Failure to detect equid herpesvirus type 1 DNA in Thoroughbred placentae and healthy new-born foals

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Declaration of originality

I, Lara Jean Brown, do hereby declare that the research presented in this dissertation was conceived and executed by myself, and apart from the normal guidance of my supervisor, I have received no assistance.

Neither the substance, nor any part of this dissertation, has been submitted in the past, nor is to be submitted for a degree at this university, nor any other university.

This dissertation is presented in fulfilment of the requirements for the MSc (Production Animal Science) in the department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria.

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Lara Jean Brown Date

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List of abbreviations

AHS: African Horse Sickness

CNS: Central nervous system

DNA: deoxyribonucleic acid

DOB: Date of birth

eCG: equine chorionic gonadotropin

EDTA: ethylenediaminetetraacetic acid

EHM: Equid herpes myeloencephalopathy

EHV-1,-2,-3,-4,-5: Equid alphaherpesvirus 1,2,3,4, and 5

ELISA: Enzyme-linked immunosorbent assay

EI: Equine Influenza

FSH: Follicle Stimulating Hormone

IFNy+: Interferon gamma

IgG: Immunoglobulin G

LAT: Latency associated transcripts

LH: Luteinising hormone

PBMC: Peripheral blood mononuclear cell

PCR: Polymerase chain reaction

qPCR: Quantitative or Real-Time Polymerase Chain Reaction

RNA: Ribonucleic acid

RT-PCR: Reverse transcriptase- polymerase chain reaction

SOP: Standard Operating Procedure

ZP: Zona pellucida

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Summary

Equid alphaherpesvirus 1 (EHV-1) is an economically important virus, associated with respiratory infection, late gestation abortion, neonatal death and myeloencephalopathy in horses. The aim of the present study was to test the hypothesis that EHV-1 is present in the nasopharynx and placentae of neonatal foals in the absence of clinical signs of infection. This would suggest that vertical transmission of virus occurs in inter-epizootic periods: such information could inform foaling management and the potential eradication of the virus by vaccination.

Samples were collected from animals resident on a single farm in the Western Cape Province, South Africa, which had not experienced a clinical outbreak of EHV-1 recently. Sterile swab samples from 71 post-partum Thoroughbred mares, their healthy full-term foals and fetal membranes were obtained and assayed for EHV-1 and EHV-4 nucleic acid using a duplex quantitative polymerase chain reaction (qPCR).

The null hypothesis for this study was that EHV-1 was not present in the nasopharynx and placentae of new-born, viable and healthy foals. As no EHV-1 or EHV-4 nucleic acid was detected on a duplex EHV-1/EHV-4 qPCR assay from the mare and foal nasal and fetal membrane swabs, the null hypothesis was accepted. It was therefore concluded that there was no detectable EHV-1 and -4 DNA in this population at the time of sampling. It was speculated that this may have been due to the cyclical nature of EHV-1 infections. The inclusion of additional breeding seasons on additional farms would be valuable for future studies.

1 General introduction

Herpesviruses affect virtually all mammals, typically have a narrow host range and have become highly adapted to their individual host species (MacLachlan and Dubovi, 2011, Schulman, 2016), although, cases of non-equid species such as polar bears and rhinoceroses becoming infected with EHV-1 and -9 have been documented (Abdelgawad et al., 2014, Greenwood et al., 2012). A key to the evolutionary success of herpesviruses has been their ability to establish a latent infection in their host species (Griffin et al., 2010), which provides a reservoir for continued transmission in the population (MacLachlan and Dubovi, 2011). In horses, multiple herpesviruses have been detected, some of which are associated with clinical disease. These include the alphaherpesviruses EHV-1,-3 and -4 and the gammaherpesviruses EHV-2 and -5. These viral pathogens are commonly referred to as the equid herpesviruses, however the term "alphaherpesvirus" is more taxonomically correct and will be used in this dissertation.

Equid alphaherpesvirus 1 and 4, have an economically significant impact on athletic and reproductive performance, with racing and breeding operations being most affected (Gilkerson et al., 1999). Respiratory disease caused by EHV-1 and -4 is seen more frequently in weanlings and yearlings (Van Maanen, 2002). In these cases, infection can lead to poor performance and a loss of training time (Gilkerson et al., 1999). Reproductive losses occur usually as a result of late-term abortions caused by EHV-1 (Gilkerson et al., 1999), but neonatal infection with EHV-1 also leads to high mortality rates (Van Maanen, 2002). Such mortalities, especially if many animals are affected, represent a significant loss to a stud farm. Treatment for affected foals also has financial implications for the owner. Outbreaks of the neurological form of EHV-1 are usually sporadic (Pusterla et al., 2009). Cases of neurological disease often

result in the death or euthanasia of the affected animal (Charlton et al., 1976, Wilsterman et al., 2011)).

While EHV-1 and -4 may cause severe disease during outbreaks, little is known about the state of the virus and its transmission during inter-epizootic periods. From an evolutionary perspective, it is reasonable to anticipate a mode of viral transmission that does not severely affect the health of the host, since to do so would markedly limit the opportunities for spreading the virus in a population. Evidence of viral shedding and transmission in healthy animals would be useful in attempting to identify and characterise such a transmission strategy.

Kydd and co-workers found that the presence of an MHC class 1 B2 allele was a significant risk factor in cases of fetal loss, regardless of EHV-1 status of the fetus. This study raises the idea that abortion due to EHV-1 could, in fact, be an accident due to the presence of this allele (Kydd et al., 2016). Abortion due to EHV-1 infection could be associated with the presence of this allele and not a primary means of viral spread. Other mechanisms of transmission, including vertical transmission, could be more important to consider. In a study by Gardiner and co-workers, EHV-1 was isolated from the chorioallantois of experimentally infected mares, at 270-290 days gestation. Two foals were born slightly prematurely (days 307 and 317) and shed EHV 1 for the first week of life (Gardiner et al., 2012).

The aforementioned studies support further investigation of the possibility of vertical transmission of the virus. The detection of viral antigen in samples from apparently healthy mares and foals could provide insight into viral strategies for vertical transmission and assist in the development of control strategies.

In order to achieve this, the present study tested for the presence of EHV-1 and -4 nucleic acid in clinically healthy neonatal foals and their fetal membranes. Diagnostic samples were collected at parturition from a year's crop of foals resident on a single Thoroughbred stud farm. The null hypothesis tested was that EHV-1 was not present in the nasopharynx and placentae of healthy neonatal foals.

In addition to describing the methodology used in testing the research hypothesis, aspects of the physiology of equine pregnancy, as well as the structure and development of equine fetal membranes are discussed, to better inform the reader of the reproductive effects of EHV-1. Furthermore, the mechanisms by which the virus evades the host's immune responses are discussed, to provide further insight into the host-virus interaction that has evolved over the millennia.

2 Literature review

The literature describing equid herpesviruses is extensive. The principle focus of this dissertation is on EHV-1 and -4, but brief mention will be given to other equid herpesviruses.

2.1 Equine pregnancy

2.1.1 Overview

The average gestation period of the horse, measured from the end of oestrus to the onset of parturition, is 336 ±20 days (Wooding and Burton, 2008).

A recent review described the equine placenta as diffuse, microcotyledonary and epitheliochorial (Allen and Wilsher, 2009). As in all eutherian mammals, the placenta allows for nutrient and gas exchange between the developing fetus and the dam (Cross, 2005).

2.1.2 Development of the equine conceptus and placenta

2.1.2.1 Early events following fertilisation

In the horse, fertilisation occurs in the ampulla of the Fallopian tube. Six to six and a half days post ovulation, the equine embryo enters the uterus as a late morula or early blastocyst. An acellular capsule develops around the late morula or early blastocyst coincident with uterine entry. Initially, this capsule lies between the trophectoderm and the zona pellucida (ZP), but after the blastocyst hatches on day 7, the ZP disappears and the blastocyst capsule becomes the outermost structure (Stout et al., 2005). The blastocyst capsule allows the embryo to withstand myometrial contractions and to maintain its spherical shape as it moves continually within the uterus, contacting the endometrium to release the maternal recognition of pregnancy signal which blocks the release of endometrial $PGF_2\alpha$ (Allen and Wilsher, 2009, Allen, 2000), thus preserving the primary corpus luteum (CL). On day 16 or 17 post-ovulation,

the embryo fixes within the base of one of the uterine horns and the capsule begins to attenuate, becoming discontinuous at around day 23 and disappearing completely by day 28. (Allen and Wilsher, 2009).

2.1.2.2 The chorionic girdle and endometrial cups: formation and function

Between days 20 and 30 of gestation, the allantois forms as an outpouching of the embryonic hindgut. It fuses with the chorion to form the allantochorion and enlarges until it eventually surrounds the yolk sac which it replaces as the primary interface with the endometrium (Allen and Wilsher, 2009). At day 25 of gestation, the chorionic girdle can first be identified. Histologically, between days 20 and 30, the chorion consists of low columnar epithelioid trophoblast cells, and the chorionic girdle consists of a series of folds in the trophoblast layer (Allen and Wilsher, 2009, Allen, 2000). The chorionic girdle provides the invasive trophoblast cells that form the endometrial cups, which are a circle of temporary endocrine organs that form in the endometrium surrounding the conceptus. The endometrial cups form from days 35 to 37 of gestation when the chorionic girdle cells detach from the conceptus and invade the maternal endometrium (Allen and Wilsher, 2009). The chorionic girdle cells enlarge and become binucleate, cease to invade and establish themselves as the equine chorionic gonadotrophin (eCG)-producing endometrial cups. The endometrial cups are one to two cm wide and vary considerably in length (Allen and Wilsher, 2009).

The endometrial cups can be identified histologically between days 40 and 42 as densely packed masses of large, binucleate cells with some blood and lymph vessel involvement (Allen and Wilsher, 2009, Allen, 2000). Over the next 60 to 80 days, the endometrial glands continue to enlarge and become more complex, secreting “uterine milk” which is rich in growth factors. The endometrial cups secrete eCG, a hormone with luteinising hormone (LH) -like biological

activity in the mare. In combination with 10 to 12-day cycles of follicle stimulating hormone (FSH) release, eCG is responsible for the luteinization and ovulation of secondary follicles (Allen and Wilsher, 2009). The resultant accessory corpora lutea help ensure sufficient secretion of ovarian progesterone to maintain pregnancy until placental production of progesterone takes over between days 70 and 120 of gestation (Allen and Wilsher, 2009).

At a histological level, from days 37 to 42, lymphocytes accumulate in the stroma surrounding the endometrial cups, but these lymphocyte numbers decrease from days 45 to 60(Allen and Wilsher, 2009). After day 60, the leukocyte numbers increase again, and mostly consist of CD4+ and CD8+ lymphocytes (Allen and Wilsher, 2009), with smaller numbers of plasma cells, macrophages and eosinophils (Allen, 2000). By days 100 to 120 of gestation, the endometrial cups have degenerated and sloughed off. The underlying mechanism causing the death of the endometrial cup cells is unclear (Allen, 2000)

2.1.2.3 Implantation and development of the allantochorion

By day 35, the embryo has completed organogenesis. Implantation of the placenta begins at around day 40 of gestation and microcotyledons develop to enable haemotrophic materno-fetal exchange.

Histologically, interdigitation of the microvillus border of low columnar trophoblasts of the allantochorion with outgrowths of the endometrium allows for stable attachment to the endometrium (Allen and Wilsher, 2009, Allen, 2000). Capillary beds develop in the fetal mesoderm and endometrial stroma, and glandular secretions accumulate at the endometrial surface. Areolae develop in these areas of glandular accumulation and consist of elongated trophoblast cells that form pseudostratified layers (Allen and Wilsher, 2009).

The fetus and fetal membranes fill the gravid horn of the uterus and uterine body by days 55 to 60 of gestation and by days 80 to 85 have expanded further to fill the entire uterus. Between days 60 to 80 of gestation, there is a deepening of the chorioallantoic-endometrial interdigitation. These interdigitations become increasing complex by forming secondary and tertiary branches until mature microcotyledons are formed by approximately 120 days of gestation (Allen and Wilsher, 2009). Figure 1 shows the structure of the equid microcotyledon.

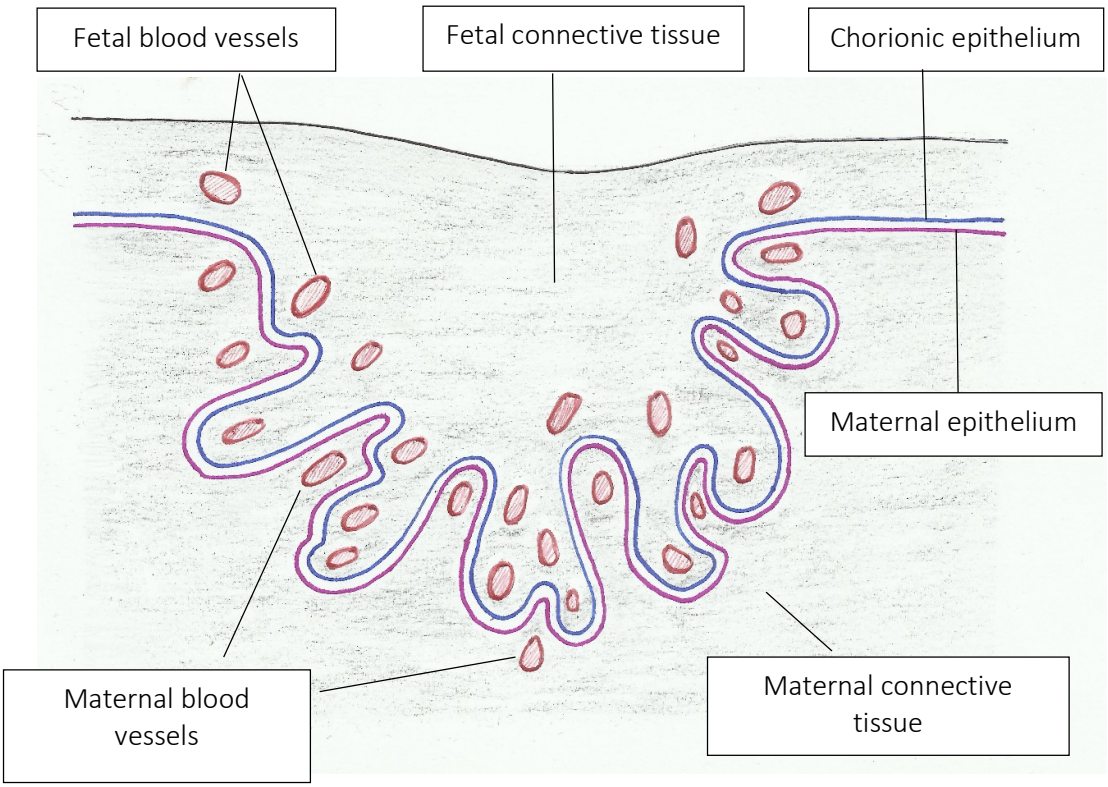

Figure 1: Diagram of the basic structure of the equine microcotyledon

Illustration by B.G. Snow (adapted from McGeady et al., 2013, figure 10.17, page 106)

Histologically, the interdigitating villi branch and lengthen to form a mature microcotyledon (Allen, 2000). Each microcotyledon is supplied by a maternal artery and a placental vein and so acts as the primary haemotrophic exchange unit (Allen, 2000).

2.2 Taxonomy of herpesviruses in domestic species

Within the order *Herpesvirales,* there are three families; namely the *Alloherpesviridae*, the *Malacoherpesviridae* and the *Herpesviridae*. The *Herpesviridae* includes all mammal-, bird- and reptile-associated herpesviruses (Davison, 2010). There are three subfamilies in the family *Herpesviridae: alpha-, beta- and gammaherpesvirinae* (Schulman, 2016, Welch et al., 1992, Davison, 2002). The subfamily *Alphaherpesvirinae* includes six species of herpesvirus that are associated with Equidae: EHV -1,-3,-4,-6, -8 and -9 (Schulman, 2016, Welch et al., 1992). The *Gammaherpesvirinae* includes three species associated with the Equidae: EHV -2, -5 and -7. Horses act as natural hosts for EHV-1,-2,-3,-4 and -5 (Patel and Heldens, 2005).

2.3 Herpesvirus structure

2.3.1 Functions of the glycoproteins

All herpesviruses are enveloped and contain a linear and double-stranded DNA genome inside a protein capsid (MacLachlan and Dubovi, 2011, Davison, 2002, Davison, 2010, Griffin et al., 2010). The envelope consists of lipoprotein and glycoprotein spikes (MacLachlan and Dubovi, 2011, Davison, 2002). There are 11 glycosylated polypeptides in the viral envelope, the most numerous of which are the major capsid proteins, the tegument protein and five glycoproteins designated as gB, gC, gD, gM and gp300 (Allen et al., 2004) (Figure 2).

The glycoproteins have several important functions. They facilitate viral attachment to and entry into the target cell, as well as determining the tropism of the virus. Cell-to-cell spread and pathogenesis of viral infection are reliant on these glycoprotein spikes (see 2.5.1 below). The induction of the host's humoral immune response is determined by these glycoproteins (Allen et al., 2004). Glycoproteins, B, C and D are important because they serve as targets for

the antibodies involved in virus neutralisation (Allen et al., 2004). The glycoproteins involved in replication of the virus are gB, gD, gH, gL and gK. Certain glycoproteins are not required for growth within the host cell (gC, gE, gG, gI, gM, g300) but are essential to the infectious process within the host, since experimental deletion of these genes results in a decrease in virulence (Allen et al., 2004).

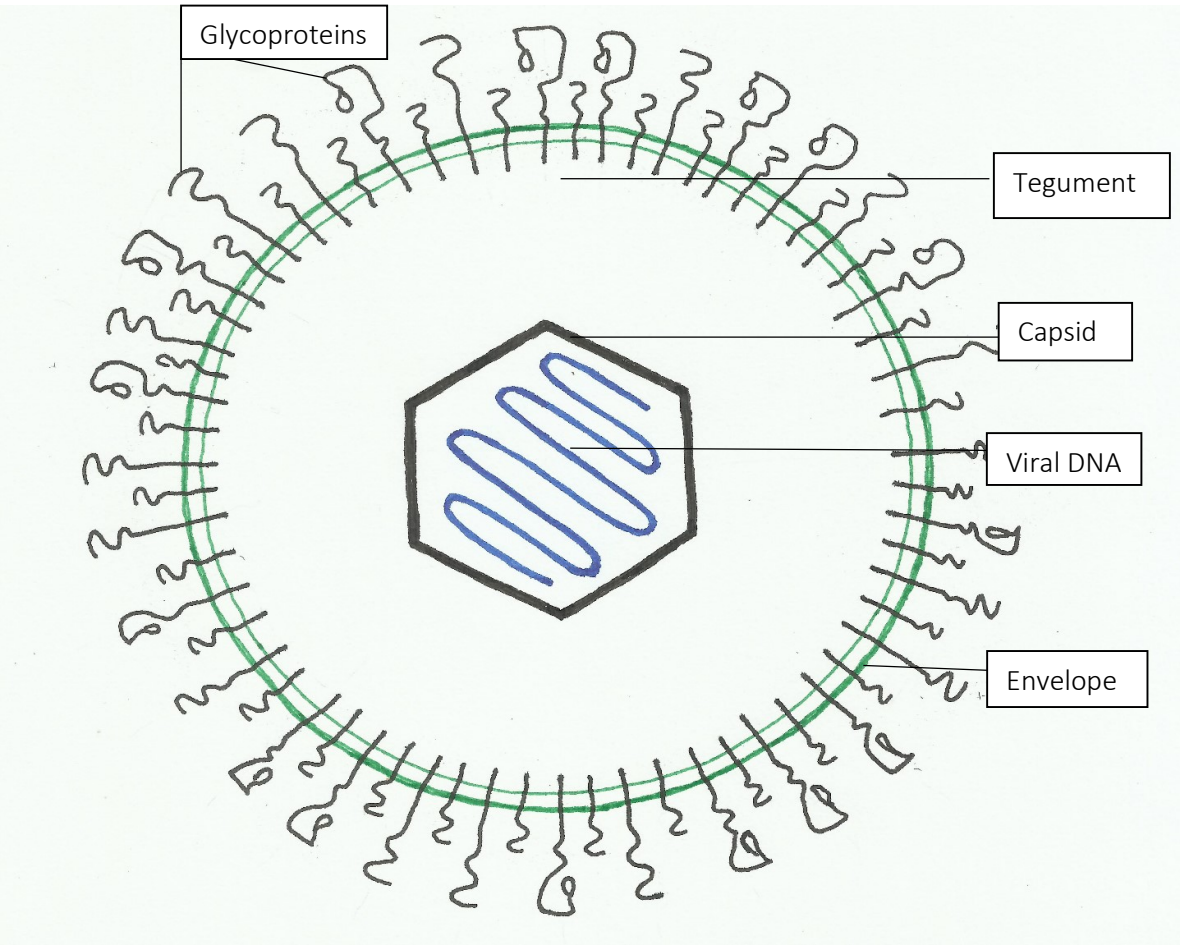

Figure 2: Diagram showing the basic structure of the herpesviridae

Illustration by B.G Snow. (Adapted from MacLachlan et al, 2011, Fenner's Veterinary Virology, Figure 9.1 MacLachlan and Dubovi, 2011; and Paillot et al, 2008, Equine herpes virus-1: virus, immunity and vaccines, Figure 1B).

2.4 Virus-host interaction and immunology

When a horse is exposed to EHV-1, both the humoral and cellular immune responses are essential in the body's response to the infection (Rusli et al., 2014). In the upper respiratory tract, the presence of mucosal and serum virus neutralising antibody decreases the amount and duration of viral shedding following experimental infection (Rusli et al., 2014).

Cytotoxic T- lymphocytes kill cell-associated virus. Following experimental infection, increases in several immune system components are detectable during days 10 to 15 post-infection (Paillot et al., 2007) in the lungs and the blood (Rusli et al., 2014) and include viral antigen-driven CD4+ T lymphocytes and virus-specific CD8+ cytotoxic T lymphocyte precursors (Allen et al., 2004, Kydd et al., 2006). During the three to six month period post-infection, there is an increased level of serum virus neutralising antibody, herpesvirus reactive natural killer (lymphocyte) cell activity (Allen et al., 2004) and MHC class 1 restricted cytotoxic activity which is mediated by cytotoxic T lymphocytes (Kydd etc al., 2006).

Equid herpesviruses have developed several strategies to evade detection by the immune system and these are summarised in Table 1.

Table 1: Summary of the mechanisms by which EHV-1 evades the immune response in vitro

(Rusli et al., 2014)

Immune defence mechanism	Viral evasion strategy in infected cells
Antibody-dependent response	1. Suppresses viral antigen expression on the cell surface
Cellular immune response	1. Downregulates MHC class 1 expression 2. Blocks transport of viral peptides to MHC class 1 molecules in the endoplasmic reticulum 3. Blocks transporter associated with the host cell's antigen processing protein.

2.4.1 EHV-1 specific immunology in foals

While the basic immune components are similar in mature foals and in adult horses, there are distinct differences in young foals in their responses to vaccination and challenge with infectious virus.

After birth, the foal is exposed to a high novel antigen load and thus a high level of immune regulation is required. Ingestion of colostrum is essential for the passive transfer of immunoglobulins and cytokines from dam to foal. While the foal's immunoglobulin G (IgG) profile is initially similar to that of the mare's colostrum, there is a gradual decline in IgG concentration. Thereafter, the foal starts to produce its own IgG. Cytokines, produced by different T helper cell subsets, are important in regulating the adaptive immunity of the foal to disease and vaccination. The T cell cytokine responses are delayed in foals in the first three to 12 months of life (Perkins and Wagner, 2015).

In a study by Paillot and co-workers (2007), the production of interferon gamma (IFNγ) by peripheral blood mononuclear cells (PBMC) in foals (aged eight months on average) was found

to be low when challenged with EHV-1. However, the frequency of IFNγ PBMC increased between 66 and 120 days post-infection. The peak levels detected in the foals in this study were low compared to the peak levels in pregnant mares post-infection in the same study. The researchers suggested that the most likely explanation for these lower interferon gamma positive PBMC numbers, was that the experimental challenge was the naïve foals' first exposure to EHV-1, such that primary immune responses were generated (Paillot et al., 2007).

In contrast, yearlings and pregnant mares were previously primed by natural infection, and experimental infection therefore stimulated an anamnestic response. In foals and young horses, IFNγ is produced by the $CD8^+$ cytotoxic T cells, whereas the $CD8^-$ cells are responsible for the majority of its production in older horses (Perkins and Wagner, 2015).

Natural EHV-1 and -4 infections of foals in early life have also been studied. In foals, the most likely source of infection prior to weaning is through reactivation of infection in their dams, or other mares in the herd (Allen et al., 2004, Patel and Heldens, 2005).

In one study, serological monitoring of foals immediately before weaning using a type-specific ELISA to differentiate between EHV-1 and -4, revealed the presence of antibodies to both serotypes. The detection of an increased prevalence of EHV-1 antibody positive foals was outside the period within which a foal can absorb maternal antibodies from the intestine. This showed active infection within this group of foals. It was further observed that these infected foals spread the virus to other foals in the group; this spread continued into the weaning period (Gilkerson et al., 1999).

In another study that used PCR as a means of detecting viral DNA, five of the six foals that tested positive for EHV-1 DNA were free of clinical signs of disease. The single foal showing a purulent nasal discharge had a concurrent infection with *Rhodococcus equi* (Foote et al., 2004).

2.4.2 Latency

A review of the literature on latent infection with EHV-1 in the horse population suggested that more than 50% of the horse population was latently infected with EHV-1 (Brown et al., 2007). It has been suggested that shedding of the virus through reactivation of latent infection is an important biological source of the virus (Brown et al., 2007, Allen et al., 2004).

The conditions required for the reactivation of latent infection are poorly defined, but reviews repeatedly cite stressful conditions including travel, handling and weaning (Van Maanen, 2002) as key to the reactivation and subsequent shedding of virus (Walter et al., 2013). Other workers however reported that horses that had undergone a long period of transportation had a low level of recrudescence of infection (Pusterla et al., 2009). The causal association between the physiological stress response induced by an environmental or management stressor is currently undefined. Increased faecal cortisol metabolite levels have been suggested as a helpful indicator of stress in horses (Schulman, 2016) however the *in vivo* evidence linking high cortisol concentrations with reactivation of EHV-1 infection is currently absent.

The establishment of latency is a key feature of all herpesvirus infections (Dunowska, 2016). EHV -1 becomes latent in the trigeminal ganglia, peripheral blood mononuclear cells and lymph nodes (Slater et al., 1994). EHV -1 and -4 viruses were identified by polymerase chain reaction (PCR) in the trigeminal ganglion and lymph nodes associated with the respiratory tract of several horses (Edington et al., 1994). Subsequently Baxi and co-workers isolated EHV-1 from trigeminal ganglia samples using co-cultivation methods. In a study of 40 abattoir horses, the most common site of EHV-1 and -4 detection by PCR was the lymph nodes of the respiratory tract (Baxi et al., 1995). The primary site of latency of EHV-4 was found to be in the trigeminal ganglia (Allen et al., 2004). Reactivation of latent virus in

leukocytes allows it to disseminate rapidly through the vasculature and lymphatics (Welch et al., 1992). During the period of latency, EHV-1 is harboured in the CD5+and CD8+ T lymphocytes and can be found in an estimated one in every 50 000 PBMC (Chesters et al., 1997, Patel and Heldens, 2005)

In latently infected lymphocytes, viral protein expression is suppressed and immunological detection is avoided (Allen et al., 2004). The details of viral reactivation from latency are poorly understood, but the reactivation and subsequent shedding of the virus may depend on the site of reactivation. For example, reactivation in the respiratory epithelium may lead to shedding of virus in nasal secretions.

Reactivation may occur within the resident lymphocytes of the pregnant endometrium which allows for possible transfer to the endothelium, with subsequent vascular damage resulting in abortion (Dunowska, 2016, Allen et al., 2004). Shedding of the virus following reactivation can occur over one to three weeks; the amount of virus shed during this time varies among individuals (Dunowska, 2016). Horses may not necessarily show clinical signs, shedding virus silently, complicating control measures (Allen et al., 2004). Reactivation of infection with subsequent shedding provides an opportunity for transmission to other horses, allowing for persistence of EHV-1 and occurrence of new infections within closed herds (Van Maanen, 2002).

2.5 Equid alphaherpesviruses 1 and 4

The alphaherpesvirus, EHV-1, is considered the most important viral cause of abortion in horses and is assumed enzootic in the global equine population (MacLachlan and Dubovi, 2011). In some outbreaks of EHV-1- and -4-associated disease, particular viral strains have been identified (McFadden et al., 2016). Different viral strains show varied abortigenic

potential (Walter et al., 2013, Gardiner et al, 2012). Hypervirulent strains exist, such as EHV-1 Ab4 and Army 183, although the exact genetic basis of this hypervirulence is currently unknown (Allen et al., 2004). These strains exhibit marked endotheliotropism and cause high rates of abortion and neurological disease in experimental infections (Allen et al., 2004). For example, the Ab4 strain is capable of inducing severe vascular injury in the endometrium (Smith et al., 1992). Equid alphaherpesvirus 1 can also cause equine herpesvirus myeloencephalopathy (EHM) (Walter et al., 2013), which can affect individual animals but is more commonly epizootic (Friday et al., 2000). Different strains of EHV-1 vary in their neuropathogenicity: in one study, a single nucleotide polymorphism in the DNA polymerase gene (ORF30) was found to have a significant association with pathogenicity (Nugent et al., 2006). This genotype (G_{2254}/D_{752} Pol) increased in prevalence from 3.3% in the 1960s to 19.4% in the 2000s (Perkins et al., 2009, Walter et al., 2013). This marker may have predictive value for the ability of an isolate to cause neurological signs (Nugent et al., 2006). The findings of a study on an outbreak of EHM in New Zealand also identified this polymorphism as being strongly associated with neuropathogenicity (McFadden et al., 2016)

2.5.1 Pathogenesis of EHV-1 and -4

2.5.1.1 Respiratory disease with EHV-1 and -4 infection

The route of primary infection for both EHV-1 and EHV-4 is via the respiratory tract (MacLachlan and Dubovi, 2011) following inhalation of infected aerosols, contact with infected secretions (Rusli et al., 2014), contact with virus-shedding horses, or contact with an aborted fetus or fetal membranes (Allen et al., 2004).

After initial infection, the virus replicates in the epithelium of the upper respiratory tract, or conjunctivae. It then moves to the draining lymph nodes, in which replication continues (Rusli

et al., 2014, Allen et al., 2004). Within 24 hours, EHV-1-infected mononuclear cells can be detected in the sinuses and parenchyma of the lymph nodes associated with the respiratory tract. Viral antigen is detected primarily in CD8+ lymphocytes and in some CD4+ lymphocytes. It is also detected in 20 to 25% of monocytes (Kydd et al., 2006) (Wilsterman et al., 2011). Wilsterman and co-workers demonstrated, in vivo, that EHV-1 could be detected in all PBMC subpopulations and found that the CD8+ lymphocytes were most often infected (Wilsterman et al., 2011). Occasionally, EHV-4 infections are associated with a leukocyte-associated viraemia (Allen et al., 2004).

Virus-infected cells can be detected by PCR in the trigeminal ganglion within 48 hours of initial infection (Slater et al., 1994, Allen et al., 2004). Naive horses may shed the virus from the nasopharynx for 15 days after first exposure, whereas previously exposed horses typically shed for only two to four days (Allen et al., 2004) but Burgess and co-workers detected viral shedding via qPCR in horses up to 9 days after EHM was diagnosed in the individual horses (Burgess et al., 2012).

2.5.1.2 Abortion with EHV-1 infection

Amplification in the lymph nodes leads to a leukocyte-associated viraemia which can then affect the endothelium in the uterus (Allen et al., 2004, Rusli et al., 2014, Lunn et al., 2009). The duration of this viraemia can be at least 14 days (Lunn et al., 2009). The cell-associated viraemia, involving the CD5+ and CD8+ T lymphocytes, is a prerequisite for abortion but does not necessarily lead to an abortion (Warner, 1987). The pathogenesis of late-term abortion (abortion in the second half of the third trimester) relates to infection of the endothelial cells of the uterine blood vessels, which allows for transmission of the virus from the mare to the fetus (Kimura et al., 2004) or placental infarction and detachment (Smith et al., 1992).

It has been suggested that an important factor in uteroplacental transmission of infection is vasculitis and thrombosis of affected blood vessels (Smith and Borchers, 2001, Patel and Heldens, 2005). The vasculitis affects primarily arterioles in the glandular layer of the endometrium, particularly at the base of the microcotyledons (Allen et al., 2004, Lunn et al., 2009). Within nine to 13 days post-experimental infection, widespread thromboischaemic necrosis of the microcotyledons can be identified (Allen et al., 2004).

In a study by Smith and Borchers, an *in situ* hybridisation technique identified viral DNA in necrotic tissue in infarcted microcotyledons and within the endometrial glands. These areas of infarction are suspected to be important in the movement of the virus across the placental barrier (Smith and Borchers, 2001) because the virus has been found to be more concentrated in areas associated with infarction (Gerst et al., 2003). Infection of the placental trophoblast is thought to occur by cell-to-cell spread and facilitates infection of the chorioallantoic endothelium and, subsequently, the fetal organs (Gerst et al., 2003).

If virus-induced lesions are widespread, the mare aborts prior to transplacental spread of the virus via placental detachment. If the degree of damage is less severe, the virus can reach and infect the fetus, with the mare giving birth to a live, but EHV-1 infected foal (Patel and Heldens, 2005, Allen et al., 2004, Szeredi et al., 2003). In a study by Gardiner and co-workers, qPCR was used to quantify the viral load in aborted foetuses. The fetuses and their fetal membranes showed no gross lesions and it was found that viral loads were higher in fetal lung and liver than in the placenta (Gardiner et al., 2012). In a study by Kimura and Hasebe, EHV-1 antigen was detected in the bronchioalveolar epithelial cells and in the alveolar macrophages in the fetal lung samples tested. No EHV-1 antigen-positive cells were found in placental samples in this study (Kimura et al., 2004). However, a study by Smith and co-workers, demonstrated

EHV-1 antigen in the endothelial cells of the endometrial blood vessels of experimentally infected pregnant mares. In one of the mares, antigen was detected in the chorion and the fetus (Smith et al., 1992). In a study by Gardiner and co-workers, viral quantification was performed on the fetuses and fetal membranes of experimentally infected mares. Fetal lung, liver and spleen samples contained significantly higher viral loads compared to the chorioallantois. Significantly, virus could be detected from the chorioallantois of all mares included in the study, even those that gave birth to live foals that developed normally (Gardiner et al., 2012).

2.5.1.3 Neurological disease with EHV-1 infection

In experimentally infected horses, the incubation period for neurological disease was six to 10 days following respiratory infection (Charlton et al., 1976, Friday et al., 2000). The pathogenesis of neurological disease is thought to be similar to that of abortion. Thus, due to the strong endotheliotropism of virulent strains of EHV-1, vasculitis and subsequent thrombosis can occur in the CNS, with resultant ischaemic damage and myelomalacia (Friday et al., 2000). This leads to a diffuse myeloencephalopathy (Pusterla et al., 2009). In a study by Burgess and co-workers, it was found that virus shedding of EHV-1 via nasal secretions could occur for at least nine days after the onset of clinical signs of neurological disease (Burgess et al., 2012).

2.5.2 Clinical signs of EHV-1 and -4infection

2.5.2.1 Respiratory disease of EHV-1 and -4 infection

Clinical signs of EHV-1 infection in the respiratory system in naive animals include pyrexia, anorexia, nasal discharge and conjunctivitis with an increase in submandibular lymph node size (Powell, 1987, Sutton et al., 1998, Patel and Heldens, 2005). In older animals with previous

exposure to the virus, infection is often clinically inapparent (Powell, 1987). Equid alphaherpesvirus 4 causes an acute respiratory infection that affects foals at around two months of age most commonly, as their maternal immunity wanes (MacLachlan and Dubovi, 2011), but it can also affect yearlings (Pusterla et al., 2009). In foals, infection can progress to lower airway disease. Affected foals present as depressed and anorexic, with signs of tachypnoea and dyspnoea (Warner, 1987, Allen et al., 2004).

In a study on ponies infected experimentally with EHV-1, bronchoscopy performed up to day 11 post-infection noted hyperaemia in linear streaks in the nasopharynx and tracheal mucosa (Sutton et al., 1998). During recovery from the respiratory syndrome caused by EHV-1, clinical signs can mimic those seen with IAD (inflammatory airway disease) or RAO (recurrent airway obstruction) (Allen et al., 2004).

'Pulmonary vasculotropic EHV-1 infection' is a peracute but rare form of the disease with a high mortality rate seen in young adult horses. Affected animals are pyrexic and experience respiratory distress (Allen et al., 2004). Infection with EHV-4 has a similar presentation, with affected horses becoming anorexic and pyrexic. Enlargement of the lymph nodes and nasal discharge are also noted. Secondary bacterial infections can complicate recovery (Van Maanen, 2002).

2.5.2.2 Ocular disease in EHV-1 infection

Equid alphaherpesvirus 1 can infect choroidal endothelium within the eye, which can cause extensive retinal damage, resulting in blindness (Allen et al., 2004).

2.5.2.3 Reproductive disease in EHV-1 infection

Equid alphaherpesvirus 1 infection can cause late-term abortions, which in a broodmare population may be epizootic or sporadic (Gilkerson et al., 1999). Typically, abortions can occur as early as five months of gestation, but most occur from between eight months to term (Kydd et al., 2006). The aborted fetuses typically show limited autolysis (MacLachlan and Dubovi, 2011).

In experimentally infected pregnant mares (using EHV-1 Ab4 strain), abortion occurred within nine to 14 days after intranasal inoculation (Smith et al., 1992). Infected mares may show pyrexia 10 to 15 days prior to the abortion (Walter et al., 2013). The placenta is usually expelled with the fetus. Occasionally, if the onset of the abortion is rapid, the placenta may fail to rupture at the cervical star and no eversion occurs, resulting in a 'red bag' delivery. Typically, the fetus is dead upon delivery due to early separation of the placenta or dies soon afterwards due to pulmonary lesions caused by the virus (Allen et al., 2004, Powell, 1987). Abortion caused by EHV-1 infection usually does not affect the future reproductive performance of the mare (Allen et al., 2004).

2.5.2.4 Neonatal disease in EHV-1 infection

Foals born with EHV-1 infection are usually diseased at birth or show signs of illness within 24 hours (Allen et al., 2004). Affected foals show clinical signs of weakness, jaundice (Van Maanen, 2002), diarrhoea (Allen et al., 2004) and respiratory distress. Mortality rates are high in such cases (Van Maanen, 2002). On haematological examination, neutropaenia and lymphopaenia are usually detected (Murray et al., 1998). In a study by Chavatte and co-workers, examination of the bone marrow revealed depletion of mature neutrophils and an

increase in atypical lymphocytes (Chavatte et al., 1991). Clinical signs in affected mares prior to foaling may be absent (Allen et al., 2004).

2.5.2.5 Neurological disease in EHV-1 infection

In cases of the neurological form of EHV-1 infection, signs develop in the second week following respiratory infection with EHV-1 (Allen, 2002) and are preceded by pyrexia (Walter et al., 2013, Pusterla et al., 2009). The neurological form of EHV-1 infection can manifest with variable clinical signs; from mild ataxia in the hind limbs to complete fore- and hindlimb paralysis, with recumbency (Van Maanen, 2002, Walter et al., 2013). Subcutaneous oedema of the limbs is seen occasionally (Warner, 1987). Upper motor neuron signs can also develop (Walter et al., 2013). A decrease in bladder and anal tone may occur (Warner, 1987), resulting in urinary incontinence and the inability to expel faeces (Pusterla et al., 2009).

2.5.3 Pathological findings in EHV-1 and -4 infections

2.5.3.1 Respiratory disease in EHV-1 and -4 infections

On post-mortem examination following fulminant respiratory disease in foals, there is necrosis and ulceration of the nasal mucosa (Allen et al., 2004). Multifocal areas of necrotising bronchiolitis are noted and histopathology examination shows intranuclear inclusion bodies in the epithelial cells of the nasal mucosa, upper airways and conjunctiva (Allen et al., 2004, Pycock, 1997).

2.5.3.2 Ocular disease in EHV-1 infection

Uveitis and focal or diffuse chorioretinal lesions have been noted following respiratory infection with EHV-1 (Allen et al., 2004). In one study, it was found that between 50 and 90 % of experimentally infected horses developed chorioretinal lesions (Hussey et al., 2013).

2.5.3.3 Aborted fetuses in EHV-1 infection

Typical post mortem findings of fetuses aborted at more than five months of gestation include icterus and distended lungs, hydrothorax and ascites, with foci of necrosis in the liver and adrenal glands (Allen et al., 2004, MacLachlan and Dubovi, 2011, Powell, 1987). In the spleen, white pulp necrosis is often demonstrated (MacLachlan and Dubovi, 2011). In fetuses aborted at less than five months of gestation, there is marked autolysis (Allen et al., 2004).

2.5.3.4 Neonatal disease in EHV-1 infection

In cases of perinatal mortality, interstitial pneumonia, consolidation and oedema of the lungs are noted (Allen et al., 2004, Van Maanen, 2002). There is typically depletion of the thymic and splenic lymphocytes (Van Maanen, 2002). On histopathology, necrotic hepatocytes are often noted (Allen et al., 2004).

2.5.3.5 Neurological disease in EHV-1 infection

In encephalomyelopathy, replication of the virus in the endothelial cells of the arterioles in the brain causes a non-suppurative vasculitis associated with thrombosis, which ultimately causes focal ischaemic necrosis of brain tissue (MacLachlan and Dubovi, 2011, Allen et al., 2004, Welch et al., 1992). In a study conducted following an abortion storm due to EHV-1 infection in naturally infected horses, post mortem examinations were performed on those animals that developed neurological signs. Equid alphaherpesvirus type 1 was isolated from the brains and lungs of these horses with extensive foci of malacia in the brain and spinal cord (Charlton et al., 1976).

2.6 Other equine herpesviruses

2.6.1 Equid gammaherpesvirus 2 and 5

The gammaherpesviruses EHV-2 and -5 have an unknown clinical significance (Bell et al., 2006). These two viruses are more closely related to each other than to other herpesviruses (Telford et al., 1993). They have a double stranded DNA genome and are genetically and antigenically distinct from the alpha-herpesviruses (Allen and Murray, 2004). Equid gammaherpesvirus 2 is a slow-growing, cytopathogenic gammaherpesvirus (Borchers et al., 1997). Infection with EHV-2 is widespread and is more prevalent than EHV-5 (Telford et al., 1993, Nordengrahn et al., 2002), but co-infection with both viruses is commonly seen (Bell et al., 2006). Equid gammaherpesvirus 2 has been isolated from both clinically healthy horses and from horses showing a variety of clinical signs (Telford et al., 1993, Nordengrahn et al., 2002). These include upper respiratory tract infections, pyrexia and an increase in lymph node size (Telford et al., 1993, Bell et al., 2006, Nordengrahn et al., 2002) as well as keratoconjunctivitis (Nordengrahn et al., 2002), and may predispose foals to *Rhodococcusequi* infection (Bell et al., 2006, Nordengrahn et al., 2002). Equid gammaherpesvirus 2 can cause a latent infection in B lymphocytes and may be isolated in PBMC from up to 90% of apparently healthy horses (Bell et al., 2006). Recently, it was found that experimental infection with EHV-5 caused pulmonary fibrosis in horses (Williams et al., 2013).

2.6.2 Equid alphaherpesvirus 3

Infection of the male and female genital mucosa by EHV-3 may cause equine coital exanthema. Sexual rest is advised for affected animals (Simpson, 1987). Good hygiene practices should be observed to reduce opportunity for spread (Pycock, 1997). Treatment, if necessary, is symptomatic (Simpson, 1987).

2.7 Diagnosis of herpesvirus infections

Diagnosis of EHV-1 infection is usually focused on rapid diagnostic testing first performed in an outbreak situation. Rapid methods include PCR and immunofluorescent testing (Allen et al., 2004). Confirmatory, but slower, procedures such as virus isolation and histopathology may then be undertaken (Allen et al., 2004). Diagnosis in cases of respiratory disease can be made through the use of nasopharyngeal swabs for virus isolation, ELISA or PCR testing (Van Maanen, 2002). In the case of an abortion, diagnosis can be performed via gross pathology; using immunohistochemistry (IHC); virus specific PCR or by virus isolation (MacLachlan and Dubovi, 2011)

The techniques described below are discussed specifically with regard to EHV-1 and -4. However, some of these techniques are also applicable to the diagnosis of EHV -2, -3 and -5 with EHV-2 and -5 detected using virus isolation as well as molecular diagnostic techniques such as PCR (Allen and Murray, 2004). EHV-3 is commonly diagnosed based on clinical examination, but a confirmatory PCR can also be performed (Sprayberry and Robinson, 2014).

2.7.1 Histopathology

In abortion cases, fetal tissue samples include the liver, lung, spleen, thymus, adrenal glands and kidneys for histopathology (MacLachlan and Dubovi, 2011, Chopin, 2010). Fetal membranes are also examined (MacLachlan and Dubovi, 2011, Chopin, 2010). On histopathological examination, areas of necrosis can be detected in the liver and spleen (Walter et al., 2013); eosinophilic intranuclear inclusion bodies can be additional indicators of disease and may be useful in making a diagnosis in cases of abortion or neurological disease (Allen et al., 2004). In cases of EHV myeloencephalopathy, evidence of vasculitis, congestion and ischaemic damage in the CNS assist in making a diagnosis (Friday et al., 2000).

2.7.2 Virus isolation

During acute infections, EHV-1 and -4 can be isolated from a variety of sample types including nasal swabs (Welch et al., 1992). The virus can be detected in PBMC for up to three weeks post-infection (Welch et al., 1992). In a study of neonatal EHV-1 infection by Murray and co-workers, virus isolation was used in combination with other techniques to reach a diagnosis (Murray et al., 1998). Although virus isolation is a good confirmatory method, this process is time consuming and expensive (Carvalho et al., 2000) but does yield infectious virus for future DNA sequencing and epidemiological analysis.

2.7.3 Immunostaining techniques

Immunohistochemistry, immunofluorescent antibody staining and immunoperoxidase (IP) staining are all similar techniques with slight differences in their application.

In a study by Smith and co-workers, immunohistochemical techniques were used to detect EHV-1 antigen in fetal membranes (Smith et al., 1992). In a similar study, antigen was identified mainly in the cytoplasm of various cell types and, in most cases, these cells were distributed throughout the fetal membranes (Szeredi et al., 2003). Immunohistochemistry has also been used to identify EHV-1 antigen in the neurons and astrocytes of horses affected by EHM (Schultheiss et al., 1997).

Immunofluorescent antibody staining techniques involve the staining of specimens with fluorescein-labelled antibody to detect the presence of viral antigen (Minnich and Ray, 1980). Direct immunofluorescent detection of EHV-1 and -4, particularly on snap frozen tissues can be used to make a preliminary diagnosis. Immunofluorescent antibody techniques were used to identify EHV-1 antigen in the lungs, pharyngeal mucosa and adrenal glands as well as the intestines, trigeminal ganglion and kidneys in the case of a fatal, non-neurological EHV-1

infection of a filly (Del Piero et al., 2000). Immunofluorescent antibody staining and indirect IP techniques have been used to identify cells containing EHV-1 antigen in the brain and spinal cord (Friday et al., 2000). One study using an indirect IP technique to identify EHV-1 antigen in formalin-fixed fetal tissue was found to be sensitive and specific for EHV-1 (Schultheiss et al., 1993). In this study, the IP test was as sensitive as immunofluorescent antibody testing and out-performed immunofluorescent antibody testing in autolysed tissue (Schultheiss et al., 1993).

2.7.4 Serology

If fetal tissue is unavailable in cases of abortion, serological methods may confirm recent exposure of mares to EHV-1 (MacLachlan and Dubovi, 2011). Such methods include enzyme-linked immunosorbent assay (ELISA), complement fixation and virus neutralisation in naive animals. Due to antigenic similarities between EHV-1 and EHV-4, serological testing may not be able to distinguish between these two species (Friday et al., 2000, Dunowska, 2016). Additionally, prior vaccination of the horse may cause antibody titre elevations that may be indistinguishable from natural infection; knowledge of the horse's vaccination history is therefore essential for meaningful interpretation (Allen et al., 2004). Samples should be taken during the period of acute illness and again two to three weeks later. A significant rise (a four-fold increase or more) (Pusterla et al., 2009) in virus-specific antibody titres can indicate that an infection occurred (Allen et al., 2004). It is important to note that detection of EHV-1 antibodies in the CSF of horses with myeloencephalopathy is due to protein leakage into the CSF following vasculitis, rather than intrathecal antibody production (Pusterla et al., 2009).

2.7.5 Molecular diagnostics

2.7.5.1 Polymerase chain reaction

Polymerase chain reaction is an *in vitro* method of synthesising DNA sequences using primers and is a common diagnostic method for viral nucleic acid detection. It is the diagnostic test of choice for EHV-1 detection because it is highly sensitive and specific (Lunn et al., 2009). Such a method is essential in cases where a virus is not easily cultured, is inactive or complexed with antibodies, or in the case of a latently infected animal. A thermocycler is used to control temperature of the reaction and repeated cycles amplify specific DNA sequences (MacLachlan and Dubovi, 2011). Conventional PCR uses gel electrophoresis as a post-PCR step to visualise the PCR product. With this method there is a risk of product carry over (Pusterla et al., 2006) which is the contamination of a PCR with amplified DNA from a previous test. As PCR can create many amplicons of a DNA sequence, any minute contamination can cause significant problems (Kwok, 1990).

2.7.5.2 Quantitative PCR

Quantitative PCR or Real-Time PCR(qPCR) is a second-generation PCR system. This method performs DNA amplification and detection within a closed tube system (Pusterla et al., 2006) and quantifies viral DNA. Hydrolysis qPCR makes use of the 5'-exonuclease activity of *Taq* DNA polymerase to hydrolyse a labelled probe during the amplification of target DNA. A fluorogenic probe is hybridised to the target gene. When the probe is hydrolysed, the probe fluoresces. This fluorescence is captured by a camera and a graph is plotted (Diallo et al., 2006). There are no wet-lab components in the analysis, which reduces the post-PCR handling steps and eliminates the risk of product carryover, as well as reducing the risk of false positives (Pusterla et al., 2006). The qPCR assay is rapid, producing reliable results within three to four hours,

including sample preparation time (Diallo et al., 2006). The results are also highly reproducible (Pusterla et al., 2006). In the case of abortion, fetal lung and liver are suitable samples to be submitted for qPCR testing (Diallo et al., 2006). Type-specific primers are used to detect and distinguish between EHV-1 and -4 (Allen et al., 2004).

To detect latently infected animals on post-mortem examination, nested PCR techniques can be used and, when used in combination with qPCR, allow for the quantification of viral loads. This has several advantages: it indicates the disease stage, aids in monitoring response to therapy and allows for better assessment of exposure of 'in-contact' horses (Pusterla et al., 2009). Reverse transcriptase-PCR (RT-PCR) has been used to detect EHV-1 latency associated transcript (LAT) sequences in latently infected cells (Chesters et al., 1997, Allen et al., 2004).

2.8 Differential diagnoses for EHV-1

Whenever respiratory, neurological or neonatal disease and late-term abortion are identified in equids, the herpesviruses, particularly EHV-1 should be ruled out. Differential diagnoses for these syndromes are presented in Table 2.

Table 2: Differential diagnoses for EHV-1 infection

Form of disease	Infectious cause	Non-infectious cause
Respiratory disease	Equine influenza (Allen et al., 2004) African horse sickness (Allen et al., 2004)	Inflammatory airway disease (IAD) or recurrent airway obstruction (RAO) (Léguillette, 2003)
Abortion	Bacterial placentitis due to *Streptococcus zooepidemicus, Leptospira* spp. and *E.coli*. (Hong et al., 1993b) Equine viral arteritis (Givens and Marley, 2008) Equine babesiosis (Allen et al., 2004, Givens and Marley, 2008)	Chronic endometritis (Vanroose et al., 2000) Stress due to pain, for example gastrointestinal colic (Vanroose et al., 2000) Umbilical cord torsion (Hong et al., 1993a)
Neonatal disease	Septicaemia (Allen et al., 2004) Equine babesiosis (Allen et al., 2004)	Neonatal isoerythrolysis (Allen et al., 2004) Failure of colostral transfer (Clabough et al., 1991)
Neurological disease (EHM)	Equine encephalosis (Allen et al., 2004) Rabies (Allen et al., 2004)	Trauma (Allen et al., 2004)

2.9 Management of equid herpesviruses in breeding populations

2.9.1 Prevention of infection

2.9.1.1 Animal husbandry and on-farm preventive care

Equid alphaherpesvirus 1 infection control measures focus on broodmare management and vaccination (MacLachlan and Dubovi, 2011). Vaccination alone cannot be relied upon but should be supported by good management practices (Allen et al., 2004, Allen, 2002).

Stud farm management should ensure separate paddocks for different age categories, with yearlings, weanlings, pregnant mares and mares with foals kept separately and attended by dedicated staff (Allen et al., 2004, Allen, 2002, Chopin, 2010). Pregnant mares of similar gestational stage should be kept in groups as small as is practically possible (Allen et al., 2004), without 'nose-to-nose' contact, with minimal stress (Pusterla et al., 2009). All newly introduced animals must be isolated for three weeks, and newly introduced late-pregnant mares should be kept separate until after foaling (Schulman et al., 2015). Foster mares brought onto the farm should be isolated from other mares until EHV-1 is ruled out as the cause of the loss of their foals (Van Maanen, 2002).

In a veterinary hospital, strict isolation and barrier nursing of any cases showing pyrexia and with a history of recent abortion or neurological signs must be practised until EHV-1 has been excluded (Pusterla et al., 2009). Stables that have housed infected horses should be thoroughly disinfected and left unused for three weeks (Van Maanen, 2002). Disinfectant options include hypochlorite, iodophores, phenols and formalin (Van Maanen, 2002).

2.9.1.2 Vaccination

Vaccination against EHV-1 and -4 aims to increase humoral and cell mediated immunity and thus limit nasopharyngeal virus shedding, cell-associated viraemia and endothelial cell infection (Kydd et al., 2006). In pregnant mares it reduces nasal shedding, cell-associated viraemia and potential abortion (Kydd et al., 2006, Allen et al., 2004). In a field study, vaccination in the face of an outbreak was not associated with any adverse effects and in conjunction with biosecurity measures, most likely reduced abortion-associated losses (Schulman et al., 2015).

Vaccines that are available currently have limitations. In pregnant mares, multiple boosters are required during the course of each pregnancy (Allen et al., 2004) and are typically administered at five, seven and nine months of gestation (Pycock, 1997). Conventional inactivated vaccines provide partial clinical and virological protection against EHV-1 and -4 by stimulating humoral immune responses (virus neutralising antibody) which reduces the amount and duration of virus shed from the nasopharynx. However, vaccines do not prevent the development of a cell-associated viraemia following infection and therefore cannot predictably prevent abortion (Minke et al., 2004). In a 2010 study, a commercially available modified-live vaccine was compared with a commercially available inactivated vaccine. The trial showed that both vaccines provided some protection by decreasing clinical signs of respiratory disease and viral shedding post-infection. The inactivated vaccine was found to reduce viraemia post-infection. The modified-live vaccine reduced clinical signs more than three times as effectively as the inactivated vaccine counterpart (Goehring et al., 2010). The ideal vaccine against EHV-1 and -4 should prevent or reduce respiratory infection and the development of viraemia following challenge infection (Minke et al., 2004). Currently there are no available vaccines that claim to

prevent the development of EHM, largely due to the unavailability of a reliable experimental infection model (Pusterla et al., 2009).

2.9.2 Infection control following a suspected outbreak

In the event of any abortion on a stud farm, an early diagnosis is critical. An infectious cause should be presumed at the outset to ensure all necessary precautions are taken until laboratory results can exclude EHV-1 (Schulman et al., 2015). The affected mare must be isolated (Allen et al., 2004, Chopin, 2010, Pycock, 1997). The aborted fetus and fetal membranes should be sealed in impermeable containers and removed to avoid further contamination of the environment (Allen, 2002, Schulman et al., 2015). The area should be disinfected and staff, vehicle and animal movement restrictions put in place (Chopin, 2010). All in-contact animals should also be isolated—if possible they should be further subdivided into smaller groups to reduce the opportunity for horizontal transmission should further abortions occur. Daily checking of rectal temperature has been shown to be helpful in identifying infected animals with fever but not yet showing other clinical signs (Walter et al., 2013). Group mates should be kept within sight and hearing of the aborting mares to reduce stress (Schulman et al., 2015), and should remain isolated until 30 days after the last abortion (Chopin, 2010).

Mares that are confirmed to have aborted due to EHV-1 infection should be only be re-bred on their second post-abortion oestrus (Van Maanen, 2002, Allen et al., 2004, Allen, 2002). Ideally, different staff should attend isolation areas and must wear a new set of protective clothing between areas (Walter et al., 2013, Allen et al., 2004).

Fomites can be an important means of spreading the virus (Allen, 2002). Both EHV-1 and -4 are labile in the environment but have been shown experimentally to survive for up to seven

days if dried onto wood or rope (Allen et al., 2004). The viruses are killed by heat, lipid solvents and disinfectants (Allen et al., 2004) such as hypochlorite, iodophores, phenols and formalin (Van Maanen, 2002).

In an outbreak of neurological disease, all horse movements should be suspended for at least three weeks (Van Maanen, 2002). Exposed animal groups should be isolated (Pusterla et al., 2009, Van Maanen, 2002). All animals on the farm should be monitored for clinical signs. Serological surveillance could be considered as an additional means of monitoring exposure (Van Maanen, 2002).

2.10 Treatment of EHV-1 infection

Treatment relies on symptomatic and supportive care and in certain cases of respiratory disease, the use of antibiotics, to treat secondary bacterial infections.

2.10.1 Symptomatic treatment of fever, respiratory symptoms and abortion

Treatment for respiratory disease includes the use of antipyretics and prophylactic antibiotics. In the case of abortion, the mare should be treated if necessary for retained fetal membranes and their endotoxic sequelae (Van Maanen, 2002). Treatment of neonatal EHV-1 infection focuses on symptomatic and supportive care. The use of anti-inflammatory drugs, prophylactic antibiotics and acyclovir has been reported (Murray et al., 1998).

2.10.2 Symptomatic treatment of EHV-1 myeloencephalopathy

In the treatment of neurological disease, the aim is to reduce inflammation and ischaemic injury, using non-steroidal anti-inflammatory drugs such as flunixin meglumine (Friday et al., 2000, Pusterla et al., 2009). The use of corticosteroids is controversial (Pusterla et al., 2009). Neurological disease requires good nursing care and monitoring. Animals should be kept standing whenever possible using supportive body slings. Non-slip flooring and good bedding are essential adjuncts to treatment. Elevation of food and water is usually necessary (Pusterla et al., 2009). Recumbent animals should be maintained in a sternal position, with head and leg protection. Manual expression of the bladder or catheterisation may be required and the prevention of urine scald is a priority. Manual evacuation of the rectum or the use of laxatives may also be required (Pusterla et al., 2009). Euthanasia within 48 hours is a frequent outcome in unresponsive recumbent cases, because the prognosis is poor (Allen, 2002, Van Maanen, 2002) and the animals' welfare must be considered (McFadden et al., 2016).

2.10.3 Antiviral agents

Like vaccination, antiviral drugs are unable to prevent infection completely (Maxwell, 2017). Acyclovir is an acyclic nucleoside analogue antiviral drug that has been used in treatment of adult and neonatal horses during outbreaks of EHV-1 infection (Friday et al., 2000, Murray et al., 1998). Treatment protocols are extrapolated from human medicine as studies in horses are limited, although oral dosing with antiviral agents is not associated with adverse effects (Murray et al., 1998, Friday et al., 2000, Bentz et al., 2006). Administration of acyclovir does not prevent viral latency (Murray et al., 1998). While a study has been done on the intravenous administration of valganciclovir, it was found that bioavailability varied greatly between horses (Carmichael et al., 2013). Valganciclovir more potently inhibits EHV-1 but the dosages required are often prohibitively costly (Maxwell, 2017). Valacyclovir has been used to treat horses experimentally infected with neuropathogenic strains of EHV-1. The horses did not develop EHM but still showed signs of ataxia (Maxwell, 2017).

3 Research question

3.1 Aim

To determine the presence of EHV -1 and -4 viral DNA in samples collected from Thoroughbred mares, their healthy newborn foals and their placentae during the 2016-2017 southern hemisphere foaling season.

3.2 Hypotheses

3.2.1 Null hypothesis

Equid alphaherpesvirus 1 and 4 DNA are not present in the nasopharynx and placentae of new-born, viable and healthy foals.

3.2.2 Alternative hypothesis

Equid alphaherpesvirus 1 and 4 DNA are present in the nasopharynx and placentae of new-born, viable and healthy foals.

4 Materials and Methods

4.1 Study design and ethical approval

The study described in this dissertation was a cross-sectional observational study, as used commonly to determine prevalence of a particular disease (Mann, 2003). Ethical approval was granted by the Animal Ethics Committee of the University of Pretoria, project number V109-16.

4.2 Study location and population

The study was conducted on the Moutonshoek Stud Farm near Piketberg, in the Western Cape Province, Republic of South Africa (GPS co-ordinates: 32°43'01.0" S, 18°42'19.0" E).

The study population consisted of 71 Thoroughbred mares and their foals. At the time of the study, the ages of the mares ranged from five to 19 years and the group included maiden and multiparous mares, ranging from one to 12 parities (see Appendix A for specific population details). The farm experienced an abortion storm due to EHV-1 in 2007. Of the 71 mares included in this study, five had been present on the farm at the time of the abortion storm. None of these five horses aborted in 2007. An additional mare present on the farm in 2007 was barren in 2016 and was not included in our study.

4.3 Housing and feeding

On the study farm, pregnant mares were housed in grass paddocks adjacent to the foaling stables. Supplementary feed was given year-round: oat hay (*Secale cereale)* and lucerne *(Medicago sativa)* provided in winter and teff hay *(Eragrostis tef)* and lucerne in the summer.

4.3.1 Foaling management

The mares and their foals were moved from the foaling stables within eight hours of foaling, then placed in paddocks adjacent to the foaling stables. They were typically moved to larger paddocks and ultimately grouped with other mares and foals of a similar age.

4.3.2 Routine care

4.3.2.1 Vaccinations

Pregnant mares were vaccinated against EHV-1 using Pneumabort-K inactivated vaccine (Zoetis, Sandton, South Africa) intramuscularly at 5, 7 and 9 months of gestation. Foals were vaccinated against *Lawsonia intracellularis* using Enterisol Ileitis (Boehringer Ingelheim, Randburg, South Africa) one month before weaning and again at weaning, and against equine influenza (EI) at six and seven months of age using ProteqFlu-Te (Merial, Midrand, South Africa) intramuscularly. Adult horses were vaccinated against African horse sickness (Onderstepoort Biological Products, Onderstepoort, South Africa) and EI annually.

4.3.2.2 Deworming

Foals were dewormed monthly until six months of age and again at nine and 12 months of age. The farm used a combination of the following deworming products for the foals: ivermectin and praziquantel (Pegaforte: Ciplavet, Cape Town, South Africa), moxidectin and praziquantel (Pegaquest: Ciplavet), piperazine and pyrantel pamoate (Pegasol: Ciplavet). Broodmares, yearlings, geldings and stallions were dewormed every six months using ivermectin and praziquantel (Equimax: Virbac, Centurion, South Africa and Pegaforte: Ciplavet) and moxidectin and praziquantel (Equest gel + tape: Zoetis, Sandton, South Africa).

4.4 Sampling procedures

4.4.1 Hygiene and safety considerations

A new pair of gloves was worn for each animal to reduce the opportunity for cross-contamination. All needles and scalpel blades were disposed of using a designated sharps container. Disposal of biological waste was managed through the systems already in place on the farm. Following sampling, fetal membranes were removed to a central disposal area and burned within 24 hours. All gloves and cotton wool not contaminated with blood or other biological material were disposed of in the normal refuse system.

4.4.2 Sample labelling and tracking

Each mare was assigned a unique number at the time of foaling. This number was subsequently recorded with her microchip identity number. The table of sample collection is shown in Appendix B. Each sample collected from a mare, her foal and its fetal membranes was labelled with a barcode with the unique number and a letter indicating the sample type. For example, (a) indicated the mare blood sample and (b) the foal blood sample. This same number and letter were marked on the sample container in indelible ink (Appendix C).

4.4.3 Blood sample collection

4.4.3.1 Animal restraint

For sampling, all mares were restrained using a halter. Fractious animals were restrained using a combination of halter and lip twitch. Foals were restrained manually in a standing position adjacent to their dams by an experienced assistant.

4.4.3.2 Equipment used

For blood sampling, BD Vacutainer® (Becton Dickinson, Johannesburg, South Africa) serum and EDTA tubes were used with either a 20 or 18 gauge, 1.5 inch needle for jugular venepuncture. Sterile 10cm plastic shafted, cotton-tipped swabs were used for collection of both nasal and fetal membrane samples.

4.4.3.3 Blood collection procedure

Blood was collected by jugular venepuncture in the lower third of the neck. In foals, all blood collection was performed from the right jugular vein to reduce the risk of development of left recurrent laryngeal nerve hemiplegia. Prior to venepuncture, the skin was not shaved, but was thoroughly cleaned with an alcohol-soaked cotton wool swab. The EDTA blood sample was labelled and refrigerated at approximately 5 °C.

4.4.3.4 Timing of blood collection

Blood samples were collected from the mare and foal approximately eight hours after foaling, in keeping with the farm's routine post-foaling sampling of foals for serum IgG testing.

4.4.3.5 Nasal swab collection

Nasal swabs were taken from both mare and foal concurrent with blood collection. The nasal swab was inserted carefully, to its full length, into each nostril and rubbed against the mucosa and then rapidly replaced and sealed within its plastic sleeve, without virus transport medium. Each sleeve was labelled and refrigerated at approximately 5 °C until transfer to the laboratory.

4.4.4 Fetal membrane sample collection

Fetal membranes were sampled immediately following their expulsion after parturition, to reduce potential contamination (Figure 3).

Figure 3: Fetal membrane sampling *(Photograph by R. Bettison)*

The fetal membranes were placed on a plastic table for examination and sample collection. This table was thoroughly cleaned between successive samples using a sodium hypochlorite solution (0.5% dilution) and was left in bright sunlight during daylight hours.

For each sample, a layer of newspaper, followed by a layer of disposable, industrial strength plastic sheeting was used to reduce potential cross-contamination. Unused disposable plastic rectal palpation sleeves were worn by the sample collector, over which an additional unused set of disposable latex gloves was worn.

Prior to sampling, fetal membranes were inspected and any pathology or apparent abnormality was noted. After inspection the amnion was removed for disposal. The allantochorion was laid out in a standardised manner with the smooth (allantoic) surface to the outside in an F-shape with the pregnant horn at the top and cervical pole at the bottom. A new scalpel blade was used to incise the allantochorion from the ruptured cervical star area, and the placenta was then inverted to expose the chorionic (villous) surface. A dry swab was rubbed vigorously over the villous surface to sample successively from the three selected sites (i- pregnant horn, ii- non-pregnant horn, iii- body of uterus, as shown in Figure 4).

Two tissue samples were excised from each of sites (i), (ii) and (iii), with one of each paired sample placed in a 10% buffered formalin container, and the other in an empty sterile plastic sample bottle (Figure 5). The dry swab was sealed within its sleeve, labelled and refrigerated. The sample bottle containing tissue alone, was labelled and placed in a freezer at -18 °C and the bottle containing tissue in formalin was labelled and stored at room temperature away from direct sunlight.

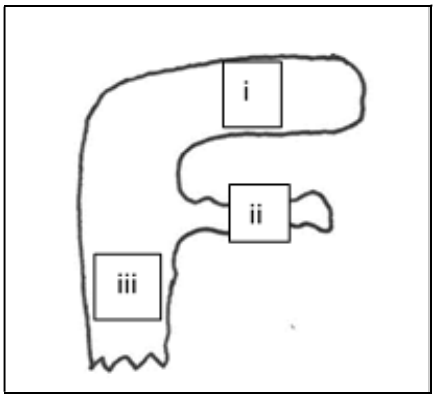

Figure 4: Placental tissue sampling sites

i: pregnant horn. ii: non-pregnant horn. iii: body of the uterus

Figure 5: Placental sample collection procedures

Image A displays the use of a dry swab to sample the villous surface of the placenta. In Image B, excised tissue samples are placed in a sterile sample bottle, and in 10% buffered formalin.
Photographs by R. Bettison

4.4.5 Sample transport

Samples for PCR analysis were removed from 5 °C storage, placed in a sealed polystyrene container to protect from sunlight and transported overnight by road to the laboratory.

4.5 Sample analysis

Duplex qPCR was performed for EHV-1 and EHV-4.

4.5.1 Sample preparation (extraction)

Nasal and placental swabs were agitated in 0.5ml of 0.1M phosphate buffered saline (PBS) (pH 7.4) in a 1.5ml Eppendorf tube for five seconds. The samples were then centrifuged for 1 minute at 13000 RPM (10000G) using a desktop centrifuge. The supernatant was removed from each sample container and was discarded in order to concentrate cellular material and antigen, if present. Thereafter 100 µl of distilled water was added to each container. The samples were then agitated and placed in a temperature-controlled heat block (dry bath).

The 0.1ml PCR plates were prepared in a separate section of the laboratory. The master mix (17 µl per sample) was placed into each sample well of the PCR plate and a foil seal was placed over the plate. The prepared samples (3 µl) were then added to the individual wells of the plate by introducing the pipette tip through the foil seal. Lastly, the positive and negative controls were added. Nucleic acids extracted from EHV-1 and EHV-4 reference viral cultures obtained from the Equine Virology Research Laboratory, University of Pretoria, were used as positive controls. Endonuclease-free water was used as a negative control.

The qPCR was performed according to the manufacturer's guidelines and followed the Standard Operating Procedure (SOP) of the Veterinary Genetics Laboratory using the Applied Biosystems™ Thermo Fischer Scientific StepOnePlus™ Real-Time PCR System (Appendix D). A

cut off value of 40 cycles (C_t) was assigned for the detection of viral DNA in the prepared samples.

5 Results

The qPCR failed to detect EHV-1orEHV-4 nucleic acid in any nasal swabs collected from the study population of 71 mares and their foals, or from their fetal membranes (see Table 3). The positive control used in the qPCR procedure tested positive for viral DNA and the negative control tested negative. For detailed results see Appendices E, F and G.

Since EHV-1 and -4 are respiratory viruses, the failure to detect viral shedding suggested that a cell associated viraemia in the tested horses was unlikely, and consequently blood samples for serology and viral detection were not tested.

Table 3: Summary of results of EHV-1 and EHV-4 specific qPCR obtained from 71 mares, their foals and fetal membranes

Type of sample	**Nº. samples**	**Nº. samples positive for EHV-1 DNA**	**Nº. samples positive for EHV-4 DNA**
Positive control	1	1	1
Negative control	1	0	0
Mare- nasal swab	71	0	0
Foal- nasal swab	71	0	0
Fetal membrane swab	71	0	0

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6 Discussion

Data from this study supported the null hypothesis statement that EHV-1 nucleic acid was not present in samples obtained from healthy new born foals or their fetal membranes.

Furthermore, no EHV-4 was detected in any of the nasal and fetal membrane swab samples taken from the study population. It was concluded that there was no active infection with either EHV-1or EHV-4 detectable by qPCR at the time of sampling.

Various factors may have contributed to the absence of detectable EHV-1 and -4 DNA in this study population. Improper transport of samples to the laboratory may have resulted in a false negative result due to destruction of viral DNA. However, in this study, transport was unlikely to have affected our results, as previous studies using the same sampling and transport system, extraction methods and PCR assay have recovered positive results for EHV-1 and -4 from similar samples (Schulman et al., 2014, Badenhorst et al., 2015). A false negative result due to the assay system was considered unlikely, because the positive control gave a positive reading but no EHV-1 or -4 viral DNA was detected in a relatively large sample set.

A reported EHV-1 abortion-associated epizootic occurred on the same farm in 2007, with nine of the then 30 resident pregnant broodmares aborting (Schulman et al., 2016). The current study included five mares who, although present, did not abort during the 2007 outbreak. An additional mare, present during the previous outbreak, was also resident on the farm but was not sampled due her barren status in 2016. Therefore, previous exposure of some of the sampled mares to the virus had most likely occurred. Assuming a likely previous exposure to the virus, the mares in the current study may simply not have demonstrated viral recrudescence with subsequent viraemia and shedding (Dunowska, 2016). The total number

of broodmares on the farm had increased significantly since 2007, and the percentage of latently infected mares was unknown at the time of the study. Furthermore, the farm's protocol of frequent vaccination of pregnant mares subsequent to the 2007 outbreak may have suppressed viral reactivation and shedding, a suggestion which is supported by Minke et al. (2004), Goehring et al. (2010) and Pusterla et al. (2016).

To find active shedding of virus in animals that are possibly latently infected, presents a challenge well documented in the literature. In a study by Pusterla and co-workers, it was found that there was a low detection rate of EHV-1 in adult horses, even among those showing pyrexia and respiratory signs (Pusterla et al., 2016). Carr and co-workers conducted a study of 124 hospitalised, critically ill horses, to determine the risk of EHV-1 reactivation and shedding in a hospital environment. No evidence of EHV-1 shedding was detected in this study, although low levels of latency could not be excluded (Carr et al., 2011). Sonis and co-workers also concluded from their study of hospitalised, febrile horses, that nasal shedding of EHV-1 and -4 were rare events as only one of 64 febrile horses was PCR- positive for EHV-4 and none were positive for EHV-1 (Sonis and Goehring, 2013).

In light of these prior research findings and considering that animals used in the present trial were healthy, besides the possible stress of impending partus, it is likely that a far larger sample size would be required to detect random recrudescence. Nonetheless, our failure to detect EHV-1 DNA at the time of foaling in a population of animals that had previously experienced an abortion outbreak supports the findings of Pusterla et al., 2016, Carr et al., 2011 and Sonis and Goehring, 2013.

In performing the molecular diagnostics, the improper handling of positive controls may potentially increase the risk of false positives. This risk was addressed in our study by utilising separate rooms for primer preparation, sample processing and sample testing.

Compared to viral isolation, PCR is more sensitive for detection of viral DNA (Lunn et al., 2009), however a major disadvantage of conventional PCR is the risk of product carryover. This risk was reduced by using qPCR in the current study (Pusterla et al., 2006). If viral DNA had been detected by qPCR in our study, the frozen tissue samples were available for virus isolation as well as molecular sequencing.

All mares and foals sampled were resident on a single farm and consequently were exposed to the same management system. Animal movement, feeding, routine care and handling were consistent across all groups, minimising the potential confounding effects of varying management and husbandry methods.

The opportunity for the development of chronic, low-grade infections through reactivation of latency is an effective strategy that EHV-1 uses to maintain itself within the global horse population (Brown et al., 2007, Allen et al., 2004). Arguably, it would appear to be against the best interests of a virus to cause the death of its host, and initiating abortion would create a 'dead end' in viral replication. An EHV-1 positive abortion or neonatal death may benefit horizontal transmission as the abortus or neonate creates a source of infection. However, a seemingly superior viral evolutionary strategy may be to disseminate virus via the birth of an infected, but viable foal. This may result in immediate infection of vulnerable animals in the same cohort but may also permit the development of latency; future reactivation events, might then continue to disseminate the virus to an even wider population of horses.

In a recent preliminary study, there was a strong correlation between the presence of an MHC class 1 B2 allele and pregnancy loss in horses, which was present regardless of the EHV-1 status of the fetus (Kydd et al., 2016). Despite the numerous and multifactorial causes of abortion, the presence of this allele was found to be a statistically significant risk factor (Kydd et al., 2016). While this association needs further study, it raises the interesting idea that perhaps abortion in cases of EHV-1 is not a specific viral propagation strategy, but rather an accident in mares carrying this particular allele. Alternatively, it may be a reflection of the complexity of the interactions between the virus and its host. *In vitro,* MHC class 1 alleles form an important part of the virus's life cycle, with viral glycoprotein D using this allele as an entry receptor (Kydd et al., 2016). Therefore, horses with different MHC class 1 alleles may affect efficient viral entry and lower the ability of the virus to replicate and cause severe disease. MHC class I also plays a key role in the development of the host's immune responses, particularly virus specific, cytotoxic lymphocyte activity (Kydd et al., 2016). Currently, any variation in the role of individual MHC class I alleles in the efficient stimulation of EHV-1 specific immune responses is unknown.

The purpose of this study was to examine critically the currently-accepted routes of EHV-1 horizontal dissemination, questioning the virus' underlying evolutionary strategy by considering that vertical transmission may occur silently and that virus-associated abortions are merely an accidental manifestation.

Several studies have reported the time point between birth and weaning at which foals became EHV-1 and -4 positive (Gilkerson et al., 1999, Foote et al., 2004). Foote and co-workers (2004) showed the presence of EHV-1 and EHV-4 DNA in nasal swabs from a group of foals, some of which were as young as 11 days. The young age at which these foals seroconverted has two

potential explanations. First, a very rapid post-partum infection and seroconversion, despite the presence of maternally derived antibody. Alternatively, as a result of vertical transmission, intrauterine priming may have occurred, leading to rapid seroconversion on exposure immediately after birth.

During an EHV-1 abortion storm, EHV-1 was identified by virus isolation in four out of 39 foals aged 7–9 days, three of which showed no clinical signs (Mumford et al., 1987).

This repeated discovery of EHV-1 and EHV-4 DNA and infectious virus in young healthy foals was a significant factor in the justification of the present study.

The role of donkeys in the viral replication cycle warrants consideration because they may be the natural hosts for EHV-1. In a recent study, the seroprevalence of EHV-1 was much higher in donkeys and mules than in horses, and it was concluded that donkeys and mules could serve as a significant reservoir of infection (Ataseven et al., 2009). Perhaps horses, due to their proximity within a herd, became an aberrant host that the virus adapted to over time? Could horses, as is the case with polar bears and rhinoceroses (Greenwood et al., 2012, Abdelgawad et al., 2014), show more severe signs of infection because they are aberrant hosts? This hypothesis would be difficult to test and the effects of EHV-1 in a wild horse population would be difficult to study, since diseased wild animals do not typically survive long in their natural environment due to predation.

A field study sampling a single farm with a single management system over one season obviously limits extrapolation of the findings to both the South African and global horse population. On this particular farm, there was no evidence of active EHV-1 or -4 infection at the time of sampling. Given the cyclic nature of herpesviral disease, repeat sampling in successive breeding seasons or during the course of an EHV-1 outbreak may better represent

the prevalence of EHV-1 in shedding horses. An alternative approach would be to collect samples on a farm during a period where EHV-1 abortions are occurring concurrent with the birth of viable foals.

Six mares of the current study's sample mares were present during a prior EHV-1 epizootic. During this outbreak, nine of 30 pregnant mares aborted. Although impossible to definitively establish, there was a reasonable probability that a latent carrier state existed among at least these six mares of the population under study. This presented an opportunity to look for detectable viral DNA among apparently healthy animals from a presumed carrier population. Furthermore, the under-reported potential for vertical transmission of detectable EHV-1 DNA could be better defined. As is the case in most biological interactions, the triggers for viral reactivation of EHV-1 and dissemination within a population are probably more complex than a simple response to stress or the physiological milieu of late pregnancy. While the present study failed to show vertical transmission of EHV-1, such transmission was not definitively excluded. Evidence of such a mode of transmission would provide additional valuable information for more effective management of this important viral disease.

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Appendix A: Date of birth (DOB) and parity of sampled mares

Age and parity of foaling mares (n=71)					
Sample ID	**DOB**	**Parity**	**Sample ID**	**DOB**	**Parity**
LB0001	2007	3	LB0037	2005	5
LB0002	2011	1	LB0038	2006	4
LB0003	2005	5	LB0039	2008	3
LB0004	2009	2	LB0040	2006	6
LB0005	2004	6	LB0041	1997	8
LB0006	2007	2	LB0042	2008	2
LB0007	2010	3	LB0043	2006	5
LB0008	2003	3	LB0044	2006	5
LB0009	1999	9	LB0045	2003	6
LB0010	2005	4	LB0046	1998	10
LB0011	2009	1	LB0047	2009	1
LB0012	2004	5	LB0048	2007	4
LB0013	2004	5	LB0049	2004	5
LB0014	2004	5	LB0050	2007	4
LB0015	2009	1	LB0051	2003	5
LB0016	2006	3	LB0052	2005	7
LB0017	2001	1	LB0053	2001	9
LB0018	2006	5	LB0054	2008	1
LB0019	2005	5	LB0055	2004	6
LB0020	2003	7	LB0056	2002	5
LB0021	2003	7	LB0057	2003	8
LB0022	2006	4	LB0058	2005	7
LB0023	2010	2	LB0059	2009	2
LB0024	2004	7	LB0060	2002	8
LB0025	2007	1	LB0061	1997	12
LB0026	2004	5	LB0062	2005	5
LB0027	2011	1	LB0063	1998	10
LB0028	2009	3	LB0064	2005	5
LB0029	2006	6	LB0065	2007	5
LB0030	2003	9	LB0066	2008	2
LB0031	2001	9	LB0067	2008	2
LB0032	2007	4	LB0068	2007	5
LB0033	2009	3	LB0069	2002	8
LB0034	2001	6	LB0070	2004	5
LB0035	2002	8	LB0071	2004	7
LB0036	2010	1			

Appendix B: Sampling information

Sampling information					
Sample ID	**Foaling** **date and time**	**Fetal membrane** **date and time**	**Blood and nasal swab** **date and time**	**Collector**	**Comment**
LB0001	31/08/16 11.31pm	01/09/16 6pm	01/09/16 5.40pm	M. Schulman	Note delay sampling
LB0002	3/09/16 1.46am	3/09/16 2.45am	3/09/16 8.50am	L. Brown	
LB0003	3/9/16 9.25pm	3/09/16 10pm	04/09/16 6.55am	L. Brown	
LB0004	3/09/16 10.20pm	3/09/16 11.10pm	04/09/16 7.20am	L. Brown	
LB0005	7/09/16 3.25am	7/09/16 7.25am	07/09/16 11.30am	L. Brown	Placenta retained until 7.15am
LB0006	8/09/16 3.07am	8/09/16 3.30am	08/09/16 11.40am	L. Brown	Placenta expelled in field, nonpregnant
					horn partially retained. Mare on cortisone
LB0007	8/09/16 3.40am	8/09/16 4.25am	08/09/16 11.45am	L. Brown	
LB0008	12/09/16 2.06am	12/09/16 3.55am	12/09/16 10.10am	L. Brown	
LB0009	14/09/16 5.50am	14/09/16 9.20am	14/09/16 2pm	R. Bettison	
				supervised by L Brown	
LB0010	14/09/16 6.05am	14/09/16 6.35am	14/09/16 2.17pm	K. Skirmaans	
				supervised by L Brown	
LB0011	15/09/16 7.40am	15/09/16 8.07am	15/09/16 3.45pm	R. Bettison	
LB0012	15/09/16 11.15am	15/09/16 12.35pm	15/09/16 7.15pm	R. Bettison	

Sample ID	Foaling date and time	Fetal membrane date and time	Blood and nasal swab date and time	Collector	Comment
LB0013	16/09/16 2.38am	16/09/16 3.20am	16/09/16 11.05am	R. Bettison	
LB0014	17/09/16 00.15am	17/09/16 00.54am	17/09/16 8.20am	R. Bettison	Placenta expelled in field
LB0015	19/09/16 1.55am	19/09/16 2.33am	19/09/16 10.30am	R. Bettison	
LB0016	19/09/16 2.02am	19/09/16 3.09am	19/09/16 10.39am	R. Bettison	
LB0017	21/09/16 11.10pm	21/09/16 11.50am	22/09/16 07.17am	K. Skirmaans	
LB0018	22/09/16 7.40am	22/09/16 8.55am	22/09/16 3.48pm	R. Bettison	
LB0019	25/09/16 19.35pm	25/09/16 8.30pm	26/09/16 6.30am	K. Skirmaans	
LB0020	25/09/16 7.40pm	25/09/16 9.10pm	26/09/16 7am	K. Skirmaans	
LB0021	26/09/16 00.05am	26/09/16 01.30am	26/09/16 8.50am	K. Skirmaans	
LB0022	26/09/16 9.42pm	26/0916 10.40pm	27/09/16 6.40am	R. Bettison	
LB0023	29/09/16 11.10pm	30/09/16 00.20am	30/09/16 07.10am	R. Bettison	
LB0024	29/09/1611.56pm	30/09/16 00.40am	30/09/16 8.00am	R. Bettison	
LB0025	30/09/16 4.22am	30/09/16 4.50am	30/09/16 12.20pm	R. Bettison	
LB0026	2/10/16 1.20am	2/10/16 2am	2/10/16 9.20am	K. Skirmaans	
LB0027	2/10/16 11.30pm	3/10/16 00.05am	3/10/16 7.30am	R. Bettison	
LB0028	06/10/16 8.15pm	06/10/16 10.55pm	07/10/16 7.19am	R. Bettison	Dystocia- foal died. Placenta exposed
					to hands, lube, hibitane
LB0029	08/10/16 4.50am	08/10/2016 5.30am	08/10/16 1pm	L. Brown	
LB0030	09/10/16 3.20am	09/10/16 4.20am	09/10/16 11.55am	L. Brown	
LB0031	09/10/16 9.13pm	09/10/16 10.10pm	10/10/16 6.45am	R. Bettison	
LB0032	09/10/16 11.40pm	10/10/16 00.45am	10/10/16 7.44am	R. Bettison	Placenta passed in field- Placed in
					contaminated bucket
LB0033	10/10/16 00.05am	10/10/16 1am	10/10/16 8.15am	R. Bettison	
LB0034	11/10/16 00.12am	11/10/16 1.25am	11/10/16 8.20am	L. Brown	Mild placentitis. Foal subsequently died-
					PM findings: septicaemia, suspect
					Clostridial infection, pneumonia

Sample ID	Foaling date and time	Fetal membrane date and time	Blood and nasal swab date and time	Collector	Comments
LB0035	11/10/16 3.16am	11/10/16 3.45am	11/10/16 11.30am	L. Brown	
LB0036	11/10/16 11.15pm	12/10/16 00.15am	12/10/16 7.40am	L. Brown	
LB0037	12/10/16 6.45am	12/10/16 7.20am	12/10/16 3.30pm	L. Brown	
LB0038	12/10/16 11.10am	12/10/16 4.55pm	12/10/16 7.10pm	L. Brown	Placenta retained until 4.45pm- expelled in camp so contamination with grass/soil.
LB0039	12/10/16 8.22pm	12/10/16 9.22pm	13/10/16 6.25am	L. Brown	
LB0040	13/10/16 11.45pm	14/10/1 00.25am	14/10/16 7.45am	L. Brown	Mare has cleft palate
LB0041	14/10/16 7.05am	14/10/16 7.30am	14/10/10 3.30 pm	L. Brown	
LB0042	15/10/16 2.22am	15/10/16 2.40am	15/10/16 10.45am	L. Brown	
LB0043	15/10/16 8.43pm	15/10/16 9pm	16/10/16 6.35am	L. Brown	
LB0044	19/10/16 9.22pm	19/10/16 11.35pm	20/10/16 6.55am	L. Brown	Pregnant horn very inflamed
LB0045	20/10/16 9.35am	20/10/16 12.30pm	20/10/16 9.35am and 12.30pm	L. Brown	Mare aborted 1 month before due date. Placenta in sun before sampling
LB0046	20/10/16 11.25pm	20/10/16 11.55pm	20/10/16 11.55pm and 21/10/16 2.30pm (foal blood sample only)	L. Brown	Red bag delivery, foal born dead
LB0047	21/10/16 9.45am	21/10/16 10.20am	21/10/16 5.50pm	L. Brown	
LB0048	22/10/16 9.05pm	22/10/16 9.50pm	23/10/16 6.15 am	L. Brown	Half of non-pregnant horn retained
LB0049	24/10/16 6.31am	24/10/16 7.20am	24/10/16 2.35pm	L. Brown	
LB0050	25/10/16 6.02pm	25/10/16 7.19pm	26/10/16 6.25am	R. Bettison	
LB0051	27/10/16 10.45pm	27/10/16 11.40pm	28/10/16 6.50am	L. Brown	
LB0052	28/10/16 2.49pm	28/10/16 9.35pm	29/10/16 6.40am	L. Brown	
LB0053	29/10/16 3.55am	29/10/16 4.30am	29/10/16 11.30am	L. Brown	
LB0054	30/10/16 9.25pm	30/10/16 10.20pm	31/10/16 6.25am	L. Brown	
LB0055	30/10/16 10.12pm	30/10/16 10.50pm	31/10/16 6.45am	L. Brown	

Sample ID	Foaling date and time	Fetal membrane date and time	Blood and nasal swab date and time	Collector	Comments
LB0056	31/10/16 10am	31/10/16 10.28am	31/10/16 5.50pm	L. Brown	Placentitis- on treatment prior to foaling
					Placenta within entire non-pregnant horn was retained
LB0057	6/11/16 9.14am	6/11/16 10.12am	6/11/16 5.25pm	L. Brown	
LB0058	9/11/16 11.35pm	10/11/16 00.10am	10/11/16 07.40am	L. Brown	
LB0059	10/11/16 5.40am	10/11/16 6.12am	10/11/16 1.40pm	L. Brown	
LB0060	11/11/16 00.37am	11/11/16 01.20am	11/11/16 8.40am	L. Brown	
LB0061	11/11/16 9.23am	11/11/16 10am	11/11/16 5.30pm	L. Brown	
LB0062	11/11/16 8.21pm	11/11/16 10.30pm	12/11/16 6am	K. Skirmaans	
LB0063	14/11/16 12.10pm	14/11/16 1pm	14/11/16 8.15pm	L. Brown	
LB0064	14/11/16 10pm	14/11/16 10.45pm	15/11/16 6.35am	L. Brown	
LB0065	15/11/16 12.13am	15/11/16 12.56am	15/11/16 7.55am	L. Brown	
LB0066	15/11/16 01.37am	15/11/16 2.55am	15/11/16 9.55am	L. Brown	
LB0067	20/11/16 01.50am	20/11/16 2.30am	20/11/16 11am	K. Skirmaans	
LB0068	23/11/16 5.35am	23/11/16 6.14am	23/11/16 13.45pm	R. Bettison	
LB0069	24/11/16 1.20am	24/11/16 2am	24/11/16 9.40am	R. Bettison	
LB0070	29/11/16 7.10am	29/11/16 7.45am	30/11/16 12pm	R. Bettison	
LB0071	30/11/16 2.45 am	30/11/16 3.19am	30/11/16 12.15pm	R. Bettison	

Appendix C: Data collection sheet

Sample Collection Sheet

Mare name:

Microchip number:

Foaling date and time:

Sample collection date and time:

Location collected: Moutonshoek

Collector name:

Samples:

A. Mare blood sample- EDTA ☐

B. Foal blood sample- EDTA ☐

C. Mare nasal dry swab ☐

D. Foal nasal dry swab ☐

E. Placental dry swab- site i,ii,iii ☐

F. Placental sample- sample bottle- site i,ii,iii ☐

G. Placental sample site i, ii, iii in formalin ☐

Place

barcode

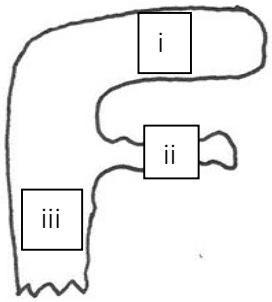

Diagram to show the 3 placental sample sites

Barcode number to be handwritten in indelible ink on sample containers

Change gloves between animals to avoid cross-contamination

Sample A, B bloods in EDTA- refrigerated

Sample C, D - refrigerated

Sample E - refrigerated

Sample F- frozen

Seal formalin container (G) with parafilm under lid

Appendix D: Standard Operating Procedure for Multiplex qPCR for EHV-1 and -4

Standard operating procedure: Multiplex qPCR for EHV-I and -4

A. Sample preparation

Agitate nasal swabs for 5 s in 0.5 ml of 0.1 M phosphate buffered saline (pH 7.4) in a 1.5 ml Eppendorf tube.

B. Nucleic acid extraction using the MagMax^{TM}Pathogen DNA/RNA kit (Life Technologies) and Kingfisher 96 Magnetic Particle Processor (Thermo FisherScientific Inc.)

Plate 1- Sample plate: add 100 µl sample to each well. Add 20 µl of beads mix (10 µl RNA binding beads, 10 µl Lysis Binding Enhancer) to sample, centrifuge for short period, then shake plate for 1 min at max setting on an IKA TTS3D shaker (Merck). Add 400 µl of Lysis Solution Mix (200 µl Lysis Binding Solution, 200 µl Isopropanol) to each sample.

Plates 2 and 3- Wash Solution 1 (300 µl/well). Plates 4 and 5- Wash Solution 2 (450 µl/well).

Plate 6. Elution Buffer (90 µl/well).

Add two positive and one negative control samples to the sample plate with Lysis Solution.

C. Duplex real-time PCR preparation

Master Mix (17 µl/sample):

Kapa Probe Fast ABI Prism@ 2X PCR Master Mix (Kapa Biosystems)	10 µl
EHV-I primer/probe mix	1µl
EHV-4 primer/probe mix	1µl
Nuclease-free water	5µl

D. Plate setup

Use a 0.1 ml PCR plate:

Add 17 µl of the Master Mix per sample.

Add 3 µl of each extracted sample to the wells individually.

E. Duplex real-time PCR

Place the plate on the real-time PCR machine and close the machine.

Select the 'Advance setup' option.

Create run name.

Select Fast run 40 min' (step 1 - 95^{0}C for 20 sec; step 2 - 95^{0}C for 1 sec, for 20 sec, step 2 repeated for 40 cycles).

Click on Run method' and change volume to 20 pl.

Click on 'Plate setup'.

Import sample sheet by clicking on file, then import, then browse and then select file.

Select targets: TaqMan Probe EHV-I and EHV-4.

Click on 'Assign targets and samples'.

Add targets to each sample.

Save run.

Ensure machine is closed and that samples are in correct rows.

Select 'Start run'.

Run lasts approximately 45 min.

Appendix E: Duplex RT-PCR results for EHV-1 and EHV-4 mare nasal swabs

Sample ID	Test date	C_t	Detection DNA EHV-1	Detection DNA EHV-4
LB001c	30/11/2016	40	Negative	Negative
LB002c	30/11/2016	40	Negative	Negative
LB003c	30/11/2016	40	Negative	Negative
LB004c	30/11/2016	40	Negative	Negative
LB005c	30/11/2016	40	Negative	Negative
LB006c	30/11/2016	40	Negative	Negative
LB007c	30/11/2016	40	Negative	Negative
LB008c	30/11/2016	40	Negative	Negative
LB009c	30/11/2016	40	Negative	Negative
LB010c	30/11/2016	40	Negative	Negative
LB011c	30/11/2016	40	Negative	Negative
LB012c	30/11/2016	40	Negative	Negative
LB013c	30/11/2016	40	Negative	Negative
LB014c	30/11/2016	40	Negative	Negative
LB015c	30/11/2016	40	Negative	Negative
LB016c	30/11/2016	40	Negative	Negative
LB017c	30/11/2016	40	Negative	Negative
LB018c	30/11/2016	40	Negative	Negative
LB019c	30/11/2016	40	Negative	Negative
LB020c	30/11/2016	40	Negative	Negative
LB021c	30/11/2016	40	Negative	Negative
LB022c	30/11/2016	40	Negative	Negative
LB023c	30/11/2016	40	Negative	Negative
LB024c	30/11/2016	40	Negative	Negative
LB025c	30/11/2016	40	Negative	Negative
LB026c	30/11/2016	40	Negative	Negative
LB027c	30/11/2016	40	Negative	Negative
LB028c	30/11/2016	40	Negative	Negative
LB029c	30/11/2016	40	Negative	Negative
LB030c	30/11/2016	40	Negative	Negative
LB031c	30/11/2016	40	Negative	Negative
LB032c	30/11/2016	40	Negative	Negative
LB033c	30/11/2016	40	Negative	Negative

Sample ID	Test date	C_t	Detection DNA EHV-1	Detection DNA EHV-4
LB034c	30/11/2016	40	Negative	Negative
LB035c	30/11/2016	40	Negative	Negative
LB036c	30/11/2016	40	Negative	Negative
LB037c	30/11/2016	40	Negative	Negative
LB038c	30/11/2016	40	Negative	Negative
LB039c	30/11/2016	40	Negative	Negative
LB040c	30/11/2016	40	Negative	Negative
LB041c	30/11/2016	40	Negative	Negative
LB042c	30/11/2016	40	Negative	Negative
LB043c	30/11/2016	40	Negative	Negative
LB044c	30/11/2016	40	Negative	Negative
LB045c	30/11/2016	40	Negative	Negative
LB046c	30/11/2016	40	Negative	Negative
LB047c	30/11/2016	40	Negative	Negative
LB048c	30/11/2016	40	Negative	Negative
LB049c	30/11/2016	40	Negative	Negative
LB050c	30/11/2016	40	Negative	Negative
LB051c	30/11/2016	40	Negative	Negative
LB052c	30/11/2016	40	Negative	Negative
LB053c	30/11/2016	40	Negative	Negative
LB054c	30/11/2016	40	Negative	Negative
LB055c	30/11/2016	40	Negative	Negative
LB056c	30/11/2016	40	Negative	Negative
LB057c	30/11/2016	40	Negative	Negative
LB058c	30/11/2016	40	Negative	Negative
LB059c	30/11/2016	40	Negative	Negative
LB060c	30/11/2016	40	Negative	Negative
LB061c	30/11/2016	40	Negative	Negative
LB062c	30/11/2016	40	Negative	Negative
LB063c	30/11/2016	40	Negative	Negative
LB064c	30/11/2016	40	Negative	Negative
LB065c	30/11/2016	40	Negative	Negative
LB066c	30/11/2016	40	Negative	Negative
LB067c	09/01/2017	40	Negative	Negative
LB068c	09/01/2017	40	Negative	Negative
LB069c	09/01/2017	40	Negative	Negative
LB070c	09/10/2017	40	Negative	Negative
LB071c	09/01/2017	40	Negative	Negative

Appendix F: Duplex RT-PCR results for EHV-1 and EHV-4 foal nasal swabs

Sample ID	Test date	C_t	Detection DNA EHV-1	Detection DNA EHV-4
LB001d	30/11/2016	40	Negative	Negative
LB002d	30/11/2016	40	Negative	Negative
LB003d	30/11/2016	40	Negative	Negative
LB004d	30/11/2016	40	Negative	Negative
LB005d	30/11/2016	40	Negative	Negative
LB006d	30/11/2016	40	Negative	Negative
LB007d	30/11/2016	40	Negative	Negative
LB008d	30/11/2016	40	Negative	Negative
LB009d	30/11/2016	40	Negative	Negative
LB010d	30/11/2016	40	Negative	Negative
LB011d	30/11/2016	40	Negative	Negative
LB012d	30/11/2016	40	Negative	Negative
LB013d	30/11/2016	40	Negative	Negative
LB014d	30/11/2016	40	Negative	Negative
LB015d	30/11/2016	40	Negative	Negative
LB016d	30/11/2016	40	Negative	Negative
LB017d	30/11/2016	40	Negative	Negative
LB018d	30/11/2016	40	Negative	Negative
LB019d	30/11/2016	40	Negative	Negative
LB020d	30/11/2016	40	Negative	Negative
LB021d	30/11/2016	40	Negative	Negative
LB022d	30/11/2016	40	Negative	Negative
LB023d	30/11/2016	40	Negative	Negative
LB024d	30/11/2016	40	Negative	Negative
LB025d	30/11/2016	40	Negative	Negative
LB026d	30/11/2016	40	Negative	Negative
LB027d	30/11/2016	40	Negative	Negative
LB028d	30/11/2016	40	Negative	Negative
LB029d	30/11/2016	40	Negative	Negative
LB030d	30/11/2016	40	Negative	Negative
LB031d	30/11/2016	40	Negative	Negative
LB032d	30/11/2016	40	Negative	Negative
LB033d	30/11/2016	40	Negative	Negative

Sample ID	Test date	C_t	Detection DNA EHV-1	Detection DNA EHV-4
LB034d	30/11/2016	40	Negative	Negative
LB035d	30/11/2016	40	Negative	Negative
LB036d	30/11/2016	40	Negative	Negative
LB037d	30/11/2016	40	Negative	Negative
LB038d	30/11/2016	40	Negative	Negative
LB039d	30/11/2016	40	Negative	Negative
LB040d	30/11/2016	40	Negative	Negative
LB041d	30/11/2016	40	Negative	Negative
LB042d	30/11/2016	40	Negative	Negative
LB043d	30/11/2016	40	Negative	Negative
LB044d	30/11/2016	40	Negative	Negative
LB045d	30/11/2016	40	Negative	Negative
LB046d	30/11/2016	40	Negative	Negative
LB047d	30/11/2016	40	Negative	Negative
LB048d	30/11/2016	40	Negative	Negative
LB049d	30/11/2016	40	Negative	Negative
LB050d	30/11/2016	40	Negative	Negative
LB051d	30/11/2016	40	Negative	Negative
LB052d	30/11/2016	40	Negative	Negative
LB053d	30/11/2016	40	Negative	Negative
LB054d	30/11/2016	40	Negative	Negative
LB055d	30/11/2016	40	Negative	Negative
LB056d	30/11/2016	40	Negative	Negative
LB057d	30/11/2016	40	Negative	Negative
LB058d	30/11/2016	40	Negative	Negative
LB059d	30/11/2016	40	Negative	Negative
LB060d	30/11/2016	40	Negative	Negative
LB061d	30/11/2016	40	Negative	Negative
LB062d	30/11/2016	40	Negative	Negative
LB063d	30/11/2016	40	Negative	Negative
LB064d	30/11/2016	40	Negative	Negative
LB065d	30/11/2016	40	Negative	Negative
LB066d	30/11/2016	40	Negative	Negative
LB067d	09/01/2017	40	Negative	Negative
LB068d	09/01/2017	40	Negative	Negative
LB069d	09/01/2017	40	Negative	Negative
LB070d	09/01/2017	40	Negative	Negative
LB071d	09/01/2017	40	Negative	Negative

Appendix G: Duplex RT-PCR results for EHV-1 and EHV-4 placental swabs

Sample ID	Test date	C_t	Detection DNA EHV-1	Detection DNA EHV-4
LB001e	30/11/2016	40	Negative	Negative
LB002e	30/11/2016	40	Negative	Negative
LB003e	30/11/2016	40	Negative	Negative
LB004e	30/11/2016	40	Negative	Negative
LB005e	30/11/2016	40	Negative	Negative
LB006e	30/11/2016	40	Negative	Negative
LB007e	30/11/2016	40	Negative	Negative
LB008e	30/11/2016	40	Negative	Negative
LB009e	30/11/2016	40	Negative	Negative
LB010e	30/11/2016	40	Negative	Negative
LB011e	30/11/2016	40	Negative	Negative
LB012e	30/11/2016	40	Negative	Negative
LB013e	30/11/2016	40	Negative	Negative
LB014e	30/11/2016	40	Negative	Negative
LB015e	30/11/2016	40	Negative	Negative
LB016e	30/11/2016	40	Negative	Negative
LB017e	30/11/2016	40	Negative	Negative
LB018e	30/11/2016	40	Negative	Negative
LB019e	30/11/2016	40	Negative	Negative
LB020e	30/11/2016	40	Negative	Negative
LB021e	30/11/2016	40	Negative	Negative
LB022e	30/11/2016	40	Negative	Negative
LB023e	30/11/2016	40	Negative	Negative
LB024e	30/11/2016	40	Negative	Negative
LB025e	30/11/2016	40	Negative	Negative
LB026e	30/11/2016	40	Negative	Negative
LB027e	30/11/2016	40	Negative	Negative
LB028e	30/11/2016	40	Negative	Negative
LB029e	30/11/2016	40	Negative	Negative
LB030e	30/11/2016	40	Negative	Negative
LB031e	30/11/2016	40	Negative	Negative
LB032e	30/11/2016	40	Negative	Negative

Sample number	Test date	C_t	Detection viral DNA Virus probe EHV 1	Detection viral DNA Virus probe EHV 4
LB033e	30/11/2016	40	Negative	Negative
LB034e	30/11/2016	40	Negative	Negative
LB035e	30/11/2016	40	Negative	Negative
LB036e	30/11/2016	40	Negative	Negative
LB037e	30/11/2016	40	Negative	Negative
LB038e	30/11/2016	40	Negative	Negative
LB039e	30/11/2016	40	Negative	Negative
LB040e	30/11/2016	40	Negative	Negative
LB041e	30/11/2016	40	Negative	Negative
LB042e	30/11/2016	40	Negative	Negative
LB043e	30/11/2016	40	Negative	Negative
LB044e	30/11/2016	40	Negative	Negative
LB045e	30/11/2016	40	Negative	Negative
LB046e	30/11/2016	40	Negative	Negative
LB047e	30/11/2016	40	Negative	Negative
LB048e	30/11/2016	40	Negative	Negative
LB049e	30/11/2016	40	Negative	Negative
LB050e	30/11/2016	40	Negative	Negative
LB051e	30/11/2016	40	Negative	Negative
LB052e	30/11/2016	40	Negative	Negative
LB053e	30/11/2016	40	Negative	Negative
LB054e	30/11/2016	40	Negative	Negative
LB055e	30/11/2016	40	Negative	Negative
LB056e	30/11/2016	40	Negative	Negative
LB057e	30/11/2016	40	Negative	Negative
LB058e	30/11/2016	40	Negative	Negative
LB059e	30/11/2016	40	Negative	Negative
LB060e	30/11/2016	40	Negative	Negative
LB061e	30/11/2016	40	Negative	Negative
LB062e	30/11/2016	40	Negative	Negative
LB063e	30/11/2016	40	Negative	Negative
LB064e	30/11/2016	40	Negative	Negative
LB065e	30/11/2016	40	Negative	Negative
LB066e	30/11/2016	40	Negative	Negative
LB067e	09/01/2017	40	Negative	Negative
LB068e	09/01/2017	40	Negative	Negative
LB069e	09/01/2017	40	Negative	Negative
LB070e	09/01/2017	40	Negative	Negative
LB071e	09/01/2017	40	Negative	Negative