Equine respiratory viruses in New Zealand

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Abstract

Equine respiratory disease has been recognised as an important cause of wastage resulting in financial loss for the equine industry worldwide. Limited studies have been conducted on equine respiratory viruses in New Zealand, particularly within the past ten years. As such, the objective of the present study was to determine 1) which respiratory viruses circulate among horses from selected New Zealand locations and 2) whether or not infection with any of the viruses identified was associated with clinical disease. A survey was conducted on 85 horses to detect the presence of viruses known to be associated with equine respiratory disease. Nasal swabs were taken from 52 horses with signs of respiratory disease and from 33 healthy horses. Horses were sampled from within the Manawatu and Hawkes Bay regions by convenience.

Species specific PCR was performed directly on nasal swabs. The only viruses detected were equine herpesviruses (EHV) types 1, 2, 4 and 5. Of the 52 horses with respiratory disease, 3 tested positive for EHV-1, 14 for EHV-4, 23 for EHV-2 and 26 tested positive for EHV-5. Of the 33 healthy horses 2 tested positive for EHV-2, one of which also tested positive for EHV-5. Over all, the detection of herpesviruses was significantly associated with respiratory disease (p value <0.0001). Detection of individual virus species (EHV-2, EHV-4 or EHV-5) was also significantly associated with respiratory disease (p value 0.0002, 0.0006, <0.0001, respectively). The sample size was not large enough to evaluate the significance of EHV-1 detection and respiratory disease.

Virus isolation performed on the samples from the 52 horses with respiratory disease detected EHV types 1, 2, 4 and 5. No viruses were detected from the 33 samples of healthy horses. There was poor correlation between virus isolation and PCR results, particularly with regard to EHV-4.

This work gives a recent contribution to the knowledge of equine respiratory viruses in New Zealand. Although the sampling was performed by convenience, the results suggest an association between equine herpesviruses types 2, 4 and 5 and equine respiratory disease.

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List of publications

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Abbreviations

7TMR	Seven trans-membrane receptor
Ab	Antibody
bp	Base pair
CF	Complement fixation
CFU	Colony forming units
CPE	Cytopathic effect
CTL	Cytotoxic T-lymphocyte
DIG	Digoxigenin
DMEM	Dulbecco's modified eagle medium
DNTPs	Deoxynucleoside-5' –triphosphate
EAdV	Equine Adenovirus
EAV	Equine arteritis virus
EDTA	Ethylenediamine tetra-acetic acid
EFK	Equine fetal kidney (cells)
EHV	Equine herpesvirus
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscope
ERAV	Equine rhinitis A-virus
ERBV	Equine rhinitis B-virus
EtBr	Ethidium bromide
FBS	Fetal bovine serum
FMDV	Foot and mouth disease virus

gB	Glycoprotein B
GM	Growth medium
IAD	Inflammatory airway disease
Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
LRT	Lower respiratory tract
MHC	Major histocompatibility complex
MM	Maintenance media
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RK-13	Rabbit kidney -13 (cells)
RT-PCR	Reverse transcriptase -PCR
SDS	Sodium dodecyl sulphate
VERO	African green monkey (cells)
VN	Virus neutralization

Chapter 1 Literature review

This review outlines the current literature on equine respiratory disease, specifically; the risk factors for disease development, the means of disease detection and the limitations to equine respiratory disease research. The focus is on equine respiratory viruses, including; equine herpes viruses, picornaviruses and adenoviruses. The classification, properties, pathogenesis, immune responses, isolation and detection and epidemiology is outlined for each virus species. In addition, Equine arteritis virus and influenza virus, bacteria and mycoplasmas are also reviewed briefly.

1.1 Equine respiratory disease

Equine respiratory disease has become an area of intense research interest, due to the impact that even minor inflammation of the respiratory tract can have on the success of high performance horses (Viel, 2009). A study at Thistledown racetrack showed that increased tracheal mucus significantly reduced racing performance, therefore, likely had an effect on lung function (Holcombe and Ducharme, 2004). Horses are known to be obligate nasal airway breathers, so any change to the upper airway will result in an immediate decrease in exercise capacity (Sellon and Long, 2007). As such, respiratory disease is recognised as an important cause of "wastage" in the equine industry worldwide (More, 1999, Bailey, 1998, Perkins, 2005). The term "wastage" refers to both time and financial losses due to injury or disease which results in lost training days, prolonged spelling or prevention of racing and early retirement. Equine respiratory disease was found to be the second most important cause of wastage in a study on New Zealand Thoroughbreds in training (Perkins et al., 2004). In this study respiratory disease was the cause of 15.6% of 1058 unplanned interruptions in training or racing**.** This was similar to a study in Australia where equine respiratory disease was also the second most important cause of wastage, with 15.8% of days lost due to respiratory illness (Bailey, 1998). However, the Michigan Equine Monitoring System ranked respiratory problems as having the third greatest impact on the average days lost for performance (Kaneene et al., 1997). Regardless of ranking, respiratory disease remains a problem for the equine industry, as horses require rest of up to 4 weeks for the full resolution of

clinical and subclinical signs of disease; exercise prior to full resolution can potentially result in recurrent episodes of respiratory disease (McKenzie, 2010).

Equine respiratory disease is often poorly characterised in clinical practice as diseased horses can display a variety of clinical signs with vast differences in severity (Christley et al., 2001b). This has led to variations in the definition of respiratory disease between studies. Many regarded horses as affected by respiratory disease if they showed one or more of the following clinical signs: coughing, nasal or ocular discharge, varying degrees of in-appetence, fever, enlargement of submandibular/ retropharyngeal lymph nodes or poor performance as subjectively assessed by their trainers or owner (Mumford et al., 1998, Mumford and Rossdale, 1980, Dunowska et al., 2002b, Ruszczyk et al., 2004, Pusterla et al., 2011, Bell et al., 2006a, Carman et al., 1997, Diaz-Mendez et al., 2010). Others only considered specific clinical signs such as coughing (Christley et al., 2001b), nasal discharge and fever (Dynon et al., 2007) or increased amounts of tracheal fluid (Robinson et al., 2006). One investigation into respiratory disease among Thoroughbred horses in training found that horses spent 33% of their time with likely impaired performance due to subclinical disease (Burrell et al., 1996). Therefore studies that rely on the observation of overt clinical signs to identify diseased individuals may have underestimated the prevalence of disease in their study population.

Some authors have reported a seasonal pattern associated with the development of equine respiratory disease. Studies in the UK found the highest rate of equine respiratory disease to occur in months February- April and the lowest rate in August-October (Wood et al., 2005b, Burrell et al., 1996). Similarly, in a study in Brazil it was found that the occurrence of respiratory disease was higher in summer months (Ribas et al., 2009). This seasonal pattern may be influenced by; weather changes affecting the activity of infectious agents, levels of dust and pollen in the air, seasonal training and racing patterns or time of foaling (Wood et al., 2005b, Cohen, 1994). In contrast, other studies found no seasonal pattern associated with respiratory disease (Robinson et al., 2006, Christley et al., 2001b). In one of these studies the prevalence of subclinical respiratory disease was found to be just over 20% in both summer and winter (Robinson et al., 2006). However, there was a relationship between increased occurrence of respiratory disease and horses being housed outdoors in winter, while in summer housing outdoors appeared to be protective. Interestingly, persistent subclinical disease

was observed in older horses, 62.5% of horses over 20 years of age between summer and autumn. This contrasts with findings by Wood et al., (2005b) and Newton et al., (2003) who found that the risk of developing disease in horses decreased after 2 years of age and this risk continued to decrease with increasing age. They postulated that this observation was likely explained by immunity increasing with age through increased exposure to a wide range of microorganisms. In another study age was significantly associated with disease categories based on clinical signs (Pusterla et al., 2011). Horses less than one year of age mainly displayed fever and nasal discharge, while horses more than one year of age either displayed a combination of fever and nasal discharge, nasal discharge alone or fever alone.

To date very few studies have considered the effect of gender on the risks for development of respiratory disease in horses. In an Australian study of risk factors for coughing, gender was not found to be significantly associated with disease after controlling for other factors, although at a univariable level colts and females were at apparently increased risk compared to geldings (Christley et al., 1999). In the study by Newton et al., (2003), male horses, either geldings or colts, appeared to have a decreased risk of clinically apparent respiratory disease compared to females, although gender was not found to be a significant risk factor in the final models.

1.2 Pathogens associated with equine respiratory disease

1.2.1 Equine herpes viruses

Order: ***Herpesvirales***

Family: ***Herpesviridae***

-Subfamily *Alphaherpesvirinae*
Genus *Varicellovirus*
Species - Equid herpesvirus 1
-Equid herpesvirus 4

-Subfamily *Gammaherpesvirinae*
Genus *Percavirus*
Species - Equid herpesvirus 2
- Equid herpesvirus 5

(Davison et al., 2009)

Classification

Herpesviruses belong to the order *Herpesvirales* and have been divided up into three families; *Alloherpesviridae, Herpesviridae* and *Malacoherpesviridae (Davison et al., 2009).* Viruses associated with equine respiratory disease belong to two subfamilies within the family *Herpesviridae: Alphaherpesvirinae* and *Gammaherpesvirinae.* Member of the two subfamilies differ in their tissue tropism, pathogenicity and behaviour in cell culture; *(Paillot et al., 2008).* Equid herpesvirus 1 (EHV-1) and Equid herpesvirus 4 (EHV-4) are members of *Alphaherpesvirinae.* Alphaherpesviruses are characterised by a variable host range, short reproductive cycle, rapid spread in cell culture, efficient destruction of infected cells and the establishment of latent infection in sensory ganglia (Roizman, 1996). Equid herpesvirus 2 (EHV-2) and Equid herpesvirus 5 (EHV-5) are members of *Gammaherpesvirinae.* In contrast to members of *Alphaherpesvirinae,* Gamma-herpesviruses usually have a limited host range and latency is established in lymphoid tissue, usually either T or B lymphocytes (Roizman, 1996).

Properties

Herpesviruses are large spherical to pleomorphic viruses measuring 120-200 nm in diameter (Granzow et al., 2001). They consist of an icosahedral capsid comprised of 162 capsomers, a structured tegument and an envelope with morphologically indistinct glycoprotein spikes. The double stranded linear DNA genome is between 130-230 kb in length, composed of a unique long region and a unique short region, flanked by two inverted repeat sequences. The herpesvirus genome contains between 70-100 genes, including genes coding for structural proteins, enzymes and proteins for virus replication and release (Field et al., 2006, Elia et al., 2006, Paillot et al., 2008, Roizman, 1996).

Within the herpesvirus genome there are many genes which encode products that interact with host's innate, adaptive and inflammatory immune responses (Field et al., 2006). These interactions interfere with processes such as antibody dependent cell lysis, cytotoxic T lymphocyte cell lysis, natural killer cell mediated lysis and the host cytokine network. As such, the different members of the family *Herpesviridae* are able to elude the host immune system using different evasion strategies (Van der Meulen et al., 2006). An important aspect of immune evasion shared by all herpesviruses is the two distinct phases of infection, lytic and latent infection (Vider-shalit et al., 2007). Lytic or productive infection is characterised by extensive gene expression resulting in virus production and release through host cell lysis. In contrast, latent or non-productive infection is characterised by the expression of very few genes, and the herpesvirus genome is maintained as a circular episome in the host cell. The molecular mechanisms and sites of latency differ between herpesviruses. However, for all herpesviruses, latency allows the establishment of a lifelong infection in which the virus periodically undergoes phase switch between latent and lytic cycles as induced by physiological conditions (Knipe et al., 2001, Vider-shalit et al., 2007, Lannello et al., 2006). The diversity of immune evasion strategies of herpesviruses in combination with the shared ability to establish latency poses a significant problem for prevention, diagnosis and treatment of herpesvirus infections and diseases (Field et al., 2006).

Equid herpesvirus 1 and 4

Equid herpesviruses 1 and 4 initially replicate in respiratory epithelial cells, but EHV-1 infection may spread by cell associated viremia to placental, fetal and central nervous system tissues (Harless and Pusterla, 2006, Patel and Heldens, 2005). As such, EHV-1 has been found to cause respiratory disease, abortion (sporadic abortion and abortion storms), neonatal foal death and neurological disease, while EHV-4 has been mainly associated with respiratory disease (Nugent et al., 2006, Leon et al., 2008). Equid herpesviruses 1 and 4 are contracted by droplet inhalation or through exposure to environmental contamination by respiratory secretions, as well as direct contact with fomites such as placental tissues and aborted fetuses (Paillot et al., 2005, Harless and Pusterla, 2006). Respiratory infection by EHV-1 and EHV-4 begins with an incubation period of 3-10 days before the onset of any clinical signs of disease (Harless and Pusterla, 2006, Patel et al., 2003), followed by shedding of large quantities of virus particles (Six et al., 2001).The severity of disease caused by infection with EHV-1 and 4 is influenced by a range of factors including age, physical condition and the immune status of the horse, and whether the infection is primary, reinfection or reactivation of a latent virus. In addition, the pathogenic potential of the infecting virus can change the severity of disease (Nugent et al., 2006, Paillot et al., 2007).

Pathogenesis

Equid herpesvirus 1 and EHV-4 have been demonstrated to cause respiratory tract disease, particularly in young horses and foals (Allen and Bryans, 1986, Wood et al., 2005b, Burrell et al., 1996). Several studies (Powell et al., 1978, Thomson, 1978, Burrell et al., 1996) in which respiratory disease was defined as the presence of nasal discharge, found an association between EHV-1 infection and respiratory tract disease. Another study, where signs of disease were defined as acute or mucopurulent nasal discharge or rectal temperature exceeding 38·8°C, found that respiratory disease was significantly associated with sero-conversion to EHV-1 ($P<0·01$) or EHV-4 ($P<0·01$). In contrast, an Australian case control study, which investigated coughing, failed to demonstrate an association between EHV-1 or EHV-4

and disease (Christley et al., 1999, Christley et al., 2001b). This is in agreement with a more recent study, which failed to demonstrate a significant association between clinically apparent respiratory disease in young racehorses and infection with EHV-1 or EHV-4. In this study, disease was indicated by sudden onset of coughing (during exercise or at rest), serous or mucopurulent nasal discharge or rectal temperature ≥ 38.6 ◦C (Newton et al., 2003). Despite being a specific but insensitive measure of lower respiratory tract disease (specificity 84%, sensitivity 38%) (Burrell et al., 1996) coughing may not be an appropriate disease definition when looking at EHV-1 and EHV-4 infection. A recent study in the USA investigated the overt clinical signs of respiratory disease among horses with upper respiratory tract disease or acute neurological disease and found a lack of coughing to be strongly associated with EHV-1 and EHV-4 infections (P=0.001) (Pusterla et al., 2011). In contrast to findings by Newton et al., (2003) and Crabb and studdert (1996), Pusterla et al., (2011)also found a significantly higher prevalence of EHV-1 in older horses (16 to 20 years old) compared to younger horses. Although, EHV-4 was significantly associated with disease in horses aged less than one year old. In addition the presence of nasal discharge was significantly associated with EHV-1 infection (P=0.0001).

Equid herpesvirus 1 is the most common cause of infectious abortion in horses worldwide (Leon et al., 2008). Infection of pregnant mares can induce late-gestation abortion, stillbirth, or weak neonatal foals. Infected foals which are born alive show severe respiratory disease and subsequent respiratory failure usually occurs within a few days due to secondary bacterial infections (Paillot et al., 2008). In one Turkish study, 36 neonatal foals were found to be positive for EHV-1 DNA, 5 of which died within 48 hours of birth (Tekelioglu et al., 2006). Equid herpesvirus 1 reaches the reproductive tract through cell-associated viraemia following primary infection or reactivation of the latent virus, and infects endometrial endothelial cells. This leads to a premature separation of the placenta from the endometrium and finally death of the fetus. If EHV-1 is transferred to the fetus, extensive multi-organ infection and a wide range of lesions can be observed. Mares can abort months or years after primary EHV-1 infection due to the reactivation of the latent virus. However abortion

of consecutive pregnancies due to EHV-1 infection is rare (Crabb and Studdert, 1996, Smith and Borchers, 2001, Slater et al., 2006).

Infection with different strains of EHV-1 can have a major impact on the outcome of disease. Infection with neuropathic EHV-1 can cause a variety of clinical signs, from mild to severe including subclinical infection, mild hind limb ataxia, and urinary incompetence to blindness and quadriplegia as reported by Studdert et al., (2003). In the USA there were 25 laboratory-confirmed outbreaks of EHV-1 paralytic disease reported to state agencies between the years 2000-2006. Of the 452 horses, exposed to neuropathogenic EHV-1, 26% developed clinical signs of disease and 29% of these required euthanasia (Allen et al., 2008). Neurological disease is often observed after respiratory disease and clinical signs usually appear 1 week after infection with subsequent gradual recovery or death (Crabb and Studdert, 1996). Studies suggest that EHV-1 neurological disease is associated with a single nucleotide polymorphism in the EHV-1 DNA polymerase gene, encoded by open reading frame 30 (ORF30) (Nugent et al., 2006, Goodman et al., 2007, Perkins et al., 2009). Based on these findings, EHV-1 strains which possess guanine (G2254) instead of adenine (A2254) at this site, thereby replacing amino acid asparagine with an aspartic acid, are considered to have neuropathogenic potential. After the statistical analysis of ORF30 from 176 EHV-1 isolates, the odds of neurological disease among horses infected with mutant genotype EHV-1 was demonstrated to be 162 times greater than that of horses infected with the wild type EHV-1 (Perkins et al., 2009). Equid herpesvirus 1 strains with the mutant genotype have been shown to replicate more efficiently *in vivo* and produce significantly higher viral loads in leucocytes than the wild type (Allen and Breathnach, 2006). It is believed that this increased replicative capacity enhances the ability of the virus to infect capillary endothelial cells, leading to an interference with the blood supply to the central nervous system and the development of neurological signs (Allen et al., 2008, Allen and Breathnach, 2006). However the idea that a single genetic substitution is solely responsible for the development of neurological disease is overly simplistic and the laboratory diagnosis of a virulence phenotype based on a single substitution should be interpreted with caution. The evidence supporting the association between this substitution and EHV-

1 neurological disease is derived from analysis of a relatively small region of ORF30 (10% of the DNA polymerase gene) (Nugent et al., 2006). In one survey (Perkins et al., 2009), 24% of horses with neurological disease possessed the wild type strain of EHV-1 (ORF 30) suggesting that there may be many other substitutions with the capability of enhancing viral replication rates *in-vivo*. Also host and environmental factors have been shown to have a significant impact on clinical outcome following exposure to EHV-1 (Goehring et al., 2006, Goehring et al., 2009). Allen et al., (2008) found that older horses (>20 years), were 8 times more likely to develop neurological disease when experimentally exposed to the highly neuropathogenic T953 (G2254) strain of EHV-1 compared to younger horses (<15 years), when exposed under identical conditions. In a study in Australia 5/10 lactating mares with foals at foot developed clinical signs of neurological disease and it was postulated that having a foal at foot was a risk factor for the development of neurological disease (Studdert et al., 2003). While these studies suggest that the development of neurological disease may be influenced by a variety of virus, host and environmental factors, this area of research has not been fully investigated.

Immune responses and interactions

Equid herpesvirus 1 has been found to cause a more a severe disease than EHV-4 in experimentally infected horses (Patel et al., 2004), although disease was less severe overall than what is observed in the field. With regards to experimental infection, some authors (Hannant et al., 1993, Heldens et al., 2001a) have observed viraemia after intranasal EHV-1 challenge, despite the presence of circulating virus neutralizing antibodies, while other studies (Patel et al., 2004, Dolby et al., 1995) did not record viraemia. In the above studies a significant reduction in virus shed in nasal mucus was observed between samplings, possibly due to locally produced virus neutralizing antibodies. In a recent study (Breathnach et al., 2001) observed that intranasally administered live EHV-1 vaccine did not elicit mucosal antibody production whereas a field isolate, administered in the same way, resulted in virus specific local IgA response with EHV-1 neutralising activity. This reveals a gap in the literature on the mechanisms of protective immunity in EHV-1 infection.
Infection with EHV-1 initiates various responses of the immune system including;

antibody, cytokine and cellular immune responses. These immune responses to infection with EHV-1 are described in detail by Kydd et al., (2006). Briefly, assays developed to measure serum antibodies with virus neutralizing (VN) and complement fixing (CF) activity have demonstrated that the level of both VN and CF antibodies start to rise approximately 2 weeks after field or experimental infection with EHV-1 (Breathnach et al., 2001). Virus neutralizing antibodies persist for up to 1 year after infection (Allen et al., 1992). Breathnach et al., (2001) also found that primarily IgA virus specific antibodies, which elicit virus neutralizing activity, were secreted following experimental intranasal infection with strain A183. In contrast, CF antibodies are short-lived, usually becoming undetectable by 3 months after infection (Thomson et al., 1976). A limited number of reports are available on cytokine responses that follow EHV-1 infection. Pedersen et al., (2002) used a monoclonal antibody which cross reacts with equine interferon gamma (IFN g) to demonstrate that synthesis of the type II IFNɣ was increased in both CD4+ and CD8+ peripheral blood T lymphocytes collected at 10 days post-infection (Breathnach et al., 2005). In addition, this paper also discussed that in other species, IFNɣ has been associated with the evolution of a Th1 biased immune response leading to the development of cytotoxic T-lymphocyte (CTL) response. Proliferation of lymphocytes is a prerequisite for the development of immune responses and so has been used frequently as an indicator of immune status (Crabb and Studdert, 1996).

Equid herpesvirus 1 has developed an array of evasion strategies in order to interfere with these mechanisms allowing recurrent disease throughout a horses life time (Van der Meulen et al., 2006). *In-vitro* studies have shown EHV-1 to interfere with the expression of viral proteins on the surface of a majority of peripheral blood mononuclear cells (PBMC) during lytic infection, despite extensive intracellular expression of virus proteins. This renders virally infected cells insensitive to Ab-dependent complement-mediated cell lysis and Ab-dependent cell-mediated cytotoxicity (Van der Meulen et al., 2003, Van der Meulen et al., 2006). Evasion of the complement cascade also requires interference of Ab-independent complement pathway. Equid herpesvirus 1 produces the envelope protein gC which has been shown to interfere with the alternative complement pathway by binding the third

factor of complement C3 (Huemer et al., 1995). Also EHV-1 has been shown to down regulate MHC class 1 expression in virus infected cells, interfering with the CTL mediated cell lysis (Ambagala et al., 2004, Rappocciolo et al., 2003, Ambagala et al., 2005). Down regulation, instead of complete elimination, of MHC class 1 expression is thought to prevent recognition of virus infected cells by natural killer cells (Orange et al., 2002). Another method of EHV-1 evasion of the host immune response is the interference with the host cytokine network. The production of EHV-1 envelope protein gG is thought to result in inhibition of chemokine-mediated inflammatory response by binding chemokines, blocking their interaction with cell receptors (Bryant et al., 2003).

Isolation and Detection

Equid herpesvirus 1 can be cultivated on a range of different cells including; equine fetal kidney cells, lamb kidney and rabbit kidney cells. Equine herpesvirus 4 is typically isolated from only cells of equine origin (Sellon and Long, 2007). In a study conducted by Whalley et al., (2007), it was found that inoculating EHV-4 onto RK cells expressing glycoprotein D of EHV-1 (RKgD1) allowed infection to develop, observed as clusters of rounded cells. Furthermore this infectivity could be passaged in RKgD1 cells and the progeny virus was able to infect single RK cells, consistent with EHV-4 acquiring EHV-1 gD from the complementing cell line. This is consistent with gD homologues being major determinants of host cell tropism and raise the possibility that gD may be a factor in the differential pathogenicity of EHV-1 and EHV-4. Interestingly EHV-1 is able to infect cells through both endocytic and non-endocytic pathways and the mechanism of entry used is cell type dependent. Originally EHV-1 was thought to exclusively enter cells through plasma membrane fusion, as is the case with equine dermis and RK-13 cells (Van de Walle et al., 2008). However EHV-1 has now been shown to enter equine brain micro vascular endothelial cells through caveolar endocytosis (Hasebe et al., 2009). This entry pathway is particularly important in the infection of central nervous system cells and the development of neurological disease. While virus isolation remains the gold standard for EHV-1 and EHV-4 detection, variations of standard PCR techniques have become a fast reliable way for identification, detection and strain differentiation of

latent and lytic EHV-1 and 4 infections (Harless and Pusterla, 2006). Standard PCR techniques have been established to distinguish EHV-1 from EHV-4 in a one-step reaction (Lawrence et al., 1994, Kirisawa et al., 1993) and real-time PCR has been shown to detect as few as 10^0 copies of EHV-1 DNA in aborted fetuses (Elia et al., 2006, Perkins et al., 2008). Furthermore, real-time Taq Man PCR can be designed to target either viral DNA or viral mRNA. Latent infection can be distinguished from lytic infection by the absence of viral mRNA coding for structural proteins in cells that are positive for EHV-1/4 genomic DNA (Harless and Pusterla, 2006). Interestingly, in one study, 50% of horses were shown to harbour latent EHV-1 infection in the mandibular lymph node, identified via nested PCR after pre-amplification enrichment for target sequences. The viral DNA load was below the detection threshold for conventional template preparation PCR techniques. This result suggests that studies using conventional template preparation for detection of the latent EHV infections by PCR may have underestimated the true prevalence of such infections in the horse (Allen, 2006).

Epidemiology

In the field serological evidence suggests the reactivation of latent EHV-1 infection in mares is the source of infection for foals (Gilkerson et al., 1999a). In this study, foals became infected with EHV-1 from only 30 days of age by horizontal transmission of EHV-1 from their dams, with subsequent foal-to-foal spread. Similarly, in a Serological survey in New Zealand the prevalence of EHV-1 specific antibodies was found to increase with age, 29% in 6-12 month old horses and 48% in 13-24 month old horses (Donald, 1998).

Equid herpesviruses 1 and 4 are endemic in horse populations throughout the world. Equid herpesvirus 1 was first isolated in Kentucky in 1932 (Allen and Bryans, 1986). At that time the cross reactivity of EHV-1 and 4 did not allow for differentiation between the two viruses. Since then, EHV-1 and 4 infections have been reported in domestic horse populations worldwide including Europe, America, Japan, Australia and New Zealand (Pronost et al., 2010, Gryspeerdt et al., 2011, Gilkerson et al., 1999b, Dunowska et al., 2002b, Ataseven et al., 2010a, Yasunaga et al., 1998, Perkins et al., 2009). Equid herpesvirus 1 has also been reported to infect wild equids

including onager (*Equus hemionus onager*) and zebra species (*Equus grevyi* and *Equus burchelli*), as well as non-equine species including captive Thomson's gazelle (*Gazella thomsoni*), cattle, alpacas and llamas (Ibrahim et al., 2007, Ghanem et al., 2008, Kennedy et al., 1996, Chowdhury et al., 1988, Crandell et al., 1988, Rebhun et al., 1988). In Australia, between 1995 and 1997 two studies were conducted on large Thoroughbred stud farms in the Hunter Valley of New South Wales. The prevalence of EHV-1 antibody was determined to be 26.2% in mares and 11.4% in foals, while EHV-4 antibody was found in >99% of mares and foals (Gilkerson et al., 1999a, Gilkerson et al., 1999b). A serological survey of Thoroughbred horses in New Zealand in 1998 showed that approximately 70% of horses over the age of 2 years had EHV-1 specific antibodies (Donald, 1998). Similarly, a serological survey conducted in New Zealand in 2002 found 72% of horses and 100% of foals to be positive for EHV-1 and/or EHV-4 antibodies on at least one occasion. Also, there was evidence of recent infection with EHV-1 in 78% of the foals tested (Dunowska et al., 2002a, Dunowska et al., 2002b). A study conducted in Japan, found that at least a fourfold rise for EHV-1 and EHV-4 gG antibodies occurred in 37.5% of horses and 11.25% of horses respectively during winter months (Yasunaga et al., 1998). They concluded that EHV-4 was only a sporadic cause of respiratory disease while EHV-1 was responsible for epizootic respiratory disease among race horses in the winter season. Although both EHV-1 and EHV-4 cause respiratory disease and can establish viremia, EHV-4 has rarely been associated with abortion or paresis (Patel and Heldens, 2005). In contrast, EHV-1 is a known cause of abortion outbreaks (storms) and neurological disease. The incidence of abortion associated with EHV-1 infection is well documented in Kentucky. The highest incidence was recorded in 1963 as 17.3/1000 pregnant mares, but decreased to <2/1000 pregnant mares as of 2002, which is thought to be due to the implementation of vaccines (Paillot et al., 2008). However not all broodmares are vaccinated and abortion outbreaks do still occur in unvaccinated populations. In 2004, an outbreak of EHV-1 abortions occurred in unvaccinated Welsh ponies. Of 11 mares abortions, four were confirmed to be due to EHV-1 infection (Irwin et al., 2007). Over the past decade, there has been an unexpected increase in the incidence of equine herpesvirus neurological disease. This apparent recent increase in the incidence of EHV-1 neurologic disease correlates

with the higher prevalence of viruses with a G2254 genotype being isolated in diagnostic laboratories (Smith et al., 2010, Perkins et al., 2009, Pronost et al., 2010, Vissani et al., 2009). A comprehensive analysis of a large panel of archived EHV-1 isolates collected from sporadic cases of equine abortion between 1951 and 2006 in Kentucky using a real-time Taq-Man allelic discrimination PCR, revealed that viruses with the G2254 neuropathogenic genotype existed at least as far back as the 1950s (Smith et al., 2010). Furthermore, such isolates increased in prevalence from 3.3% in the 1960s to 14.4% in the 1990s, with indications of an even higher incidence from 2000 onwards.

Management, prevention and Control

There is no treatment for EHV-1 or EHV-4 induced respiratory disease, abortion or neurological disease other than supportive care, minimizing stress and providing adequate time for recovery (Harless and Pusterla, 2006). Effective control of outbreaks and minimising risk of virus introduction into naïve horse populations requires a combination of good management practices and preventative vaccination (van Maanen, 2002, Minke et al., 2006). Good management practices during outbreaks of EHV-1 and 4 include isolation and quarantine of virus shedding horses and proper cleaning and disinfection of stalls and shared facilities such as troughs and feed bins (Harless and Pusterla, 2006). Vaccines available commercially consist of a range of inert or killed and live virus vaccines and usually include both EHV-1 and EHV-4 (Heldens et al., 2001b). Inert vaccinations include inactivated whole EHV-1 and sub unit vaccines, immune-stimulating complex-based vaccines, DNA vaccines and non-infectious EHV-1 L-particle vaccines. Live vaccines include live attenuated vaccines and poxvirus-based vector vaccines (Minke et al., 2006, Paillot et al., 2008). The appropriate use of these vaccines and elicited immune responses are discussed in detail in Paillot et al., (2008).

Equid herpesvirus 2 and 5

Equid herpesviruses 2 and 5 are transmitted via nasal droplet inhalation and potentially ocular secretions through horizontal transmission (Torfason et al., 2008, Borchers et al., 2006). The exact role of EHV-2 and EHV-5 in equine respiratory disease is largely debated given that it can be recovered from both healthy horses and those with signs of respiratory disease (Agius et al., 1994, Reubel et al., 1995, Franchini et al., 1997, Borchers et al., 1999, Dunowska et al., 1999, Nordengrahn et al., 2002, Bell et al., 2006a, Wang et al., 2007, Torfason et al., 2008). One explanation is that virus may be shed from sub-clinically or latently infected horses, detectable even in the absence of clinical signs (Hanson, 1988). Herpesvirus infections are characterized by lifelong latency and persistence within the host (Knipe et al., 2001). Latent EHV-2 infection has been shown to occur in B lymphocytes and both clinical and subclinical disease is thought to often occur due to the reactivation of latently infecting virus (Drummer et al., 1996). Alternatively the inconsistency of clinical disease and pathology associated with EHV-2/5 infection may be attributable to the heterogeneity of EHV-2 and EHV-5 strains (Holloway et al., 2000, Bell et al., 2006a, Sharp et al., 2007, Dunowska et al., 2000). Despite the genomic variation between EHV-2 and EHV-5, they behave similarly in cell culture with common cytopathic properties, such that different species cannot be distinguished through the observation of infected cells alone. Equid herpesvirus 2 and EHV-5 can infect a wide range of cells when grown *in-vitro* including RK13, Vero continuous cell lines, as well as primary equine, rabbit and hamster cells. In contrast to the alphaherpesviruses, gammaherpesviruses grow slowly in cell culture and CPE is usually not detected until 12-21 days post inoculation, or 3-4 passages (Allen and Murray, 2004).

Pathogenesis

The signs of respiratory disease following infection with EHV-2 include subclinical infection, conjunctivitis, pyrexia, dullness, and enlarged lymph nodes, coughing and serous to mucopurulent nasal discharge (Borchers et al., 1997, Borchers et al., 1998, Torfason et al., 2008). Clinical signs of EHV-5 infection are not clearly defined, but can include subclinical disease, nasal discharge, coughing, pharyngitis, fever and enlarged lymph nodes (Ataseven et al., 2010b, Dunowska et al., 2000) and recently was associated with equine multi-nodular pulmonary fibrosis (Williams et al., 2007,

Hart et al., 2008). Reubel et al., (1995) suggested that apart from differences in the pathogenicity of EHV-2 and EHV-5, the apparent lower prevalence of EHV-5 may be due to the existence of an alternative native cell reservoir (lymphoid tissue). In a study by Williams et al., (2007) EHV-5 DNA was found in alveolar macrophages, an uncommon location for gammaherpesviruses. The sites of latency for EHV-2 and EHV-5 are still largely unknown. Both EHV-2 and EHV-5 infect lymphocytes, EHV-2 is most commonly isolated from leucocytes, lymphoid tissues and occasionally trigeminal ganglia (Drummer et al., 1996, Edington et al., 1994, Agius et al., 1994). Equine herpesvirus-2 has also been isolated from both the peripheral and central nervous system which may indicate further sites for latency (Rizvi et al., 1997).

Genomic diversity

It was originally thought that the diversity of EHV-2 field viruses resulted from antigenic drift (Fortier et al., 2010). However, in 1997 it was shown that EHV-2 genome undergoes frequent deletions and insertions which may account for the differences in virulence of field isolates (Franchini et al., 1997). A study in 2007 showed a profound variation in both the conserved regions, thought to be stable and the hyper-variable regions of the gene encoding gB (Sharp et al., 2007). Equid herpesvirus 2 encodes three homologues of cellular seven transmembrane receptors (7TMRs): ORF74 and E6 homologues are conserved in gamma-herpesviruses genomes, while E1 is currently unique to EHV-2. To investigate genetic variability of EHV-2, Sharp et al., (2007) sequenced EHV-2 isolates open reading frames 74, E6 and E1 and compared their internal sequence variability to that of a hypervariable region of gB gene. This provided comparative data for a variable genetic locus unrelated to the 7TMR. Six 'genogroups' with approximately 10–38% difference in amino acids were revealed for E1 and four genogroups with approximately 11–27% amino acid difference for ORF74. In contrast, E6 was highly conserved, with only two genogroups identified. Also as there was no evidence for genetic linkage between the different gB, E1, ORF74 and E6 genotypes, suggesting frequent intergenic recombination occurs between different EHV-2 strains. Similarly, a study of 10 EHV-2 viruses found that based on recognition by a monoclonal antibody against the epitope on EHV-2.86/67 gB formed two distinct antigenic groups (Holloway et al.,

2000). The authors suggested that horses may become concurrently infected with EHV-2 more than one antigenic group. Supporting this conclusion, co-detection of two EHV-2 genotypes has been reported in both apparently healthy horses and horses displaying signs of respiratory disease (Browning and Studdert, 1987a, Bell et al., 2006a). Equid herpesvirus 5 was thought to be relatively homogenous until variation in the gB of EHV-5 isolates was shown in 2000 (Dunowska et al., 2000). However the biological significance of the variation in EHV-5 genotypes has not been established.

Epidemiology

Infection with EHV-2 and EHV-5 is endemic in horse populations worldwide including Europe, America, Australia and New Zealand (Plummer and Waterson, 1963, Fortier et al., 2009a, Ruszczyk et al., 2004, Ruszczyk et al., 2003, Ataseven et al., 2010b, Craig et al., 2004, Reubel et al., 1995, Dunowska et al., 2002a, Torfason et al., 2008, Diallo et al., 2008). Equine herpesvirus 2 has also been detected in wild mountain zebras in Namibia with a seroprevalence of 95% (Borchers and Frolich, 1997, Ackermann, 2006). However this study did not investigate the prevalence of EHV5. Interestingly, there is also evidence of co-infection with EHV-2 and EHV-5 in Przewalski's horses. A study in German zoos found Przewalski's wild horses were serologically positive for both EHV-2 and EHV-5 (Borchers et al., 1999). Co infection with EHV-2 and EHV-5 is commonly reported in both horses with respiratory disease and healthy horses (Browning and Studdert, 1987a, Ataseven et al., 2010b, Wang et al., 2007, Torfason et al., 2008, Fortier et al., 2009a).

Equid herpesvirus 2 and EHV-5 were first isolated in Australia (Plummer and Waterson, 1963, Turner and Studdert, 1970, Fortier et al., 2010). However there have been limited studies on the prevalence of gamma herpesviruses circulating among horses in Australia in more recent years. A study in 2005 showed that EHV-5 DNA was present in 73% of nasal swabs or peripheral blood lymphocyte (PBL) samples from 64 foals, while EHV-2 DNA was only detected in 21% of 64 foals (Wang et al., 2007). This is contrary to what has been found in other studies where EHV-2 was detected more frequently than EHV-5. In New Zealand, a longitudinal

investigation of viral infections among 18 foals was conducted. In this study EHV-2 was isolated from 155/157 (99%) of PBL samples tested and from 40/ 172 nasal swabs from 18 foals. However EHV-5 was only isolated by either PBL or nasal swab, or both, from 15 foals on 32 occasions. With one exception, all samples that were positive for EHV-5 were also positive for EHV-2 (Dunowska et al., 2002a). Another New Zealand study, by the same author, investigated viral infections among horses from outbreaks of respiratory disease and yearlings returning from the sales. In this study EHV-2 only was isolated from PBL of 61/80 (76%) horses, while EHV-5 only was isolated from 2/80 (3%) horses. In addition both EHV-2 and EHV-5 were isolated from 13/80 (16%) horses, both EHV-2 and EHV-4 from one foal, and both EHV-2 and an unidentified herpesvirus from one horse (Dunowska et al., 2002b). In Australia, EHV-2 was detected in 30%, while EHV-5 was only detected in 16% of 83 horses (Reubel et al., 1995). Foals are often infected with EHV-2 from their dams while EHV-5 seems to spread foal to foal as determined by genotyping (Bell et al., 2006a). This may be due to EHV-5 infection occurring later in life than infection with EHV-2 (Dunowska et al., 2002a, Nordengrahn et al., 2002). In a study of a cohort of mares and their foals EHV-2 infection was found to occur significantly earlier in life than infection with EHV-5 ($P = 0.01$) with 50% of foals positive for EHV-2, and only 8% of foals positive for EHV-5, at 1 month of age (Bell et al., 2006a). In a study in the USA in 1996, EHV-2 was isolated from the PBMC of 98% (68/69) of foals aged 1-8 months old (Murray et al., 1996). In this study the youngest foal to test positive for EHV-2 was 25 days old and all foals were positive for EHV-2 by 45 days old. In a study on the kinetics of EHV-2 infections in foals viral DNA was detected in 17 (35%) foals tested in the first 2–4 days of life (Dunowska et al., 2011).

Immune responses and interactions

Unlike for the alphaherpesviruses, there are limited studies on the immune response of the horse following infection with equine gammaherpesviruses. Suckling foals have been shown to rapidly attain the same levels of serum antibodies as their dams, with the levels of maternally derived antibodies declining over the first two months of life, by which time the foal is actively-producing antibodies against EHV-2

(Fortier et al., 2010). The level of neutralising antibody typically peaked after a few days after birth and largely disappeared by 6 weeks. Among the gammaherpesviruses gB is one of the most abundant epitopes and represents a major target for neutralising antibody (Agius et al., 1994, Holloway et al., 1998, Holloway et al., 2000), similar to the gG glycoprotein targeted in equine alphaherpesvirus infections (Crabb and Studdert, 1996). Immunoglobulin IgG has been shown to be critical in defence against bacterial and viral infections (Lopez et al., 2002, McGuire et al., 1997, Sheoran et al., 2000). Equine IgG subclasses were designated as IgGa, IgGb, IgGc and IgG(T) according to their serological and biochemical properties (Lunn et al., 1995, Montgomery, 1972, Sheoran et al., 1998). Molecular characterization of the equine IgG heavy chain genes has shown that IgGa corresponds to IgG1, IgGb to IgG4 and IgG7, IgGc to IgG6 and IgG(T) to IgG3 and IgG5 (Lewis et al., 2008, Wagner et al., 2004, Wagner, 2006). An investigation into the production of IgG subclasses of 41 horses following infection with equine gammaherpesviruses demonstrated induction of IgG1, IgG4/7 and IgG3/5, with IgG4/7 being the dominant subclass (Svansson et al., 2009). Similarly, other studies have reported the most abundant IgG subclass in serum of adult horses and in colostrum is IgG4/7 (Sheoran et al., 2000, Lopez et al., 2002). In addition, Svansson et al., (2009) also investigated 10 horses for cytokine expression, and the production of IFN-g was found to always exceed that of IL-4. Cytotoxic T lymphocytes and IFN-g are essential in protection against most viral diseases including EHV-1 infection (Breathnach et al., 2005, Paillot et al., 2005).

Despite the immune responses of the horse to herpesvirus infection, gammaherpesviruses have been found to have modulatory effects allowing the establishment of latency and disease. Both EHV-2 and EHV-5 have a gene homologous to interleukin 10 (IL-10) (Holloway et al., 2000, Studdert, 1996a). The viral IL-10 encoded by ORF E7 is an immune-suppressive cytokine which down regulates class II major histocompatibility complex expression resulting in immune-suppression of the host immune response (Fortier et al., 2010). Similarly, EHV-2 ORF 74 encodes an IL-8 receptor which is chemotactic for neutrophils, normally produced by the hosts macrophages and endothelial cells (Studdert, 1996a, Telford et al.,

1993). Also, EHV-2 has be shown to down regulate *in vitro* the transcription of equine monocyte chemoattractant protein 1 which is a strong chemotactic factor for monocytes and natural killer cells (Dunowska et al., 2001). Furthermore, EHV-2 can produce G coupled protein receptors which interact with cellular chemokines and are involved in signal transduction (Telford et al., 1995, Camarda et al., 1999). Immune modulation may also contribute to the role of EHV-2 and EHV-5 in disease. Equid herpesvirus 2 and EHV-5 are often detected along with other equine respiratory viruses leading to suggestions that EHV-2 and EHV-5 predispose to or reactivate infections with other viruses (Murray et al., 1996, Dunowska et al., 2002a, Purewal et al., 1992, Borchers et al., 1997, Nordengrahn et al., 2002, Wang et al., 2007). Purewal and others found that EHV-2 was able to trans-activate the intermediate early gene of EHV-1, concluding that infection with EHV-2 may reactive latent EHV-1 *in-vivo* (Purewal et al., 1992). Furthermore, the isolation of EHV-2 was found to always precede the isolation of EHV-1 and EHV-4 when co-cultivated from lymphoid tissues of 5 horses experimentally infected through intranasal injection (Welch et al., 1992a). Also, EHV-2 was proposed to predispose to respiratory tract diseases caused by some bacterial pathogens such as *Rhodococcus equi*, *Streptococcus equi* and *Streptococcus zooepidemicus* (Banbura et al., 2004, Nordengrahn et al., 1996, Jolly et al., 1986). In one of these studies vaccination with an EHV-2 subunit vaccine confered protection against pneumonia due to *Rhodococcus equi* infection (Nordengrahn et al., 1996).

1.2.2 Equine picornaviruses

Family		***Picornaviridae***	
	Genus	*Aphthovirus*	- Equine rhinitis A virus
		Erbovirus	- Equine rhinitis B virus

(Stanway et al., 2005)

Picornaviruses are small non enveloped viruses with icosahedral symmetry, approximately 30 nm in diameter. The genome consists of positive sense single stranded RNA 7-8 kb in size (Groppelli et al., 2010). Equine picornaviruses (formerly known as equine rhinoviruses ERhV) include equine rhinitis A virus (ERAV) of the genus *Aphthovirus* and Equine rhinitis B virus (ERBV) the only member of the *Erbovirus* genus (Stanway et al., 2005). Equine rhinitis A virus was formerly known as Equine rhinovirus 1 of an unassigned genus in the family picornaviridae. However, sequence analysis of the genome of two strains of ERhV-1 indicated that this virus was most closely related to "Foot-and-mouth disease virus" (FMDV) (Li et al., 1996, Wutz et al., 1996). Subsequently, ERhV-1 was renamed *Equine rhinitis A virus* (ERAV) and reclassified as a separate cluster in the genus *Aphthovirus* (Stanway et al., 2005). Similarly ERBV was formerly known as ERhV-2 of an unassigned genus in the family picornaviridae. Sequence analysis of the ERhV-2 genome indicated that it was distinct from all other picornaviruses. Accordingly, ERhV-2 was renamed ERBV and assigned as the sole member of a new genus *Erbovirus (Stanway et al., 2005)*. Some ERBV isolates have been shown to be stable at acid pH, and have been distinguished from acid-labile ERBV-1 by genomic sequence analysis. This group of ERBV was formerly known as ERhV-3 and since has been renamed ERBV-2 as the second serotype in the genus *Erbovirus (Black et al., 2005, Huang et al., 2001)*. Acid-stable picornaviruses have been isolated from the respiratory tract and oral cavity of horses, and have been labeled as a third serotype (ERBV-3) within the genus *Erbovirus (Black and Studdert, 2006, Stanway et al., 2005)*.

Both ERAV and ERBV circulate among horses worldwide, but their clinical importance requires further assessment, particularly for ERBV-2 and ERBV-3 (Huang et al., 2001, Black et al., 2007b).

Equine rhinitis A virus (ERAV)

Equine rhinitis A virus was assigned to the genus *Aphthovirus* as it was found to be more closely related to the Foot-and-mouth-disease virus (FMDV) than to other rhinoviruses (Li et al., 1996, Hinton et al., 2002). Although it is the only non FMDV in this genus, ERAV shares physicochemical properties and structural similarities with FMDV (Groppelli et al., 2010). The two viruses also share some sequence similarity; including a 16-amino acid 2A protein which was found to be 87.5% identical between ERAV and FMDV (Li et al., 1996).

Equine rhinitis A virus cell entry is rapid and largely dependent upon its acid lability. A study on the cell entry of ERAV found that the movement of the virus from the cell surface to the interior occurred within 10 minutes (Tuthill et al., 2009). This finding suggests that the entry mechanism of ERAV is via clathrin- dependent endocytosis, as seen with FMDV. Another feature of ERAV in common with FMDV is the requirement of virus acidification for cell entry. While the exact process of acidification and its role in cell entry is still largely unknown, capsid dissociation does occur during acidification and this process allows for the insertion of viral RNA into the host cell (Groppelli et al., 2010, Tuthill et al., 2009, Horsington et al., 2011).

To date there is no evidence of extensive diversity among different strains of ERAV. However, ERAV has been shown to have a broad host range both *in-vitro* and *in-vivo* (Mori et al., 2009, Li et al., 1996). *In-vivo*, ERAV primarily infects horses, although it also have been shown to be able to infect rabbits, guinea pigs and monkeys under experimental conditions, but it does not appear to spread horizontally between members of these species (Li et al., 1996). *In-vitro* Equine rhinitis A virus replicates in cells derived from several species causing CPE which consists of cells becoming rounded, refractile and dense, eventually leading to cell detachment (Ditchfield and Macpherson, 1965). However, in some studies ERAV did not always produce visible CPE in cell culture (Li et al., 1997, Black et al., 2007b). This may explain why ERAV

infection was often detected through PCR or serology, but few viral isolates were obtained from clinical material (Carman et al., 1997, Dynon et al., 2001, Dunowska et al., 2002b).

Infection with ERAV can cause subclinical disease or acute febrile disease with signs ranging from mild through to severe (Mori et al., 2009, Dunowska et al., 2002b). The primary site of infection for ERAV is the nasopharynx as is seen with FMDV. Viraemia and persistent virus shedding from the pharyngeal region, urine and faeces accompanies infection (Hartley et al., 2001, Hinton et al., 2002). Systemic infection and persistence are not characteristic of rhinovirus infections in other species, but are characteristic of FMDV (Li et al., 1996). However, unlike FMDV, ERAV disease is limited to the respiratory tract and includes clinical signs such as nasal discharge, coughing, anorexia, fever, pharyngitis and lymphadenitis (Li et al., 1996, Hartley et al., 2001). A major difference between FMDV and ERAV is the inability of the latter to cause the vesicular lesions, which are central to the pathology and significance of FMDV infections (Hinton et al., 2002). The incubation period for ERAV is 3-8 days with recovery usually within 7 days after the onset of clinical signs. When pharyngitis persists, coughing may continue for 2-3 weeks and shedding of ERAV in urine can last for more than 4 months after infection (Sellon and Long, 2007).

Equine rhinitis A virus was first isolated in the United Kingdom in 1962 (Plummer, 1962) and subsequently from the United States, Canada, Europe and Australia (Li et al., 1996, Diaz-Mendez et al., 2010, Li et al., 1997, Studdert, 1996b). In Australia the prevalence of ERAV neutralizing antibodies was shown to vary according to the age of the horse, with a maximum infection rate of approximately 50% in horses older than one year of age (Studdert and Gleeson, 1978, Hartley et al., 2001). A study in the United States of America found the prevalence of antibodies to ERAV in horses older than 4 years of age to be 83%, compared with only 39% in horses younger than 3 years of age (Holmes et al., 1978). In a New Zealand study, the prevalence of VN antibodies to ERAV was found to be only 13%, which was considerably lower than that reported in other countries (Dunowska et al., 2002b), but similar to the prevalence reported for a previous New Zealand study (Jolly et al., 1986). The

prevalence in foals reported in this study was 12.3%, while 37.7% of adult horses 1-9 years of age with history of respiratory disease were positive for ERAV antibody by a VN test. In studies in other countries, horses have often been considered in different age categories, i.e. younger than 3 and older than 4 (Holmes et al., 1978), compared to the study by Dunowska et al., (2002b), where a large proportion (78%) of horses sampled were foals and yearlings. This suggests the differences between the results of this study and those from other countries may be in fact due to different age distributions in the horse populations sampled. Also it was noted that all but two samples positive for ERAV antibodies in this study were collected from either very young foals, which most likely represented maternally-derived antibodies, or from horses 4 years of age. Differences in prevalence between countries could also be explained by differing husbandry practices. The major difference between in New Zealand and other countries, particularly those countries in the northern hemisphere, is that horses in New Zealand horses tend to spend more of the year at pasture (Dunowska et al., 2002b).

Equine rhinitis B virus (ERBV)

Equine rhinitis B virus was recently classified as the only member in the genus *Erbovirus*. There are currently three known serotypes of ERBV labelled ERBV-1, ERBV-2 and ERBV-3 (Black et al., 2007b). Until recently it was thought that ERBV, like ERAV was acid labile. However a study in 2005 unexpectedly found a group of ERBV-1 viruses that remained infectious after 1hour at pH 3.6 and have been termed acid stable ERBV-1. Cross-neutralization between acid-labile and acid stable ERBV-1 isolates does occur, indicating a close antigenic relationship despite phylogenetic and phenotypic differences. Analysis of the P1 amino acid sequences showed there was 75–82% identity between acid-stable ERBV-1 and the acid-labile ERBV-1 (Black et al., 2005). The effect and relevance of acid stability on ERBV infection and pathogenesis is unknown (Horsington et al., 2011). However, the acid stability of picornaviruses is generally an indication of the route of infection, where acid stable viruses tend to infect the gastrointestinal tract and acid labile viruses usually are associated with respiratory tract infections (Racaniello, 2007). Equine rhinitis B virus 2 was included in the *Erbovirus* genus after phylogenic analysis and sequence

analysis. It was found at the amino acid level, the P2 and P3 regions of ERBV-2 are over 95% identical with ERBV1. However, ERBV-2 was separated into a different serotype due to low amino acid identities (72 ± 5%) in the P1 region and a lack of cross neutralization with ERBV-1. The close sequence similarity between ERBV-1 and ERBV-2 suggests that they have evolved from the same ancestral strain relatively recently (Huang et al., 2001). Formally unclassified acid stable equine picornaviruses have recently been established as a third group of ERBV by sequence analysis and labelled ERBV-3. As the former name suggests these viruses are acid stable remaining infectious after 1 hour at pH 3.6. The amino acids of the P1 region of ERBV-3 isolates have a 93–96% identity with acid-stable ERBV-1 isolates, compared to only 77–78% identity acid labile ERBV-1 and 75–77% identity with ERBV-2. As such, it has been hypothesised that acid stable ERBV-1 should be included in the ERBV-3 serotype (Black and Studdert, 2006).

Equine rhinitis B virus 1 and 2 were first isolated in Switzerland in 1978 (Steck et al., 1978) and sequenced in 1996 and 2001 (Wutz et al., 1996, Huang et al., 2001). Since then, ERBV has been isolated in United Kingdom, United States of America, Canada and Australia. Serology has shown antibodies to ERBV-1 and ERBV-2 in 50-80% of the horses in these populations (Black and Studdert, 2006, Carman et al., 1997, Diaz-Mendez et al., 2010, Holmes et al., 1978, Black et al., 2007b). Considering the high prevalence of ERBV antibody among various horse populations, these viruses are rarely isolated. There is serological evidence that ERBV circulates in New Zealand at the same rate as seen overseas but it has never been isolated in cell culture (Dunowska et al., 2002b, Jolly et al., 1986).

Clinical sings of disease from ERBV infection can range from mild to severe and include fever 41°C for 1-3 days, acute nasal discharge, anorexia, oedema of the legs, lethargy, and pain and enlargment of the lymphoid tissues. However, subclinical and persistent infection is common in many cases (Black et al., 2007a, Black et al., 2007b, Mori et al., 2009). It has also been shown that dual infection of mixed serotypes and acid stability phenotypes is not uncommon. In one horse ERBV-3 was isolated from two nasal swab samples taken 6 months apart, where in the second sample acid

labile ERBV1 was also isolated demonstrating a concurrent co-infection of ERBV serotypes (Horsington et al., 2011).

Equine rhinitis B virus is known to grow poorly in cell culture and some strains show infrequent or no CPE (Mori et al., 2009). Addition of $MgCl_2$ to the media and the use of rapidly dividing cells in cell culture can improve the growth of ERBV-1 and encourage the development of more visible CPE. Equine rhinitis B virus 1 and ERBV-2 CPE can be seen as clusters of rounded cells and small plaque formations (Black et al., 2007a). In this study, RK-13 cells supported the growth of ERBV-1 and ERBV-2, while primary EFK did not support the growth of either virus (Black et al., 2007a, Horsington et al., 2011). In the study by Horsington (2011) ERBV-1 was isolated more frequently than ERBV-2 in RK13 cells grown in maintenance medium (MM) containing Eagle's minimal essential medium (MEM; Sigma–Aldrich) with 10mMNaHCO3, 50g/ml ampicillin (Sigma) and 2% (v/v) foetal bovine serum, which may indicate that ERBV-2 has different culture requirements (Horsington et al., 2011). In addition, it was noted that the growth ERBV-1 was slow and CPE took longer than 3 cell passages to form. Equine rhinitis B virus 3 culture requirements are similar to that of acid stable ERBV-1 (Black and Studdert, 2006, Horsington et al., 2011). Due to the variable nature of ERBV with respect to the development of CPE and differing cell culture requirements for the three serotypes of ERBV, virus isolation is not a reliable method of ERBV detection (Mori et al., 2009). In addition, the use of serology to identify recent exposure to ERBV is difficult as high numbers of horses have detectible antibody to ERBV in many horse populations. Also, ERBV has been isolated from horses with signs of respiratory disease without detectable antibody on two sampling occasions 2 weeks apart, and not isolated from horses showing signs of respiratory disease with detectable antibody (Black et al., 2007a).Therefore sero-conversion can be used to identify recent infection of ERBV when a 4-fold increase in antibody can be detected. Instead PCR techniques such as nested RT-PCR have been developed for fast and accurate identification of ERBV infection and serotype differentiation in horse populations. While PCR is currently the best method of detection for ERBV, no association between ERBV and respiratory disease can be made as PCR does not differentiate between live and

killed virus (Black et al., 2007a, Mori et al., 2009, Dynon et al., 2001, Horsington et al., 2011, Black et al., 2007b).

1.2.3 Equine adenoviruses

Family	***Adenoviridae***		
	Genus	*Mastadenovirus*	- Equine adenovirus A
			- Equine adenovirus B

(Harrach et al., 2011)

Equine adenoviruses (EAdV) are non-enveloped with linear double-stranded DNA 25-46 kbp in length (Davison et al., 2003). The viron has a diameter of 80-100 nm with a hexagonal outline and icosahedral symmetry (Murphy et al., 1999). Equine adenoviruses are not sensitive to chloroform or ether and are stable between pH 4 and 7 (Horner and Hunter, 1982, Konishi et al., 1977). Most of the EAdV isolates show only minor antigenic differences and constitute a single serotype labelled EAdV-1, recently changed to EAdV-A (Harrach et al., 2011). A second serotype of EAdV was found in 1982 and labelled EAdV type 2, now known as EAdV-B (Studdert and Blackney, 1982). Phylogenetic analysis showed that EAdV-A and EAdV-B do not have an immediate common ancestor, suggesting these viruses evolved separately (Reubel and Studdert, 1997).

Equine adenovirus is associated with respiratory and/or gastrointestinal infection in horses and foals (Bell et al., 2006b, Dunowska et al., 2002a), with EAdV-A predominantly affecting the upper respiratory tract while EAdV-B has mainly been associated with gastrointestinal tract infections (Maclachlan and Dubovi, 2010, Studdert and Blackney, 1982). Equine adenovirus-A is acquired by close contact via respiratory or ocular route of infection (Sellon and Long, 2007). The virus replicates in epithelial cells throughout the respiratory tract and may also infect cells in the gastrointestinal tract and be shed through faeces (Studdert, 2003). Infections caused by EAdV-A are generally considered to be mild and self-limiting and have been

detected in both healthy and sick animals (Bell et al., 2006b). For those which do develop disease, clinical signs include nasal discharge, coughing after exercise, conjunctivitis, pneumonia, enlarged submandibular lymph nodes and soft faeces, with recovery typically in 7-10 days. However, in mixed infections with other viruses or bacteria, disease can be more prolonged (Sellon and Long, 2007, Studdert, 2003, Bell et al., 2006b). In Arabian foals with severe immunodeficiency, EAdV-A can cause severe and often fatal bronchopneumonia (McCheseny and England, 1978).

While there is some data available on the incidence of EAdV-A infections (Harden et al., 1974), there are only limited data on EAdV-B prevalence in horse populations, most likely be due to the fact that it has been difficult to grow this virus in cell culture. Equine adenoviruses are highly host cell specific and can only be grown on primary cells of equine origin or equine skin fibroblast cell line (Studdert, 2003). Equine adenovirus A grows well in cell cultures and intra-nuclear inclusion bodies are typically observed in infected monolayers. In contrast, EAdV-B replicates slowly and poorly in cell cultures, and has been isolated only a few times (Horner and Hunter, 1982, Studdert and Blackney, 1982). This phenomenon of 'nonculture ability' is well known for human Adenovirus of the subgroup F which also infects the gastrointestinal tract (Reubel and Studdert, 1997). A range of assays including hemagglutination inhibition, CF and VN have been described for the detection of antibodies to EAdV (Horner and Hunter, 1982, Dunowska et al., 2002a). Of these, only VN and HI have been used to distinguish between EAdVA and EAdVB and was initially developed for the serological characterization of these viruses. The 'gold standard' for serological typing of viruses is VN, however this assay is time consuming and is therefore not routinely used for the diagnosis of equine adenovirus infections (Horner and Hunter, 1982). PCR has also been used to detect EAdV-A and EAdV-B in clinical samples (Dynon et al., 2007) but PCR has not been extensively used in epidemiological studies of equine adenovirus.

Based on serology EAdV have a worldwide distribution including New Zealand, Australia, USA, Europe, Middle East and Asia (Wilks and Studdert, 1973, Horner and Hunter, 1982, Bell et al., 2006b, Konishi et al., 1977, Herbst et al., 1992, Harden et

al., 1974).While the prevalence of EAdV-A in various horse populations has been reported (Harden et al., 1974, Dunowska et al., 2002b), very little information is available on the prevalence of EAdV-B infection among different horse populations. One study has revealed that low to moderate titers of antibodies to EAdV-A and/or EAdV-B to be present in 97/122 (79.5%) horses tested, with 54.9% (67/122) positive for EAdV-A antibodies and 39.3% (48/122) positive for EAdV-B antibodies (Giles et al., 2010). Similar results have been reported in a study undertaken by Dunowska et al. (2002b) who demonstrated that 52.5% of horse sera tested were positive for EAdV-A HI antibodies in New Zealand. While EAdV-B infections have been identified in New Zealand by serology and by virus isolation, the prevalence of EAdV-B infections in this country has not been investigated (Horner and Hunter, 1982).

1.2.4 Equine arteritis virus

Family	***Arteriviridae***		
	Genus	*Arterivirus*	- Equine arteritis virus

(Stanway et al., 2005)

Equine viral arteritis (EVA) is caused by the Equine arteritis virus (EAV), a member of the genus *Arterivirus* within the family *Arteriviridae* (Stone, 2000). Equine arteritis virus is a small, single stranded, positive sense RNA virus, with a genome 12.7 to 15.7 kb in size. The viron has a diameter of 50-70 nm and consists of an isometric core surrounded by an envelope which contains small surface projections (Glaser et al., 1997, Holyoak et al., 2008, Snijder and Meulenberg, 1998). Equine arteritis virus has been shown to replicate in a variety of cells including primary macrophage or kidney cells and continuous cell lines like Vero or RK-13 (Snijder and Meulenberg, 1998, Glaser et al., 1997). Equine arteritis virus decreases in stability with increasing temperatures, lasting a mere 20-30 minutes at 56°C, 2-3 days at 37°C and up to 75 days at 4°C with no loss in infectivity. However, EAV is easily inactivated by lipid solvents and common disinfectants or detergents, so must be handled with care (Balasuriya and MacLachlan, 2007).

Equine arteritis virus is transmitted predominantly through the respiratory route by horses in the acute phase of the disease, but is also transmitted venereally by carrier stallions (Holyoak et al., 2008, Timoney, 1992). When contracted through aerosol exposure, EAV initially replicates in pulmonary macrophages, rapidly spreading throughout the body via the circulatory system. Horses recently infected with EAV via the respiratory route shed the virus in the respiratory tract for 7-16 days (Holyoak et al., 2008). Persistently infected carrier stallions can be clinically healthy and shed the virus in their semen for years. Unlike mares, geldings, or sexually immature colts, carrier stallions are viral reservoirs and are primarily responsible for persistence of the virus in different horse populations throughout the world. The virus is primarily localized in accessory sex glands in the carrier stallion and is shed constantly in the semen. The rate of transmission of infection when bred with susceptible mares is >85%. Recovered serologically positive horses are immune and are not carriers of the virus (Tizard, 2010). There is only one serotype of EAV, although there is genetic variation among EAV isolates. The pathogenicity of different EAV genotypes can vary significantly (Stone, 2000). Infection with the virus is often subclinical and the disease has never been seen in New Zealand. Clinical signs typically begin 2-14 days after infection (Glaser et al., 1997). In an outbreak situation, clinical signs are often mild and may include, but are not limited to, pyrexia, depression, stiffness, abortion, conjunctivitis and nasal discharge. More severe clinical signs include respiratory distress, paresis, ataxia and oedema of the extremities (Huntington et al., 1990a, Glaser et al., 1997, Holyoak et al., 2008). In adults, severe clinical signs and mortality is rare. However, EAV infection causing respiratory distress can be fatal to neonates and foals (DelPiero et al., 1997, Glaser et al., 1997). Clinical signs typically persist for 2-9 days and adult mares and gelding usually make an uneventful recovery. Between 30% and 60% of infected stallions develop persistent infection and shed the virus constantly in the semen (Glaser et al., 1997, Huntington et al., 1990a). Infected mares and geldings shed EAV in nasal discharge, urine and faeces for 2-3 weeks after infection. Mares also shed the virus in vaginal secretions (McCollum et al., 1971). Recovered mares and geldings have been shown to be resistant to clinical disease after re-exposure to the virus for up to seven years and possibly for their life time (McCollum, 1969, Gerber et al., 1978).

Equine arteritis virus has a worldwide distribution including New Zealand, Australia, Europe, America, Asia and Africa (Weiss et al., 1994, Timoney, 1992, Huntington et al., 1990a, Glaser et al., 1997, Holyoak et al., 2008). Clinical disease associated with EVA has never been recorded in New Zealand, although the causative virus is known to be present (Stone, 2000). Infection with EAV was first reported in Australia and New Zealand in 1988, the disease was made notifiable in 1989 to facilitate the industry based control scheme which restricted the movement of carrier stallions, their semen and mares to bred to them (Horner and Kirkland, 2003). All age groups and breeds of horses are susceptible to infection with EAV (Stone, 2000). However, there are differences in the seroprevalence of EAV between breeds, with a high sero-prevalence found in Standardbreds compared to Thoroughbreds (Glaser et al., 1997, Huntington et al., 1990b, Timoney and McCollum, 1993). This difference could be a reflection of differences between the breeding practices, artificial insemination for Standardbreds versus natural cover for Thoroughbreds. In New Zealand, the seroprevalence of EAV dropped from 36% in 1990 to 22% in 2003 for Standardbred stallions and from 3% in 1990 to 0.2% in 2003 for Thoroughbred stallions (Horner and Kirkland, 2003). At the end of 2006 there was only one known shedder stallion in New Zealand (O'Flaherty and Reid, 2004). Although eradication of EAV from New Zealand seems possible, other animals susceptible to infection may need to be investigated as antibodies to EAV have been found in donkeys, mules, alpacas and zebras (McCollum et al., 1995, Paweska et al., 1997, Weiss et al., 1994, Weber et al., 2006).

The diagnosis of EAV can have a considerable economic consequences for both breeding and performance horse industries. Losses to breeding farms include abortion, decreased value of persistently infected stallions and denied export of infected semen. Losses to performance horse industries include disruption of training schedules, reduced competition entry or event cancelation and spread of EAV to naïve populations resulting in abortion and further spread to uninfected stallions (Holyoak et al., 2008). However EAV is a manageable disease if simple sero-surveillance and hygienic procedures are followed by horse owners, breeders and trainers (Glaser et al., 1997). There are vaccines available which are highly effective

and immunised animals can remain solidly immune for several years. In addition, immunization of naïve stallions and immature colts between 6 and 12 months of age prevents establishment of the carrier state (Holyoak et al., 2008), although it does not prevent infection with EAV (Glaser et al., 1996). Effective strategies for control and prevention have been designed using a combination of vaccination and appropriate management of infected and uninfected animals to prevent the transmission of EAV (Ruiz-Saenz, 2010, Holyoak et al., 2008).

1.2.5 Equine influenza virus

Equine influenza virus is exotic in New Zealand and so does not contribute to respiratory disease in this country (Myers and Wilson, 2006, Daly and Mumford, 2001, Jolly et al., 1986). As such, it will not be discussed in this literature review. However it is important to note that should equine influenza virus ever be introduced it would have a major impact on the New Zealand equine industry (Daly and Mumford, 2001).

1.2.6 Bacteria

There are several species of bacteria associated with equine respiratory disease including; *Streptococcus equi* ssp. *zooepidemicus* and *equi* (ssp. *equi* is the cause of the disease known as strangles)*, S. pneumoniae* and *Pasteurella* spp /*Actinobacillus* sp. (Carman et al., 1997, Wood et al., 2005b, Burrell et al., 1996, Newton et al., 2003, Ribas et al., 2009, Saif, 2010). A significant association with respiratory disease appears to be only present when total bacterial counts from tracheal washes exceed 10^3 total cfu/ml (Christley et al., 2001b) or 10^4 total cfu/ml (Burrell et al., 1996). Christley et al., (2001a) also showed that the risk of coughing decreased significantly with increasing age, with yearlings and 2-year-olds being at greatest risk. Similarly, the risk of infection with *S. zooepidemicus*, *S. pneumoniae* and *Pasteurella* spp. associated with airway inflammation was significantly lower in horses 4 years or older (Wood et al., 1993). In two longitudinal studies, the risk of inflammatory airway disease (IAD) associated with bacterial infection, particularly *S.*

zooepidemicus, decreased with age (Burrell et al., 1996, Wood et al., 1999). The longitudinal study by Wood et al. (1999) demonstrated that the incidence of specific infections decreased between 2 and 4 years of age and this was particularly marked for *S. pneumoniae*. Burrell (1996) also reported no bacterial growth in 22% of tracheal wash samples from horses with equine respiratory disease and concluded that equine respiratory disease cannot be attributed to bacterial infection alone (Burrell et al., 1996). This study observed that mixed infections in the presence of disease were common and in some cases viral infection preceded bacterial infection. This may indicate a predisposing role of viruses for secondary bacterial infections. While some bacterial species are commonly associated with respiratory disease, there still remains a fundamental question as to whether or not there is a causal relationship, as many of the bacteria associated with respiratory disease are known to be commensal organisms of the equine respiratory tract (Newton et al., 2003). Therefore, caution should be taken when interpreting results from studies investigating the role of bacteria in respiratory disease.

1.2.7 Mycoplasmas

Studies have identified the potential for *M. felis* to produce clinical signs of respiratory disease including pleuritis (Hoffman et al., 1992), pericarditis (Morley et al., 1996), LRT inflammatory disease and subclinical infection (Wood et al., 1997). *Mycoplasma felis* and mycoplasma *equirhinis* are commonly isolated from the respiratory tract of horses with clinical and subclinical respiratory disease. Newton et al. (2003) found *M. felis* to be associated with clinical respiratory disease while *M. equirhinis* was associated with subclinical disease (Newton et al., 2003). However, both *M. felis* and *M. equirhinis* were isolated from horses with acute respiratory disease in Canada, with *M. equirhinis* isolated from over 50% of horses sampled (Carman et al., 1997). In another study large numbers of *M. felis* (>10^4 cfu/ml) were detected in tracheal washes collected from British racehorses with lower respiratory tract disease, suggesting that *M. felis* may be an important pathogen in lower airway disease (Wood et al., 1997). Out of 22 horses included in this study, 19 seroconverted to *M. felis* over a two month observation period. Of these, 18 either

had increased tracheal mucus or a cough, while only 1 displayed no clinical abnormality. Of the remaining 3 horses which did not seroconvert to *M. felis,* only 1 had increased tracheal mucus. The sero-conversion of 90% (19/22) of horses in this study demonstrates the highly contagious potential of *M. felis*.

1.3 Aims and scope of the thesis

There is a substantial amount of evidence to suggest equine respiratory viruses have an influence on respiratory disease of horses and potentially a part in disease causation. However more research is required to establish the exact role that each of these viruses may have in disease development. The fact this disease is not consistently reproducible through experimental infection shows that more complex mechanisms may be involved.

The purpose of the current study was to identify viruses that circulate among horses in the lower north island of New Zealand and determine whether or not infection with selected viruses was associated with the development of respiratory disease. It has been more than ten years since the last survey of equine respiratory viruses in New Zealand horses. It was hypothesised that, the current study would complement previous New Zealand based investigations and provide further data in support of the view that viral infections are likely to pay a role in the development of respiratory disease in horses kept under New Zealand management conditions. Many studies suggest that the development of disease may be influenced by a variety of virus, host and environmental factors. To take all of these factors into account within a single study would be more than challenging and so was not undertaken. In the current study, only herpesviruses 1, 2, 4 and 5 were found to be circulating the 85 horses sampled from the lower North Island of New Zealand. Over all, the detection of herpesviruses was significantly associated with respiratory disease (p value <0.0001). Detection of individual virus species (EHV-2, EHV-4 or EHV-5) was also significantly associated with respiratory disease (p value 0.0002, 0.0006, <0.0001, respectively). The sample size was not large enough to evaluate the significance of EHV-1 detection and respiratory disease.

The main limitation of the current study is the convenience sampling strategy employed. However, this is a common difficulty where sampling relies on the good will of private owners, particularly when animals with high individual value (such as horses) are investigated.

Chapter 2 Material and Methods

2.1 Sample collection

2.1.1 Horses sampled

Nasal swabs were collected from 33 healthy horses and 52 horses with signs of respiratory disease. Signs of respiratory disease included one or more of the following: nasal discharge, fever, in-appetence, cough or poor performance as assessed by the owner. Horses with other signs of disease which may not relate to respiratory disease were recorded. Samples were collected from horses residing within the Hawkes Bay and Manawatu regions using convenience sampling. Horses sampled varied in age, sex, breed and management situations. (Appendix 1)

2.1.2 Collection of samples

Nasal swabs were collected using the Virocult transport system (Virocult, Medical Wire & Equipment Co Australasia Pty Ltd), consisting of a 15 cm long swab and a transport tube with a foam pad saturated with 1.2 mL of viral transport medium. The swab was inserted into the nostril of the horse and rubbed against the mucosal surface. The swab was then re-placed in the transport tube and saturated with the medium. The samples were kept on ice till being transported to the laboratory within 24 hours of collection. Two swabs (one from each nostril) were collected from each horse.

2.1.3 Processing of swabs

Nasal swabs were either processed immediately following arrival at the laboratory or stored at 4°C for up to 48 hours. Swabs were squeezed 30 times in the transport medium to release any viral particles into the medium. The transport medium which was then collected into a clean 1.5 mL microtube and centrifuged for 2 minutes at 13,000 rcf to pellet particulate material. The supernatant was transferred to a sterile cryotube and stored at -80°C for further use.

2.2 Virus Isolation

2.2.1 Cell culture

Three cell types were used in this study: the two continuous cell lines, rabbit kidney cells (RK-13) and green African monkey cells (Vero) and primary cells of equine origin- equine fetal kidney cells (EFK).

2.2.2 Media

The growth medium (GM) consisted of Advanced Dulbecco's Modified Eagle Medium (Adv DMEM, Invitrogen) supplemented with 2% v/v fetal bovine serum (FBS) (In Vitro Technologies), 1% v/v penicillin streptomycin (Invitrogen) and 1% v/v glutamax (Invitrogen). For the primary cells, 10% v/v FBS was used instead of 2% v/v FBS.

2.2.3 Maintenance of cells

Cell cultures were maintained following standard laboratory procedures (Freshney, 1994). Cells were removed from liquid nitrogen, thawed and transferred into a flask to which GM containing an increased percentage of FBS (5%) was then added drop wise up to 20 mL. The cells were incubated at 37°C, 5% CO_2 in a humidified incubator. The GM was removed the following day and replaced with fresh GM. The cells were sub-cultured approximately twice weekly when deemed confluent by visual observation using an inverted microscope. For sub-culturing, the cell monolayer was washed three times with warm phosphate buffered saline, pH 7.0 (PBS) and cells were disassociated using a 0.05% trypsin solution at 37°C for 1-3 minutes. The cells were monitored to determine the detachment of the monolayer from the flask and then agitated to separate the cells from one another. The cells were then immediately suspended in 10 mL of GM and split at a 1:2 volume ratio for EFK and a 1:5 volume ratio for Vero cells and RK13 cells. For seeding 24-well plates, 1 mL of freshly diluted cell suspension was added to every well. Vero and RK13 cells were used at a passage number between 30 and 150 and EFK cells were used for up to passage number five.

2.2.4 Inoculation of 24- well plates

For virus isolation, 50 µL (52 horses with respiratory disease) and 150 µL (33 healthy horses) of nasal swab transport medium was inoculated onto each of the three different cell types (Vero, RK-13 and EFK) at the time of seeding into wells on 24-well plates (one well per cell type). Three wells on each 24 well plate were left un-inoculated and used as negative controls. The plates were incubated for 7 days at 37°C in a humidified 5% CO_2 atmosphere and checked daily for the development of viral CPE. Three one-week passages were performed. Each time, the plates were freeze-thawed three times and 50 µL of cell lysate was transferred to a corresponding well on a new plate containing fresh cell suspensions.

2.2.5 Virus detection

The cell cultures were observed daily under a microscope for the presence of viral CPE. If CPE was observed, the contents of the well (including cell monolayer)was collected and passaged one more time to confirm that the CPE was due to viral infection and not due to non-specific toxicity of the samples. The samples were considered negative if no CPE was observed after three passages and these samples were not processed further. Viral isolates were identified using species specific PCR, as described in sections 2.4 and 2.5.

2.3 PCR

2.3.1 Total nucleic acid extraction

Total nucleic acids were extracted from 200 µL of sample (either viral transport medium or cell culture lysate) using the High Pure Viral Nucleic Acid Kit (Roche Applied Science) following the manufacturer's instructions. Total nucleic acids were eluted in 50 µL of elution buffer (Nuclease-free, sterile, double distilled water) and stored at -80°C until further processing.

2.3.2 cDNA preparation

cDNA was prepared from total nucleic acids using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science) following the manufacturer's instructions. Briefly, 2 µL of Random Hexamer Primer was added to 9.4 µL of the extracted nucleic acids and denatured for 10 minutes at 65°C. A master mix was prepared consisting of: reverse transcriptase buffer 1x ($MgCl_2$), protector RNase inhibitor 20U, DNTPs 1mM each, DTT 5mM and reverse transcriptase 10U. The master mix to each denatured nucleic acid-primer mix to a total volume of 20 µL. The reagents were carefully mixed and the tubes were placed in aPX2 Thermal Cycler (Thermo Electron Corporation) and incubated for 30 minutes at 55°C. The reverse transcriptase was then inactivated by heating to 85°C for 5 minutes and the tubes placed on ice to stop the reaction. The cDNA was stored at -20°C until further use.

2.3.3 PCR reactions

Total nucleic acids were extracted from each of the 50 swab samples. PCR was performed to detect the presence of EHV1/4, EHV-2, EHV-5 and EAdV DNA for each of the samples using the extracted total nucleic acids. Equine rhinitis a virus and ERBV PCRs were performed using the cDNA obtained from the extracted total nucleic acids.

The PCR primers used for this study are listed in table 1.With the exception of EHV-1 and EHV-4 PCR reactions, the standard reaction mix consisted of 1xbuffer supplied with the enzyme, 0.1 mM dNTPs, 0.2 µM of each primer, 0.04 U Taq DNA-polymerase and 0.5 µL of template nucleic acid in a total volume of 12.5 µL. The EHV-1 and EHV-4 PCR was performed as described above, but in a multiplex format, with 0.2 µM of EHV-1 forward primer, 0.2 µM of EHV-4 forward primer and 0.4 µM of EHV-1/4 reverse primer. All PCR reagents were purchased from Roche, and primers from Invitrogen. Reactions were performed in flat cap PCR tubes (Ray Lab) in a PX2 Thermal Cycler (Thermo Electron Corporation). The same cycling conditions were used for detection of all viruses, excluding EAdV which had an annealing temperature of 50°C rather than 60°C.

Cycling conditions

Initial denaturation: 94°C for 2 minutes

35 cycles of:

Denaturation- 94°C for 10 seconds

Annealing- 60°C (EAdV 50°C) for 10 seconds

Elongation- 72°C for 1 minute

Final Elongation: 72°C for 7 minutes

Hold temperature: 4°C

For multiple reactions, a PCR master mix was prepared. PCR reagents were stored in a designated clean room, using dedicated pipettes, pipette tips and reagents. Template preparation and addition to PCR tubes was performed in an area separate from the clean room and from the room where gel analysis of the PCR products was performed. All PCR reactions were performed as one-step assays, without the nested round of amplification. Positive and negative controls were included each time the test was performed. Positive controls consisted of cell cultures infected with the respective viruses and processed in the same way as the survey samples. Positive controls included; New Zealand isolates of EHV-2 (V110282), EHV-5 (13/97), EHV-1(592), EHV-4 (Horner), Canine adenovirus (CAV-1/97) and Australian isolates of ERAV (393/76) and ERBV (1436/71). Negative (water) controls were also included in each assay. The bands obtained with positive controls were sequenced to confirm their identity.

Table 1 List of primers used

PCR	*Primers*	*Target gene*	*Product size*	*Reference*
EHV-1	Forward: 5‘-GCGAGATGTGGTTGCCTAATCTCG-3’	Glycoprotein C	649bp	(Lawrence et al., 1994)
	EHV-1/4 Reverse:[1] 5‘-GAGACGGTAACGCTGGTACTGTTAA-3’			
EHV-4	Forward: 5’-ACGCACGAACAACTCAACCGATGT-3’	Glycoprotein C	507bp	(Lawrence et al., 1994)
	EHV-1/4 Reverse:[1] 5‘-GAGACGGTAACGCTGGTACTGTTAA-3’			
EHV-2	Forward: 5’-CAGTGTCTGCCAAGTTGATA-3’	Glycoprotein B	444bp	(Dynon et al., 2001)
	Reverse: 5’-ATGGTCTCGATGTCAAACAC-3’			
EHV-5	Forward: 5’-GAGACCACGTTGTCCCCG-3'	Thymidine Kinase	293bp	(Reubel et al., 1995)
	Reverse: 5'-GCTTCAAGTCCCTCATGAGC-3'			
ERAV	Forward: 5’-GAYYTGCCTGACAAGCAGGT-3’	P1-2A region	805bp	(Dynon et al., 2001)
	Reverse: 5’-TTGCTCTCAACATCTCCAGC-3’			
ERBV	Forward: 5‘-TTTTGATGCTTCACATTCTCC-3’	3D polymerase	782bp	(Black et al., 2007a)
	Reverse: 5‘-CGCTGTACCCTCGGTCCTACTC-3			
EAdV	Forward: 5’-CMAAYGCHYTVTAYGGBTCDTTTGC-3’	polymerase	425 bp	(Thomson et al., 2002)
	Reverse: 5’-CAYTCHSWSAYRAADGCBCKVGTCCA-3’			

[1]EHV-1and EHV-4 specific PCR reactions were performed as multiplex PCR reactions with all three primers used per tube

Aliquots (6 µL) of PCR products were subjected to electrophoresis through 1.25% agarose gel (Axygen Biosciences), containing x 5 ng/mL ethidium bromide (EtBr), in 0.5 x TBE buffer at 100V for 25-45 minutes. Amplicons were visualised under UV light and photographed. A molecular size marker (Fermentas Life Sciences) was included

for reference on every gel. The results were confirmed by dot blot hybridisation with digoxigenin (DIG) labelled probes specific for the individual viruses. A sample was considered positive if a product of the correct size was visible on the gel and the identity of the product confirmed by dot blot hybridisation with the virus-specific probe. Samples that appeared negative on the gel, but were positive in the dot blot hybridisation were also considered positive. The PCR was considered valid if the positive and negative controls gave the expected results.

2.4 Dot blot hybridisation

2.4.1 Preparation of dot blots

After gel analysis, PCR reaction tubes were placed in a PX2 Thermal Cycler (Thermo Electron Corporation), heated to 95°C for 10 minutes, and quickly chilled on ice. An aliquot (1 µL) of each PCR product was spotted onto a pre-marked membrane (Hybond N+, Amersham) and allowed to dry. The DNA was fixed to the membrane by UV cross linking, by placing the membrane wrapped in a saran wrap, DNA side up, in a UV cross linker for 5 minutes at 2000 µJ/cm^2. The blots were stored dry at 4°C until use. The PCR reactions for EAdV were not evaluated by dot bot hybridisation.

2.4.2 Preparation of probes

Probes were prepared using the DIG (Dioxigenin) probe synthesis kit. Virus specific DIG-labelled DNA probes were generated by incorporation of DIG into PCR products during amplification. The probes were prepared using a PCR reaction mix consisting of a 5 µL of 10x PCR buffer with $MgCl_2$, 5 µL of 10x PCR DIG mix, 1 µL of 20 µM primers (forward and reverse, see table 1), a final concentration of 2.6.5 U of Expand high fidelity enzyme mix and sterile re-purified water in a total volume of 49 µL. Control reactions were prepared in parallel, consisting of the same PCR reaction mix, with 5 µL 10x dNTP stock solution instead of PCR DIG mix. Template DNA (1 µL) was then added to the probe mix and to the DIG-control mix, and amplified in the PX2 Thermal Cycler (section 2.5.3). The results were analysed by gel electrophoresis (section 2.5.5). The product size of the template probe was compared to the probe control to confirm the incorporation of DIG in the probe template DNA.

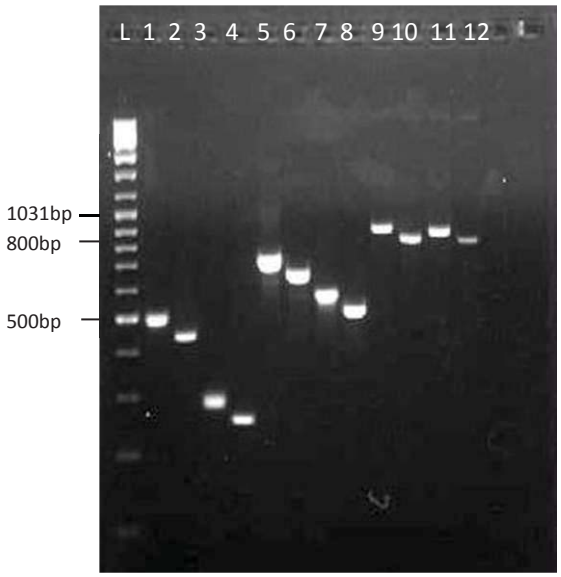

Figure 1 Gel electrophoresis photo showing the incorporation of DIG-11-dUTP into PCR products. Lane L: Gene Ruler DNA ladder (Fermentas Life Sciences)Lanes 1: EHV-2 without DIG-11-dUTP (Control) Lane 2: EHV-2 with DIG-11-dUTP Lane 3: EHV-5 control Lane 4: EHV-5 with DIG-11-dUTP Lane 5: EHV-1 control Lane 6: EHV-1 with DIG-11-dUTP Lane 7: EHV-4 control Lane 8: EHV-4 with DIG-11-dUTP Lane 9: ERAV control Lane 10: ERAV with DIG-11-dUTP Lane 11: ERBV control Lane 12 ERBV with DIG-11-dUTP.

2.4.3 Sequencing of probe controls

The bands obtained in continual reactions during preparation of DIG-labelled probes (section 2.5.2), were cut from the gel using a clean sterile blade under UV light. The gel fragments were placed in a Quantum prep 'freeze n squeeze' gel extraction spin column (Biorad) and snap frozen in liquid nitrogen. The tubes were then warmed lightly and spun at 13,000 rcf for 5 minutes. The DNA was collected at the bottom of the tube with the agarose retained in the filter. An aliquot (7µL) of eluted DNA was mixed with 3.2 pmol of one of the primers used for initial amplification in the final volume of 15 µL and submitted for sequencing to the Massey University Genome service. The sequences were compared to the sequences available in the public databases using BLAST algorithms (NCBI).

2.4.4 Hybridisation with specific probes

Hybridisation was carried out using the DIG High Prime Labelling and Detection starter Kit II (Roche Applied Science) according to manufacturer's instructions. DIG Easy Hyb working solution was preheated and 10 ml/100 cm^2 of membrane was used

for pre-hybridization of the membrane for 30minutes at the appropriate hybridisation temperature (Table 2). The appropriate DIG labelled probe was denatured by heating for 5 minutes and cooled on ice before being added to preheated DIG Easy Hyb working solution at a concentration of 2 µl/ml. The pre-hybridization solution was poured off the membrane, replaced with probe/hybridization mixture and incubated overnight in hybridization bags in a water bath weighted down with a glass plate and gently agitated at the hybridization temperature (Table 2).

2.4.5 Optimization of hybridization conditions

The starting hybridisation temperatures were calculated based on the formula in the manufacturer's instructions (Roche).

$T_m = 49.82 + 0.41 (\%G+C) – (600/L)$

$T_{opt} = T_m – 20\text{-}25°C$

The calculated hybridization temperatures were used as a starting point for optimization. The hybridisation temperature was increased from the calculated hybridization temperature until a clear signal from the virus specific product was observed with minimal cross reaction with a similar virus (EHV-1 and EHV-4, EHV-2 and EHV-5, ERAV and ERBV) and with equine DNA. Table 2 shows the optimised temperatures used for individual viruses for detection by dot blot hybridisation.

Table 2 Hybridization temperatures for virus specific DIG Labelled DNA Probes with virus specific PCR membranes

Virus	Optimised Hybridization Temperature
EHV-1	57°C
EHV-4	59°C
EHV-2	55°C
EHV-5	55°C
ERAV	52°C
ERBV	51°C

Table 3 A layout of a dot blot used for optimisation of the hybridisation temperatures for EHV-2 and EHV-5 probes. Results expected from hybridisation with the EHV-5 probe are shown.

EHV-5 Probe					
EHV-5 primers + EHV-5 template		EHV-5 primers + EHV-2 template		Equine DNA	
EHV-2 Primers + EHV-5 template		EHV-2 primers + EHV-2 template		Equine DNA	

* Black indicating the expected positive result

The dot blots for optimisation of the hybridization temperatures for EHV-2 and EHV-5 probes were identical and included amplification products of PCR with either EHV-2 or EHV-5 primers, each with either EHV-2 or EHV-5 template. In addition, 1 µl of equine DNA was also spotted on each membrane (Table 3). The layout of dot blots used for optimisation of the hybridisation temperatures for EHV-1 and EHV-4 probes is shown in Table 4.

Table 4 A layout of a dot blot used for optimisation of the hybridisation temperatures for EHV-1 and EHV-4 probes. Results expected with the EHV-1 probe are shown.

EHV-1 Probe					
EHV-1/4 primers + EHV-1 template		EHV-1/4 primers + EHV-4 template		Equine DNA	
EHV-1 Primers + EHV-1 template		EHV-1 primers + EHV-4 template		Equine DNA	
EHV-4 primers + EHV-1 template		EHV-4 primers + EHV-4 template		Equine DNA	

* Black indicating the expected positive result

2.4.6 Detection of DIG labelled probes

Probe/ Hybridization mixture was poured off and the membrane washed twice, in 2x SSC and 0.1% SDS at room temperature for 5 minutes. The membrane was then washed twice for 15 minutes in 0.5x SSC and 0.1% SDS at 65°C under constant agitation. The membrane was rinsed briefly in washing buffer and incubated for 30 minutes in blocking solution consisting of 10x blocking reagent (Roche) diluted 1:10

with Maleic acid buffer. Anti-Dioxigenin-AP (75m U/mL) was centrifuged for 5 minutes at 10,000 rpm and diluted 1:10,000 in blocking solution. The membrane was then incubated in 20 mL of diluted Anti-Dioxigenin-AP for 30 minutes. After washing twice for 15 minutes in 100 mL of washing buffer (0.1 M Maliec acid, 0.15 M NaCl; pH 7.5; 0.3% Tween 20)the membrane was equilibrated in 20 mL of detection buffer (0.1 M Tris-HCl, 0.1 M NaCl; pH 9.5) for 5 minutes. The membrane was then placed DNA face side up in a hybridisation bag and 1 mL of CSPD, was applied. After 5 minutes incubation in the dark, the membrane was exposed to the x-ray film for 25 minutes and developed using an automated x-ray film developer.

2.4.7 Re-probing

The membrane was rinsed thoroughly in double distilled water and washed twice for 15 minutes at 37°C in 0.2M NaOH containing 0.1% SDS to remove the DIG-labelled probe. The membrane was then rinsed thoroughly for 5 minutes in 2x SSC. The membrane was stored in 2 x SSC solutions and was not allowed to dry before re-probing. Re-probing was completed as described in section 2.5.4.

2.5 Statistical analysis

Probabilities were calculated using Fishers exact test. The associations show whether there was significant differences between virus positive results for the horses with respiratory disease compared to healthy horses. Results were regarded as significant if p value was ≤0.05. For calculating associations between viral infections and disease, the results of PCR and virus isolation were used in parallel.

Chapter 3 Results

3.1 Horses sampled

Counts of horses sampled stratified by age, sex and breed is shown in Table 5. Signalment data for all the horses sampled is included in Appendixes 1a and 2.

Table 5 Number (%) of horses classified as healthy or affected by respiratory disease, according to age, sex and breed, from a sample of 85 horses in the lower North Island of New Zealand

Variable	Level	Health status		P-value[3]
		Diseased[1] (*n* =52)	Healthy[2] (*n* =33)	
Age (years)	0-3	29 (56%)	7 (21%)	0.0018
	>3	23 (44%)	26 (79%)	
Sex	Female	24 (46%)	21 (64%)	0.1262
	Male	28 (54%)	12 (36%)	
Breed	Thoroughbred	48 (92%)	18 (55%)	0.0009
	Standardbred	1 (2%)	12 (36%)	
	Other	3 (6%)	3 (9%)	

1. Diseased- Horses sampled with at least one of the following; nasal discharge, ocular discharge, cough, fever, in-appetence, poor performance (as assessed by the trainer/owner)
2. Healthy- Horses sampled without any of the following; nasal discharge, ocular discharge, cough, fever, in-appetence, poor performance (as assessed by the trainer/owner)
3. P-value- The difference between the two levels for each variable; age, sex and Breed

Age distribution

The age ranges of healthy horses and horses with respiratory disease were similar, although the age distribution differed between the two age groups. Horses with respiratory disease ranged from 0-20 years of age with a median age of 3 years. Healthy horses ranged from 0-24 years of age with a median age of 7 years. More than half of the horses with respiratory disease were 3-years-old and younger, while more than 75% of healthy horses were older than 3 years of age. The most common

age of horses sampled in the healthy group was 6 years old (18%), closely followed by 1-year-olds (15%). The proportion of diseased horses was significantly greater in the 0-3 year old group than in the >3 year old group (P value 0.0018) (Table5).

Sex distribution

There was an even distribution of female and male horses sampled with respiratory disease, 46% and 54% respectively. In the healthy group 64% of horses sampled were female, while only 36% were male. However, this difference was found not to be significant (p value 0.1262) (Table 5).

Breed distribution

Thoroughbreds comprised the majority of sampled horses in both groups (92% and 55% for diseased and healthy horses, respectively). More healthy horses sampled were of Standardbred breed than diseased horses (12 and 1 respectively) and only 8% of horses sampled overall were of other breeds. There was a significant difference between the proportion of healthy horses and horses with respiratory disease for the different breed groups (P value 0.0007) (Table 5).

3.2 Detection of viruses in nasal swabs

Nasal swab samples from 52 horses with respiratory disease and 33 healthy horses were tested by virus isolation and species specific PCR. Results obtained with both tests are presented in sections 3.2.1 and 3.2.2, respectively. Data from testing are presented in Appendix 1a and 1b.

3.2.1 Virus isolation

Virus isolation was performed in cell culture on primary EFK cells and two continuous cell lines, Vero and RK13. The only viruses isolated were herpesviruses. All of the cell lysates from cultures that showed CPE tested positive for one or more of the equine herpesviruses. A total of 39 virus isolates were obtained from 22/85 (26%) horses sampled. Equine herpesvirus type 2 was the most commonly isolated virus. Of the 85 horses sampled, 12 (14%) were positive for EHV-2, 11 (13%) for EHV-4, 8 (9%) for

EHV-5 and 2 (2%) for EHV-1. No viruses were isolated from the 33 healthy horses sampled.

Multiple viruses

Most (74%) of the 85 horses sampled were virus negative by virus isolation including; all of the 33 healthy horses and over half (58%) of the 52 horses with respiratory disease. A single virus was isolated from 11 of the 52 horses with respiratory disease and 2 viruses were also isolated from a further 11 of 52 horses with respiratory disease. Figure 2 shows the number of horses positive for different combinations of herpesviruses. Three herpesviruses were never isolated from the same horse by virus isolation.

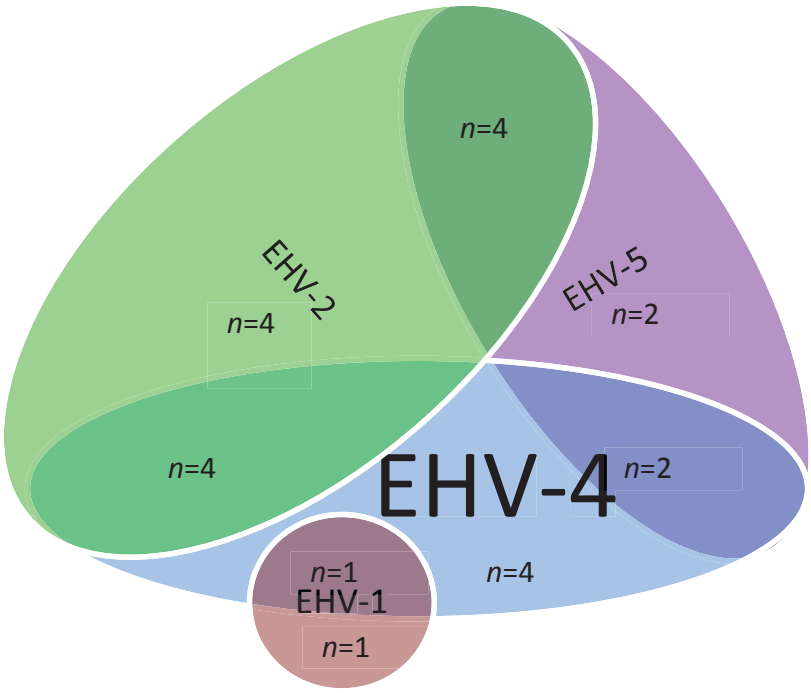

Figure 2 Schematic representation of the number of horses positive for equine herpesviruses by virus isolation. The coloured shapes represent different equine herpesviruses: EHV-2 (green), EHV-5 (purple), EHV-4 (blue) and EHV-1 (pink).

3.2.2 PCR performed directly on nasal swabs

The only viruses identified in nasal swab samples by PCR were herpesviruses. A total of 39/85 (46%) horses were positive for at least one virus species. Of these, 26 (31%) were positive for EHV-5, 23 (27%) for EHV-2, 14 (16%) for EHV-4 and 3 (4%) for EHV-

1. Only 2/33 (6%) healthy horses tested positive for viruses by PCR – 1 horse was positive for both EHV-2 and EHV-5 and another for EHV-2 only.

Multiple viruses

From the 85 horses sampled, 18 were positive for a single virus species and 18 were positive for 2 virus species. Also, there were 3 viruses detected in 5 of the 85 horses sampled. Figure 3 shows various combinations of viruses detected in nasal swabs of the 39 virus-positive horses. Equine herpesvirus type 5 was often detected in the same horse as EHV-2 (n=11) or EHV-4 (n=6). Equine herpesvirus 1 was never detected on its own, but either concurrently with EHV-4, or both EHV-4 and EHV-5. Equine herpesvirus 1 was never detected from the same horse as EHV-2.

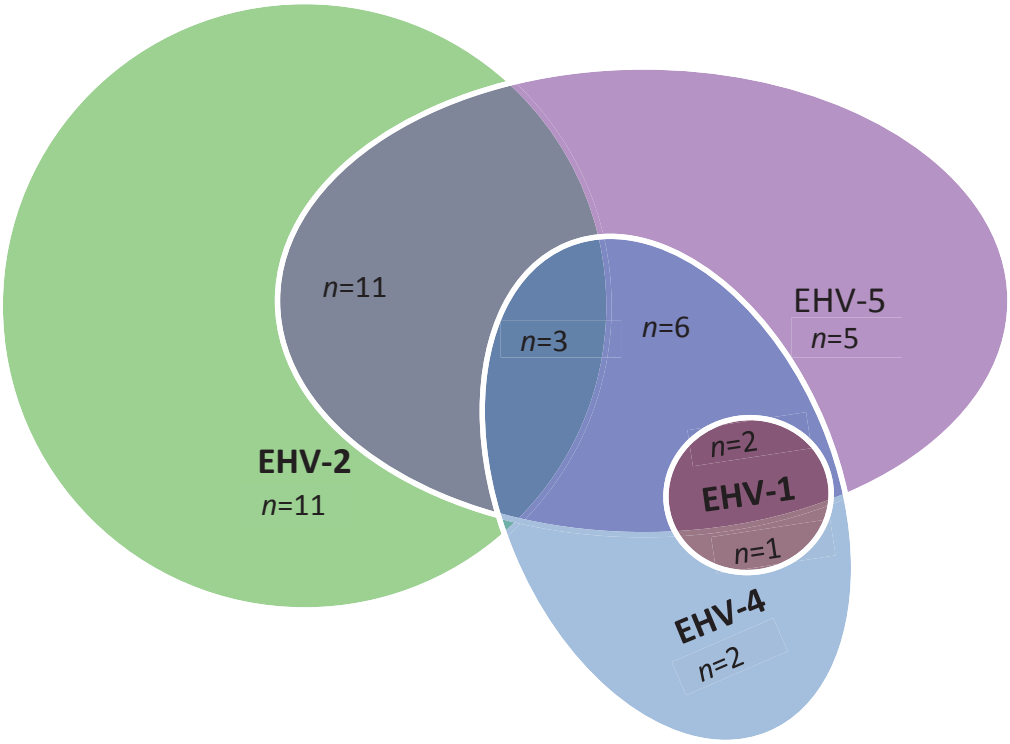

Figure 3 Schematic representation of the number of horses positive for equine herpesviruses by PCR directly on nasal swabs. The coloured shapes represent different equine herpesviruses: EHV-2 (green), EHV-5 (purple), EHV-4 (blue) and EHV-1h (pink).

3.2.3 Virus isolation and Species specific PCR results combined

Overall, when both the results of nasal swab PCR and virus isolation were considered together, a total of 43 (51%) horses sampled were positive for at least one virus.

Infection with EHV-5 was most common (28 horses), followed by EHV-2 (27 horses), EHV-4 (21 horses,) and EHV-1 (3 horses). A total of 41 (79%) of the 52 horses with respiratory disease were positive for at least one virus species. Only 2/33 (6%) healthy horses tested positive for at least one virus species.

Multiple viruses

Of the 85 horses sampled 14 horses were positive for a single herpesvirus, 22 horses were positive for 2 different herpesviruses and 7 horses were positive for 3 different herpesviruses. Figure 4 shows various combinations of virus species detected in nasal swabs of these 43 virus-positive horses. Neither EHV-4 nor EHV-5 were detected on their own; EHV-5 was detected concurrently with either EHV-2 (11 horses), EHV-4 (8 horses) or both EHV-2 and EHV-4 (5 horses), while EHV-1 was detected concurrently with either EHV-4 (1 horse), or EHV-5 and EHV-4 (2 horses). Equine herpesvirus 1 was never detected in the same horse as EHV-2.

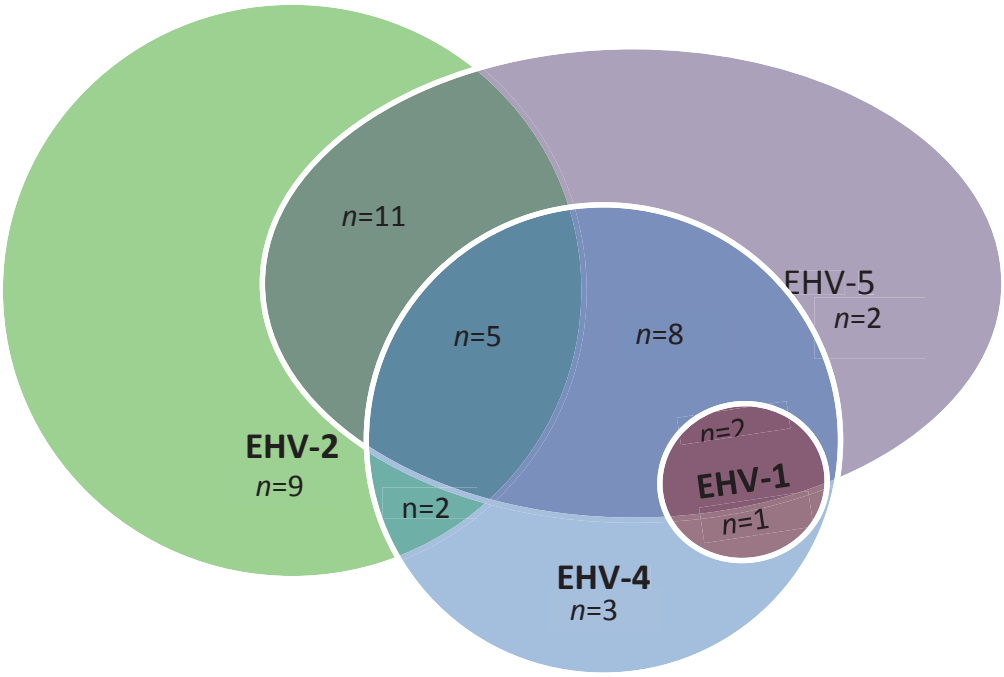

Figure 4 A Schematic representation of the number of horses positive for equine herpesviruses when combined results of virus isolation and PCR were considered. The coloured shapes represent different equine herpesviruses: EHV-2 (green), EHV-5 (purple), EHV-4 (blue) and EHV-1 (pink).

3.3 Comparison between PCR and virus isolation

A comparison between the results of virus isolation on three different cell types (EFK, Vero and RK13 cells) and species specific PCR directly on nasal swabs is presented in table 6 and sections 3.3.1 and 3.3.2.

3.3.1 Virus isolation in EFK, Vero and RK13 cells

Table 6 Virus isolation versus species specific PCR for the detection of herpesviruses from a sample of 85 horses in the lower North Island of New Zealand

Method of detection		**Number of virus positive horses (%)**			
		EHV-1 (n=3)	**EHV-2 (n=27)**	**EHV-4 (n=21)**	**EHV-5 (n=28)**
Virus isolation[1]		2 (66%)	12 (44%)	11 (52%)	8 (29%)
Viral isolates					
Cell line	*EFK*	*1 (33%)*	*9 (33%)*	*11 (52%)*	*4 (14%)*
	Vero	*2 (66%)*	*4 (15%)*	*1 (9%)*	*6 (21%)*
	RK13	*1 (33%)*	*0*	*0*	*0*
Species specific PCR[2]		3 (100%)	25 (93%)	14 (66%)	27 (96%)
Kappa level of agreement[4]		0.794	0.432	0.205	0.298

[1]Samples were regarded as positive based on the presence of viral CPE in cell culture. The identity of isolated viruses was determined by species specific PCR.
[2] Samples were regarded as positive if a band of the expected size was visible on a gel and the identity of the product was confirmed by dot-blot hybridization with the virus-specific probe or when dot-blot hybridization was positive, even if the corresponding band was not visible on the gel.
[3] Results of PCR and virus isolation were considered in parallel.
[4] Kappa-The level of agreement between PCR and virus isolation results in the detection of herpesviruses from 85 horses.

Overall, the majority of viruses (25/38) were isolated on EFK primary cells, followed by Vero (13/38) and RK13 (1/38) cells. Only one isolate of EHV-1 was detected in RK13 cells. Equine herpesvirus 1 caused CPE on day one in the first passage in all three cell types. On EFK cells EHV-2 was isolated in passage 1 for 5/9 isolates and on Vero cells 2/4 isolates in passage 2. Similarly CPE was observed for 3/4 EHV-5 isolates

in passage 1 on EFK cells and 3/6 in passage 2 on Vero cells. Equid herpesvirus 4 was only isolated on EFK cells, except for 1 isolate on Vero cells. Cytopathic effect was observed in the first passage for 8/11 isolates, including the isolate on Vero cells. Equine herpesvirus type 1 was never isolated from the same sample as either EHV-2 or EHV-5. However EHV-1 was isolated from the same sample as EHV-4 on EKF cells. Equine herpesvirus 4 and EHV-2 were isolated from the same sample on three occasions on EFK cells and EHV-4 and EHV-5 were isolated from the same sample once on EFK cells. Only EHV-2 and EHV-5 were isolated from the same sample on both EFK and Vero cells.

Equine herpesvirus 1 CPE was fast spreading with cell detachment occurring only hours after the initial plaque formation. Large plaques surrounded by refractive cells were observed on EFK cells, cell rounding and syncytia formation was observed on Vero cells and RK13 cells. The CPE observed on EFK cells from infection with EHV-4was similar to that observed for EHV-1. Equid herpesvirus 2 CPE was slow spreading and could be seen as small plaques of clustered round cells with increased refractivity which eventually detached in both EFK cells and Vero cells. Equine herpesvirus type 5 CPE looked much the same as EHV-2 CPE except that the progression of CPE appeared to develop more slowly.

3.3.2 Gel electrophoresis and Dot Blot Hybridisation

Gel electrophoresis and dot blot hybridisation with DIG-labelled virus-specific probes was used for the detection of herpesvirus-specific PCR products. All of the gel-positive samples were confirmed to represent expected herpesviruses by dot blot hybridisations with virus-specific probes. In addition, 10 gel-negative samples tested positive by dot blot hybridisation including 2 EHV-1, 4 EHV-2 and 4 EHV-5 PCR products.

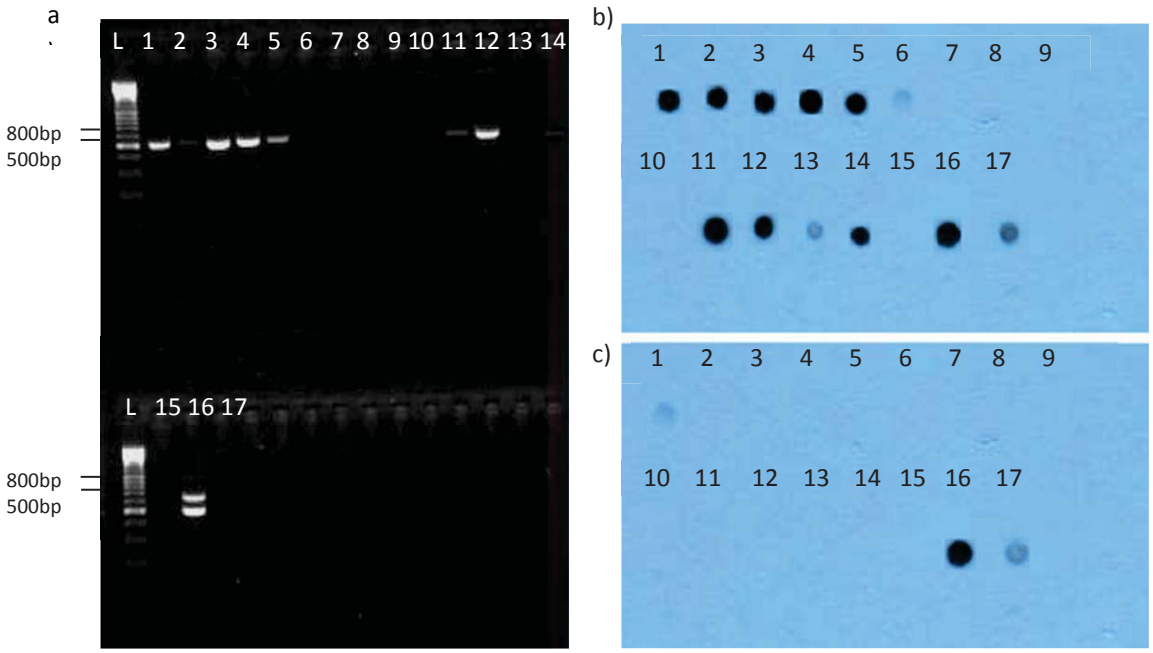

Figure 5: An example of amplification products from a PCR with EHV-1/4 primers visualised on 1.25% ethidium bromide stained gel (a) and a corresponding dot blot probed with either EHV-1 probe (b) or EHV-4 probe (c). Lane L: Gene Ruler DNA ladder (fermentas Life Sciences); Lanes 1-15: CPE positive cell lysates; Lane 16: EHV-1/4 positive control; Lane 17: Negative control (equine DNA). Expected products: EHV-1 649bp, EHV-4 507bp. Cell lysates 1-5, 11, 12 &14 are positive for EHV-4 and none are positive for EHV-1 (note bands 2, 11 & 14 are very weak, but are clearly positive on the dot blot). Dots with less or the same intensity as the negative control were considered negative.

3.3.3 Species specific PCR versus Virus isolation

Overall, correlation between results obtained by virus isolation and by PCR was poor, with almost twice as many horses having at least one herpesvirus detected in nasal swabs by species specific PCR than by virus isolation (39/85 and 22/85, respectively). For EHV1 the strength of agreement between the two tests was considered to be good (kappa 0.794) while EHV2 had only moderate agreement (0.432) and EHV4 and EHV5 had fair agreement (0.205 and 0.298 respectively) Table 6.

3.4 Association between virus detection and disease

For the purpose of this analysis the results of PCR testing and virus isolation were considered in parallel. The association between herpesvirus infection and disease is shown in Table 7.

Table 7 Association between herpesviruses, identified by PCR directly on nasal swabs and virus isolation, and respiratory disease from a sample of 85 horses in the lower North Island of New Zealand

Identification of herpesviruses		Horses		P value[3]
		Diseased[1] *n* =52	Healthy[2] *n* =33	
At least one herpesvirus species		41 (79%)	2 (6%)	<0.0001
Only one herpes virus species		13 (25%)	1 (3%)	0.0072
Multiple herpesvirus species		28 (54%)	1 (3%)	<0.0001
Individual viruses	EHV-1	3 (6%)	0	-
	EHV-2	25 (48%)	2 (6%)	<0.0001
	EHV-4	21 (40%)	0	<0.0001
	EHV-5	27 (52%)	1 (3%)	<0.0001

1. Diseased- Horses sampled with at least one of the following; nasal discharge, ocular discharge, cough, fever, in-appetence, poor performance (as assessed by the trainer/owner)
2. Healthy- Horses sampled without any of the following; nasal discharge, ocular discharge, cough, fever, in-appetence, poor performance (as assessed by the trainer/owner)

* Diseased and healthy horses were not matched in this study

3. P value- Considered a significant association when value is <0.05 (calculated using fisher's exact test)

Overall the proportion of horses positive for at least one virus by PCR or virus isolation was significantly higher for those with respiratory disease as compared with healthy ones (p value <0.0001) (Table 7). When horses with only one species or more

than one species were considered the association with respiratory disease remained significant (p value 0.0072, <0.0001 respectively). For individual herpesviruses, the proportion of virus-positive horses was higher for horses with respiratory disease than for healthy horses for EHV-5 (p value < 0.0001), EHV-2 (p value <0.0001), and EHV-4 (p value <0.0001). The association between respiratory disease and the detection of EHV-1 was not calculated due to the small number of EHV-1 positive horses (Table 7). When only the horses sampled which were 3 years or younger were considered, (29 with respiratory disease and 7 healthy horses) the association between the detection of herpesviruses and respiratory disease remained significant (p value 0.0026).

Chapter 4 Discussion

4.1 Only Herpesviruses were detected

The only viruses detected were herpesviruses, including all four species known to infect horses (EHV-1, EHV-2, EHV-4 and EHV-5). All are large, enveloped DNA viruses that belong to the sub families *Gammaherpesvirinae* (EHV-2 and EHV-5) and *Alphaherpesvirinae* (EHV-1 and EHV-4) in the newly established order *Herpesvirales* (Davison et al., 2009).

In the current study the proportion of diseased horses infected with one or more of these virus species (79%) was, in general, higher than that reported by others (Dynon et al., 2007, Pusterla et al., 2011). In a study conducted in Australia, one or more respiratory viruses were detected in 60% of nasal swabs from 20 horses with respiratory disease, by either virus isolation or PCR (Dynon et al., 2007). The higher proportion of herpesviruses detected in the current study may be due to differences in the sample size, with 52 horses sampled in the current study compared to 20 horses in the study by Dynon et al., (2007). In a larger American-based study of 761 samples tested by PCR, only 21% of nasal secretions were positive for EHV1 or EHV4 (Pusterla et al., 2011). The difference in prevalence of herpesviruses between this study and the current study may be due to a combination of factors, including differing sample sizes, and the use of only PCR as the method of virus detection rather than both PCR and virus isolation. PCR and virus isolation as methods of detection for herpesviruses is discussed later in this chapter (section 4.5).

In addition, the study by (Pusterla et al., 2011) only investigated the prevalence of EHV-1 and EHV-4, while the current study also investigated the prevalence of EHV-2 and EHV-5 among the sampled horses. As such, the higher proportion of diseased horses with detectable herpesvirus species in the current study may be attributed to the sample size, the use of both PCR and virus isolation and the inclusion of all four herpesvirus species known to infect the respiratory tract of horses in the investigation.

Of the alpha-herpesviruses, EHV-4 was found to be more prevalent in the current study than EHV-1, with EHV-4 detected in 25% of horses, while EHV-1 was the least commonly detected virus in only 4% of horses. In agreement with this result, serological studies conducted on New Zealand horse populations have reported EHV-4 to be more prevalent than EHV-1, with up to 100% of horses having antibodies to EHV-4 and approximately 70% of horses with antibodies to EHV-1 (Dunowska et al., 2002a, Dunowska et al., 2002b, Donald, 1998). Similarly, a serological study conducted in Australia by Dynon et al., (2007) reported 100% of horses to be positive for EHV-4 antibody, while EHV-1 antibody was only detected in 24% of horses. In the current study virus infection was detected by the presence of the virus or viral DNA in the nasal cavity of horses at the time of sampling. In contrast, the serological studies mentioned above investigated 'exposure' to alpha-herpesviruses, which may have occurred at any stage previous to sampling. Therefore, when compared to serological studies, the lower prevalence observed for both EHV-4 and EHV-1 in the current study was expected. In addition, similar results were reported in a study by Diallo et al., (2008) using real time PCR. In this study, 25% of horses were positive for EHV-4, while none of the 12 horses sampled were positive for EHV-1. Given the difference in sample sizes between this study and the current study, the absence of EHV-1 from a sample population of 12 horses is not unexpected. Taken together, the results of the current study are in agreement with the results of past New Zealand based studies and indicate that EHV-4 has a higher prevalence than EHV-1 among New Zealand horse populations.

Gammaherpesviruses EHV-2 and EHV-5 are often the most commonly detected herpesviruses from the horses' respiratory tract, with some authors reporting a higher prevalence of EHV-5 than EHV-2 (Craig et al., 2004, Ataseven et al., 2010b, Wang et al., 2007) and others reporting the reverse (Fu et al., 1986, Reubel et al., 1995, Murray et al., 1996, Dunowska et al., 2002b). In the current study the prevalence of EHV-5 (33%) was similar to that of EHV-2 (32%). In a previous New Zealand based study (Dunowska et al., 2002b), EHV-2 (20%) was isolated more often than EHV-5 (7%) from the nasal swabs of 82 horses. However, the higher prevalence of EHV-5 observed in the current study may be explained firstly by the differing age

of horse populations tested, as studies have shown young horses to be infected with EHV-2 earlier in life than EHV-5 (Dunowska et al., 2011, Dunowska et al., 2002a, Nordengrahn et al., 2002, Murray et al., 1996, Bell et al., 2006a). In the study by Dunowska et al (2002b) 78% of horses were 1 year or younger compared to 26% of horses in the current study. Secondly the current study used both virus isolation and PCR to detect viruses compared to only virus isolation. In the current study when only viral isolation is considered EHV-2 was isolated from the nasal swab samples of 14% of the 85 horses sampled, while EHV-5 from only 9%, suggesting the difference in results may in fact be due to the differing testing methods. Finally, one further difference in these studies is the number of sampling occasions, where the study by (Dunowska et al., 2002b) sampled horses on two occasions compared to only a single sampling occasion in the current study. However a higher prevalence of herpesviruses would be expected through multiple sampling compared to single sampling. This was not the case even when only virus isolation was considered, suggesting multiple sampling was not the cause of differing results in this example. Overall, the prevalence of EHV2 and EHV5 in horses of this study differed to other New Zealand studies. This suggests further research using multiple detection techniques is required to identify the actual prevalence of these viruses in New Zealand horse populations.

Equid herpesvirus 2 and EHV-5 are often detected along with other equine respiratory viruses leading to the idea that EHV-2 and EHV-5 may predispose or reactivate infection with other viruses (Murray et al., 1996, Dunowska et al., 2002a, Purewal et al., 1992, Borchers et al., 1997, Nordengrahn et al., 2002, Wang et al., 2007). It has been postulated that EHV-2 (and possibly EHV-5) can do this by exerting its effects on the host indirectly, through interactions with the immune system, as herpesviruses are known for their ability to modulate the immune responses of their hosts (reviewed by Van der Meulen et al., 2006). Virus-induced modification of the normal immune responses may result in immunosuppression, and subsequently predisposition to infection with other pathogens. Examination of the EHV-2 genome has shown that it encodes a number of genes potentially involved in down-regulation of the host immune responses, including an interleukin-10 homologue

(Telford et al., 1993) and three proteins with some similarity to chemokine receptors (Sharp et al., 2007, Camarda et al., 1999). Infection with EHV-2 was shown to down-regulate the expression of monocyte-chemoattractant protein 1 *in vitro* (Dunowska et al., 2001). These findings suggest that EHV-2 may modulate the immune response by interfering with the chemokine environment. Little information is currently available with regard to the genomic repertoire of EHV-5 and the potential of this virus to interact with the immune system of the horse. Similar to other gammaherpesviruses, EHV-2 establishes latency in lymphocytes (Drummer et al., 1996). The site for EHV-5 latency has not been established, but similarly to EHV-2 the virus has been isolated by co-cultivation from equine peripheral blood leucocytes (Dunowska et al., 1999) . Infection of cells directly involved in the immune responses has a clear potential to modulate such responses. Other members of the *Herpesviridae* family also encode homologues of cellular chemokines. Equid herpesvirus 1 and 4 have a homologue of glycoprotein G (gG) (Drummer et al., 1998). Glycoprotein G is unique to the Alphaherpesviruses and function to sequester chemokines and to neutralize their effects by blocking chemokine- receptor interactions and the activation of cell migration (Bryant et al., 2003). No chemokine binding has been associated with EHV-4 secreted gG yet, despite its 72% homology to EHV-1 gG (Bryant et al., 2003).

In the current study, the detection of EHV-2 and EHV-5 from horses simultaneously infected with EHV-4 is suggestive of a predisposing role for EHV-2 and EHV-5 in infection with other herpesviruses. In further support, the isolation of EHV-2 was found to always precede the isolation of EHV-1 and EHV-4 when co-cultivated from lymphoid tissues of 5 experimentally infected horses through intranasal injection (Welch et al., 1992a). Furthermore Purewal and others found that EHV-2 was able to trans-activate the intermediate early gene of EHV-1, concluding that infection with EHV-2 may reactive latent EHV-1 *in vivo* (Purewal et al., 1992). However, in the current study EHV-2 was never detected in the same horses as EHV-1. This may be explained by latent infection with EHV-2 in horses positive for EHV-1. Also samples were only taken on one occasion, so prior lytic infection with EHV-2 in horses positive for EHV-1 or future lytic infection with EHV-1 following horses positive for

EHV-2 is unknown. As such EHV-2 cannot be ruled out as a contributing factor for the activation of EHV-1.

4.2 Association between individual herpesviruses and respiratory disease

In the current study convenience sampling was used, as it is difficult to obtain a sample population for a careful epidemiological approach when researching high value animals, for example horses. As such, horses were provided for sampling by the good will of private owners. This use of convenience sampling adds a major limitation to the conclusions which can be drawn by the results of this study. Therefore, the associations made between equine herpesviruses and equine respiratory disease, discussed in this section, is relative to only horses within the sample population. Any extrapolation of these results to New Zealand horse populations made in the following section has been considered alongside biological plausibility and the results of other similar studies.

Equine herpesviruses have been detected at higher frequency from diseased horses as compared to healthy horses in several studies (Murray et al., 1996, Ruszczyk et al., 2004, Schlocker et al., 1995, Fortier et al., 2009b, Borchers et al., 1997). Of the alpha herpesviruses, EHV-1 has been demonstrated to cause respiratory tract diseases, particularly in young horses and foals (Allen and Bryans, 1986, Wood et al., 2005b, Burrell et al., 1996, Morley et al., 1996), and EHV-4 has been shown to be significantly more likely to be detected in samples from foals with marked serous or mucopurulent nasal discharge than from healthy foals (Gilkerson et al., 1994, Foote et al., 2004). Similarly, in the current study EHV-4 was detected significantly more often from horses with respiratory disease than from healthy horses (p value <0.0001), while EHV-1 was detected in only three horses with respiratory disease, but was absent among healthy horses. However due to the low sample size of this study, an association between EHV-1 detection and the presence of respiratory disease could not be made. In agreement with these results, in a study conducted in USA, EHV-4 was found to be associated with respiratory disease in a cohort of mares and their foals (p value 0.005), while EHV-1 was not detected from either diseased or healthy horses. Although the detection of EHV-1 and EHV-4 from apparently healthy

foals and horses has been described (Gilkerson et al., 1994, Marenzoni et al., 2008, Slater, 2007), the detection of EHV-1 and EHV-4 either alone or in combination with EHV-2 and/or EHV-5, from horses with respiratory disease and not from healthy horses suggests that these viruses, in particular EHV-4, were involved in development of respiratory disease among the horses sampled.

Of the gamma-herpesviruses, EHV-2 has been often associated with respiratory disease. In a study by Fortier (2009b) the detection of EHV-2 by consensus PCR in trans-tracheal wash samples collected from poor performing horses was significantly associated with disease (p value 0.0006). Similarly, in a previous New Zealand based study only EHV-2 was found to be associated with the development of respiratory disease among yearlings returning from the sales (p value 0.017) (Dunowska et al., 2002b). In the current study the proportion of EHV-2 (p value <0.0001) detected in nasal swabs was significantly higher for horses with respiratory disease than for healthy horses. Equine herpesvirus 5 has also been detected in both horses with respiratory disease and healthy horses, resulting in uncertainty in the pathogenic role of this virus (Bell et al., 2006a, Fortier et al., 2009a, Nordengrahn et al., 2002, Wang et al., 2007). However infection with EHV-5 has been suggested to predispose to secondary *Rhodococcus equi* infection (Nordengrahn et al., 1996). In addition, EHV-5 was isolated from cases of equine multinodular pulmonary fibrosis, a novel pathological entity with characteristic gross and histopathological pulmonary lesions which results in low grade fever, weight loss, and gradual exercise intolerance (Williams et al., 2007). In the current study EHV-5 was detected in diseased horses significantly more often than in healthy horses (p value <0.0001). Although, these results cannot be used to infer a causal relationship between equine herpesviruses and respiratory disease due to the use of convenience of sampling, they do suggest an aetiological involvement of EHV-2, 4 and 5 in the development of respiratory disease.

In contrast, some studies have reported no association between equine herpesviruses and equine respiratory disease (Wood et al., 2005a, Christley et al., 2001b, Newton et al., 2003, Ryu et al., 2009). Instead an association between inflammatory airway disease (IAD) and bacterial infections was reported. However,

these studies either did not consider viral causes (Ryu et al., 2009) or investigated only the role of alpha-herpesviruses EHV-1 and EHV-4, but not gamma-herpesviruses EHV-2 and 5 (Wood et al., 2005a, Christley et al., 2001b, Newton et al., 2003).

In many studies where the role of EHV-2 and EHV-5 infection was considered, samples collected from both diseased and clinically normal horses were found positive for these viruses, suggesting that EHV-2 or EHV-5 may in fact not have a causal relationship with respiratory disease (Torfason et al., 2008, Bell et al., 2006a, Dunowska et al., 2002b, Dunowska et al., 2002a, Browning and Studdert, 1987a). Alternatively, the presence of herpesviruses in both horses with and without disease may be explained by infection with different strains, resulting in varying signs of disease. Equine gamma-herpesviruses, particularly EHV-2, have been shown to have significant genetic heterogeneity between different isolates (Browning and Studdert, 1987b, Holloway et al., 2000, Dunowska et al., 2000). As such, differing biological properties between different heterogeneous groups of gamma herpesviruses could account for both the lack of an experimentally induced disease and the contradicting reports on the association of EHV-2 and EHV-5 in respiratory disease. However, a study by Brault et al., (2011) did not find any statistically significant association between respiratory disease and any specific EHV-2 genotype.

Other possible explanations for the presence of these herpesviruses in healthy horses include; detection of viruses during an incubation period or the shedding of herpesviruses from sub-clinically infected horses (Hanson, 1988). Large quantities of a virus will be shed before the onset of clinical signs (Six et al., 2001). For example EHV-1 has an incubation period of 3-10 days, during which virus is shed without the horse displaying clinical signs of disease (Harless and Pusterla, 2006) Some viruses may be continuously shed from a host in a carrier state without causing clinical signs of disease. For example; Equine arteritis virus (EAV) remains in stallions as a persistent infection with no signs of overt clinical disease. However, infected stallions are infectious to other horses when in contact with the stallion (Balasuriya et al., 2004). This ability of a carrier state is important to consider when investigating herpesviruses, as intermittent shedding occurs during the reactivation from the latent state without clinical signs of disease. Recurrent disease is common due to reactivation of latent infection, usually following stressful events, such as weaning,

transportation, castration and racing (Crabb and Studdert, 1996). Therefore the presence of gamma-herpesviruses in both horses with respiratory disease and apparently healthy horses does not necessarily suggest there is a non-causal relationship between these viruses and respiratory disease. As such EHV-2 and EHV-5 should still be considered when investigating equine respiratory disease.

To complicate the relationship between herpesviruses and respiratory disease further, more than one different virus species may be isolated from the same horse, giving rise to problems identifying the actual agent responsible for the disease (Dynon et al., 2007). A study in 1998 determined experimentally infecting horses with EHV-2 produced either no signs of respiratory disease or only mild respiratory signs (Borchers et al., 1998). This result may provide evidence against EHV-2 in disease causation or perhaps just highlights the difficulty in reproducing a multivariable disease experimentally. Multifactorial infections with other invading viruses, bacteria or fungi may be necessary for development of respiratory disease (Banbura et al., 2004, Isenburg, 1988). In the current study, mixed infections with EHV-4 and EHV-2 or/and EHV-5 were common, suggesting that infection with multiple viruses may be necessary for the development of disease. Equid herpesvirus 2 is thought to play a role in respiratory tract diseases caused by some bacterial pathogens such as *Rhodococcus equi*, *Streptococcus equi* and *Streptococcus zooepidemicus* (Banbura et al., 2004, Nordengrahn et al., 1996, Jolly et al., 1986). In one study, vaccination with an EHV-2 subunit vaccine was found to confer protection against pneumonia following infection with *Rhodococcus equi*. As such, it was concluded that EHV-2 is a predisposing factor for *R. equi* invasion of the respiratory tract (Nordengrahn et al., 1996).

In addition, the severity of respiratory disease may be influenced by age, physical condition, environmental factors and the immune status of the horse (Nugent et al., 2006, Paillot et al., 2007). More acute clinical signs are often observed in younger horses, while older horses often only have mild or subclinical infection (Crabb and Studdert, 1996). Environmental factors, such as changes in weather, food and stabling differ between horse populations and can affect the likelihood of horses

developing respiratory disease. For example, the major difference between horse management in New Zealand and other countries, particularly countries in the northern hemisphere, is that New Zealand horses tend to spend more of the year at pasture. A study looking at endotoxin concentration differences between stables and pastures showed that the mean endotoxin concentration measured in the breathing zone of stabled horses is more than 8-fold higher than that of horses kept on pasture (Berndt et al., 2010). They concluded that it is likely that endotoxin exposure plays a role in airway inflammation of stabled horses. Therefore the management of horses can have a significant impact on the likelihood of horses developing disease regardless of a known infecting agent.

Another major challenge in establishing disease causation lies in varying definitions of respiratory disease and detection methods used to identify potential causal agents in different studies. The variable nature of equine respiratory disease represents a significant problem in identifying disease causation. Respiratory disease in this study has been defined as a horse showing one or more of the following clinical signs: coughing, nasal or ocular discharge, in-appetence, fever or poor performance as subjectively assessed by their trainers or owner. Some studies have similar definitions to the current study (Mumford et al., 1998, Ruszczyk et al., 2004, Mumford and Rossdale, 1980, Pusterla et al., 2011, Bell et al., 2006a, Dunowska et al., 2002b, Carman et al., 1997), while others only look at specific clinical signs such as coughing (Christley et al., 2001b, Diaz-Mendez et al., 2010) or nasal discharge and fever (Dynon et al., 2007). Defining horses as affected by respiratory disease through observation of overt clinical signs increases the chances of incorrect classification in diseased and healthy categories. Studies have shown that many poor performing horses are affected by subclinical respiratory disease, which may not be obvious when horses are out of work (Burrell, 1985, Richard et al., 2010). Subsequently, viral infections identified in horses out of work may be incorrectly reported as not associated with disease. The detection of subclinical respiratory disease is not straight forward and requires the use of sophisticated and invasive diagnostic approaches in combination with clinical examination (Fraipont et al., 2011, Richard et al., 2010, Burrell, 1985, Sweeney et al., 1992). The current study relied on the observation of overt clinical signs of respiratory disease alone, without the aid of

endoscopic examination. However, one of the defining criteria for the placement of a horse in the diseased group was “poor performance”. While this is a subjective observation and only applies to horses currently in training, it extends the definition of disease through the placement of horses with very mild signs of respiratory disease in the diseased, rather than healthy, category.

Koch’s postulates regarding disease causation by a potential pathogen were released in 1884 and subsequently revised by virologist Thomas Rivers in 1937 to reflect the biology of viruses, which, as obligate intracellular parasites, cannot be isolated in pure culture (Rivers, 1937). In 1976 these guidelines to disease causation were further refined (Evans, 1976). More recently (Fredricks and Relman, 1996) applied these guidelines to sequence-based microbe discovery. Firstly, a nucleic acid sequence belonging to a putative pathogen should be present in most cases of an infectious disease and fewer, or no, copy numbers of pathogen-associated nucleic acid sequences should occur in hosts or tissues without disease, as was the case for herpesviruses in the current study. Secondly, with resolution of disease, the copy number of pathogen-associated nucleic acid sequences should decrease or become undetectable and when sequence detection predates disease, or sequence copy number correlates with severity of disease or pathology, the sequence-disease association is more likely to be a causal relationship. However, due to a single sampling occasion in the current study and the unknown copy number of viruses detected by using PCR and not sequencing, this guideline for causation was not met. Thirdly, the nature of the microorganism inferred from the available sequence should be consistent with the known biological characteristics of that group of organisms and tissue-sequence correlates should be sought at the cellular level and finally, these sequence-based forms of evidence for microbial causation should be reproducible. The third and final of these guidelines is consistent with herpesviruses as causative agents of respiratory disease.

There are numerous challenges in proving viruses as the etiologic causes of specific syndromes, including but not limited to: prolonged viral shedding after acute illness; latent infection and asymptomatic shedding; clinical disease in a minority of infected

individuals; recurrent symptomatic or asymptomatic infection and variable clinical presentation of disease. As such, the identification and recognition of viruses being the cause of a variable disease, such as equine respiratory disease still remains to be a substantial challenge for virologists today.

4.3 Comparison between PCR and virus isolation results

In the current study, correlation between results obtained by virus isolation and by PCR was poor, particularly for EHV-4. The poor correlation between these two tests is unlikely to be due to cross contamination between samples during processing, because negative controls included in both tests were consistently negative. Possible explanations for the discrepancy between results obtained by the two tests include differences in the amount of the original swab sample used for testing (150 µL for virus isolation versus equivalent of 2 µL for PCR) or the ability of PCR to detect non-viable viruses. The former may explain isolation of viruses from PCR-negative samples, while the latter would account for lack of virus isolation from the PCR-positive samples. Methods to identify latent infections were not used in the current study, so it is most likely that some of the latent infections present among sampled horses were detected and active and inactive forms of a virus could not be distinguished (Delwart, 2007). A study by Allen (2006) found that 50% of horses with latent EHV-1 infection, identified through enrichment based sequence capture nested PCR, had a viral load below the detection threshold for non-enriched PCR techniques, suggesting the use of non-enriched PCR techniques is likely to underestimate of the prevalence of latent EHV infections (Allen, 2006). Even so twice as many of the 85 horses sampled had at least one herpesvirus detected by species specific PCR than virus isolation (39 and 22 respectively). Similarly, an Australian study which employed both of these tests reported 17 viruses detected by PCR, while only 4 viruses were isolated in cell culture (Dynon et al., 2007). This may be explained through the detection of non-viable virus by PCR, thereby overestimating prevalence of herpesvirus infection among horse populations. In combination with virus isolation, which without co-cultivation, can only detect viable virus, thereby underestimating the prevalence of herpesviruses due to latent and subclinical

infection. This overestimation in PCR and underestimation in viral isolation can lead to major discrepancies between the two methods results.

Time of sampling is important for the successful detection of respiratory viruses, in that samples need to be taken before or during the early stages of development of clinical signs. In the current study samples were taken from horses which may have been showing clinical signs of disease for any length of time. Therefore many viruses may have not been isolated as the samples could have been taken too late in the course of disease. Furthermore horses in the early stages of infection may display no clinical signs of disease or develop only subclinical disease, leading to the possible isolation of respiratory viruses in apparently healthy horses.

In addition non-viable viruses may have been present in the nasal swabs collected if sub-optimal sample transport or storage conditions had been employed. In the current study, commercial virus-transport media were used, which have been validated by the manufacturer to support viability of a range of viruses, including herpesviruses. Furthermore, the cold chain was maintained from sample collection through to inoculation of cells. As such, the conditions of sample collection and transportation were optimal for the recovery of viable viruses. The use of non-permissive cell lines or inappropriate culture conditions may provide another explanation for lack of isolation of viruses from PCR-positive samples. In the current study, virus isolation was performed using primary EFK cells and two continuous cell lines, Vero and RK13. These cells have been successfully used for isolation of equine respiratory viruses, including EHV-1, EHV-2, EHV-4 and EHV-5 in a number of previous studies (Dynon et al., 2007, Bell et al., 2006a, Reubel et al., 1995, Dunowska et al., 2002b). In addition, all four types of equine herpesviruses were isolated in the current study, suggesting that sample collection, transport, storage and culture conditions were appropriate for isolation of these viruses. Hence, it is unlikely that the discrepancies between virus isolation and PCR can be explained entirely by the inherent differences between the two tests.

Another explanation for the lack of isolation of viruses from PCR-positive samples is the possible existence of some interactions between different equine herpesviruses *in vitro*. This is particularly relevant for horses with multiple concurrent infections, which represented the majority of virus-infected horses in the current study. Several

studies support such a possibility. For example, only EHV-2 was isolated from a horse that had been experimentally infected with EHV-1, despite the fact that the same sample tested positive for EHV-1 and EHV-4, but not for EHV-2, by PCR (Welch et al., 1992b). However, both EHV-2 and EHV-1 were isolated from other horses in the same study. In another study, an apparent inhibitory effect of EHV-2 infection on the propagation of EHV-1 in equine dermis and lymphocyte cell cultures was reported (Dutta et al., 1986). The fact that EHV-1 was detected by PCR concurrently with EHV-5 in two horses in the current study, but either EHV-1 or EHV-5, not both, were grown from these samples in cell culture may support the existence of some inhibitory effects between equine gammaherpesviruses and EHV-1. The possibility of inhibition of *in-vitro* growth of EHV-1 by EHV-2/5 is contrary to the idea that the faster growing virus (EHV-1) would be likely to overgrow the slower growing ones (EHV-2/5). Furthermore, EHV-2 was reported to be capable of trans-activating immediate early genes of EHV-1 *in vitro*, suggesting that it may activate rather, than inhibit, EHV-1 replication (Purewal et al., 1992). Finally, Whalley *et al.* (2007)reported that EHV-4 can be grown in normally non-permissive cell line, if the cells are modified to express glycoprotein D of EHV-1, suggesting that the presence of both viruses in the same sample may extend the range of permissive cells for isolation of EHV-4. In the current study, EHV-4 was isolated with EHV-1 on Vero cells from one horse. Vero cells are normally non-permissive for EHV-4, so co-infection with EHV-1 may have allowed EHV-4 grow. Overall, the results of the above studies suggest that different herpesviruses are likely to interact with each other *in-vitro*, although the exact nature of such interactions, as well as the final outcome (inhibitory versus synergistic), remain to be elucidated.

To sum up, the discrepancies between PCR and virus isolation observed in the current study likely reflect a combination of several factors including inherent differences between the two tests employed, as well as some, as yet poorly defined, interactions between different herpesviruses during *in-vitro* growth. The fact that a majority of virus-positive horses in the current study were infected with multiple viruses underscores the possible importance of the latter, and may provide one explanation for the discrepancies between our results and the results of others, who employed both testing methods in their studies. For example, Diallo et al., (2008)

reported a good correlation between PCR and virus isolation results, but only 25% of the horses sampled in that study were positive for more than one virus.

An important implication from the above discussion is that testing results for some individual horses would have been different, had we used only one testing method. In addition, virus isolation results would have been markedly different if only one type of cells had been used. A single cell line is often used for virus isolation for financial reasons, with RK13 cells typically being used in diagnostic laboratories for isolation of equine respiratory viruses, particularly in cases where EHV-1 is of main concern (Sellon and Long, 2007). The RK13 cells in our study supported the growth of EHV-1 from only 1/3 PCR-positive samples. Also, none of the EHV-2/5 viruses were isolated in RK13 cells. This is in contrast to the previous New Zealand based studies, in which the majority of EHV-2 and EHV-5 isolates were obtained on RK13 cells (Dunowska, 1999). The reasons for the poor performance of the RK13 cells in the current study remain unclear. Possibilities include high passage number of cells (more than 100) or possible contamination of cells with non-cytopathic agents such as *Mycoplasma* species. The cells were not tested for *Mycoplasma* during the study, because they appeared healthy and grew well. In conclusion, the concurrent use of both PCR and virus isolation, including the use of multiple cell types for the latter, should be recommended for applications where detection of all infected animals (high sensitivity) is important.

4.4 The role of other viruses in equine respiratory disease

Equine rhinitis A virus, ERBV and EAdV were not isolated from samples collected in the current study despite serological evidence these viruses circulate New Zealand horses (Jolly et al., 1986, Dunowska et al., 2002b). This suggests that these viruses were not involved in development of respiratory disease in horses sampled in the current study. The absence of infection with these viruses may have been due to a relatively low number of horses (n=85) sampled within one geographical area. As such, the sample was not representative of the entire population of horses in New Zealand. However, in a previous New Zealand based study ERAV, ERBV and EAdV were not isolated despite serological evidence that these viruses were circulating

among the foals and horses sampled (Dunowska et al., 2002b, Dunowska et al., 2002a). Virus isolation alone cannot be considered a valid diagnostic tool for the detection of rhinitis viruses, due to the variable nature of these viruses in the development of CPE. In particular, ERBV is known to grow poorly in cell culture and some strains show infrequent or no CPE, thought to be due to the different serotypes of ERBV (ERBV1-3) having different cell culture requirements (Horsington et al., 2011, Mori et al., 2009). The detection of non-cytopathic viruses by virus isolation is both impractical and time consuming. Successful virus isolation often requires multiple blind passages and subsequent confirmation through additional techniques such as electron microscopy and immunofluorescence for non-cytopathic strains. In the current study only samples where CPE was visible in cell culture were analysed further to identify the infecting virus. As such infecting ERAV, ERBV and EAdV which did not cause CPE would have gone undetected. To account for non-cytopathic or non-culturable viruses all nasal swabs were tested for the presence of equine rhinitis viruses and adenoviruses DNA by virus-specific RT-PCR. Equine rhinitis viruses and equine adenoviruses were not detected in any of the samples by RT-PCR suggesting that horses were in fact not shedding these viruses at the time of sample collection.

4.5 Potential role of unknown pathogens

The large diversity of known viruses, other pathogens and non-infectious agents which are involved in the development of respiratory disease suggests there is potential for as yet unknown viruses which may also contribute to disease (Fortier et al., 2009b, Newton et al., 2003, Borchers et al., 1997, Christley et al., 2001b, Murray et al., 1996). The fact that many viruses are known to be involved in this disease, but not all cases have an identified cause leads to the idea of as yet undiscovered viruses being associated with equine respiratory disease. In the current study 13/52 horses with respiratory disease did not have a corresponding infection with any of the known respiratory viruses tested for by PCR and virus isolation. Similarly, in a study in Australia by Dynon et al., (2007), 8/20 horses with acute febrile upper respiratory disease that was clinically assessed to have a viral origin had no detectable virus by either virus isolation or PCR. While the absence of detectable viruses may be

attributable to incorrect timing of sample collection or other known causes of respiratory disease, such as bacteria or mycoplasmas, there is potential for unknown viruses to be involved in the development of respiratory disease as only known equine respiratory viruses were investigated.

Furthermore, while conventional methods for virus detection, such as virus isolation and PCR, have been used successfully for identifying new viruses, these methods have limitations regarding systematic virus discovery including; only being able to detect viruses which can replicate on a commonly used cell line or display clinical symptoms in the host that gives some information about the infecting virus. Many viruses cannot be amplified in cell culture, or will not exhibit characteristic cytopathic effects during their growth (Sellon and Long, 2007). PCR targeting conserved genetic regions can be used to detect variants of known viruses, but may not be able to detect more divergent or completely novel viruses for which no sequence data exists (Rose, 2005). Recently, the use of high-throughput sequencing technology has led to the identification of several novel respiratory viruses in humans, animals and plants including; parvovirus, metapneumoviruses, bocaviruses and polyomaviruses (Lau et al., 2008, Allander et al., 2001, van den Hoogen et al., 2001, Allander et al., 2005, Allander et al., 2007, Gaynor et al., 2007, Ng et al., 2011, Lau et al., 2011, Lauck et al., 2011) . The Novel human parvovirus, provisionally named human bocavirus, in a retrospective clinical study was associated with lower respiratory tract infections in children (Allander et al., 2005). Sequence independent single primer amplification was used in the discovery of two novel viruses, bovine parvoviruses 2 and 3, found to be frequent contaminants of commercial bovine serum (Allander et al., 2001). There is currently an absence of investigations into important equine diseases, respiratory or otherwise, utilizing metagenomic methods for the discovery of novel viruses which may or may not be involved in diseases, such as equine respiratory disease. Therefore, the probability of finding new viruses associated with multifactorial diseases, such as equine respiratory disease is high.

Chapter 5 Conclusion and future research

Taken together, the results of the current study indicate that equine herpesviruses including EHV-2, EHV-5 and EHV-4, played a role in development of respiratory disease among the sampled horses. However, the exact nature of their involvement remains to be established. In addition to their possible role as primary respiratory pathogens, these viruses might have exerted their effects indirectly, via the modulation of the host immune system. If the latter were true, the clinical signs observed might have been due to secondary bacterial infections. This aspect was not investigated as part of the current study. Our results suggest that further research to elucidate the complex interactions between herpesviruses and the horse is warranted. Understanding such interactions would help to decipher the exact role that these viruses play in development of equine respiratory disease, including their role in subclinical IAD and poor performance syndrome. In the long-term, such knowledge would help to design the most appropriate disease prevention strategies. The vaccination status of the horses sampled was not recorded as part of the current study. However, the significantly higher rate of EHV-4 infection observed in horses with respiratory disease compared with healthy horses suggests that vaccination against EHV-4 may be a useful tool to consider in control of respiratory disease. There are currently no vaccines available against EHV-2 and EHV-5. As such, good biosecurity and management practices that minimize stress and hence, reactivation of latent herpesvirus infections, remain the bases for current recommendations for prevention of infectious equine respiratory disease (Slater, 2007, Traub-Dargatz et al., 2004). Furthermore investigation into the potential presence of unknown respiratory viruses using recently developed virus discovery tools may contribute to our understanding of the causative agents important for the development of clinical and subclinical equine respiratory disease.

Appendices

Appendix 1- Horses with respiratory disease

a) Sample population information

Horse ID	Age (years)	Breed	Sex	Sample collection	Signs of disease
ET01	11	TB	F	17-Jun-08	Nasal discharge
ET02	7	Other	M	16-Jun-08	Nasal discharge
ET03	1	SB	M	26-Jun-08	Cough/Nasal discharge
ET04	1	TB	M	24-Jul-08	Nasal discharge/ Poor performance
ET05	3	TB	F	24-Jul-08	Nasal discharge
ET06	<1	TB	M	29-Jul-08	Acute Nasal discharge
ET07	<1	TB	M	29-Jul-08	Nasal discharge
ET08	3	TB	M	4-Aug-08	Cough/Nasal discharge/Fever
ET09	8	TB	F	7-Aug-08	In-appetent/Nasal discharge
ET10	5	TB	M	11-Aug-08	Nasal discharge/ Poor performance
ET11	1	TB	M	25-Aug-08	Depressed/Nasal discharge
ET12	1	TB	M	25-Aug-08	Acute Nasal discharge
ET13	1	TB	F	25-Aug-08	Nasal discharge
ET14	10	Other	M	18-Aug-08	Nasal discharge/ Poor performance
ET15	1	TB	M	17-Sep-08	Nasal/Ocular discharge
ET16	1	TB	F	17-Sep-08	Acute Nasal discharge
ET17	3	TB	M	8-Oct-08	Nasal discharge/ Poor performance
ET18	12	Other	F	13-Nov-08	Cough/Nasal discharge
ET19	3	TB	F	2-Dec-08	In-appetent/ Poor performance
ET20	2	TB	M	2-Dec-08	Cough/Nasal discharge
ET21	1	TB	F	7-Feb-09	Fever/Cough/ Nasal discharge
ET22	13	TB	F	15-Mar-09	Nasal discharge
ET23	15	TB	M	15-Mar-09	Nasal/ Ocular discharge
ET24	2	TB	F	15-Mar-09	Nasal/ Ocular discharge
ET25	3	TB	F	15-Mar-09	Nasal/ Ocular discharge
ET26	5	TB	F	15-Mar-09	Nasal discharge
ET27	10	TB	M	15-Mar-09	Nasal discharge/ In-appetent/ Poor performance
ET28	20	TB	F	15-Mar-09	Nasal discharge/ Poor performance
ET29	<1	TB	F	15-Mar-09	Nasal discharge
ET30	6	TB	M	15-Mar-09	Nasal discharge
ET31	5	TB	F	15-Mar-09	Nasal discharge
ET32	1	TB	M	30-Mar-09	Fever and Severe Anaemia
ET33	11	TB	F	7-Apr-09	Nasal discharge/ Coughing
ET34	9	TB	F	7-Apr-09	Nasal discharge/ Poor performance

Horse ID	Age (years)	Breed	Sex	Sample collection	Signs of disease
ET35	17	TB	F	7-Apr-09	Nasal/ Ocular discharge
ET36	8	TB	F	7-Apr-09	Nasal discharge
ET37	7	TB	M	18-Apr-09	Nasal discharge
ET38	18	TB	F	20-Apr-09	Nasal discharge/ Poor performance
ET39	9	TB	M	23-Apr-09	Nasal discharge
ET40	14	TB	M	23-Apr-09	Nasal discharge/Cough/ Temperature
ET41	5	TB	M	14-Jul-09	Nasal discharge/ Poor performance
ET42	3	TB	M	14-Jul-09	Nasal discharge/ Cough
ET43	3	TB	M	14-Jul-09	Nasal discharge
ET44	4	TB	M	14-Jul-09	Nasal discharge/ Poor performance
ET45	<1	TB	F	14-Jul-09	Nasal/ Ocular discharge
ET46	<1	TB	M	14-Jul-09	Nasal/ Ocular discharge
ET47	<1	TB	M	14-Jul-09	Nasal/ Ocular discharge
ET48	1	TB	M	14-Jul-09	Nasal/ Ocular discharge
ET49	1	TB	M	14-Jul-09	Nasal/ Ocular discharge
ET50	3	TB	F	14-Jul-09	Nasal discharge/ Poor performance
ET51	3	TB	F	30-Jul-09	Nasal discharge/ Cough
ET52	2	TB	F	30-Jul-09	Nasal discharge

b) Laboratory results

Horse ID	EHV1			EHV2			EHV4			EHV5			ERAV ERBV EAdV
	PCR	Virus Isolation	Cell Line	PCR	Virus Isolation	Cell Line	PCR	Virus Isolation	Cell Line	PCR	Virus Isolation	Cell Line	
ET01	-	-	-	+ve	-	-	-	-	-	-	-	-	-
ET02	-	-	-	+ve	-	-	-	+ve	EFK	+ve	+ve	Vero	-
ET03	-	-	-	-	-	-	-	-	-	-	-	-	-
ET04	-	-	-	-	-	-	-	-	-	+ve	-	-	-
ET05	-	-	-	+ve	+ve	Vero	-	-	-	-	-	-	-
ET06	-	-	-	-	+ve	EFK/ Vero	-	-	-	+ve	+ve	Vero	-
ET07	-	-	-	+ve	-	-	-	-	-	-	-	-	-
ET08	-	-	-	-	-	-	-	+ve	EFK	-	-	-	-
ET09	-	-	-	+ve	-	-	-	-	-	+ve	-	-	-
ET10	-	-	-	+ve	-	-	-	-	-	-	-	-	-
ET11	+ve	-	-	-	-	-	+ve	-	-	+ve	+ve	EFK/ Vero	-
ET12	+ve	+ve	Vero/ RK13	-	-	-	+ve	-	-	+ve	-	-	-
ET13	-	-	-	-	-	-	+ve	+ve	EFK	+ve	+ve	EFK	-
ET14	-	-	-	+ve	-	-	-	-	-	+ve	-	-	-

Horse ID	EHV1			EHV2			EHV4			EHV5			ERAV ERBV EAdV
	PCR	Virus Isolation	Cell Line	PCR	Virus Isolation	Cell Line	PCR	Virus Isolation	Cell Line	PCR	Virus Isolation	Cell Line	
ET15	-	-	-	+ve	-	-	-	-	-	+ve	-	-	-
ET16	-	-	-	-	-	-	+ve	-	-	+ve	-	-	-
ET17	-	-	-	-	-	-	-	-	-	-	-	-	-
ET18	-	-	-	-	-	-	+ve	-	-	-	-	-	-
ET19	-	-	-	+ve	+ve	EFK	-	-	-	-	-	-	-
ET20	-	-	-	-	-	-	+ve	-	-	-	-	-	-
ET21	+ve	+ve	EFK/ Vero	-	-	-	+ve	+ve	EFK/Vero	-	-	-	-
ET22	-	-	-	-	-	-	-	-	-	-	-	-	-
ET23	-	-	-	+ve	-	-	-	-	-	+ve	-	-	-
ET24	-	-	-	+ve	+ve	EFK	-	+ve	EFK	+ve	-	-	-
ET25	-	-	-	+ve	-	-	-	-	-	+ve	-	-	-
ET26	-	-	-	-	-	-	-	-	-	-	-	-	-
ET27	-	-	-	+ve	-	-	-	-	-	-	-	-	-
ET28	-	-	-	-	-	-	-	+ve	EFK	+ve	-	-	-
ET29	-	-	-	-	-	-	-	+ve	EFK	+ve	-	-	-
ET30	-	-	-	+ve	+ve	EFK	-	+ve	EFK	-	-	-	-
ET31	-	-	-	-	-	-	-	-	-	-	-	-	-
ET32	-	-	-	+ve	+ve	EFK	-	+ve	EFK	-	-	-	-
ET33	-	-	-	+ve	+ve	EFK	-	-	-	-	-	-	-
ET34	-	-	-	-	-	-	-	-	-	+ve	-	-	-
ET35	-	-	-	-	+ve	EFK	-	-	-	-	+ve	EFK/Vero	-
ET36	-	-	-	-	-	-	-	-	-	-	-	-	-
ET37	-	-	-	-	-	-	-	-	-	-	-	-	-
ET38	-	-	-	-	-	-	-	-	-	-	-	-	-
ET39	-	-	-	+ve	+ve	EFK	-	-	-	-	-	-	-
ET40	-	-	-	+ve	+ve	EFK	-	-	-	+ve	+ve	EFK	-
ET41	-	-	-	-	-	-	-	-	-	-	-	-	-
ET42	-	-	-	-	-	-	+ve	-	-	+ve	-	-	-
ET43	-	-	-	-	-	-	+ve	-	-	+ve	+ve	Vero	-
ET44	-	-	-	-	-	-	-	-	-	-	-	-	-
ET45	-	-	-	+ve	+ve	Vero	-	-	-	+ve	+ve	Vero	-
ET46	-	-	-	+ve	-	-	+ve	-	-	+ve	-	-	-
ET47	-	-	-	+ve	-	-	-	-	-	+ve	-	-	-
ET48	-	-	-	+ve	-	-	+ve	-	-	+ve	-	-	-
ET49	-	-	-	+ve	+ve	Vero	+ve	+ve	EFK	+ve	-	-	-
ET50	-	-	-	-	-	-	+ve	+ve	EFK	+ve	-	-	-
ET51	-	-	-	-	-	-	-	-	-	-	-	-	-
ET52	-	-	-	-	-	-	+ve	-	-	+ve	-	-	-

Appendix 2- Sample information and lab results for Healthy horses

Horse ID	Age (years)	Breed	Sex	Sample collection	PCR				
					EHV1	EHV2	EHV4	EHV5	ERAV/ERBV EAdV
ETH01	11	TB	F	4-Apr-09	-	-	-	-	-
ETH02	6	TB	F	4-Apr-09	-	-	-	-	-
ETH03	12	TB	M	5-Jul-09	-	-	-	-	-
ETH04	5	TB	F	5-Jul-09	-	-	-	-	-
ETH05	3	TB	M	30-Jul-09	-	-	-	-	-
ETH06	6	TB	M	30-Jul-09	-	-	-	-	-
ETH07	6	TB	M	30-Jul-09	-	-	-	-	-
ETH08	7	TB	F	30-Jul-09	-	-	-	-	-
ETH09	3	TB	M	30-Jul-09	-	-	-	-	-
ETH10	<1	TB	M	30-Jul-09	-	-	-	-	-
ETH11	<1	TB	F	7-Apr-09	-	+ve	-	+ve	-
ETH12	<1	TB	F	7-Apr-09	-	-	-	-	-
ETH13	<1	TB	M	7-Apr-09	-	-	-	-	-
ETH14	<1	TB	F	7-Apr-09	-	-	-	-	-
ETH15	9	TB	F	7-Apr-09	-	-	-	-	-
ETH16	5	SB	M	15-Mar-10	-	-	-	-	-
ETH17	6	SB	F	15-Mar-10	-	-	-	-	-
ETH18	8	SB	F	15-Mar-10	-	-	-	-	-
ETH19	12	SB	M	15-Mar-10	-	-	-	-	-
ETH20	20	SB	F	15-Mar-10	-	-	-	-	-
ETH21	14	SB	M	15-Mar-10	-	-	-	-	-
ETH22	15	Other	F	15-Mar-10	-	-	-	-	-
ETH23	4	SB	F	20-Mar-10	-	-	-	-	-
ETH24	10	SB	F	20-Mar-10	-	-	-	-	-
ETH25	8	SB	F	20-Mar-10	-	-	-	-	-
ETH26	14	TB	F	20-Mar-10	-	-	-	-	-
ETH27	18	TB	F	20-Mar-10	-	-	-	-	-
ETH28	18	SB	F	20-Mar-10	-	-	-	-	-
ETH29	24	SB	F	23-Mar-10	-	-	-	-	-
ETH30	14	Other	F	23-Mar-10	-	+ve	-	-	-
ETH31	12	Other	M	23-Mar-10	-	-	-	-	-
ETH32	6	SB	M	23-Mar-10	-	-	-	-	-
ETH33	6	TB	F	23-Mar-10	-	-	-	-	-

Note- No viruses were detected by virus isolation from the 33 healthy horses sampled.

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