of New Zealand. It was estimated that 96 horses would need to be sampled based on an expected EHV-1 infection prevalence of 50%, a 95% level of confidence and a precision of +/- 10% of the estimated true value (Thrushfield, 1995).

Sample processing, DNA purification and initial verification of the test methods were done at the School of Veterinary Studies (SoVS, former Institute of Veterinary, Animal and Biomedical Sciences), Massey University, Palmerston North. Further assay optimisation and testing of samples were carried out at the Animal Health Laboratory-Wallaceville, Biosecurity New Zealand, Ministry for Primary Industries. PCR amplicons from EHV-1 positive samples were sent for sequencing to Massey Genome Service to determine the genotype of the virus.

2.2 *Sample collection*

Samples were collected from carcasses of horses that have been slaughtered for unrelated reasons (injury or retirement) by the local huntsmen. Heads of slaughtered horses were either dissected on site or transported to Massey University if the distance was within two-hour drive. Two sample types were collected from each head: retropharyngeal lymph nodes (RLN) and submandibular lymph nodes (SLN). The dissection and collection of samples were performed by a veterinarian participating in the project (Patricia Pearce), who had been trained in the procedure by a board-certified pathologist. Information on individual horse such as age, sex, breed, vaccination status and international travel history were obtained from owners or trainers and captured on a data sheet (Appendix 1).

Small pieces of tissue (approximately 0.5 cm in each dimension) collected from RLN and SLN were placed in containers containing 2.5 mL RNA later solution (Life Technologies) each. Thus, two sample containers were collected from each horse: one with RLN sample and one with SLN sample. Each container contained 2 pieces of tissue. The containers were labelled with horse identification number, date of collection and type of sample. Sampling was done aseptically using disposable gloves and disposable blades, which were changed between animals. Tissue samples in RNA later solution were transported

chilled to either Massey University or to a temporary storage facility as soon as feasible. Samples were then stored at -20°C until processed.

2.3 *DNA extraction*

All DNA extractions were performed at the Virology laboratory, SoVS in a biological safety cabinet. Care was taken to avoid cross-contamination between lymph node tissues from different horses including the use of separate disposable instruments for each sample. Each tissue sample was placed in a petri dish, blotted with paper towel to remove excess RNA later solution, cut into small pieces and weighed. Approximately 200 to 250 mg of a minced lymph node tissue was placed in an individual 1.5 mL metal tube (DNAture, NZ) with two stainless steel beads (SKF Palmerston North, New Zealand). The metal tube was then sealed with a rubber stopper, briefly immersed in liquid nitrogen, and the content homogenised for one minute using a Mini Beadbeater-16 (Biospec).

An EZNA HP Tissue DNA Maxi Kit (Omega Bio-tek) was used to extract DNA from the samples following manufacturer's instructions, with some modifications. Briefly, tissue lysis (MTL) buffer (1 mL) was added to each tube after the initial homogenisation and samples were homogenised in a Mini Beadbeater-16 for an additional 30 seconds. Homogenates were then transferred to 50 mL conical centrifuge tubes (Greiner Bio-One). After each use, metal beads, metal tubes and lids for homogenisation were washed and soaked in Trigene (Ceva Animal Health Pty Ltd) disinfectant for at least 1 hour, rinsed with 70% ethanol and washed twice with RNase free water. The treated beads, tubes and rubber lids were air dried and autoclaved before use.

Eight millilitres of high salt buffer (MTL1) containing the cationic detergent CTAB (cetyltrimethyl ammonium bromide) and 300 µL of Proteinase K were added to each tube with tissue homogenate and vortexed at a maximum speed for 15 seconds. Digestion of homogenates was done overnight in a waterbath at 55°C. The following day (day 2), 9 mL of chloroform:isoamyl alcohol (24:1) was added to each tube. The tubes were vortexed for 15 seconds, followed by centrifugation at 3,220 × *g* for 6 minutes at room temperature. After centrifugation, the upper aqueous phase containing DNA (~6 mL) was transferred to a new 50 mL conical tube, avoiding the milky interface containing contaminants and inhibitors. For samples that did not generate enough aqueous phase, another 2 mL of MTL buffer was added, followed by vortexing for 15 seconds, centrifugation at 3,220 x *g* for 10 minutes, and collection of the aqueous phase. An equal volume of a kit-provided BL buffer was then added to the aqueous phase to provide suitable DNA binding conditions to Omega Bio-tek's HiBind® matrix. The contents were mixed, and the tubes incubated in a water bath for 10 minutes at 70°C.

The HiBind® DNA Maxi Columns (Omega Bio-tek) were pre-equilibrated by applying 3 mL of 3M NaOH to each column, followed by centrifugation at 3,220 × *g* for 3 minutes. A volume of 100% ethanol equal to the volume of the aqueous phase (~6 mL) was then added to each sample tube. The samples were vortexed at maximum speed for 30 seconds, and the content of each tube (~18 mL) was applied to a pre-equilibrated HiBind® DNA Maxi Column. Following centrifugation of sample in the HiBind® column at 3,220 × *g* for 6 minutes at room temperature, the filtrate was discarded, and the centrifugation step was repeated with the remainder of the sample until the entire content of the tube was processed.

The kit-provided HBC buffer (10 mL) was then applied to each column, and the samples were centrifuged at 3,220 × *g* for 6 minutes at room temperature. The HBC buffer contains guanidine hydrochloride that removes residual proteins while isopropanol enhances the binding of nucleic acids to the membrane. The filtrates were discarded, and DNA bound to the columns was washed with 15 mL of the supplied wash buffer. The washing was done by centrifugation at 3,220 × *g* for 6 minutes. The filtrate was discarded, and DNA was washed for the second time in the same manner, with the exception that the tubes were centrifuged for 10 minutes to remove excess ethanol from the column.

Each dry column was then transferred to a new 50 mL tube for the elution step. The elution buffer (10mM Tris buffer, pH 8.5) was pre-warmed at 60°C, added to the centre of the column and left for 5 minutes at room temperature. Elution was done twice using 2 mL of the elution buffer each time, for a total volume of 4 mL. Both eluents containing DNA were collected into the same tube by centrifugation at 3,220 × *g* for 6 minutes at room temperature. The quantity and quality of the DNA were analysed using a Nanodrop spectrophotometer (Thermo Scientific). DNA was stored at -20°C until use.

2.4 *DNA concentration*

The aim was to obtain at least 240 µg DNA at a minimum concentration of 360 ng/µL from each sample. Samples with DNA concentration lower than 360 ng/µL were further concentrated using Microcon® 30K (Merck Millipore Ltd) centrifugal filter device. Briefly, the filter device was rinsed with distilled water before use. DNA was concentrated by adding 500 µL of DNA to Microcon® filter device. The tube was sealed with the attached cap and spun at 14,000 × *g* for 20 minutes. The concentrated DNA was collected by inverting the filter device into a new tube followed by centrifugation at 1,000 × *g* for 3 minutes. This process was repeated until the entire 4 mL of extracted DNA was concentrated or until the required amount of DNA at the desired concentration was obtained.

2.5 *EHV-1 detection method*

DNA enrichment by magnetic bead sequence-capture method and the subsequent nested PCR analysis used in this study was based on previously published protocols (Allen, 2006; Dunowska et al., 2015). The testing was performed at the Animal Health Laboratory (AHL-Wallaceville). The first step of the project was to verify that the method was transferred satisfactorily from Massey virology laboratory to AHL-Wallaceville laboratory.

2.5.1 Primary PCR

Primary conventional PCR for EHV-1 was performed using the primer pair ORF30.F: GTGGACGG-TACCCCGGAC and ORF30.R: GTGGGGATTCGCGCCCTCACC. This primer pair amplifies a 256 bp fragment of the ORF30 DNA polymerase gene. Each 20 µL PCR reaction consisted of 4 µL 1× HotFirePol PCR mix (Solis Biodyne) with 2 mM final concentration of $MgCl_2$, 0.4 µM of each forward and reverse primer, water and template (either 5 µL bead suspension from DNA enrichment step or 1 µL of EHV-1 DNA). Amplification was done on an AB Veriti 96-well Thermal Cycler (Thermo Fisher Scientific) with the following conditions: initial denaturation at 95°C for 15 minutes followed by 35 cycles of 10 second denaturation at 95°C; 30 seconds of annealing at 60°C; and 20 seconds of elongation at 72°C; with the final extension step at 72°C for 5 minutes.

2.5.2 EHV-1 qPCR

Quantitative PCR reactions were performed in a SYBR Green format in the Bio-rad CFX96 (Bio-Rad) machine. Each 10 µL reaction consisted of 5 µL 1× Luminaris Green (ThermoFisher) master mix, 0.4 µM of each primer (EHV-1 F1: GGGAGCAAAGGTTCTA-GACC and EHV-1 R1: AGCCAGTCGCGCAGCAAGATG), water and template (either 2 µL primary PCR amplicon diluted 1:10 in water or 1 µL of EHV-1 DNA). The conditions for the EHV-1 qPCR were the same as the primary PCR, with the exception that the melt from 55°C to 95°C, increment of 5°C for 15 seconds was added at the end of each run. Samples were considered positive for EHV-1 DNA if the amplification curve crossed the threshold and the melting peak was within 1°C of the melting peak of the positive control. Automatic threshold detection settings were used.

2.5.3 Assay controls

Assay controls employed in each step of the EHV-1 detection method are illustrated in Figure 3. Primary PCR run included a positive control 1 (PC-1, EHV-1.592 DNA obtained from cell culture lysate), a no template control 1 (NTC-1, beads from enrichment reaction carried out with water instead of the sample) and no template control 2 (NTC-2, water) (Figure 3). Assay controls for the nested qPCR consisted of PC-1 (carried over from the primary PCR), an additional positive control 2 (PC-2, EHV-1 DNA), NTC-1

(carried over from DNA enrichment step), NTC-2 (carried over from primary PCR) and an additional no template control 3 (NTC-3, water).

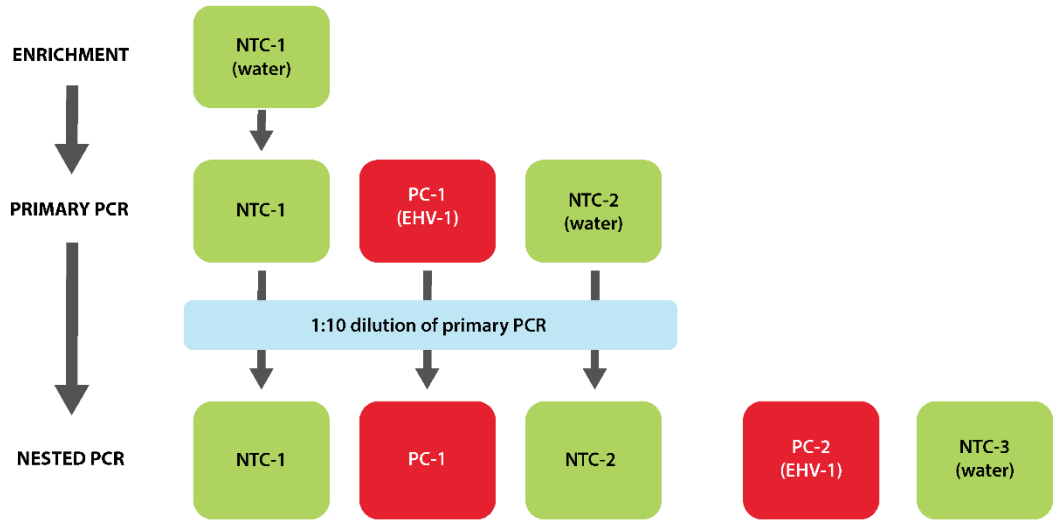

Figure 3. Summary of controls introduced at each step of the protocol from enrichment to nested PCR: negative non-template control (NTC) in green and positive control (PC) in red.

2.5.4 Validation of EHV-1 qPCR

To demonstrate the efficiency, reproducibility and analytical sensitivity of the EHV-1 qPCR step (section 2.5.2), two standard curve experiments were conducted using 1 µL of each of the 10-fold dilutions of EHV-1.592 DNA (10^{-1} to 10^{-9}) as a template done in triplicate. Lysate of a cell culture that had been infected with EHV-1 was used as a source of viral DNA.

2.5.5 Verification of sequence capture DNA enrichment

During verification of the DNA enrichment method, all reaction volumes were scaled down 10-fold in comparison with the published protocol (Dunowska et al., 2015). Retropharyngeal lymph nodes that had been determined to be negative for EHV-1, based on results from the previous study (Dunowska et al., 2015) were used as starting materials for the validation experiments. One µL from each EHV-1.592 dilution (10^{-1} to 10^{-5}) was spiked into each of the five tubes containing 24 µg equine DNA extracted from EHV-1 negative lymph nodes. An additional tube with water which was un-spiked was included as a no template control (NTC-1). To determine the detectable levels of the EHV-1 DNA in the preparation that was used for spiking, the DNA was diluted 10-fold (10^{-1} to 10^{-8}), and 1 µL of each dilution was used as a template in the primary EHV-1 PCR (section 2.5.1), followed by nested qPCR (section 2.5.2) using 2 µL of the primary PCR product diluted 1:10 with water. Each spiked sample was then digested with 48 units of Bgl-II restriction enzyme (New England BioLabs) in supplied NEBuffer 3. Digestion was done overnight at

37°C in a total volume of 75 µL. The following day (day 2), digested samples were denatured at 100°C for 10 minutes using a heat block and hybridised with 1 µL (0.24 picomol) of biotinylated capture oligonucleotide probe (Biotyn-CCG TAA ACG GAG TTC CAT ATC ACC) at 60°C while shaking gently at 200 rpm (Vortemp shaker, Labnet) for 24 hrs. The oligonucleotide probe (Allen et al., 2008), was complementary to the ORF30 EHV-1 gene encoding the viral DNA polymerase.

EHV-1 DNA was captured using pre-washed streptavidin coated magnetic beads (Dynabeads M280, Invitrogen), as per manufacturer's instruction. Briefly, a desired volume of beads was removed from the vial and washed 3 times with one volume (or at least 1 mL) of 1 x binding and washing buffer (1x BWB: 5 mM Tris–HCl pH 7.5, 0.5 mM EDTA, 1M NaCl). Each time, the beads were vortexed for 5 seconds, placed on a magnet (DynaMag-2, Thermo Fisher Scientific) for 3 minutes and supernatant removed. After the 3rd wash, the beads were resuspended in two times the initial volume of 2x BWB (10mM Tris HCl pH 7.5, 1mM EDTA, 2M NaCl) to make up a final concentration of 5 µg/µL. After hybridisation (day 3), an equal volume (75 µL) of 2x BWB containing 6 µg (1.2 µL) pre-washed magnetic beads, was added to each tube. The mixture was then incubated at 43°C with gentle shaking at 200 rpm for 6 hours. Magnetic beads were immobilised on a magnet for 3 minutes. The supernatant was removed, and the beads were washed thrice with 30 µL 1× BWB. Each time, the beads were immobilised on the magnet for 3 minutes to allow removal of the supernatant. After the last wash, beads were re-suspended in 20 µL water and used as template for EHV-1 primary PCR reaction (section 2.5.1), followed by nested qPCR (section 2.5.2).

2.6 *Processing of survey samples*

Survey samples were analysed for the presence of EHV-1 genome using the validated magnetic bead, sequence capture DNA enrichment followed by the nested EHV-1 qPCR detection protocol (Figure 4). The amount of starting DNA for digestion was 240 µg, with all the reagents proportionally scaled up. Samples were processed in batches of 8 to 9 samples. All controls (Figure 3) were included when processing each batch of the samples.

2.6.1 Sequence capture DNA enrichment

Lymph node DNA (240 µg) samples were dispensed into individual 1.5 mL tubes. Each sample was digested with 480 units of Bgl-II restriction enzyme in supplied NEBuffer 3 in a total volume of 750 µL. Digestion was performed overnight at 37°C. The following day, DNA was denatured at 100°C in a heat block for 10 minutes and a freshly diluted biotinylated capture oligonucleotide probe (1 µL containing 2.4 picomoles of DNA) was added to each sample. The samples were placed in a Vortemp shaking incubator at 60°C for 24 hours. This step allowed for the hybridisation of the biotinylated capture oligonucleotide probe (section 2.5.5) to any EHV-1 DNA that may have been present in the samples.

After the hybridisation step, the biotinylated oligonucleotide probe hybridised to EHV-1 DNA was captured by adding 2x BWB including 60 µg (12 µL in 2× BWB) streptavidin coated magnetic beads (Dynabeads M280) in a total volume of 1500 µL in each reaction tube. Binding was done by incubating the mixture at 43°C with gentle shaking for 6 hours in a Vortemp instrument. The magnetic beads in the sample tubes were immobilised on a magnet for 3 minutes. After immobilization, the supernatant was aspirated followed by washing the beads with 30 µL of 1× BWB three times for 3 minutes each time. After the final wash, beads from each sample were re-suspended in 20 µL water and used as a template for the EHV-1 nested PCR. Water, as no template control (NTC), was carried through all the steps of enrichment, alongside each set of samples (Figure 3).

2.6.2 EHV-1 PCR and genotyping

The bead suspension from each sample was divided into two 10 µL aliquots (aliquots A and B). Each aliquot was tested separately in EHV-1 primary PCR performed at different times. The primary EHV-1 PCR amplification was performed in a 50 µL reaction volume. Each reaction consisted of 10 µL of 5× HotFirePol PCR master mix (Solis Biodyne) with 10mM $MgCl_2$, 1 µL (0.4 µM) of each forward and reverse primer, 28 µL water and 10 µL template (aliquot A or B). Amplification was performed using AB Veriti 96 Thermal Cycler (Thermo Fisher Scientific) with the same conditions as those described in section 2.5.1. Primary PCR amplicons diluted 1:10 in water (2 µL) were then used as a template in the nested qPCR assay (section 2.5.2) in duplicate. Thus, 4 nested qPCR reactions were performed for each original tissue sample. Assay controls described in section 2.5.3 were included.

Nested qPCR reactions were considered positive for EHV-1 DNA if Cq value was generated with a corresponding melting peak within 1°C of the melting peak of the positive control (EHV-1.592 DNA). The assay was considered valid if all controls showed the expected results. Lymph node samples were considered positive for EHV-1 DNA if sequences of the DNA polymerase gene were retrieved from the samples as indicated by at least one positive EHV-1 nested qPCR assay.

Nested qPCR was repeated for all positive samples. An aliquot (5 µL) from each product was subjected to gel electrophoresis through a 0.1 % gelred (Biotium) stained gel. The remaining volume of each product was cleaned-up using ExoProStar S (GE Healthcare Life Sciences) to remove unincorporated primers and deoxynucleotide triphosphates (dNTPs). The purified products were sent for DNA sequencing to Massey Genome Service (Massey University, Palmerston North). Each duplicate was sequenced separately. Assembly and editing of DNA sequences were done using the Geneious prime Software (Biomatters, Ltd). The genotype of the EHV-1 was determined based on the presence of A or G nucleotide at position 2254 of the DNA polymerase gene using GenBank accession AY464052 (EHV-1 stain V592) for ORF30 N752 variant and AY665713 (EHV-1 strain AB4) for ORF30 D752 variant as reference sequences.

2.7 *Data analysis*

A horse was classified as infected with EHV-1 if the target viral DNA polymerase gene was detected in either RLN or SLN. Frequency of EHV-1 detection was calculated as the proportion of infected horses in the study sample. Univariate association of EHV-1 infection and each categorical variable such as age, breed, sex was analysed using χ^2 test. For all statistical analyses using Microsoft office excel, values of $p \leq 0.05$ were considered significant.

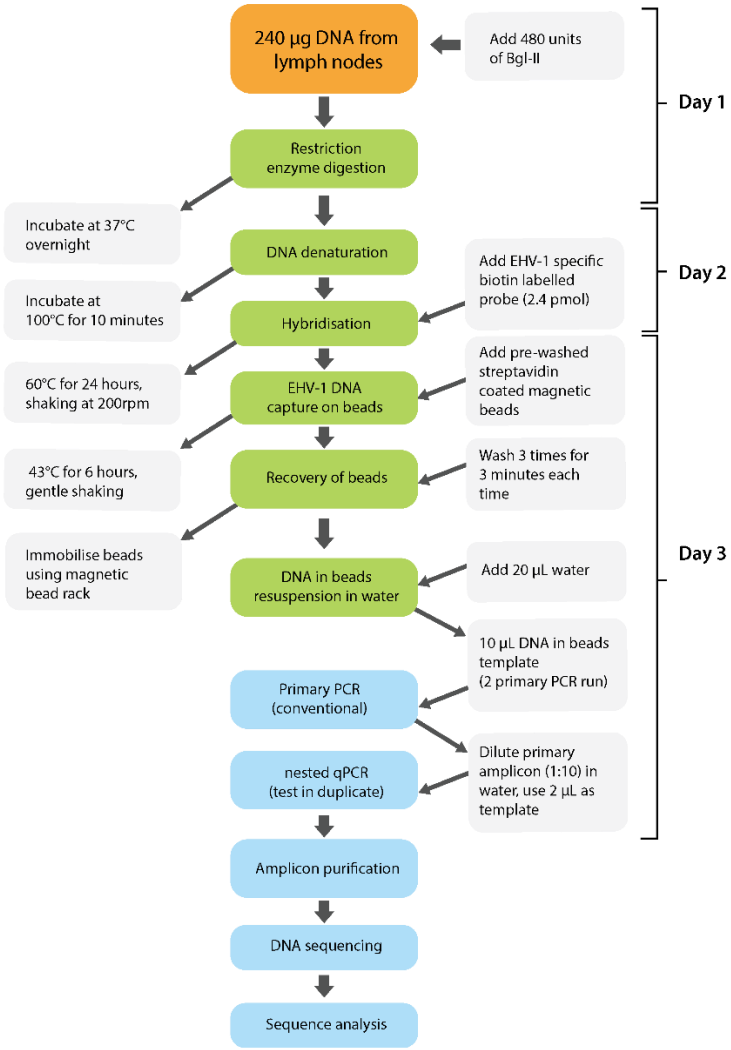

Figure 4. Summary of the methodology used to detect EHV-1 sequences in lymph node tissues from horses following slaughter. Large-scale DNA extraction (orange) was followed by magnetic bead, sequence capture DNA enrichment (green) and PCR-based detection and sequence analyses (blue). Details about experimental protocol at each step are shown in grey boxes.

3 Results

3.1 *Study population*

Sixty-three horses, slaughtered over a period of nine months either due to old age or injury, were included in this study (Appendix 2). These comprised all horses that were available via participating huntsmen during the study period. Sampled horses originated from the Manawatu-Wanganui region in the central North Island of New Zealand (Figure 5). Thirty-three horses came from the districts of Rangitikei, including the towns of Marton (*n*=14), Whangaehu (*n*=6), Bulls (*n*=5), Mangaweka (*n*=2), Turakina (*n*=2), Hunterville (*n*=1), Rata (*n*=1) and others (*n*=2). Nine horses came from Tararua (*n*=3), Ruapehu (*n*=3), Whanganui (*n*=2) and Manawatu (*n*=1). The origin of 21 horses was not recorded.

The study group consisted of females (*n*=30), geldings (*n*=25), an intact male (*n*=1) and horses with unknown gender (*n*=7). The median age of the horses was six years (range from 2 to 30 years) (Appendix 2), with an average of 10.4 years. Majority of horses sampled were Thoroughbreds (*n*=47) with the remaining horses classified as sport horses (*n*=4), ponies (*n*=4), Arabs (*n*=1) or Standardbreds (*n*=1) and unknown (*n*=6). More than half of the studied horses (*n*=49) might have had travelled locally and had contacts with other horses during their racing, showjumping or polo cross careers (Appendix 2). One horse (ID no. 37) was born in Australia and travelled to Hong Kong for racing, while four had unknown birthplace and unknown history of international travel. The rest of the sampled horses (*n*=58) were born in New Zealand and had no history of international travel. Only three horses (ID no. 3, 4 & 20) had history of vaccination but with no details available. The rest were either unvaccinated (*n*=43) or had unknown (*n*=17) vaccination history.

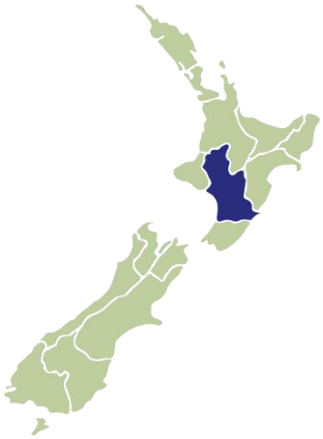

Figure 5. Manawatu-Wanganui Region in the North Island of New Zealand.

3.2 *Validation of EHV-1 qPCR*

Equine herpesvirus 1 DNA was detected from 10^{-1} to 10^{-4} serial dilutions of DNA extracted from EHV-1 infected cell culture lysate, when 1 µL was used directly as a template in qPCR, not in a nested format (Figure 6). The mean efficiency was 96.05 % with linearity of (R^2) 0.995. The intra-run % coefficient of variance (CV) ranged from 0.074 to 1.034 for different dilutions of the template (Table 1).

3.3 *Analytical sensitivity of the EHV-1 detection method*

Equine herpesvirus 1 DNA was detected from the same dilutions (10^{-1} to 10^{-4}) as those positive directly by qPCR, using 1 µL template for primary PCR followed by nested qPCR (Table 2), indicating that 10^{-4} dilution was the highest dilution containing detectable EHV-1 DNA. Hence, 10^{-4} dilution was presumed to contain between 1 and 9 copies of EHV-1 DNA per µL. Following spiking of EHV-1 DNA into the large excess (24 µg) of equine DNA, EHV-1 was recovered from samples that have been spiked with 1 µL of EHV-1 DNA at 10^{-1} and 10^{-2} dilutions but not from samples spiked with 10^{-3} to 10^{-5} (Table 3). Hence, it was estimated that the methodology employed was sensitive enough to recover between 10^2 and 10^3 copies of EHV-1 DNA in a background of a large excess of host DNA. The water sample carried through the enrichment and detection process (NTC-1) remained negative for EHV-1 DNA.

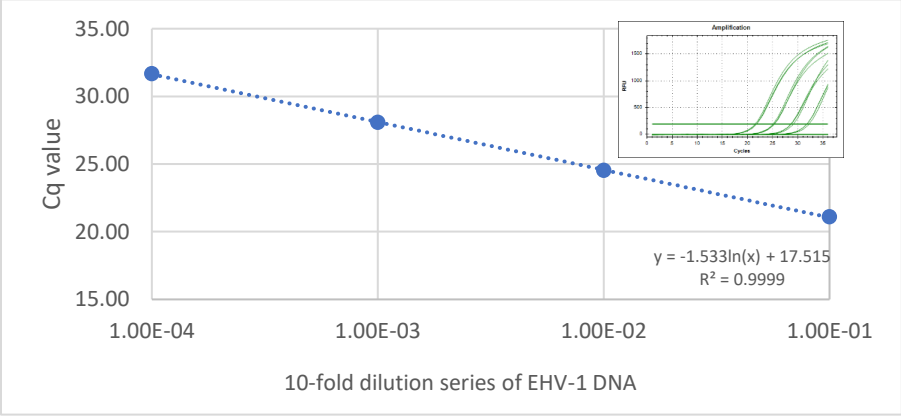

Figure 6. EHV-1 standard curve generated based on results of EHV-1 qPCR using 1 µL of 10-fold dilutions of EHV-1 DNA as a template. Cycle thresholds (Cq) are mean values from two runs of the qPCR.

Table 1. Standard curve prepared using serial dilutions of EHV-1 DNA extracted from the infected cell culture lysate as a template in EHV-1 specific quantitative PCR. Repeatability and reproducibility of the EHV-1 qPCR in two experiments.

EHV-1.592 dilution	Run 1						Run 2					
	Replicate 1	Replicate 2	Replicate 3	Cq mean*	SD	CV	Replicate 1	Replicate 2	Replicate 3	Cq mean	SD	CV
10^{-1}	21.72	21.44	21.67	21.61	0.149	0.691	20.56	20.57	20.59	20.57	0.015	0.074
10^{-2}	25.05	25.28	25.21	25.18	0.118	0.468	23.83	23.87	23.87	23.86	0.023	0.097
10^{-3}	28.62	28.54	29.09	28.75	0.297	1.034	27.38	27.34	27.44	27.39	0.050	0.184
10^{-4}	31.74	31.75	32.18	31.89	0.251	0.788	31.44	31.75	31.17	31.45	0.290	0.923
10^{-5}	-	-	-	-	-	-	-	-	-	-	-	-
10^{-6}	-	-	-	-	-	-	-	-	-	-	-	-
10^{-7}	-	-	-	-	-	-	-	-	-	-	-	-
10^{-8}	-	-	-	-	-	-	-	-	-	-	-	-

	Run 1	Run 2	mean
Efficiency (%)	94.9	97.2	96.05
R^2	0.998	0.992	0.995
slope	-3.45	-3.39	-3.42

* Cq: quantification cycle; SD: standard deviation; CV: coefficient of variance

***Table 2. EHV-1 nested qPCR limit of detection, 1 µL of EHV-1 DNA was used as template* in primary PCR followed by nested qPCR.**

EHV-1.592 dilution	Cq value	Melt temp	Cq value	Melt temp	Cq value	Melt temp	Cq value	Melt temp
10^{-1}	6.18	81.5	7.10	81.5	7.11	81.5	8.64	81.5
10^{-2}	7.29	81.5	8.48	81.5	8.74	81.5	9.01	81.5
10^{-3}	11.13	81.5	11.82	81.5	11.69	81.5	12.18	81.5
10^{-4}	15.89	81.5	16.07	81.5	15.89	81.5	17.05	81.5
10^{-5}	NA	None	NA	None	NA	None	NA	None
10^{-6}	NA	None	NA	None	NA	None	NA	None
10^{-7}	NA	None	NA	None	NA	None	NA	None
10^{-8}	NA	None	NA	None	NA	None	NA	None
NTC	NA	None	NA	None	NA	None	NA	None

Table 3. Recovery of EHV-1 DNA from a large excess of EHV-1 negative DNA (24 µg) following spiking with 1 µL of 10-fold dilutions (10^{-1} to 10^{-5}) of EHV-1 DNA using target DNA enrichment by magnetic bead sequence-capture method followed by nested qPCR).

EHV-1 "spiked" LN* DNA	Run 1		Run 2	
	Cq value	Melt temp	Cq value	Melt temp
LN + EHV-1 10^{-1}	5.72	81.5	5.63	81.5
LN + EHV-1 10^{-2}	7.77	81.5	9.82	81.5
LN + EHV-1 10^{-3}	neg	none	neg	none
LN + EHV-1 10^{-4}	neg	none	neg	none
LN + EHV-1 10^{-5}	neg	none	neg	none
Water	neg	none	neg	none

*LN= 24 µg lymph node DNA was spiked with 1 µL of corresponding EHV-1.592 serial dilution point.

3.4 *Frequency of EHV-1 infection*

Sixty-three RLN and sixty-two SLN were processed for large volume of DNA extraction. On one occasion (ID no. 66), only RLN was available. Most of the extracted DNA samples (*n*=55) had to be further concentrated to obtain 240 µg DNA at a concentration of ≥360 ng/µL to become suitable for the downstream EHV-1 detection method.

Individual test results are shown in Table 4. Overall, 6/63 (9.5%) of horses sampled were positive for EHV-1 DNA in RLN samples (ID no. 8, 41, 44, 58, 59 & 63). Of those, 3 horses were also positive for EHV-1 DNA in SLN samples (ID no. 44, 58 & 59). The remaining horses were negative for EHV-1 DNA in both RLN and SLN samples. All controls showed the expected results.

There was no significant difference (p = 0.88) between ages of horses positive (mean, 11.33 years) and negative (mean 10.81 years) for EHV-1 DNA. There was no significant difference (p = 0.65, $\chi 2$ = 0.205) between TB and other breeds of horses positive or negative for EHV-1 DNA. Also, there was no significant difference between the sex of horses positive or negative for EHV-1 DNA (p= 0.85, $\chi 2$ = 0.035). Therefore, there was no association between EHV-1 positivity and age, sex, or breed of the animals sampled (Table 5).

Nested EHV-1 PCR positive 256-bp product was demonstrated in a gel electrophoresis run (Figure 7). Based on the sequence analysis of the amplicons, all EHV-1 positive horses harboured the ORF30 N752 genotype, consistent with the non-neuropathogenic or abortigenic EHV-1 (Figure 8). The D752 genotype was not detected in any of the samples.

Table 4. Detection of EHV-1 DNA in RLN and SLN using bead-based target sequence capture followed by nested EHV-1 qPCR.

Horse ID	EHV-1 DNA																			
	RLN-A				RLN-B						SLN-A				SLN-B					
	Rep 1		Rep 2		Rep 1		Rep 2		Result	Genotype	Rep 1		Rep 2		Rep 1		Rep 2		Result	Genotype
	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp			Cq	Melt temp	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp		
1	-		-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
2	-		-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
3	-		-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
4	-		-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
6	-		-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
7	-		-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
8	20.5	82.0	20.41	81.5	-	-	-	-	+	N752 (A2254)	-	-	-	-	-	-	-	-	-	
9	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
10	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
11	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
13	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
14	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
15	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
16	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
17	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
18	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
19	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
20	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	

Horse ID	EHV-1 DNA																			
	RLN-A				RLN-B						SLN-A				SLN-B					
	Rep 1		Rep 2		Rep 1		Rep 2		Result	Genotype	Rep 1		Rep 2		Rep 1		Rep 2		Result	Genotype
	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp			Cq	Melt temp	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp		
21	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
22	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
23	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
24	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
25	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
26	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
27	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
28	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
29	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
30	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
31	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
32	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
33	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
34	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
35	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
36	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
37	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
38	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
39	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
41	17.18	None	15.31	81.5	-	-	-	-	+	N752 (A2254)	-	-	-	-	-	-	-	-	-	

Horse ID	EHV-1 DNA																			
	RLN-A				RLN-B				Result	Genotype	SLN-A				SLN-B				Result	Genotype
	Rep 1		Rep 2		Rep 1		Rep 2				Rep 1		Rep 2		Rep 1		Rep 2			
	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp			Cq	Melt temp	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp		
42	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
43	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
44	7.95	81.5	7.43	81.5	9.37	81.5	9.17	82.0	+	N752 (A2254)	6.87	82.0	6.95	82.0	16.18	82.0	16.19	82.0	+	N752 (A2254)
45	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
46	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
47	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
48	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
49	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
50	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
51	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
52	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
53	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
54	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
55	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
56	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
57	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
58	11.15	81.5	11.02	81.5	7.96	81.5	7.85	81.5	+	N752 (A2254)	8.46	81.5	8.47	82.0	-	-	-	-	+	N752 (A2254)
59	10.25	81.5	10.12	81.5	9.96	81.5	9.74	81.5	+	N752 (A2254)	10.58	81.5	11.48	82.0	14.79	82.0	14.79	82.0	+	N752 (A2254)
60	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	

Horse ID	EHV-1 DNA																			
	RLN-A				RLN-B				Result	Genotype	SLN-A				SLN-B				Result	Genotype
	Rep 1		Rep 2		Rep 1		Rep 2				Rep 1		Rep 2		Rep 1		Rep 2			
	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp			Cq	Melt temp	Cq	Melt temp	Cq	Melt temp	Cq	Melt temp		
61	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
62	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
63	11.11	81.5	11.01	81.5	-	-	-	-	+	N752 (A2254)	-	-	-	-	-	-	-	-	-	
64	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
65	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	
66	-	-	-	-	-	-	-	-	-		*		*		*		*		*	

Abbreviations: Rep replicate; **+** positive; **-** negative; * no sample

Table 5. Age, breed and sex of horses included in the study, stratified by EHV-1 testing results.

	EHV1 positive	**EHV1 negative**	**at $p \leq 0.05$**
Age (years):			
≤5	0	22	
6 to 10	4	14	
11 to 15	0	4	
>16	2	13	
Unknown	0	4	
Mean age	11.33	10.81	$p = 0.88$
Breed/Use:			
Thoroughbred	5	42	
Farm Hack	1	4	
Sport Horse	0	4	
Pony	0	4	
Arab	0	1	
Standardbred	0	1	
Unknown	0	1	
χ^2	0.205		$p = 0.65$
Sex:			
Female	3	27	
Castrated male	3	22	
Male	0	1	
Unknown	0	7	
χ^2	0.035		$p = 0.85$

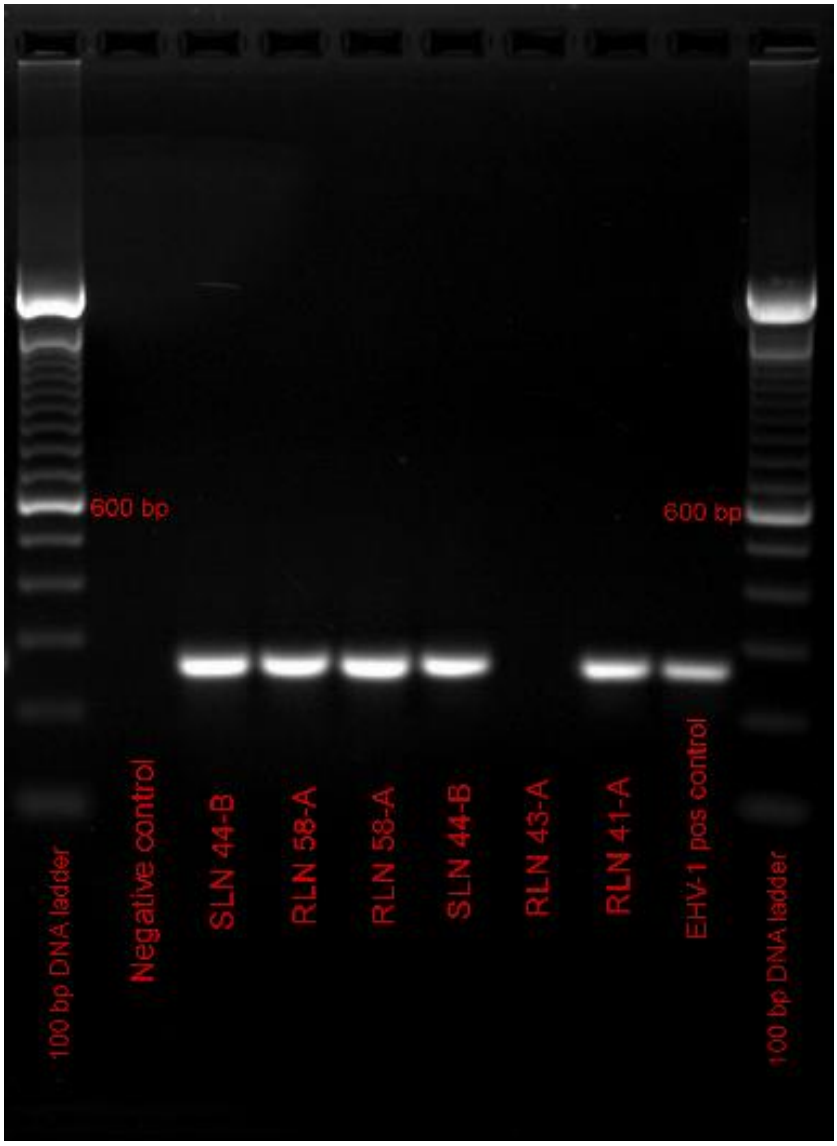

Figure 7. An example of EHV-1 amplicon from nested qPCR from selected lymph node (LN) samples as labelled on the gel. The amplicons were subjected to electrophoresis through 1.5% agarose gel stained with Gel Red (Biotium). Negative (water) and positive (EHV-1 DNA) controls are labelled. The 100 bp DNA ladders (Thermo Fisher Scientific) are visible at both ends.

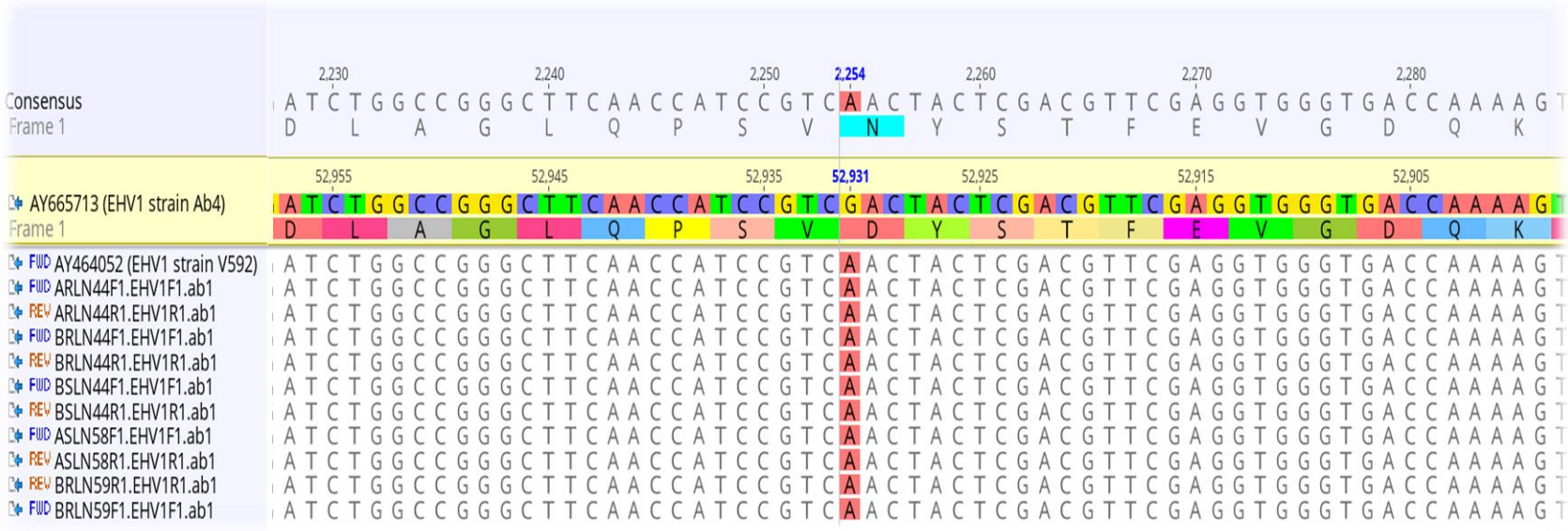

Figure 8. Sequences of EHV-1 positive amplicons with nucleotide A at position 2254 of the DNA polymerase gene using GenBank accession [AY464052](AY464052) (EHV-1 stain V592) for ORF30 N752 variant and [AY665713](AY665713) (EHV-1 strain AB4) for ORF30 D752 variant as reference sequences.

4 Discussion

In New Zealand, little is known about the current frequency of EHV-1 infections among various horse populations, particularly the D752 genotype. The D752 genotype was recovered for the first time in 2012 from a trigeminal ganglion of one of the 52 horses tested at the meat processing plant (Dunowska et al., 2015) and was implicated in the first outbreak of EHM in Waikato in 2014 (McFadden et al., 2016). Understanding the epidemiology of EHV-1 infection in equine population is useful for the development of a robust disease control program thus, the aim of the current study was to investigate the frequency of EHV-1 infection among selected population of horses from the North Island of New Zealand and to determine whether the frequency of infection with D752 genotype has increased following the 2014 EHM outbreak. The study complements the data from a similar investigation conducted in the South Island in 2012 (Dunowska et al., 2015).

The frequency of EHV-1 infection among the sampled horses (*n*=63) was 9.5%. The horses sampled originated from the Manawatu-Wanganui region in the central North Island of New Zealand. However, these horses were likely to have travelled to other places during their lives, which may have increased their chance of EHV-1 infection through contact with actively infected horses. As an example, the EHV-1 respiratory infection epidemic in Thoroughbreds at the Ritto Training Centre in Japan illustrated this scenario of disease transmission (Matsumura et al., 1994). In New Zealand, the widespread prevalence of EHV-1 infection in Thoroughbreds (>24 months) was previously demonstrated based on serology (Donald, 1998), with 70% of sampled horses testing positive for EHV-1 specific antibodies. Since horses presumably remain EHV-1 infected for life, it is unlikely that the frequency of latent EHV-1 infection differs significantly between TB from different locations within the North Island of New Zealand, including Waikato where the first outbreak of EHM took place. However, only 63 horses out of 96 desired sample size were available for sampling during the study period. As such, the results presented may not be representative of a larger population of horses beyond that sampled.

The frequency of EHV-1 detection among sampled horses was lower than in the survey conducted in 2012 where horses from various New Zealand locations slaughtered at the Gore processing plant in the South Island were tested for EHV-1 (Dunowska et al., 2015). In the 2012 survey, 32.7% of 52 horses were positive for EHV-1 in RLN samples. One possible explanation for this discrepancy is that horses at the processing plant were yarded with other horses of about 50 to 100 animals in the holding pen before they were slaughtered. Such stressful condition may have triggered the re-activation of latent EHV-1 infection and subsequent spread of the virus to other horses which resulted to higher frequencies of EHV-1 detection. Detection of EHV-1 DNA in lymph nodes of 11 serologically negative horses in that study

may support this view. In contrast, the horses in the current study comprised of horses transported to and processed by local huntsmen, usually on individual basis, without the stress associated with the hold period at the slaughter facility. Although transport also constitutes a possible stressor, a very small proportion of horses (between 0.7 and 1.2%) sampled following a long international transport were shedding EHV-1 in their nasal secretion (Pusterla et al., 2009a; Smith et al., 2018), indicating that transport alone may not be enough of a stressor to facilitate recrudescence of EHV-1. Even if recrudescence did occur in some horses following a comparatively short transport to the huntsmen, there was no opportunity for the horizontal spread of the virus to other animals. In addition, over 50% of the horses had recent histories of having lived in an isolated subpopulation on farm for many years rather than regularly mixing with a wider population through travel or competition. EHV-1 recrudescence may likely to occur in stressful conditions such as weaning, castration, re-grouping or mixing, long travel, strenuous training or during disease state (Pusterla et al., 2009a).

Another possible explanation for the differences in frequency of EHV-1 detection between the 2012 and the current study is the sensitivity of the EHV-1 detection. Based on the validation experiments performed prior to each study, the current protocol was approximately 100-fold less sensitive than that used in 2012. The reasons for this discrepancy in the performance of the method are unclear, as both studies used principally the same methodology, with minor differences such as brands of the PCR machines and other equipment used, a brand of the qPCR master mix, or the number of freeze-thawed cycles that the biotinylated probe may have been exposed to. Although low amounts of EHV-1 DNA (10^2 to 10^3 copies, equivalent to 0.05 to 0.5 pg) were still recovered from a very large excess of host DNA (240 µg), horses with an estimated concentration of EHV-1 DNA of between 1 and 100 copies/240 µg of host DNA would have been detected in the 2012 study, and not in the current one.

The frequency of EHV-1 detection in the current study was generally lower than what was reported overseas. This could be due to variations between sampled populations sampled or differences in the methods used including the amount and source of the starting material. Between 17.7% (26/147) (Pusterla et al., 2010b) and 25.7% (18/70) (Pusterla et al., 2012b) of horses examined following euthanasia at the University of California in the USA were positive for latent EHV-1 using PCR-based detection without prior magnetic bead based enrichment. Horses sampled in the former study consisted of racing Thoroughbreds that sustained catastrophic injuries while racing, and as such were considerably different to the population of horses sampled in the current study. Horses sampled in the later study comprised a variety of horses submitted for necropsy over a period of 14 months. Hence, these horses represented a population comparable to the population sampled in the current study, but with a median age of 13 years, as opposed to 6 years, which may have influenced the results. Half of the horses (20/40, 50%) tested in a UK-based study were found to be latently infected with EHV-1 based on analysis of a variety of tissues by co-cultivation and PCR (Edington et al., 1994) and a similar proportion (71/132, 54%) of latent infections

was detected among horses submitted for necropsy to the Kentucky University in the USA (Allen et al., 2008). The authors of the latter study used the same protocol as in the current study, but with a ten-fold higher quantity of DNA (2.4 mg) extracted from SLN as a starting material. It was shown by the same group in a separate study (Allen, 2006) that even with 2.4 mg of DNA not all PCR replicates from the same sample were positive for EHV-1. This suggested that SLN of some horses harboured EHV-1 DNA at a concentration that was close to the detection threshold of the assay. As the latter is limited only by the amount of the starting material used, it is likely that the percentage of horses positive for EHV-1 DNA in the current study would have been higher than 9.5% if we had used a larger amount of DNA in the assay. In support of this conclusion, approximately 70% of adult (<2 years old) Thoroughbred horses in one study (Donald, 1998), and 42/66 (66.7%) of horses in another New Zealand based study (Dunowska et al., 2002a) were serologically positive for EHV-1, suggesting that EHV-1 infection is common among New Zealand horses.

The EHV-1 sequences detected, most likely represented latent viruses based on the sampling sites (Allen, 2006). Equine herpesvirus 1 establishes latency in trigeminal ganglia, lymphoid tissues draining the respiratory tract and CD5+ and CD8+ lymphocytes (Baxi et al., 1995; Edington et al., 1985; Smith et al., 1998; Welch et al., 1992). During latency, there is a limited transcription and expression of viral genes. Unfortunately, detection of latent EHV-1 infection is challenging, especially in live animals due to inaccessibility of sites for testing with low levels of viral DNA present (Balasuriya et al., 2015; Edington et al., 1994). Detection of EHV-1 DNA in biopsy samples of submandibular lymph nodes (SLN) has been proposed as one possibility to overcome this problem (Allen, 2006; Allen et al., 2008; Dunowska et al., 2015). Assays such as *in-situ* hybridisation using a digoxigenin-labelled EHV-1 BamHI E fragment probe; and PCR and southern hybridisation to detect the presence of expressed LATs (IE 63 or IE 64) in affected cells have been used to demonstrate EHV-1 latency (Baxi et al., 1995; Chesters et al., 1997). However, a negative result does preclude absence of latent EHV-1, as the virus could have been present in the sample a very low copy number, below the detection limits of the method used. The latency can be confirmed in tissues positive for EHV-1 DNA if the same tissues are negative from RNA for late structural protein genes (e.g. gB gene) using reverse transcription (RT) qPCR (Allen et al., 2008; Pusterla et al., 2010b, 2012b). This was not done in the current study. As such, it is not possible to definitively confirm the status of EHV-1 infection among sampled horses.

Our data suggest that the presence, or levels, of EHV-1 DNA differ between different tissues in latently infected horses. In agreement with this conclusion, Chester et al (1997) detected latent EHV-1 in bronchial lymph nodes from 4/5 ponies by co-cultivation and from all five ponies by PCR/RT-PCR, but in none of the trigeminal ganglia from the same ponies. The lymph nodes draining the respiratory tract were also the only sample type from which EHV-1 was recovered by co-cultivation and PCR in another study (Edington et al., 1994), although EHV-1 was also detected in trigeminal ganglia of some horses by PCR only. Other investigators reported detection of latent EHV-1 more often in trigeminal ganglia than in

lymphoid tissues (Pusterla et al., 2010b, 2012b). Retropharyngeal lymph nodes were tested in the current study in order to provide data comparable to that from the 2012 study (Dunowska et al., 2015) where RLN and trigeminal ganglia were used. In that study, EHV-1 DNA was detected in RLN only of 16 horses and in trigeminal ganglia only of one additional horse. Testing of SLN was included as these, unlike RLN, are accessible for sampling in a live horse and have therefore been used by others (Allen, 2006; Allen et al., 2008). The protocol used in the current study involved concentration of viral DNA and multiple rounds of amplification. Negative controls were introduced at each step of the protocol (enrichment, primary PCR, nested PCR). All remained negative, which indicates that discrepant results for testing RLN and SLN samples from three horses included in the study were real and unlikely to be a result of cross-contamination between samples. Altogether, our data from this and from the previous study (Dunowska et al., 2015) suggest that RLN are the preferred sample choice for detection of latent EHV-1.

The factors that determine at which site EHV-1 establishes latency following primary infection are currently unknown. Interestingly, the only sample positive for D752 genotype in the 2012 study was a trigeminal ganglion of one horse. Latent EHV-1 with D752 genotype was also preferentially detected in trigeminal ganglia in other studies (Pusterla et al., 2010b, 2012b), although in both studies the D752 viruses were also detected, albeit at a lower frequency, from lymph node samples. In each of the above two studies only one horses was positive for D752 genotype in both lymph node and trigeminal ganglia samples. The remaining horses were positive for EHV-1 D752 sequences either in trigeminal ganglia only (11 and 6 horses, respectively) or in the lymph nodes only (4 and 5 horses, respectively). More research is needed to understand whether the genotype of the virus influences the site of latency, the levels of viral DNA within the tissues, and the likelihood of recrudescence. Based on the limited data available, one cannot exclude the possibility that viruses with D752 genotype prefer to establish latency in trigeminal ganglia, and thus our sampling strategy may have not been optimal for detection of those viruses. None-the-less, other authors detected viruses with D752 genotype within the lymphoid tissues (Allen et al., 2008; Pusterla et al., 2010b, 2012b), so lack of detection of EHV-1 sequences with D752 genotype in the current study suggest that infection with this genotype remains rare in New Zealand. This finding agrees with the overseas data where ORF30 N752 genotype was detected more commonly than D752 genotype, despite occasional reports of EHM cases with D752 involvement (Perkins et al., 2009; Pusterla et al., 2012b).

Alternatively, the sites of latency and the levels of EHV-1 DNA may be related to the horse's age. In the population sampled, horses that were positive for EHV-1 in both RLN and SLN tended to be younger than horses that were positive in the RLN only, although the numbers were too small to draw any solid conclusions. The fact that horses with EHV-1 positive SLN were generally younger than horses with EHV-1 positive trigeminal ganglia in another study (Pusterla et al., 2010b) suggest that some age-related predilection for the site of latency may exist, a notion that warrants further investigation.

In conclusion, this study demonstrated that EHV-1 continues to circulate among horses in New Zealand. The RLN appear to be the sample of choice for detection of EHV-1 DNA in the sampled horses. If live animals are tested using SLN biopsy it should be kept in mind that negative results do not fully rule-out the presence of latent EHV-1 at other sites inaccessible for testing. The detected frequency of latent EHV-1 infection (9.5%) may be an under-estimation, as some of the latently infected horses may have harboured EHV-1 DNA at the levels beyond the sensitivity limit of the assay used. Importantly, none of the EHV-1 positive horses harboured EHV-1 with ORF30 D752 genotype. Together with detection of only one horse positive for EHV-1 D752 genotype in the previous study (Dunowska et al., 2015), these findings suggest that infection with D752 genotype is not common among New Zealand horses. The importance of ORF30 N/D752 genotype should not, however, be over-interpreted, as the markers for neurovirulence are most likely more complex than this single amino acid substitution, and viruses with either genotype have been recovered from EHM cases in the past (Cuxson et al., 2014; Kydd et al., 2012; Pronost et al., 2010).

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6 Appendix

6.1 *Appendix 1. Data sheet template used to record the details of the sampled horse.*

EHV-1 surveillance project 2015
Sample submission form

Location of animal at the time of euthanasia:
.....

Date: ……

Animal Details

Animal ID:.....(format EHV1_2015/001)
Name:.....
Breed:..... **Age: ..... Brand:.....**

Sex: stallion ☐ gelding ☐ mare ☐

EHV-1 vaccination status within the past 6 months:
Not vaccinated ☐ Vaccinated ☐ Unknown ☐
If vaccinated:
Date(s) vaccinated: .....
Brand of vaccine: .....

How familiar are you with this horse's history?
Entire life ☐
Since *(date)***.....**

Was the horse been born in New Zealand? Yes ☐ **No** ☐

Has the horse travelled internationally? Yes ☐ **No** ☐
If so, what countries……

What other horses was this horse in contact with?

Sport horses at shows:	**Yes** ☐	**No** ☐
Racehorses in training:	**Yes** ☐	**No** ☐
Broodmares at stud:	**Yes** ☐	**No** ☐
Yearlings at stud:	**Yes** ☐	**No** ☐
Other horses:	**Yes** ☐	**No** ☐

Work/training/use history:
Please provide as many details as available (use to back side of this form):

6.2 *Appendix 2. Horses included in the study population.*

Horse ID	Breed	Age	Sex	Born In NZ	Origin of horses	International travel	Vaccinated	Use
1	TB	4	F	Y	unknown	N	N	TB racing
2	TB	5	MC	Y	Rangitikei	N	N	TB racing
3	TB	8	F	Y	Turakina	N	Y	TB racing
4	TB	15	F	Y	unknown	N	Y	TB racing Home stud (Broodmare)
6	TB	5	unknown	Y	Marton	N	N	TB racing
7	TB	7	unknown	Y	Rangitikei	N	N	TB racing
8	TB	16	F	Y	unknown	N	N	TB racing
9	TB	10	F	Y	Bulls	N	N	TB racing Sport horse
10	TB	4	F	Y	Bulls	N	N	TB prep
11	NZ sport	>20	F	Y	Marton	N	N	Hunting
13	TB	18	F	Y	Woodville	N	N	TB racing Broodmare
14	NZ sport	30	MC	Y	unknown	N	N	Showjumping Hunting
15	Pony	6	MC	Y	unknown	N	unknown	Showjumping Hunting
16	TB	8	F	unknown	Taumaranui	unknown	unknown	TB prep Polo
17	TB	7	MC	unknown	Taumaranui	unknown	unknown	Polo
18	TB	5	F	Y	Taumaranui	unknown	unknown	Polo
19	TB	6	F	Y	unknown	N	unknown	TB prep Polo
20	TB	unknown	F	Y	unknown	N	Y	TB racing Broodmare
21	TB	7	MC	Y	unknown	N	unknown	TB racing
22	Arab	10	MC	Y	Marton	N	unknown	endurance
23	TB	5	F	Y	Maxwell	N	unknown	TB racing Polo
24	Farm hack	4	MC	Y	unknown	N	unknown	Farm hack
25	Farm hack	20	F	Y	unknown	N	unknown	Farm hack

Horse ID	Breed	Age	Sex	Born In NZ	Origin of horses	International travel	Vaccinated	Use
26	TB	4	MC	Y	Marton	N	N	TB racing
27	TB	5	MC	Y	Marton	N	N	TB racing
28	TB	2	F	Y	Marton	N	N	TB racing
29	TB	6	F	Y	Whangaehu	N	N	TB racing
30	TB	6	MC	Y	Feilding	N	N	TB racing
31	TB	2	M	Y	unknown	N	N	Stud farm
32	Farm hack	25	F	Y	unknown	N	unknown	Farm hack
33	TB	3	F	Y	unknown	N	N	Dairy farm
34	TB	4	MC	Y	Marton	N	N	TB prep
35	TB	20	F	Y	Marton	N	N	Broodmare
36	TB	3	F	Y	unknown	N	N	TB prep
37	TB	8	MC	N (Australia)	Marton	Y	N	TB racing (Hong Kong)
38	Pony	25	F	Y	Turakina	N	N	Farm hack Hunting
39	SB	unknown	MC	Y	unkown	N	unknown	unknown
41	Farm hack	25	F	Y	Mangaweka	N	unknown	Farm hack
42	TB	6	F	Y	Whangaehu	N	N	TB racing
43	TB	12	F	Y	unknown	N	N	TB racing Broodmare
44	TB	6	MC	Y	Bulls	N	N	TB racing
45	TB	2	MC	Y	Hunterville	N	N	TB and SB racing Home Farm
46	TB	20	MC	Y	unknown	N	N	Dressage
47	TB	2	MC	Y	unknown	N	N	TB prep (racing)
48	TB	3	unknown	Y	unknown	N	N	TB racing
49	TB	3	F	Y	unknown	N	N	TB racing
50	TB	aged	F	Y	Whangaehu	N	N	TB racing broodmare (dairy farm)
51	Pony	30	unknown	Y	Marton	N	N	Farm hack
52	NZ sport	26	MC	Y	Rata	N	unknown	hunting
53	TB	5	MC	Y	Whangaehu	N	N	TB racing
54	TB	5	F	Y	Marton	N	N	TB racing
55	TB	7	MC	Y	Marton	N	N	TB racing
56	sports	14	MC	Y	unknown	N	N	Hunting
57	TB	5	MC	Y	Woodville	N	N	TB racing

Horse ID	Breed	Age	Sex	Born In NZ	Origin of horses	International travel	Vaccinated	Use
58	TB	9	MC	Y	Woodville	N	N	TB racing
59	TB	6	F	Y	Marton	N	N	TB racing
60	Pony	unknown	unknown	unknown	Maxwell	unknown	unknown	unknown
61	Farm hack	>20	MC	Y	Mangaweka	N	unknown	Farm hack
62	TB	11	F	Y	Marton	N	N	TB racing, farm stud
63	TB	6	MC	Y	Bulls	N	N	TB prep stables
64	TB	< 5	unknown	Y	Bulls	N	N	TB racing stables
65	TB	aged	F	Y	Whangaehu	N	N	TB racing broodmare dairy farm)
66	unknown	unknown	unknown	unknown	unknown	unknown	unknown	unknown

Abbreviations: Thoroughbred (TB), male (M), female (F), male castrated (MC), Yes (Y), No (N). TB racing indicates horses that raced. TB prep denotes horses that were in race training/preparation but did not race.