Breeder Demographic Survey and Molecular Period Prevalence of Equid Alphaherpesvirus 1 in Healthy Ontario Broodmares

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Carina J. Cooper

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ABSTRACT

BREEDER DEMOGRAPHIC SURVEY AND MOLECULAR PERIOD PREVALENCE OF EQUID ALPHAHERPESVIRUS 1 IN HEALTHY ONTARIO BROODMARES

Carina J. Cooper
University of Guelph, 2020

Advisors:
Brandon N. Lillie
Luis G. Arroyo

Equid alphaherpesvirus 1 (EHV-1) causes disease in all ages of horses, from abortion and neonatal death to encephalomyelopathy in adults. The main objective of this thesis was to investigate the current opinions of breeders in Ontario, provide prevalence estimates, and describe current vaccine use for EHV-1, which have not been studied. A survey of 88 breeders in Ontario indicated 72.7 % currently vaccinate their herds, and most consult veterinarians for assistance only when encountering abortions, not respiratory disease. Most herds were vaccinated either twice annually or using a "pre-foaling protocol".

In a follow up study, a period prevalence estimate was generated by sampling 381 broodmares from 42 farms across Ontario every two months from December 2016 through October 2017. Samples were collected from the nose, blood and vaginal mucosa and analysed using droplet digital PCR (ddPCR). A total of 85.0 % of broodmares were positive for EHV-1 at some point during the sampling period, and vagina was identified as a source of EHV-1, with viral DNA collected more often from the vagina than the nose. Using mixed logistic regression models, we found no association between virus presence and age or breed. Nasal shedding occurred more often in pregnant mares, and vaccination was only associated with reduced virus in blood samples. When describing the virus neutralization titer response in this population, 32.3 % of

mares did not respond to vaccination, and 48.5 % of all mares had no biologically significant changes in titers over the sampling period. Using IgG subisotyping, differing immune responses were observed depending on vaccine product most recently administered. There was no association between IgG subisotype and neutralizing virus titers, nor was there an association with serological titers and ddPCR positivity. Titers did generally increase with age and were typically highest in Standardbred and Thoroughbreds.

In conclusion, although most respondents were concerned about EHV-1 enough to vaccinate, many did not, leaving the population at risk for outbreaks. Although vaccines were widely used, they were not consistent in their immune stimulation, potentially creating a false sense of security among breeders. Furthermore, most broodmares harbour the virus and shed intermittently.

Dedication

This thesis is dedicated to 3 very important people who supported me and gave up so much of their time to see me succeed and complete this thesis. Without each of you, I would be lost and not nearly as proud of the final product.

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Declaration of Work Performed

All of the work described in this thesis was performed by me, with the following exceptions:

Chapter 3: Sampling was performed by the farm owners, Dr. Luis Arroyo, me with the assistance of visiting students Dr. Bruna Parapinski and Veridianna Cunha, and summer students, Drs. Michaela Botts, and Bailey Fuller. DNA isolation and preparation of PCR samples was done by Drs. Jutta Hammermueller, Michaela Botts, and Bailey Fuller, as well as me. The droplet digital PCR analysis was performed by the Animal Health Laboratory at the University of Guelph (Guelph, ON, Canada).

Chapter 4: Virus neutralization of serum samples was performed by Keenan Mullaney under the supervision of Dr. Roger Maes at the Diagnostic Center for Public and Animal Health at Michigan State University (Lansing, MI, USA). The IgG subisotype ELISA was performed by Drs. Jutta Hammermueller, Bailey Fuller, and Solstice Pecile, a visiting student laboratory technician, and me.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AAEP	American Association of Equine Practitioners
ACC	Animal Care Committee
AHL	Animal Health Laboratory
AIC	Akaike's information criterion
Asn	asparagine
Asp	aspartic acid
AUP	Animal Utilization Protocol
BLUPS	best linear unbiased predictors
CD	cluster of differentiation
CI	confidence interval
CMI	cell-mediated immunity
Ct	cycle threshold
CTL	cytotoxic T lymphocytes
ddPCR	droplet digital polymerase chain reaction
DNA	deoxyribonucleic acid
dsDNA	double-stranded deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
eGAPDH	equine glyceraldehyde 3-phosphate dehydrogenase
EHM	equine herpesvirus myeloencephalopathy
EHV-1	equid alphaherpesvirus 1
ELA	equine leukocyte antigen
ELISA	enzyme-linked immunosorbent assay
ETIF	equine α-trans-inducing factor
FBS	fetal bovine serum
g	glycoprotein
HA	hemagglutinin
HHV	human alphaherpesvirus
HSV	herpes simplex virus
ICC	intra-class correlation coefficient
ICP0	infected cell polypeptide 0
ICTV	International Committee of Taxonomy of Viruses
IE	immediate early
IFAT	indirect fluorescent antibody test
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IQR	interquartile range

IRF	IFN regulatory transcription factor
JNK	c-Jun N-terminal kinase
LAMP	loop-mediated isothermal amplification
LAT	latency associated transcript
LN	lymph node
mAbs	monoclonal antibodies
Mal/TIRAP	MyD88-adapter-like
MHC	major histocompatability complex
miRNA	micro-ribonucleic acid
MLV	modified-live vaccines
mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation primary response 88
NFκB	nuclear factor-κB
NK	natural killer
OMAFRA	Ontario Ministry of Agriculture, Food, and Rural Affairs
OR	odds ratio
ORF	open reading frames
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PMN	polymorphonuclear cells
qPCR	quantitative PCR
RBC	red blood cell
REB	Research Ethics Board
RK13	rabbit kidney cells
RNA	ribonucleic acid
RT-PCR	reverse transcription -PCR
SMLN	submandibular lymph node
ssDNA	single-stranded deoxyribonucleic acid
TGFβ	transforming growth factor-β
Th	T-helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF	tumor necrosis factor receptor-associated factor
UL	unique long
URL	uniform resource locator
US	unique short
USA	United States of America
VN	virus neutralization
WBC	white blood cell

List of Appendices

Chapter 1 Literature Review

1.1 Introduction

Equid alphaherpesvirus 1 (EHV-1) has been associated with diseases affecting horses of all ages, from abortion and perinatal death, to respiratory disease and meningoencephalitis in adults. Infection may first occur in foals from their dams, leading to seroconversion and even latent infections without necessarily causing overt disease (Gilkerson *et al.* 1999a & b). Further exposure throughout the life of the horse occurs from novel infections or from reactivation of latent virus due to stress or other unknown stimulation (Pusterla *et al.*2010a). Some articles have stated that EHV-1 presence is essentially ubiquitous in equine populations with prevalence estimates up to 88 % depending on the study population (Carvalho *et al.* 2000), and that shedding may occur intermittently (Allen *et al.* 2008, Foote *et al.* 2004). No studies have performed a cross-sectional investigation to determine the EHV-1 prevalence being harboured by a healthy population. With newly implemented identification techniques, the number of total EHV-1 diagnoses made on submitted samples (from respiratory, abortion, and one encephalitis case) appeared to be increasing in Ontario, Canada as recently as 2012 compared to previous years (Carman *et al.* 2012). At the same time, some farms that have endemic EHV-1 and vaccinate regularly, have sporadic shedders and encounter clinical disease at any given time, suggesting that individual horses may have different risks of clinical disease depending on their own virus reactivation (Brown *et al.*2007).

Although vaccination helps, the cornerstone of protection still lies with management because exposure cannot be entirely prevented. Vaccines have been used since the 1960s, yet outbreaks of EHV-1-associated diseases continue to occur (Chowdury *et al.* 1986, Barrandeguy *et al.* 2002, Burgess *et al.* 2012). In addition, the neuropathogenic strain of EHV-1, which is associated with a greater risk of neurological disease and causes abortions (Nugent *et al.* 2006), has been associated with outbreaks as recently as 2019 in Ontario, Canada, and there is still no vaccine licensed for protection against neurological disease. The current guidelines recommend vaccination of the general population at least bi-annually, and pregnant mares at 5, 7, and 9-months gestation, in addition to a multivalent vaccine given at 30-days prior to foaling (AAEP

2018). These recommendations are based on vaccine studies where researchers have noted protection when given shortly before exposure (Bresgen *et al.* 2012, Foote *et al.* 2002, Paillot *et al.* 2008, Heldens *et al.* 2001). However, there has been some evidence that vaccines are not as effective against abortion as previously touted (Bürki *et al.* 1990, Foote *et al.* 2004). Frequent vaccination in broodmares has even been detrimental to the circulating protective antibody levels (Wagner *et al.* 2015), further bringing into question the efficacy of current vaccination programs.

In Ontario, the extent to which EHV-1 impacts the breeding industry has not previously been studied. The actual prevalence estimates of latent and active infections are currently unknown in Ontario, or anywhere in Canada, with data about this population coming from reports on isolated incidents of disease or outbreaks. Currently, the number of horse breeders who vaccinate against EHV-1 is unknown, as is the EHV-1 status of the broodmare population, except for those who have experienced disease or the few who have been tested due to suspected exposure. It would be impossible to discuss the risk of EHV-1 in Ontario without knowledge of the underlying prevalence within the population and the level of concern within the industry. Vaccination is thought to be widespread, as veterinarians lead the herd health activities of most farms in Canada, but the response to the vaccines and quality of protection are unknown in this population. To investigate this problem, we need to understand the virus pathogenesis, immune responses to current available vaccines, and the prevalence within this population.

1.2 Herpesvirus

1.2.1. General characteristics and classification

Herpesvirales is a large order that is characterized by viral morphology and behaviour. Herpesviruses have managed to infect all investigated vertebrate species, and even insects and mollusks. The genome characteristics have little to do with the classification of the virus into the order, rather the typical icosahedral shape and latency are key taxonomic determinants. The International Committee of Taxonomy of Viruses (ICTV) has divided the *Herpesvirales* order into 3 families depending on the species they infect: *Herpesviridae* (mammals, birds and reptiles), *Alloherpesviridae* (fish and frogs), and *Malacoherpesviridae* (bivalve oyster). *Herpesviridae* are then further subdivided into 3 subfamilies: *Alpha-, Beta-, and Gammaherpesvirinae* (ICTV 2017). Herpesviruses have co-evolved with their hosts in order to adapt to the complicated host immune system created to expel foreign deoxyribonucleic acid (DNA). Much of our current knowledge is extrapolated from research on human alphaherpesvirus 1 (HHV1, previously known as herpes simplex virus (HSV)) of humans (Maclachlan & Dubovi 2010). Genes, proteins, and mechanisms of action have been compared and contrasted in other herpesviruses to grow our understanding of the viruses exponentially.

1.2.2. Virus genomic structure and proteins

The genome of all herpesviruses is made of double-stranded deoxyribonucleic acid (dsDNA) from 125-295 kbp in length and forms the core of the virion. From 70 to over 200 open reading frames (ORFs) have been isolated from various herpesvirus strains, interspersed between microRNA (miRNA) genes, and there remain segments of DNA with unknown functions. The structure of the genome varies between viruses and families in the presence, number, and order of the repeated sequences, the unique long (UL) sequences, and the unique short (US) sequences. To add confusion to the puzzle, these sequences can be “direct” or “inverted” and found internally (between other sequences) or terminally. The combinations of sequences have allowed herpesviruses to be divided into four general groups based genomic structure: 1) long direct terminal repeat at each end, 2) multiple short repeated direct terminal repeats at each end, 3) terminal and internal repeats that may be direct or inverted and create a UL and US sequence,

and 4) a combination of long and short, inverted and direct, internal and terminal repeats with UL and US sequences (Maclachlan & Dubovi 2010). *Alphaherpesvirinae* are typically in this latter format.

The genome is then packaged into the 16-sided icosahedral capsid and surrounded by a lipid bilayer envelope. This envelope contains glycoproteins which are commonly investigated in genome and immunologic studies. An amorphous proteinaceous material which contains capsid- and envelope-associated proteins called the "tegument" is present between the inner capsid and outer envelope membranes. The tegument can be associated with the inner capsid or outer envelope membranes (Guo *et al.* 2010, Owen *et al.* 2015). The function of many proteins within the tegument is unknown, however some have been shown to serve as preformed materials or scaffolding to speed up replication within host cells, while others help with transportation, manipulation of the host environment, or immune evasion. There are approximately 40 protein-coding genes that are unchanged in mammalian and avian herpesviruses, with these genes coding for capsid, membrane, and tegument proteins, elements for replication and virion assembly, and some host-control proteins. These genes have been divided into "immediate early (IE)" or α, "early" or β, and "late" or γ groups, depending on when during transcription they are activated (Guo *et al.* 2010, Telford *et al.* 1992, Owen *et al.* 2015, Ibáñez *et al.* 2018). Gene homology has been essential in understanding the mechanisms of immune modulation of newer mammalian herpesviruses, such as equid alphaherpesvirus 1 (EHV-1) (Telford *et al.* 1992).

1.2.3. Replication

When the virus prepares to enter a host cell, glycoproteins (g) on the envelope surface (gB, gD, gH, and gL) assist with binding and fusion with the cell membrane, and release of the capsid into the cytoplasm (Ibáñez *et al.* 2018, Poelaert *et al.* 2019). The capsid is carried to the nucleus along microtubules in a bi-directional and "saltatory movement" (Owen *et al.* 2015). Upon arrival near the nucleus, the virus core is injected through a nuclear pore with the aid of tegument proteins (Owen *et al.* 2015). The viral genome is circularized inside the host nucleus (Strang & Stow 2007). Once the viral genome is exposed to host transcription factors, the genome is activated in a sequential manner to begin the replication cycle. To begin viral replication, seven gene products are required: an origin-binding protein, an ssDNA-binding protein, a DNA polymerase with two subunits, and a helicase-primase complex of three gene products (Gulati *et*

al. 2015). In addition, IE proteins, equine α-trans-inducing factor (ETIF), a late tegument protein, and four other early proteins are also needed to initiate the cascade (Charvat *et al.* 2011, Gulati *et al.* 2015). The use of a "genome activation cascade" ensures that virion genome replication occurs first and generates a certain amount of DNA prior to the shift to protein and scaffold production. For example, ETIF activates the IE genes, which then act as transcription factors for the early and late genes, but ETIF also acts to downregulate the promoter for the late gene gK to ensure a sequential genome activation (Kydd *et al.* 2006, Kim *et al.* 2012). The IE genes begin gene expression and promote early gene transcription. The early genes form the DNA replication complex and modify host cell metabolism, and the late genes are expressed only after viral DNA synthesis has begun and produce the structural proteins. New DNA is put into preformed immature capsids based on a scaffold formed on the inner nuclear membrane of the cell (Granzow *et al.* 2001, Turcotte *et al.* 2005, Lv *et al.* 2019). The nuclear envelopment complex containing the viral integrin proteins pUL31 and pUL34 binds to the inner nuclear membrane and promotes vesicle formation (Johnson & Baines 2011, Lv *et al.* 2019). Viral pUS3 and protein kinase C solubilize the nuclear membrane allowing the capsid to move through (Johnson & Baines 2011). These same proteins are left behind in the outer nuclear membrane when the capsid is de-enveloped and released into the cytoplasm. Glycoprotein gB and the gH-gL dimer are involved in virus membrane fusion and egress through the nuclear membrane but do not appear critical, as virus particles were still present on cell surfaces when infected with deletion mutants (Johnson& Baines 2011). Tegument proteins accumulate on the surface of cytoplasmic capsids (Granzow *et al.* 2001), and more are added when the capsid undergoes a second envelopment and vesicle formation (Turcotte *et al.* 2005). The second envelopment occurs via the Golgi apparatus and involves the tegument-coated virus capsid moving through the membranes of the Golgi itself (*cis*-Golgi and medial Golgi) and via the trans-Golgi network and/or endosomes (Johnson& Baines 2011, Lv *et al.* 2019). EHV-1, unlike HHV1, does not appear to utilize endosomes or lysosomes (Granzow *et al.* 2001). Glycoprotein gD and the gE-gI dimer interact with tegument protein and other glycoproteins to stimulate movement to the perinuclear membranes and secondary envelopment in the Golgi (Johnson & Baines 2011, Owen *et al.* 2015). Once loaded into endosomes, the virus may exit the cell through exocytosis rather than cell lysis, and, in the case of HHV1, can be found attached to the outer surface of the cell (Johnson & Baines 2011). Cell lysis occurs when two enveloped viruses are found within the

cytoplasm after having budded from the trans-Golgi network or endosomes within the cell, disrupting the cell functions and matrix, or due to host immune-induced cell apoptosis. A detailed review on herpesvirus egress can be found in Lv *et al*. (2019) or Tandon & von Einem (2020).

1.2.4. Herpesvirus immune evasion

Herpesviruses work in three ways to effectively evade the host immune system: 1) they directly downregulate the host cell function and immune response, 2) they can spread between cells without entering the extracellular environment, and 3) they can undergo latency, surviving within cells and preventing the cell's senescence (Oladunni *et al.* 2019).

The innate immune system is the first line of defense against any invading virus. One facet of the innate response is activated when intracellular receptors sensitive to foreign DNA, RNA, or proteins respond by activating interferons (IFNs) (Oladunni *et al.* 2019). Once stimulated, IFNs trigger chemotaxis of immune cells, differentiation of the adaptive immune system, as well as apoptosis and autophagy of the infected cell. Based on research in HHV1, several IE genes and tegument proteins have been shown to regulate the IFN and apoptotic pathways. A few examples of the proteins and their mechanisms are given here to demonstrate the various pathways inhibited by herpesviruses, but a detailed review on the immune system evasion of HHV1 can be found elsewhere (Gulati *et al.* 2015, Sarkar 2015, Su *et al.* 2016).

- Infected cell polypeptide 0 (ICP0) - reduces the Toll-like receptor (TLR)-2 inflammatory response via reduction of myeloid differentiation primary response 88 (MyD88) and MyD88-adapter-like (Mal/TIRAP) responses (van Lint *et al.* 2010). ICP0 is also capable of binding p65 thereby preventing nuclear translocation and reducing nuclear factor-κB (NFκB) response (Zhang *et al.* 2013). This protein plays a role in latency establishment as well (Pan *et al.* 2014, Gulati *et al.* 2015).
- US3 – limits the TLR2 response by reducing tumor necrosis factor (TNF) receptor-associated factor (TRAF)-6 ubiquitination, and dimerization of IFN regulatory transcription factor (IRF)-3 and p65, thereby reducing NFκB response.

- US11 - an RNA binding tegument protein interacts with endogenous retinoic acid inducible gene (RIG)-I and melanoma differentiation-associated protein (MDA)-5 preventing their complex formation and limiting the production of IFNβ.
- UL36 - essential for replication and conserved across the Herpesviridae family because it contains a deubiquitinase (DUB) that inhibits proteins requiring ubiquitination to exert their function thereby reducing IFNβ production.
- UL41 - an endoribonuclease that targets the messenger RNA (mRNA) of IFN-stimulating genes like viperin, zinc finger antiviral protein, and tetherin, and prevents their stimulation of IFN production.

Once cells are infected, HHV1 and other herpesviruses, including EHV-1, can direct virus-laden vesicles to cell-cell junctions in polarized cells. In epithelial cells, for example, HHV1 can be found on the lateral surfaces, in direct contact with the neighboring cells (Owen *et al.* 2015). This movement is aided by the gE-gI dimer, but other glycoproteins, such gB, gD, gM, and gK, also participate in cell-to-cell spreading (as reviewed in Khusro *et al*. 2020). Removal of the gE-gI dimer rendered EHV-1 avirulent in young horses (Matsumura *et al.* 1998). Cell-to-cell spread, or "bridging" was confirmed in EHV-1 as well when the virus passed into neighboring cells in the face of neutralizing antibody (Goehring *et al.* 2011, Spiesschaert *et al.* 2015, Poelaert *et al.* 2019). As early as 1974, Notkins concluded that cell-mediated immunity (CMI) may be the most important defense against EHV dissemination because of this cell-to-cell transfer, which was only suspected at the time (Notkins 1974).

Due to an unknown stimulus, herpesviruses may not replicate and erupt from the cell, but instead undergo "latency". Latency is the state of virus presence in an inactive form within a host cell, and the ultimate demonstration of immune evasion. Herpesviruses demonstrate a tropism for latency in slow growing or static cells like neurons (e.g. the trigeminal ganglion), lymph nodes (LNs), and circulating leukocytes, making antemortem identification of carriers challenging (Edington *et al.* 1994, Chesters *et al.* 1997, Ma *et al.* 2013, Rusli *et al.* 2014). Latency of EHV-1 has also been confirmed in the draining LNs of the respiratory tract and the trigeminal ganglion (Edington *et al.* 1994), and in peripheral blood mononuclear cells (PBMCs) (Chesters *et al.* 1997).

Studies regarding latency often depended on extrapolation from reactivation experiments and reverse engineering to make assumptions on how latency was established. What is known so far is that herpesviruses enter the latent state by translocating the viral genome to the cell's nucleus, circularizing it, and maintaining it as an episome using histones (Mellerick & Fraser 1987, Preston & Efstathiou 2007, Gulati *et al.* 2015). The acetylated and methylated histones cover the promoter regions of lytic genes (Cliffe & Knipe 2008, Cliffe *et al.* 2017). Although the entire viral genome is present in the infected cell, only latency-associated transcript (LAT) RNAs, associated with IE genes, may be detectable (Chesters *et al.* 1997). The function of LATs has long remained elusive but recent research has provided evidence on host cell response modulation and downregulation of host cell apoptosis (You *et al.* 2017), preventing superinfection of cells with multiple herpesviruses (Mador *et al.* 2003), and possibly preventing IE and ICP0 gene expression (Gulati *et al.* 2015). To support the latter theory, latency has been induced by inhibiting the function of the IE proteins (Kydd *et al.* 2006). It has been found that EHV-1 contains two LATs, encoded by genes 63 and 64 (Edington *et al.* 1994, Chesters *et al.* 1997, Borchers *et al.* 1999, Vargas-Bermudez *et al.* 2018), that can effectively decrease the major histocompatibility complex (MHC) I receptor on lymphocytes (Ma *et al.* 2012), and prevent apoptosis of infected cells (Proft *et al.* 2016). In addition to LATs, it appears that microRNAs are also involved in the persistence of latency (as reviewed by Gulati *et al* (2015). MicroRNAs can target certain genes and silence them, preventing the virus from replicating and can downregulate cellular defense genes as well (Riaz *et al.* 2014). Therefore, in order to persist without detection in their host, herpesviruses infect cells that do not replicate (such a neurons) to avoid cellular mechanisms of defense, form episomes to quiet their gene replication, actively inhibit apoptosis of infected cells, and produce LATs and microRNAs that further inhibit their own gene expression as well as host defense signals.

1.2.5. Reactivation and recrudescence

Latently infected cells can be reactivated under specific conditions, to cause either subclinical reactivation, with or without shedding and vasculitis, or to cause clinical disease known as "recrudescence" (Patel & Heldens 2005). The stimuli associated with reactivation and recrudescence are typically cellular stressors and require the cellular activation of c-Jun N-terminal kinase (JNK) signaling, a stress-response pathway (Cliffe *et al.* 2017). Unlike *de novo*

infection, when a latent virus is reactivated, it requires an epigenetic switch to work around the histones and begin generating the viral proteins needed for DNA synthesis (Cliffe *et al.* 2017). Unlike the original infection, there is no gene cascade meaning IE, early, and late genes are replicated simultaneously, and this initial burst of replication occurs without the demethylation of histones (Cliffe *et al.* 2017). A histone methyl/phospho switch, correlating with increased JNK activity, has been described where phosphorylation of serine residues is able to overcome repressive lysine modifications (Cliffe *et al.* 2015). When a certain threshold of viral genes has been crossed, viral replication continues in the form of a cascade as seen in *de novo* infections. This means that all transcription factors are required, and histones are demethylated to allow access to the entire genome (Cliffe *et al.* 2015).

Reactivation of EHV-1 has been successfully induced by corticosteroid administration in both cell culture and in horses (Edington *et al.* 1985, Gibson *et al.* 1992, Slater *et al.* 1994), however true recrudescence, involving viremia, shedding, and clinical disease, has been much more sporadic, even in these clinical trial environments (Pusterla *et al.* 2010a). Environmental factors thought to cause stress, and therefore viral reactivation have been investigated, including travel, illness, colic, and sales, but all have been relatively inconsistent, despite each having been involved in outbreaks of clinical disease in the past (Pusterla *et al.* 2009c, Carr *et al.* 2011, Sonis & Goehring 2013, Carlson *et al.* 2013, Badenhorst *et al.* 2015). Therefore, the prediction of which cells will endure viral recrudescence, and which undergo virus propagation or lysis, is unknown (Cliffe *et al.* 2015).

1.3 Equid alphaherpesvirus 1

1.3.1. History

EHV-1 is part of the *Alphaherpesvirinae / Varicellovirinae* genus, along with EHV- 3, 4, 8 and 9, bovine herpesviruses 1 and 5, and pseudorabies virus (PRV) (ICTV 2017). It is important to note these other viruses that are classified similarly to EHV-1, as many details regarding pathogenesis, viral proteins, and immunomodulation are similar and therefore extrapolated in the research.

EHV-1 infection is a significant problem in the equine industry due to its ability to cause disease in all life stages of horses, from late-term abortions and neonatal deaths, to respiratory disease in young and myeloencephalopathy in adults (Wohlsein *et al.* 2011). As a cause of abortion, EHV has been described as early as 1932, as cited in "The Herpesviruses" (Roizman 1983), and has been associated with neurologic conditions since 1942. Due to the very similar immunologic responses elicited by EHV-1 and 4, they were originally thought to be two variations of the same virus (occasionally referred to a "fetal, F" and "respiratory, R" strains, or subtype 1 and 2) (Studdert 1983, Edington *et al.* 1985, Meyer *et al.* 1987). It was not until Studdert *et al*. (1981), described the different sequence fingerprints using restriction endonucleases that it became evident that "EHV-1" was actually two viruses: EHV-1 and EHV-4, the fetal and respiratory subtypes, respectively. In 1987, Meyer *et al*. showed that EHV-1 and 4 both caused respiratory disease, but EHV-1 was the more prevalent herpesvirus associated with equine abortion and myeloencephalopathy, and EHV-4 was only associated with those conditions on very rare occasions. However, the differentiation of the two viruses was not officially recognized until Roizman *et al* (1992) published an updated taxonomy. Data thereafter were much more specific to either EHV-1 or EHV-4, and often the studies would investigate both viruses together, and draw parallels to further elucidate the nuances of their pathogeneses and behaviour.

1.3.2. Replication and pathology

Briefly, a common pathogenesis of EHV starts with an aerosol spread of particles from a carrier, and mucosal infection of the naïve recipient. It is believed that initial infection may occur as early as the first month of life since passive immunity does not prevent infection, and exposure is from the dam herself (Gilkerson *et al.* 1999a, Foote *et al.* 2004). EHV-1 infection behaves like

most herpesviruses initially, replicating in the respiratory tract mucosa, causing cell lysis, and shedding. It can then infect local mononuclear cells, predominantly monocytes and T lymphocytes (Van de Walle *et al.* 2009) and cluster of differentiation (CD) 172a+ monocytes (Poelaert *et al.* 2019), who leave the epithelial surface, penetrate through the basement membrane (Vandekerckhove *et al.* 2011), and either return to the LN or enter the systemic circulation creating a viremia (Poelaert *et al.* 2019). Replication can occur in the LN as well, as demonstrated by the presence of viral DNA, late proteins in these organs and successful co-cultivation of lymphoid tissues in cell cultures (Welch *et al.* 1992, Rusli *et al.* 2014). T cells that are CD4+ appear more susceptible to EHV-1 infection than CD8+ cells, possibly because CD4 is part of the receptor to which EHV-1 binds (Goodman *et al.* 2006, Vandekerckhove *et al.* 2011, Poelaert *et al.* 2019). Direct virus transmission from infected T cells to endothelial cells *in vitro* then occurs with the aid of gB, pUS53, and LFA1 by forming of a viral synapse in the infected T cell adjacent to the bound target cell (Smith *et al.* 2002, Spiesschaert *et al.* 2015, Proft *et al.* 2016, Poelaert *et al.* 2019). Both T-cell polarization and dynein transport mechanisms are used to move capsid from the nucleus through the host cell and to the viral synapse (Smith *et al.* 2002, Poelaert *et al.* 2019). The mechanisms, to the extent of our current knowledge are discussed in Poelaert *et al*. (2019).

Although there have been some studies assessing isolated groups or outbreaks to determine EHV viral kinetics, none have identified a consistent correlation between the index case and proximity to other animals, or viral load shed and actual clinical disease development in new cases (Van Maanen *et al*. 2000, Pusterla *et al*. 2009b, Damiani *et al*. 2014, Bannai *et al*. 2014). When focusing on viral loads specifically, in both clinical disease and exposed cases, no clear cut-off of viral particle number has been identified that guarantees disease or propagation (Pusterla *et al*. 2008b, Pusterla *et al*. 2009a, Pusterla *et al*. 2009b, Estell *et al*. 2015). Also, outbreak studies have identified multiple strains in the same exposed population, suggesting that it is not always a propagation of the index virus to naïve horses, but may either be a multivirus exposure, or recrudescence of latent virus in the stressed population (Pusterla *et al*. 2009b). This may explain the variety of disease presentations seen in outbreaks situations.

1.3.2.1. EHV-1 associated diseases

When aerosols are inhaled, the epithelia of the upper respiratory tract, pharynx, turbinates, soft palate, and trachea are invaded, and viral replication and cell lysis ensues. The incubation period for respiratory disease is approximately 2-10 days, but fevers and shedding can occur within 24 hours after infection under experimental conditions (Matsumura *et al.* 1992). Clinical signs from lytic lesions are observed and environmental contamination occurs via viral shedding (Kydd *et al*. 1994). Respiratory signs associated with EHV-1 include: pyrexia, anorexia, conjunctivitis, rhinitis and nasal discharge (Van Maanen *et al*. 2001, Patel & Heldens 2005).

Equine alphaherpesvirus viremia and mononuclear cell infection allow the virus to reach distant sites, including the nervous system and reproductive organs. Abortions typically occur during late pregnancy following pyrexia or respiratory disease (Edington *et al*. 1991, Matsumura *et al.* 1992). The virus replicates in endothelial cells causing vasculitis, which causes the additional clinical signs: abortion, neurologic disease, chorioretinopathy and even epididymal lesions (Tearle *et al*. 1996, Smith *et al*. 2004, Borchers *et al*. 2006, Soboll Hussey *et al*. 2013, Gulati *et al*. 2015). The incubation period prior to abortive signs has been described between 9- and 120-days post infection (Matsumura *et al.* 1992). Abortion typically occurs between 2-12 weeks after infection, during the last trimester of gestation, depending on the severity of the vasculitis induced (Smith *et al*. 2004). In males, EHV-1 has been found in seminal fluids and the epididymis (Tearle *et al*. 1996, Hebia-Fellah *et al*. 2009), and in females, in the uterus after a sterile abortion and even having crossed the placenta and infected the fetus (Smith *et al*. 2004). Occasionally, the fetus may be born but survives only a short period (often less than 24 hours) due to respiratory compromise (Paillot *et al*. 2008). Although EHV-4 has also been associated with abortion, it is a very uncommon occurrence, and the mechanism is not well understood (Patel & Heldens 2005).

The neurologic disease caused by EHV-1, equine herpesvirus myeloencephalopathy (EHM), follows an incubation period of approximately 6-10 days. This neurologic disease, a relatively uncommon outcome of EHV-1 infection, involves multifocal lesions leading to rapidly progressing lethargy, hind limb ataxia and recumbency, loss of anal tone, and urinary incontinence (van Maanen *et al*. 2001, Allen *et al*. 2008, Soboll Hussey *et al*. 2013). Chorioretinal lesions such as retinal degeneration and choroiditis, appearing as multifocal, small,

donut-shaped lesions close to the optic disc have also been seen (Whitwell & Blunden 1992, Soboll Hussey *et al*. 2013, Holz *et al.* 2019). EHM has been associated with a mutation of the 752nd amino acid, which is presumed to enhance viral tropism to neuronal cells, but the wild type is also involved in neurologic outbreaks both independent from and as a co-infection with the mutant strain (Nugent *et al*. 2006, Perkins *et al*. 2009, Pusterla *et al*. 2009b). A shift from adenine to guanine at base position 2254 (within ORF 30) creates a substitution from asparagine (Asn, N) to aspartic acid (Asp, D) of amino acid 752 in the catalytic subunit (polymerase) of the DNA replication complex (Nugent *et al*. 2006, Allen *et al*. 2008). The odds of having EHM with the D752 (mutant) strain are 490 times greater than those with a N752 (wildtype) strain (Perkins *et al*. 2009), and 86 % of horses with EHM had the neuropathogenic strain (Nugent *et al*. 2006). Initial assays using the D752 (mutant) strain were associated with prolonged viremia, and therefore more likely to affect the CNS vascular endothelium (Allen & Breathnach 2006, Nugent *et al*. 2006). In support of the increased mutant virulence, it has been shown that the mutant strain was faster and more efficient at infecting monocytic cells from explants (Vandekerckhove *et al*. 2011). A recent experimental study confirmed increased viremia and shedding associated with the D752 variant reported by Allen and Breathnach (2006) and added that low levels of IFNγ were present in horses affected by EHM (Holz *et al*. 2017). Infections with the mutant D752 variant led to a four-fold increase in nasal EHV-1 shedding compared to infections with the N752 (wildtype) variant, thus the neuropathogenic strain is associated with an increased virulence and very high amounts of viral particle shed from the nose (Franz *et al*. 2017). Clinically, shedding after EHM has a prolonged duration that may be reduced with valacyclovir administration (Pusterla *et al.* 2009b), but this treatment has been controversial due to inconsistent benefits and results (Maxwell *et al*. 2017, Garré *et al*. 2009).

1.4 Immunology

1.4.1. Host defenses

Every host has an innate and an adaptive immunity to potential threats of disease. Innate immunity is largely reliant on barriers, sentinel cells that remove foreign pathogens, and soluble proteins, such as complement and non-specific antibodies (See section 1.2.4 Herpesvirus Immune Evasion). The innate system can signal for help if infections occur, leading to activation of the adaptive immune system, including cell-mediated immunity and/or antibody-mediated (humoral) immunity.

Viral antigens found in the respiratory tract after inhalation or mucosal contact are processed by resident macrophages and presented on the MHC II. This antigen-MHC II complex is seen by CD4+ T cells, a macrophage or dendritic cell in a regional draining LN, and they produce interleukin-12 (IL-12) to call natural killer (NK) cells to the site of infection (Steinbach *et al*. 1998). In turn, NK cells produce IFNγ that promotes CD4+ T cell's differentiation to the T helper 1 (Th1) phenotype and enhances their activation. When host cells become infected with the virus, viral peptides are mounted on MHC I molecule in the endoplasmic reticulum. CD8+ T cells bind to specific viral antigen-MHC I complexes on the surface of infected cells, get activated by IL-2 produced by the Th1-cells, and are driven to proliferate into cytotoxic T lymphocytes (CTLs) (Coombs *et al*. 2006). CTLs destroy target cells through TNFα secretion or via the apoptosis-promoter Fas and attract more immune cells to the area by secreting IFNγ. Meanwhile, B-cells bind to the antigen and become antibody-secreting plasma cells in the presence of IFNγ. In the presence of IL-4, produced in response to EHV-1 infection of antigen presenting cells, CD4+ T cells differentiate into T helper 2 (Th2) and produce more IL-4 to enhance antibody production by the plasma cells. Now both the humoral and CMI pathways are activated and are specific to the viral pathogen. Of note, there are actually seven different types of CD4+ T cells that have been identified, each with different cytokines and regulatory functions, but little is known of their role in EHV-1 response (as reviewed by Luckheeram *et al*. 2012).

The role of the antibody is to limit free virus spread or, by binding to foreign surface proteins, to stimulate the CMI response by signalling that a cell is infected (Kydd *et al*. 2006, Kydd *et al*. 2016). Immunoglobulin G (IgG) is the most prevalent antibody and is a key responder to viruses

(Lewis *et al*. 2008, Tizard 2017). There are seven subclasses of IgG with different capabilities for binding antigens and stimulating immune responses (See Table 1.1 below) (Lewis *et al*. 2008). Interestingly, IgG subclasses are produced at different times, with IgG3 and 5 first detectable within five to eight weeks after birth, but IgG4 and 7 not being detected for 16-20 weeks (Holznagel *et al*. 2003, Wagner *et al*. 2017). This is why maternal colostrum, rich in antibodies and other immune factors, is so critical to the success of mammalian offspring as it provides IgGs that have been primed prior to parturition. The most prevalent IgG subisotypes in adult serum are IgG4 and IgG7, followed by IgG3 and IgG5, then IgG1, and lastly IgG6 and IgG2. All subisotypes stimulated peripheral blood leukocytes, except IgG2 and 6. When binding complement, IgG3 was most effective followed by IgG1, IgG4 and IgG7 (Lewis *et al*. 2008).

Once the initial viral infection is resolved, B and T cells differentiate into memory cells, which takes approximately four-to-five days (Smith *et al*. 2004, Wagner *et al*. 2017). Antibody and memory cell formation depend on the quality of the host's initial response to exposure or previous vaccination, meaning immune priming post-vaccination is very variable (Wagner *et al*. 2017).

The importance of the innate immune system against EHV-1 is not to be overlooked. When re-infecting specific pathogen-free foals 61 days after initial infection using the same *Ab4* strain, no clinical signs, viremia, or virus shedding were identified (Gibson *et al*. 1992). Unlike in adult horses, it is theorized that protection was primarily due to high IFN and NK cell activity in the three-to-four-month-old specific pathogen-free foals (Gibson *et al*. 1992). The age of the foals in these studies is important because newborn foals are inherently deficient in IFNγ, with marked inability to express the IFNγ gene until three months of age, and it only reaches adult levels after one year (Breathnach *et al*. 2006).

Table 1.1: New vs old nomenclature of IgG subclasses of horses, adapted from Lewis et al. (2008).

New nomenclature	Old Nomenclature
IgG 1	IgGa
IgG 2 (Tizard 2017), 6 (Wagner 2006)	IgGc
IgG 3/5	IgG(T)
IgG 4/7	IgGb

1.4.1.1. Cellular response to EHV-1

The most effective cells at fighting an EHV infection are CTLs who destroy virus-infected cells. Having co-adapted to its host, EHV-1 has found methods to smother initial T-cell responses by infecting them, reducing IFNγ production (Wagner *et al*. 2017) and then downregulating the MHC I presence on the cell surface (Ma *et al.*, 2013). In fact, EHV-1 has been found in all subpopulations of PBMC, but $CD8^+$ T lymphocytes (CTL precursors) and B lymphocytes were most frequently infected (Wilsterman *et al*. 2011). As demonstrated in three-to-four-month-old specific pathogen-free foals, PBMC levels typically first decline between 2-7 days-post-infection, and then increase around 10-13 days-post-infection, before returning to pre-infection levels by day 20 (Gibson *et al*. 1992). Similar immunosuppression with rebound lymphocytosis is seen in adult animals (Allen *et al*. 1995, Soboll Hussey *et al*. 2011, Perkins *et al*. 2019). Further describing this initial immunosuppression, Perkins *et al*. (2019) identified little IFNγ and few circulating $CD4^+$ and $CD8^+$ T cells one week after infection with the *Ab4* strain. In addition to the quantitative immunosuppression of the PBMCs, increased levels of transforming growth factor-β1 (TGF-β1), a known regulator and suppressor of T, B and NK cells, were found in EHV-1 exposed sera, especially in horses who became clinically ill (Charan *et al*. 1997). This was the first-time cytokine function had been investigated in EHV-1 infection. Pro-inflammatory (IL-1, TNF-α) and regulator (TGF-β) cytokines were increased within two days after *Ab4* strain infection, along with similar T-cell suppression (Soboll Hussey *et al*. 2011). The decline in non-specific T-cell function was able to last at least 40 days post EHV-1 *V592* inoculation (Hannant *et al*. 1991). The difference in severity and duration of immunosuppression may be due to differences in the challenge strains used.

Following early PBMC suppression, the next phase involves an increase in circulating PBMC levels characterized by predominantly MHC-1-restricted $CD8^+$ or "cytotoxic", and IFNγ producing T cells (Lunn *et al*. 1991, Allen *et al*. 1995, Allen *et al*. 2008, Paillot *et al*. 2008). An increase in CD4+ lymphocytes also occurred one week post inoculation, but these appeared to be T helper cells, producing IL-2 and acting as antigen presenting cells to promote the differentiation of CTLs (Allen *et al*. 1995). The EHV-1-specific CTLs continued to lyse virus-infected target cells for at least 6 weeks, with the peak activity occurring at two-to-three weeks post inoculation (Allen *et al*. 1995). In adult ponies and horses, those who had high levels of CTL precursors were better protected against challenge infection, suggesting an effective memory function of lymphocytes (O'Neill *et al*. 1999, Kydd *et al*. 2003).

1.4.1.2. Unifying the immunoglobulin, cytokine, and CMI responses to EHV-1

Unlike many other viruses, once EHV-1 infects a host, very little virus is found outside of host cells, limiting the efficacy of the humoral response. This is consistent with experimental studies that found neutralizing antibodies alone were insufficient to prevent disease in subjects succumbing to illness and viremia despite vaccination and the production of high virus neutralization (VN) titers (Stokes & Wardley 1988). A better analysis of the humoral response was sought and found when IgG subisotyping was popularized. Researchers have also looked at cytokine and IgG antibodies as proxies for humoral and CMI responses.

The various arms of the immune system use cytokines to coordinate responses to pathogens. Some cytokines promote the CMI response, whereas others enhance antibody production. The CD4+ T helper cell family is divided based on the cytokines they produce. Instead of these CD4+ T cells being thought of as 7 distinct subpopulations, they may in fact represent a continuum, with the Th1 and Th2 responses being the polar ends (Horohov 2000, Hegazy *et al*. 2010). Key cytokines produced by Th1 cells are IFNγ and IL-2, as well as the pro-inflammatory mediator, TNFα. Both IFNγ and IL-2 stimulate the differentiation of CD8+ cytotoxic T cells, as well as the activity of NK cells and polymorphonuclear cells (PMNs). Therefore, the Th1 response is most associated with CMI. In addition, IFNγ is in turn produced by the CD8+ effector cells and NK cells to further enhance immune stimulation (Guidotti & Chisari 2001). Direct measurement of CTL activity is laborious and time consuming, therefore surrogates were sought to simplify the investigations. When IFNγ was used as a surrogate for CTL activity in

horses, increased levels were associated with protection from equine influenza virus-associated disease and virus shedding (Paillot *et al*. 2006). The Th1 response with elevated IFNγ has been associated with protection from viruses in other species as well (Woolums *et al*. 2003, Meier *et al*. 2004, Aranyos *et al*. 2016).

Th2 cells produce predominantly IL-4, -5, and -13 (Hegazy *et al*. 2010). Originally, IL-4 was thought to just enhance the activity of plasma cells to generate antibody and limit the activity of Th1 cells. Depending on the timing of exposure, IL-4 can actually augment CTL differentiation and MHC-restricted cytotoxicity when added five-to-six days post Equine Influenza virus exposure (Horohov *et al*. 1988). In contrast, when added at the time of antigen exposure, IL-4 suppressed the CTL activity, confirming both a stimulatory and regulatory effect (Horohov *et al*. 1988). The other cytokines produced by Th2 cells have roles in antibody induction as well, especially IgE, or in modulation of the immune response (as reviewed by Horohov *et al*. 1988 and Luckheeram *et al*. 2012). Therefore, the Th2 response is typically associated with humoral immunity and regulation.

In addition to lymphocytes and cytokines, IgG subisotypes appear have associations to specific immune responses as well. There is a debate in the literature about whether the IgGb (IgG4/7) response is directly protective or a proxy of the CMI protection. An IgG(T) (IgG 3/5) dominated response was found when ponies were vaccinated against equine influenza virus with the alum-adjuvanted Fluvac™ (Fort Dodge, now Zoetis), but was not associated with protection when challenged (Nelson *et al*. 1998). This was in contrast to infected ponies, who mounted an IgGa (IgG 1), IgGb (IgG 4/7) and IgA response and had reduced clinical signs upon reinfection (Nelson *et al*. 1998, Soboll *et al*. 2003). In addition, DNA vaccination of horses with the hemagglutinin (HA) gene resulted in a similar clinical protection that was associated with IgGa (IgG1) and IgGb (IgG 4/7), but without a mucosal IgA response (Soboll *et al*. 2003). When investigating cytokine production, infected horses had significant amounts of IFNγ and IL-2, whereas the vaccinated group did not, and instead had higher levels of IL-4 with some increase in IL-2 (Nelson *et al*. 1998). These results are in line with another study where a DNA vaccine containing the HA gene stimulated a Th1 cytokine response (along with increased IgGa and IgGb) (Lunn *et al*. 1999). These data suggest an association between IgGa (IgG 1) and IgGb (IgG 4/7) and the Th1 response, as demonstrated by increased IFNγ and IL-2, and an association

between the IgG(T) (IgG 3/5) levels and a Th2 response with IL-4 (Horohov 2000). That IgG 4/7 is in and of itself protective remains unclear.

Using the results from equine influenza virus investigations, research has been done with EHV-1 to see if a similar pattern could be found. As indicated above, infection with EHV-1 induces MHC-1-restricted CD8+ or "cytotoxic" cell proliferation, followed by a CD4+ helper cell increased with IL-2 production (Allen *et al*. 1995, Lunn *et al*. 1991). The measurement of IFNγ was first performed in response to EHV-1 infection in 2005, and increased production in response to viral infection was seen in horses, as had been described in other species (Breathnach *et al*. 2005). As expected, more CD4+, CD5+, and CD8+ cells produced IFNγ after EHV-1 infection, whereas the B-cell population did not, and the increased IFNγ production was associated with increased CTL activity (Breathnach *et al*. 2005, Allen *et al*. 2008). As Nelson *et al*. 1998 and Soboll *et al*. 2003 had found with equine influenza, Breathnach *et al.* reported that vaccination did not induce a IgA response, but did reduce the clinical signs seen and duration of viremia after infection with EHV-1 *Army183* (Breathnach *et al.* 2001). A study comparing young and naïve to primed ponies found that previously EHV-1-infected ponies had a high IFNγ/low IL-4 response when re-infected (Coombs *et al*. 2006). The young group began the study with low IFNγ and IL-4 but both increased throughout the study post infection. A ratio of IFNγ/IL4 was also used to summarize these findings: the primed group had consistently high ratio values and the naïve group was consistently low (Coombs *et al*. 2006). This study concluded that the immune response in the older, exposed group was polarized towards a Th1 response compared to a non-polarized response in the young naïve group. Because only the immune older group, with the polarized response, had lymphoproliferation in response to EHV-1, it was also suspected that the polarized IFNγ response supported lymphoproliferation, and therefore immunity to challenge infection (Coombs *et al*. 2006). These studies also lend support to the notion that IFNγ can be used as an indirect indicator of CTL activity in EHV-1 research as it had for equine influenza. Evidence that IgGb (4/7) is protective and associated with IFNγ production comes from series of vaccine studies which applied the vaccinate to the nasal mucosa and elicited a high local and systemic antibody responses when compared to the wildtype strain infection (Wimer *et al*. 2018). These antibody responses were dominated by EHV-1-specific IgG 4/7 antibodies and IFNα locally, and a weak peripheral T-cell response (Wimer *et al*. 2018). When these horses were later challenge-infected by the wildtype *Ab4* strain, a pre-existing high *local* IgG 4/7 antibody level in

the nasal mucosa appeared to prevent EHV-1 infection in respiratory epithelium as indicated by no viral shedding or IFNα presence, leading the authors to conclude IgG 4/7 had a protective effect in the nose (Perkins *et al*. 2019). This study also confirmed that a high IgG 4/7 level was associated with increased IFNγ and CTL numbers systemically. However, another study contradicted the notion that IgG 4/7 was protective when IgGb (4/7) was not correlated with IFNγ production, and protection from clinical disease was not consistent (Soboll Hussey *et al*. 2011, Goodman *et al*. 2012). All studies agree, however, that IgG(T) (IgG 3/5) is considered part of the Th2 response (Hooper-McGrevy *et al*. 2003, Goodman *et al*. 2006, Soboll Hussey *et al*. 2011, Perkins *et al*. 2019). Taken together, these findings demonstrate the difficulty in relying solely on systemic humoral immune responses for predicting protection from an intracellular pathogen like EHV-1, as high IgGb (IgG 4/7) subisotype may reduce respiratory disease and neutralize free virus but is not always able to prevent viremia and secondary clinical manifestations. In addition, Kydd *et al.* (2003) reported that the amount of complement fixing antibodies in blood was not associated with protection from strain *Ab4* infection, but a high pre-infection CTL level was. These contradictions also lend evidence that the immune response may differ in efficacy when comparing the outcome at the level of the local mucosae compared to systemically.

The antibody response to EHV-1 and vaccination is also assessed using VN assays. Vaccines were initially touted as protective when they generated an increased VN titer. However, as described above, it was quickly agreed that a high VN titer did not guarantee protection, and in some instances, infection and outbreaks occurred in the face of high titers (Thomson *et al.*1979). To better understand the humoral response to EHV-1, horses were infected with the *Ab4* strain and IgGb (IgG 4/7) responses were found to parallel VN titers, while IgGa (IgG 1) responses were of a shorter duration compared to VN responses, and the IgG(T) (IgG 3/5) response was low throughout (Soboll Hussey *et al*. 2011). Because low pre-infection IgG(T) (IgG 3/5) titers were present but VN was negligible, IgG(T) likely does not contribute to the VN values (Soboll Hussey *et al*. 2011). Similar results were described when infection with the *Army 183* strain of EHV-1 was compared to a subunit vaccine response (Soboll *et al*. 2006), and when vaccinated and exposed horses were compared (Goodman *et al*. 2012). The VN and IgG 4/7 values may be useful in differentiating vaccination from exposure, as a vaccinated and exposed group of horses

had significantly higher VN and IgG 4/7 titers than a recently vaccinated group (Goodman *et al*. 2012).

Using the IgG subisotypes to qualify the immune response generated has led to ratio exploration, such as IgGa (1): IgGb (4/7) (Hooper-McGrevy *et al*. 2003), IgGb (4/7): IgGa (1) (Perkins *et al*. 2019), IgG(T) (3/5): IgGb (4/7) (Goodman *et al*. 2006), and IgGb (4/7): IgG(T) (3/5) (Soboll Hussey *et al*. 2011). Investigating the IgG ratio IgGa(1) or b (4/7) versus T (3/5) responses should parallel the IFNγ: IL-4 ratio of cytokine response suggested by Coombs *et al*. (Coombs *et al*. 2006). When investigating the intracellular bacterium *Rhodococcus equi*, a higher IgGa (1): IgGb (4/7) ratio was protective, and they concluded that IgGa (1) was most associated with a Th1 response (Hooper-McGrevy *et al*. 2003). With EHV-1, an IgG $4/7^{high}$/IgG 1^{low} pattern was present in horses that were protected (Perkins *et al*. 2019). Goodman *et al*. (2006) suggested that IgGb was more predictive than IgGa for antiviral protection, and this was supported by fewer clinical signs in horses with high IgGb (4/7): IgG(T) (3/5) ratios after vaccination with an ORF1/2 deletion strain (Soboll Hussey *et al*. 2011). Although the Th1 and CMI/CTL response appears dominant and associated with protective immunity in all referenced studies, the IgG subisotypes were different, indicating that the type of intracellular pathogen (bacterium vs virus) may stimulate yet another facet of the CMI system and IgG subisotype association.

Based on these studies, it is possible to conclude that CMI, IFNγ and IgGa (1), and IgGb (4/7) should be elicited for good vaccine-induced immunity. When testing the humoral immune responses, IgGb (4/7) correlates well with VN titers in experimental models but may not be sufficient in ensuring protection. Furthermore, a strong IgGa (1) and IgGb (4/7) response is typically seen with virus infection as are the induction of cytotoxic T cells and the increase of IFNγ and IL-2 responses. All of these have been associated with better protection from subsequent exposure. The IgG(T) (3/5) and IL-4 responses appear to occur more in response to vaccination and are not associated with protection from clinical disease. Therefore, it follows that IgGa (1) and IgGb (4/7) are likely associated with the Th1-cell and CMI responses with their respective cytokines IL-2 and IFNγ, whereas IgG(T) (3/5) is associated more with a Th2-cell response and IL-4.

1.4.2. Response to currently available vaccines

EHV-1 vaccination has been practiced since the 1960s, and all vaccines available commercially at the time of this writing appear to have been developed prior to 1980. A literature review on the topic of current vaccines and their origins is difficult, as many of the original archived articles have not been digitized or are no longer available to the public. For this reason, this literature review begins in 1990, unless referenced otherwise, and earlier articles will be cited from the more recent reference.

In order to mount an effective defense against infection, both arms of the adapted immune system, humoral and CMI, must be activated. Only three vaccines, all inactivated/killed, are currently labeled as protective for EHV-1 abortion: Pneumabort K + 1b™, and Equip EHV1,4 ™ (a.k.a. Duvaxyn-1,4™) from Zoetis, and Prodigy™ from Merck. Other vaccines, such as Prevaccinol™ were licensed for protection from EHV-1-associated abortion but later removed the claim. There are several other vaccines that contain EHV-1 and 4 antigens, typically at lower levels, that are labeled for the aid in protection against respiratory disease (e.g. Rhinomune™, Calvenza™ and Vetera™ from Boehringer Ingelheim, Prestige™ from Merck, Innovator™ from Zoetis). None of the currently available vaccines are labeled for the prevention of EHM (See Table 1.2 for more details). Interestingly, compared with other viruses, EHV is considered antigenically stable, and studies looking at the strains found in various countries find minimal antigen drift over time and geographically (Bryant *et al*. 2018). Therefore, in theory, the original vaccines should be as effective now as they were when first developed.

Many EHV-1 strains have been isolated and documented since the beginning of the 20th century. Wildtype strains such as the *Kentucky-D* and *RacL* strains, isolated from abortions, *Army 183* a respiratory isolate, and the neurologic isolates *Ab4* and *Findlay OH03* (a.k.a. *Kentucky T953*) are still used in vaccine challenge studies for their respective diseases (Turtinen *et al*. 1981, Goodman *et al*. 2006, Paillot *et al*. 2008, Goehring *et al*. 2010, Shakya *et al.* 2017, Bryant *et al.* 2018). The first vaccines available for EHV-1 were modified-live vaccines (MLV) derived from some of these pathogenic strains (Rosas *et al.* 2006). As the name implies, MLVs are typically attenuated to limit the disease they produce but need to replicate within the host to stimulate both humoral and CMI responses. Two popular vaccine strains that were originally sold as MLVs to prevent abortion, are *RacH* (Mayr & Pette 1968) and *Army 183* (Bürki 1988). Of note, the *RacH*

strain was found to contain two mutations: G2254, which has been associated with EHM, and C2258 that may be masking the neurologic tropism because this strain only seems to induce abortions (Smith 2013). The *RacH* strain, which was passaged 256 times in porcine kidney cells from the abortive wildtype *RacL* strain (Mayr & Pette 1968), is still used in the MLV vaccine "Rhinomune™" from Boehringer Ingelheim, which was originally licensed as "Prevaccinol™" for the aid in protection against abortion until 2001. In 1977, a study using the Prevaccinol™ vaccine assessed the response in various age groups as well as pregnant mares (Gerber *et al*. 1977). Young horses, 18 to 21-months-old, had a two-to-eightfold increase in VN titers which declined drastically by 13 weeks post vaccination, the time when the CMI response was first observed. However, weanlings between six and eight-months of age showed little VN increase but had a CMI response, especially after revaccination. In late-term pregnant mares, the CMI was reduced but antibody levels were elevated after EHV-1 vaccination (Gerber *et al*. 1977). That maximum CMI suppression occurs in the final months of pregnancy has been found in other species as well, as a natural progression of pregnancy (Oliveira *et al*. 2012) and coincides with the timing of most EHV-1-associated abortions. The only pregnant mare that had an increase in CMI response was only six months pregnant (Gerber *et al*. 1977). Attenuated vaccines using *RacH* were optimized in 1979 after comparing the immune response generated by different adjuvants (Thomson *et al.* 1979). Alhydrogel and "adjuvant 65" were able to reduce the duration of shedding from seven days to four, but yearlings mounted no discernable increase in VN after repeated vaccination. Some foals and yearlings did, however, show an immune response to vaccination when complement fixation and lymphocyte stimulation were performed. Despite these responses, none of the vaccinated horses were fully protected from clinical disease, pyrexia and nasal discharge (Thomson *et al.* 1979).

Although safer, inactivated vaccines only stimulate humoral immune responses, limiting their efficacy against infection (Paillot *et al*. 2008). Because of concern regarding abortions following administration of the MLV Prevaccinol™ vaccine (Overstreet v. Norden Laboratories, Inc 1982) breeders and veterinarians in Kentucky, USA used a whole virus inactivated using formalin, hoping to prevent vaccine-associated disease. The *Army 183* strain used in the development of "Pneumabort K™", distributed by Fort Dodge (now Zoetis) was actually isolated from the respiratory tract of a young horse from Virginia, United States of America (USA) in 1941 (Bryans & Allen 1982, Chowdury *et al*. 1986, Allen & Bryans 1986, Bürki 1988). Bryans and

Allen confirmed efficacy of this vaccinate when EHV-1-associated abortion frequency was only 14 of 8638 vaccinated mares in Kentucky, compared to 140 of 20 732 unvaccinated mares (Bryans & Allen 1982). Vaccine strains sold commercially today for pregnant mares have all been killed to avoid iatrogenic EHV-1 infection from reversion of the attenuated strains. More recently, the Equip EHV-11,4™ vaccine (rebranded from Duvaxyn 1,4™ in Europe) contains inactivated strain *EHV 438/77* isolated from an aborted fetus and a carboxypolymer adjuvant, and is licensed for the protection against abortion, but it is not available in North America. In 1996, Prodigy™ (Merck) was patented in the USA specifically to protect broodmares from abortion and respiratory disease using the EHV-1 strain *AB69* derived from the infected lung tissue of an aborted fetus and a proprietary adjuvant Havlogen® (Intervet 2018). Since Prodigy™ in 1996, no new vaccines have been licensed to protect against EHV-1, and most vaccine strains are from isolates collected before 1970.

There is a lot of debate as to vaccine efficacy in the literature, with the variety of populations, vaccines, and challenge strains making outcomes difficult to compare. Some authors have stated that vaccination has been associated with the decline in abortions (Frymus *et al*. 1986, Bryans & Allen1982), although even these references indicated that there were mixed immune responses to vaccination, and outbreaks still occurred in vaccinated herds. Frymus *et al*. (1986) compared the abortion rate on six Polish stud farms prior to and after implementing a vaccination program using Prevaccinol™. After 3763 pregnancies from 1975-1982, only 8.9 % fetal loss occurred, compared to 11.8 % in the 5 foaling seasons previous. However, only data from one foaling season is provided regarding EHV-1-associated losses, detailing 76 fetal deaths, of which 50 were submitted for testing and 16 were positive for EHV-1. No other data is provided from previous breeding seasons, limiting the strength of their conclusions. This article does reference previous analyses from a referral laboratory where, of pre-vaccination aborted fetuses submitted from 1949 to 1971, 288 or 28.6 % contained EHV-1. This was compared to aborted fetus submissions after vaccination programs were implemented (from 1972-1978) and only 20 (7.8 %) were positive for EHV-1. This data was used to further support the conclusion that the

EHV-1 vaccine (Prevaccinol™ specifically) was essential in reducing EHV-1-associated abortions in Poland (Frymus *et al*. 1986).

Other authors were suspicious of the efficacy of available vaccines and vaccination protocols to prevent abortion in mares or disease in foals. Bürki *et al.* (1990) used the *Piber 178/63* abortive strain (isolated from an abortion outbreak on the Piber stud farm in Austria (Chowdury *et al*. 1986) to challenge pregnant mares vaccinated with a series of either Prevaccinol™ or Pneumabort K™. Only 11 of 18 vaccinated mares developed a VN titer ≥1:32, 3/5 aborted despite Pneumabort K™ vaccination, and 2/5 aborted in the Prevaccinol™ group. Similarly, Burrows *et al*. (1984) had 3/7 pony mares abort after vaccinating with Pneumabort K™ when using a British EHV-1 isolate for the challenge strain.

In a follow up study, Bürki *et al*. (1991) monitored the vaccination program within the Piber herd for 4 years using three different vaccine products and found a reduced abortion rate but could not confidently link vaccination with the improvement. In the summary analysis comparing Prevaccinol™ ("L-vaccine") and Pneumabort K™ ("K-vaccine"), Bürki *et al*. (1990 & 1991) referenced other failed attempts at developing serological responses or immunity to EHV-1, and concluded that the "live-virus vaccine was remarkably ineffective" for antibody priming in young horses and that viremia occurred with an alarming frequency regardless of vaccine used. This study supported the protocol involving vaccination at 5, 7, and 9 months in pregnant broodmares to maximize immunity with Pneumabort K™. It was also recommended to discontinue use of Prevaccinol™ as it was ineffective at mounting a satisfactory immune response or protection, and instead to use Pneumabort K™ to prime the immune system of young horses, and then to switch to another vaccine product "Resequin F" (from Hoechst AG, Germany, now Aventis, and no longer available) (Bürki *et al*. 1991).

A more recent study reported anti-abortive efficacy of a killed vaccine (Duvaxyn™), using 30 weanlings and 9 three-year-old pregnant pony mares, against the *Ab4* strain (Heldens *et al*. 2001). A challenge was administered two weeks after the booster vaccine in weanlings and four weeks after the third vaccine in mares. No significant reduction in fever duration or prevention of respiratory signs was noted in weanlings or pregnant mares, contrary to the author's own concluding statements (Heldens *et al*. 2001). Duration of viral shedding from the nose was significantly reduced to 5.2 days versus 10 days in unvaccinated weanlings, but it had required

both boosters in order for an adequate VN titer to develop. In pregnant mares, VN titers were not significantly increased after vaccination and neither viremia nor shedding was prevented. Of note, only 1/5 vaccinated mares aborted, whereas all four control mares aborted 15-65 days post infection (Heldens *et al*. 2001). This study further concluded that there was a negative association between increasing complement fixation and VN titers and the duration of viral shedding (Heldens *et al*. 2001).. In 2003, a study comparing vaccinated mares (also using Duvaxyn^{TM}) and previously infected mares, found that 4/5 vaccinated mares foaled normally, but that previous infection and high CTL levels were more protective, with all mares foaling normally and showing no evidence of clinical disease (Kydd *et al.* 2003). Even more recently, a vaccine challenge study comparable to that by Bürki in 1988, also found nasal virus shedding was reduced from 18 to 8 and 12 days, using Rhinomune^{TM} and Pneumabort K^{TM}, respectively, but significant VN titer increases after each vaccine booster did not occur (Goehring *et al*. 2010). The benefit of reducing viremia with vaccination consistent with findings from Breathnach *et al.* (2001) who also used Rhinomune^{TM} and Pneumabort K^{TM}. In 1988, Bürki had commented that antibody does not prevent EHV-1 infection therefore other means should be developed to impede viremia (Bürki *et al*. 1988). This sentiment is consistent with previous conclusions regarding the ineffective titers and CMI from vaccines to prevent EHV-1 infection or shedding from 1969 (Bryans 1969) and 1977 (Gerber *et al*. 1977), yet the study by Goehring *et al*. (2010) concluded that vaccination could lower the level of viremia, but that viremia lasted for a similar duration. This difference may be explained by the different populations used or by the different responses to the challenge virus.

Dr. Tadeusz Frymus stated eloquently that "field results are of great importance but they must be collected over several seasons since the result of short-term field observations are difficult to interpret due to the cyclical character of naturally occurring EHV-1 epizootics" (Frymus *et al*. 1986). A retrospective study assessing foal infection rates prior to and after increased farm vaccination, found a similar foal infection rate and attributed it to shedding by the mares early in life, as had Gilkerson *et al*. in 1999a & b (Foote *et al*. 2003). The study concluded however, that the differences in the prevalence of new infection between 1995 and 2000 was likely a reflection of seasonal, nutritional, and other management factors, rather than a result of increased vaccination (Foote *et al*. 2003). A follow-up to this study confirmed that, despite adoption of widespread vaccination, foals continued to be infected with EHV-1 at a similar rate (Foote *et al*.

2004). In Germany, a large organic study compared the existing protocol to a new vaccination protocol to determine if the abortion rate could be decreased on stud farms where EHV-1 was endemic (Bresgen *et al*. 2012). Some mares had previously been vaccinated with either Duvaxyn™ (Zoetis) or Prevaccinol™ (Boehringer Ingelheim) therefore 3 groups were created: Prevaccinol™/Prevaccinol™, Duvaxyn™/Duvaxyn™, and Prevaccinol™/Duvaxyn™. Duvaxyn™ was administered per label instructions at 5, 7, and 9-months gestation, whereas Prevaccinol™ was administered at 5 and 8-months arbitrarily as it was not labeled for abortion prevention. When VN titers were compared, the Prevaccinol™ groups had higher titers compared to the Duvaxyn™, in contrast to previous studies where the MLVs typically produced little to no significant rise in VN levels (Bürki *et al*. 1991). Unfortunately, regardless of vaccine used, 9 of 55 abortions were still caused by EHV-1 (Bresgen *et al*. 2012). Rather than full immunity, vaccination has been providing incomplete protection as seen by repeated outbreaks within vaccinated herds (Barrandeguy *et al*. 2002, Kydd *et al*. 2006). A study from Japan analyzed respiratory disease rates and "winter pyrexias" in 3- and 4-year-old Thoroughbreds, at 2 local racetracks over a 14-year period to determine vaccine efficacy of a new formalin-inactivated *HH-1* strain vaccine with aluminum chloride adjuvant (Bannai *et al*. 2014). Despite a peak vaccination rate of 95 % and a decrease in the overall rate of disease occurrence, intermittent outbreaks were still reported. The conclusions indicated that a prophylaxis rate of 79.3-85.3 % would be sufficient to control epizootics, but they admit that these results may not be extrapolated to other populations, such as breeding farms. They also acknowledge that sporadic outbreaks still occurred, and there were years where the incidence rates were similar to pre-vaccination years (Bannai *et al*. 2014).

The manufacturer recommendations of repeated vaccination during the at-risk period for pregnant mares comes from the decline of antibody and VN levels within two months of vaccination (as referenced in Bürki *et al*. 1988 and 1991). Repeated vaccination was investigated using Calvenza™ (Boehringer Ingelheim), a killed vaccine safe for use in pregnant mares but not labeled for the prevention of abortion, in a naïve herd of pregnant Icelandic mares (Wagner *et al*. 2015). The first two vaccinations yielded increases in IgG-specific antibodies after day-0 and day-30. The antibody levels declined from 30 days to 6 months despite additional vaccinations. Only 6 months after the initial vaccination was the booster vaccination met with an increase, albeit smaller, in antibody levels (Wagner *et al*. 2015). A previous study testing the same

vaccine, found no significant increase in VN levels after 2 doses of Calvenza™ (Holmes *et al*. 2006). These differences may be because of the studies using a natural exposed group versus naïve, or because of the different assays used to assess antibody levels. In fact, Holmes *et al*. found no significant difference in VN levels between horses vaccinated with Prestige™ or Fluvac™ (now part of Innovator™, Merck) either (Holmes *et al*. 2006). Only Prodigy™ (Merck) produced a significantly higher VN titer compared to Pneumabort K™ (Boehringer Ingelheim) and was only able to produce a second VN increase after the third vaccination (Holmes *et al*. 2006). The VN titer for Pneumabort K™ did not increase at all after the initial vaccination in this population (Holmes *et al*. 2006). Furthermore, Bresgen *et al*. were also unable to find significant increases in VN titers with multiple vaccine boosters using Prevaccinol™ and Duvaxyn™ after an initial increase (Bresgen *et al*. 2012). The lack of VN rise after additional vaccination with Pneumabort K™ and Prevaccinol™ was in contrast to the study from Goehring *et al*. (2010), when the VN titers did increase with each booster vaccination. Therefore, vaccine response is most certainly vaccine product-specific, and moreover, the results for each product may be conflicting depending on the population studied, boosters administered, and assays used.

In 2002, the concept of "non-responders" or "persistently seropositive" horses was presented as a possible explanation for vaccine failures in certain horses (Foote *et al*. 2002). In this study, up to 30 % of horses did not appear to respond significantly to repeated vaccination with Duvaxyn™ (Foote *et al*. 2002). Although this concept does not appear to have been revisited specifically, Mumford *et al*. (2003) reported only 35 %, and Bannai *et al*. (2014) found 33 % of horses seroconverted after vaccination in one study, and 20 % seroconverted in another (Bannai *et al*. 2019). More evidence of horses not responding to repeat vaccination occurred when racehorses were vaccinated monthly three times and produced no additional increase in VN titers (Bannai *et al*. 2014). The aim of this frequent vaccination protocol was to improve the number of horses who seroconverted, yet only an additional 2-3 % of horses seroconverted with additional vaccinations (Bannai *et al*. 2014, Bannai *et al*. 2019). A unique study on Old Kladruber and Thoroughbred horses identified microsatellites that appeared to be strongly associated with an individual's responsiveness to *Rhodococcus equ*i and *Lawsonia intracellularis* vaccination (Horín *et al*. 2004). This research was adapted to determine the response to Duvaxyn™ vaccination (Rusek *et al*. 2013). After dividing the Old Kladruber mare population by coat color, the enzyme-linked immunosorbent assay (ELISA) titer response to seasonal vaccination was

assessed. Mares were considered “low-responders” if their titer remained below average: low-responders represented 44.6 % and 48.5 % of the grey and black coat subpopulations, respectively. When the microsatellites were analyzed, TKY325 was significantly associated with high or average responders with black coats, whereas TKY343 was associated with low responders with grey coats. It is not clear what this means but the authors are suggested that an association between these microsatellite markers and the MHC-linked microsatellite *UM011* may play a role in determining the quality of the immune response to vaccination (Rusek *et al*. 2013). Although not a vaccine study, Thoroughbred mares with the MHC class I *B2* allele had an increased risk of foal loss, regardless of EHV-1 involvement in the abortion (Kydd *et al*. 2016). Four of the five mares who aborted due to EHV-1 in this study were carriers of the *B2* allele. The data on equine genomic involvement with EHV-1 or vaccine response in general is paltry but may have important implications in future vaccine development.

Kydd *et al*. (2006) summarized the shortcomings of current vaccinations, indicating inadequate CMI induction and short-lived mucosal antibody stimulation, as well as poor correlation between antibody levels and protection from clinical disease. As was concluded by Frymus in 1986 and Bürki in 1988, it is possible that efficacy of vaccination of pregnant mares may be hindered by the nature of the herpesvirus, including the behaviour during the latent phase, but also by the pattern of natural reactivation that occurs regardless of vaccination status. Vaccinating more frequently is currently the best option with the vaccines available, in an attempt to maximize immunity during the at-risk period and prevent novel infection. Frequent vaccination is inadequate based on the evidence of “non-responders” and continued outbreaks. Further to the findings by Rusek *et al*. (2013) and Kydd *et al.* (2016) in horses, research in vaccinomics (the combination of immunogenetics and immunogenomics) is just budding in human literature (Poland *et al*. 2007). New data is emerging that humans do not respond to vaccines consistently and that unique “immunograms” may be required to truly assert if protective humoral response has been achieved (Corrales-Aguilar *et al*. 2016). Therefore, unless a very specific immune stimulating vaccine can be developed to prevent infection in neonates and naïve horses, or better protect from natural exposure, it would seem that the focus needs to be on dealing with virus reactivation within latently infected hosts and manipulating the host immune response more effectively towards CMI.

Table 1.2: Currently available vaccines in North America.

The immune response to the current available vaccines was best described by the 2003 AAEP session presented by Holmes *et al*. (published in Holmes *et al.* 2006). A complete discussion regarding vaccines available in Europe and the USA can be found in Chapter 4 of Recent Advances in Animal Virology (Gulati *et al*. 2019) or Patel & Heldens (2005)

Company	Product	Strain	Type + adjuvant used	Labelled Safe in Pregnant mares	References
Boehringer-Ingelheim	Calvenza EHV/EI	"unique respiratory isolate"	Killed + carbimmune (carboxypolymer) adjuvant	Yes	Holmes *et al*. 2003, Holmes *et al*. 2006
	Rhinommune (USA only)	RacH	Modified Live	Yes > 2month gestation	Witherspoon 1984
	Vetera line *multivalent	Unknown	Killed + carbimmune (carboxypolymer) adjuvant	Yes	Holmes *et al*. 2006
Zoetis	Pneumabort K +1b	Army 183, "EHV 1P and 1B"	Killed + squalene (oil emulsion) adjuvant	Yes	Bryans & Allen 1982, Chowdury 1986, Bürki 1988, Holmes *et al*. 2006
	Innovator/Fluvac line *multivalent	Unknown	Killed + MetaStim® (oil emulsion) adjuvant	No	Holmes *et al*. 2006, Davis *et al*. 2014
Merck	Prodigy	AB69	Killed + Havlogen® (carboxypolymer) adjuvant	Yes	Holmes *et al*. 2006, Intervet 2018
	Prestige line *multivalent	AB69	Killed + Havlogen® adjuvant	No	Intervet 2018

1.5 Prevalence studies

The reported prevalence of EHV-1 in horses has been as high as 88 % but varies with the sample population and method of detection (Carvalho *et al*. 2000). Some articles have stated that EHV-1 presence is essentially ubiquitous in equine populations (Allen *et al*. 2008). A very thorough cross-sectional study, performed in New South Wales, provided indirect evidence through increased antibody levels that up to 81 % of 90-day-old foals were exposed to or infected with EHV-1, and 22 % of all weanling foals examined were considered positive on ELISA (Gilkerson *et al*. 1999b). Other groups have had relative difficulty isolating the virus: Wang *et al*. (2007) found 12.5 % of horses infected in the Australian population sampled, while Carman and co-workers (1997) found 0 % of samples were positive for EHV-1 in the respiratory disease cases submitted for testing in Ontario between 1987 and 1995. In contrast to ELISAs that quantify the immune response, polymerase chain reaction (PCR) under-estimate exposure due to intermittent shedding. A chronological summary of key articles describing EHV-1 prevalence studies in horses around the world is found in Appendix 1 and 2 and includes outbreak studies and investigations into the prevalence of the neuropathogenic strain as well.

1.5.1. Populations studied and sampling

When dealing with herpesvirus infection, "prevalence" can be assessed based on the living population via seroconversion, active mucosal shedding, viremia or latency within peripheral blood mononuclear cells, or based on postmortem sampling using abortions, and examination of ganglia and lymphatic tissues.

In live horses, the early prevalence assessments relied on antibody ELISAs and seroconversion to confirm exposure to EHV-1. With foals being born naïve to EHV-1, seroconversion was blamed on viral shedding from the dam (Gilkerson *et al*. 1999a). Twenty-four percent of foals were already seropositive by nine days of age from maternally derived antibody in the colostrum, but 81.8 % were positive by 90 days of age (Gilkerson *et al*. 1999b). A more recent study found 27 % (64/237) of foals were seropositive and 6 foals had EHV-1 DNA in nasal samples despite most (5/6) coming from vaccinated dams (Foote *et al.* 2004).

With the advent of PCR, blood and nasal swabbing have been used more frequently to identify infected horses, adding to the serology evidence. Nasopharyngeal and blood samples were recommended to identify clinical cases of EHV-1, and for cross-sectional studies (& b). Nasopharyngeal samples appeared more sensitive than blood, and Pusterla *et al*. (2008a) noted a risk of shedding contagion from subclinical horses with and without evidence of viremia. Nasal swabbing was confirmed as a good alternative to nasopharyngeal swabbing, making sample collection by practitioners much easier during field and outbreak investigations (Pusterla *et al.* 2008a & b). Knowing that corticosteroids could cause reactivation of latent virus and that there was evidence of subclinical shedding in diverse populations, researchers began looking at "stressful situations" (Gibson *et al.* 1992, Pusterla *et al.* 2010a). The stressors were chosen based on historical outbreaks having occurred at showing or sales events, after travel, and other situations that could be stressful physiologically. For example, upon entering the quarantine facility in Davis, California after long-distance travel, only 1 % of the horses were PCR positive for EHV-1, and 15.2 % were seropositive (Pusterla *et al.* 2009c). For competition purposes, most horses require recent vaccination against EHV-1/4 prior to travel. For this reason, only the horses who had seroconverted while in quarantine and those who were PCR-positive were combined to produce a final prevalence of 2.6 % after transportation (Pusterla *et al.* 2009c). Pregnant broodmares at a sales barn were tested for increased cortisol in feces, and yet only 1 of the mares shed EHV-1 (Schulman *et al.* 2014). When sick horses in a referral hospital were tested for EHV-1, none were found positive despite having fevers, colic, or other signs of systemic illness (Carr *et al.* 2011, Sonis & Goehring 2013). Horses with acute respiratory disease were sampled across the United States, and only 3 % were positive for EHV-1 DNA in nasal swabs or blood samples (Pusterla *et al.* 2011). During outbreaks of neurologic and respiratory disease in 2006, 25 % of horses in the vicinity of the index case were positive for EHV-1, but only the index case and one other horse possessed the neuropathogenic mutant strain (Pusterla *et al.* 2009b). Although physiologically sound, it appears that common stressful situations were unable to reactivate EHV-1 consistently, despite outbreaks of disease having occurred spontaneously in these environments.

The earliest study regarding abortion available for this literature review reported an abortion rate of 11.8 % on the largest breeding farm in Germany (Frymus *et al.* 1986). Prevalence of EHV was determined based on pathology seen in aborted tissues, finding evidence of EHV lesions in

28.6 % of abortions. The percentage of abortion in the last year examined in that study was only 7.8 % and led to the widespread belief that vaccination is protective and reduces abortions associated with EHV-1 (Frymus *et al.*1986). When the causes of abortions were assessed in Sweden 35/63 samples from 29 aborted fetuses were positive for EHV-1 (Ballagi-Pordany *et al.* 1990), while 16 % of aborted or perinatal deaths in France were associated with this pathogen (Léon *et al.* 2008). More locally, the University of Guelph's Animal Health Laboratory (AHL) receives submissions from aborted fetuses and placentas, mostly from around Ontario, Canada. In 2004, they reported that EHV-1 was by far the most significant infectious cause for abortion, affecting 18 % of all submitted cases (McEwen & Carman 2004). With newly implemented identification techniques, the number of total EHV-1 diagnoses made on submitted samples (from respiratory, abortion, and one encephalitis case) was seen to be increasing in Ontario as recently as 2012 compared to previous years (Carman *et al.* 2012).

On post-mortem examination, more difficult to access locations such as ganglia, LNs, and the central nervous system that can be more difficult to access can be compared in order to better understand latent infection. Bronchial LNs were thought to be a reservoir of latent respiratory virus DNA, and indeed 30 % of samples collected from the United Kingdom were positive for EHV-1 after co-cultivation with equine embryonic kidney cells (Edington *et al.* 1994). When assessing horses entering Brazilian abattoirs, up to 88 % of various samples collected over six years were positive for EHV-1 (Carvalho *et al.* 2000). Of mares in Kentucky, some previously infected with EHV-1 experimentally, 72 % were positive for EHV DNA in the submandibular lymph node (SMLN), suggesting it would be a good source to identify latency within the population (Allen 2006). A cross-sectional study from Kentucky identified that 54 % of broodmares examined at postmortem were positive for EHV-1 in at least one tissue sample, and again the SMLN was most likely to contain viral DNA in addition to the trigeminal ganglion (Allen 2008). In horses that died or were euthanized for causes other than respiratory or neurological signs, 15 % of SMLN samples were positive for EHV-1 DNA (Pusterla *et al.* 2010b). A similar study two years later found 25.7 % of Thoroughbreds positive for EHV-1 (Pusterla *et al.* 2012). This study was in line with all others in that the trigeminal ganglion, SMLN, and bronchial LNs were the most likely to contain viral DNA.

1.5.2. Laboratory reports

Consolidated reference laboratory reports have been helpful in assessing EHV-1 in large regions. For example, Frymus *et al*. (1986) were able to describe the change in EHV-1 prevalence in samples received prior to, and after, vaccination was introduced in Poland. Some laboratories archive samples allowing for large studies describing the emergence of new viral strains. Since the identification of the neuropathogenic strain of EHV-1 in 2006, a theory has emerged that the number of outbreaks has increased, and they have been more devastating (Lunn *et al.* 2009, Smith *et al.* 2010). After re-testing EHV-1 isolates from 1984-2007 in New York, the neuropathogenic strain was identified in 11 % of samples, 76 % of which were from EHM cases (Perkins *et al.* 2009). Among samples collected in Kentucky from 1951-2006, the mutant strain D752 was identified as early as the 1950s, and the rate of mutant isolation increased from 3.3 % to 19.4 % in 2006 (Smith *et al.* 2010). In Poland, where EHM had not been diagnosed prior to 2018, two abortive samples first tested positive for the neurologic variant in a retrospective analysis in 2015, increasing national concern for an EHM outbreak in the future (Stasiak *et al.* 2015). Bryant *et al*. (2018) investigated the restriction patterns (microsatellites) of archived EHV-1 viruses and divided the strains into 13 clades, based on polymorphisms, with nine clades isolated predominantly from abortions, and five clades associated with neurologic isolates. Overall, there was very little antigenic drift between isolates over time, with sequence conservation being over 99 % (Bryant *et al*. 2018).

1.5.3. Diagnostics

What is interesting regarding the prevalence studies performed, is the variety of assays used as technology developed. Certain antibody assays cannot differentiate well between EHV-1 and 4 due to strong cross-reactivity, and thereby produced unclear results early on (Crabb *et al.* 1995); the growing concern over EHV-1 and the well-established cross-reactivity with the milder EHV-4 prompted the development of new diagnostics to differentiate the viruses using structure-specific ELISAs and PCR (Crabb *et al.* 1995, Elia *et al.* 2006, Hussey *et al.* 2006, Allen *et al.* 2008, Nemoto *et al.* 2011). With the advent of PCR tests, the use of a specific glycoprotein gene as a target has become more popular than restriction endonuclease-generated genome segments to confirm presence of EHV-1 DNA (Balasuriya *et al.* 2015). Currently, the most popular

method of EHV-1 detection uses the gB gene, a late structural protein described in 2006 (Elia *et al.* 2006, Hussey *et al.* 2006, Diallo *et al.* 2006, Diallo *et al.* 2007). The presence of this protein's gene in larger numbers would suggest that the virus is actively replicating or has replicated within the host as it relates closely to the presence of clinical disease. The assay developed by Hussey *et al.* (2006) reported the ability to detect as few as 6 copies of the plasmid-derived EHV1 gB gene. The use of the gB gene was also compared to cellular housekeeping genes such as equine glyceraldehyde 3-phosphate dehydrogenase (eGAPDH) (Pusterla *et al.* 2009a & b, Pusterla *et al.* 2010a, Estell *et al.* 2015). Specifically, for blood, β-actin has been used, with values expressed as the log of EHV-1 gB DNA copies/106 β-actin copies (Goehring *et al.* 2010). Because of the low sensitivity of quantitative real-time PCR, Burgess *et al*. (2012) divided horses sampled into very high, high, medium, low, and very low cycle threshold (Ct) categories. Those horses with at least a medium or greater Ct were considered to be a high infectious risk to other horses (Burgess *et al.* 2012).

To improve the detection sensitivity, pre-amplifying and precipitation of DNA prior to PCR is now performed (Pusterla *et al.* 2010b, Pusterla *et al.* 2012). Because of the "relatively high" limit of detection using the original PCR method of approximately 100 genome copies of EHV-1, multiple-step PCR and amplification techniques have been investigated, four of which are described below. When compared to conventional PCR, the magnetic bead-based, sequence-capture, nested PCR method improved viral identification by 100 % (Allen *et al.* 2008). Loop-mediated isothermal amplification (LAMP) with heat on nasal swabs followed by fast-cycling PCR for gC gene was able to identify samples that were considered negative by conventional PCR (Nemoto *et al.* 2011). The University of California at Davis has implemented a precipitation step using 5 M sodium chloride, 5 mg/ml glycogen, and absolute ethanol, then incubating overnight and washing prior to the amplification step (DNA Engine Dyad thermocycler, Peltier Thermal Cycler; Bio-Rad Laboratories) (Pusterla *et al.* 2010b). Since the recognition of the neuropathogenic EHV-1 strain, an ORF30 (Pol)-specific PCR has been used for detection of viral nucleic acid in aborted tissues and compared to conventional nested PCR targeting gB (Walter *et al.* 2013, Perkins *et al.* 2009). Multiplex PCR analyses have also been described to identify EHV-1 and EHV-4 (Diallo *et al.* 2007) and all 5 equine herpesviruses (EHV 1-5) (Wang *et al.* 2007). The use of EvaGreen has demonstrated promise by distinguishing several target genes in one tube with more affinity than the usual SYBR Green I dye and may be

used in clinical laboratories in the future to differentiate strains during co-infection situations (Hu *et al.* 2014). Each of these methods are labor intensive and time consuming, especially as more assays are trying to determine the presence of multiple strains per sample.

Lastly, the identification of latently infected individuals in a population is very difficult as the virus circulates silently within the host and only transcribes LATs, generates no immune response as no viral proteins are detected, and because latently infected cells are rare. However, it is the latently infected horse population that is likely causing most of the virus spread as they move and mingle unrestricted among susceptible horses. Molecular techniques to investigate EHV latency involves reverse transcription (RT)-PCR to detect the presence or absence of mRNA (Borchers *et al.* 1999, Allen *et al.* 2008, Pusterla *et al.* 2012) and *in-situ* hybridization (Baxi *et al.* 1995). However, the co-cultivation of infected PBMC with lymphoid tissue remains the gold standard, albeit time consuming and tedious.

One diagnostic assay that has not been investigated to date is droplet digital PCR (ddPCR). Real-time quantitative PCR (qPCR) introduced the use of fluorescent probes to better monitor the target gene amplification after each cycle. An arbitrary Ct was created by each laboratory to signal the point at which the fluorescence signal had increased above baseline, or background, and an external calibration curve is required to normalize the fluorescence seen, usually created by a housekeeping gene (Hindson *et al.* 2011). Reports of minimal thresholds indicate that qPCR requires at least 10, but more likely 100 copies of viral DNA to produce a reliable signal (Burgess *et al.* 2012). These issues are not present when using ddPCR, as it uses volumetrically defined water-in-oil droplet partitions to count the number of absolute probe-positive versus negative wells. The measurement of absolute concentration does not need a normalization curve, instead using a Poisson distribution (Lillsunde Larsson & Helenius 2017). Conventional PCR is susceptible to generate inaccurate Ct values when samples have background contamination and low concentrations of target DNA as the samples must be diluted or amplified. ddPCR is therefore superior in this situation as it does not rely on dilution to reduce the effect of contamination on the result quality and each droplet is individually analyzed for the presence of DNA (Taylor *et al.* 2017).

The benefits of ddPCR in samples with low concentrations of DNA or viral particles have been repeatedly touted (Hayden *et al.* 2013, Aizawa *et al.* 2016, Taylor *et al.* 2017, Lillsunde Larsson

& Helenius 2017). The sensitivity and specificity of conventional PCR is poorly defined, therefore modifications, such as “nested PCR”, are required to improve the detection limits (Wang *et al.* 2007). Other groups have attempted to circumvent these limitations by pre-amplifying or using ultrasensitive magnetic bead-based assays to purify samples prior to qPCR analysis (Allen 2006, Allen *et al.* 2008), adding time and cost to the procedure. New TaqMan probes are considered more sensitive and specific but are usually associated with increased costs and are time-consuming to synthesize. The ddPCR uses existing TaqMan or other sensitive PCR probes, thereby reducing the labour involved with PCR design. As with any PCR assay, ddPCR relies on the assumption that the target is randomly distributed and that there is a clear difference between positive and negative droplet partitions. The optimal number of copies per partition is approximately 1.5-1.6, corresponding to approximately 80 % positive partitions (Vynck *et al.* 2016). Although this would be ideal, ddPCR provides a very precise measurement over a large range and has better technical reproducibility than traditional qPCR (Huggett *et al.* 2015).

1.6 Conclusions, research objectives, and hypotheses

Considering the research on EHV-1 has been ongoing for almost 100 years, new advances have been slow to reach the public, allowing continued outbreaks and inadequate protection in the horse populations. Big strides have been made in genomic sequencing of the virus, first by separating EHV-1 from the immunologically similar EHV-4, then by identifying a point mutation associated with neurologic outcomes. Diagnostics have also improved significantly, allowing simple and rapid identification of carriers and shedding in field situations using sampling of accessible areas like the nose or peripheral blood. Unfortunately, despite these advancements, the actual population prevalence of EHV-1 in primary horse populations (i.e. outside of clinics or diagnostic laboratories) remains unknown because cross-sectional analyses involving multiple stables have not been performed, and identification of latent infection remains difficult. A better understanding of the frequency of latency, and whether it is novel infection or recrudescence that affects horses during disease is critical in determining the appropriate method for future research on prevalence and response to immunization. Without clarity on either of these facets, it is very difficult to manage disease or protect broodmares from abortion. As isolation of populations during outbreaks and frequent testing of in-contact neighbours to

determine spread is currently recommended, regardless of clinical signs, it would be important to understand the baseline prevalence within the horse population, so as not to over interpret these results. A proper baseline may also confer more confidence in statements regarding viral load and contagion. Furthermore, it is becoming more evident that individual genetics play a significant role in terms of the immune response and clinical disease present after exposure to EHV-1, a feature that has been discussed infrequently in the literature.

There remains a large gap in information made available to the public regarding the efficacy of current vaccines and vaccination against EHV-1 in general. One of the difficulties with the investigation on current vaccine products used is that they were created in the 1960s and 1970s, and strains for this period are still used in their production. Similarly, many of the virulent strains used in challenge studies are also isolates from that time, which have been propagated in cell lines, potentially undergoing multiple freeze-thaw cycles. Unfortunately, many articles before 1990 are not digitized and some of the printed archived materials are not available to the public, making these articles essentially inaccessible. Proprietary secrecy also limits the ability to understand the vaccinates and the studies that support or contradict their benefits. It would be ideal to access the original articles isolating both the wildtype strains from cases and outbreaks, as well as their derived vaccinates, to better assess the original research and data produced. The fact that these vaccines are still used today with the same inadequate protective responses is disconcerting and warrants a fresh look. Although this thesis did not focus on vaccine development, a number of new vaccines appear promising in the literature. It is surprising that no new vaccines have been made commercially available.

These limitations underpin the objectives of this dissertation: the first objective of this thesis was to describe the current state of EHV-1 in the equine industry of Ontario, Canada. To achieve this goal, management and business demographics of Ontario and their use of vaccines against EHV-1 were explored. Thereafter, the current prevalence of EHV-1 and the potential sources of viral shedding in broodmares were assessed, comparing nasal, vaginal, and blood samples using ddPCR. Lastly, a serological investigation analyzed various risk factors, including vaccination, on the individual immunoglobulin (Ig) G levels and virus particle presence. The research hypothesis was that most broodmares are infected with EHV-1 and that their titers were not linearly associated with vaccination and ddPCR status. This information will be useful to

develop new approaches to better control this significant pathogen and change the way we currently think about EHV-1 vaccination.

Chapter 2 Survey of the Equine Broodmare Industry, Abortion, and Equine Herpesvirus 1 Vaccination in Ontario (2014–2015)

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Carina J. Cooper, Luis G. Arroyo, David L. Pearl, Joanne Hewson, Brandon N. Lillie.

Department of Pathobiology (Cooper, Lillie), Department of Clinical Studies (Arroyo, Hewson), and Department of Population Medicine (Pearl), Ontario Veterinary College, University of Guelph, 50 Stone Rd W, Guelph, Ontario N1G 2W1.

Address all correspondence to Dr. Brandon Lillie; e-mail: blillie@uoguelph.ca

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2.1 Abstract

A survey of Ontario horse breeders was conducted in 2016 and retrospectively asked farm-level management questions regarding herd sizes, vaccination, respiratory disease and abortion. A total of 88 farm owners completed the survey, reporting 857 broodmares. Using logistic regression modelling, management influences on vaccine use and the reporting of respiratory disease or abortion was investigated. Having veterinary records and the reasons for breeding were significantly associated with the odds of an abortion even after controlling for broodmare herd size. The odds of having respiratory illness on the farm were significantly greater if the farm was the primary source of income even after controlling for farm size. Respondents with primary breeding operations were more likely to utilize vaccines against EHV-1, independent of herd size. Veterinarians were more involved with abortions than with respiratory disease leaving a significant gap in the opportunities for client education.

Enquête de l’industrie poulinière, l’avortement et l’utilisation du vaccin contre l’herpèsvirus équin-1 en Ontario

Abstrait (French): Une enquête auprès des éleveurs de chevaux de l'Ontario a été menée en 2016 et a posé rétrospectivement des questions de gestion au niveau de l’écurie concernant la taille des troupeaux, la vaccination, les maladies respiratoires et l'avortement. Au total, 88 propriétaires d’écuries ont répondu à l'enquête avec 857 juments poulinières. Utilisant des modèles de régression logistique, l’influence du style gestionnaire sur l'utilisation des vaccins et la déclaration des maladies respiratoires ou de l'avortement a été étudiée. La probabilité d’avoir un avortement était significativement associée avec l'utilisation des dossiers vétérinaires et la raison de l’élevage. Les probabilités de souffrir de maladies respiratoires au niveau de la ferme étaient significativement plus élevées si l’écurie était la principale source de revenu. Les répondants ayant des établissements d'élevage principales étaient plus probables d'utiliser des vaccins contre HVE-1. Les vétérinaires participaient plus dans les avortements que dans les maladies respiratoires, laissant un écart important pour l’éducation des clients.

2.2 Introduction

Reproductive efficiency and low abortion rates are good indicators of success in the equine breeding industry (Bosh *et al.* 2009), while outbreaks causing abortions on equine breeding farms can cause catastrophic loss of time, money, and life in both mares and foals, as was evidenced by mare reproductive loss syndrome experienced by breeders in Kentucky in 2001 (Riddle 2003). Regions that are heavily reliant on horse breeding will use seasonal analysis of reproductive performance outcomes such as pregnancy rate and live foal rate to ensure a healthy industry, and to raise alarms during potential outbreaks (Bosh *et al.* 2009, Morris & Allen 2002). Despite improvements in management knowledge and in conception rates, abortions are still the leading causing of financial loss to the equine industry (Morris & Allen 2002). Overall abortion rates per breeding season have ranged from 9.1 % to 21.7 % (Chevalier-Clement 1989, Morris & Allen 2002, Bosh *et al.* 2009, Langlois *et al.* 2012, Miyakoshi *et al.* 2012).

Known host risk factors for equine abortion that have been previously investigated included: age, breed, parity, method of insemination, rate of twinning (Schnobrich *et al.* 2013), racing or competition (Sairanen *et al.* 2011, Langlois & Blouin 2004), vaccination (Barrandeguy *et al.* 2002, Bosh *et al.* 2009), and endocrine disease (Hennek*et al.* 1984, Fradinho *et al.* 2014, Burns 2016). Environmental factors that have been investigated include season of breeding, light exposure, and stress (Langlois *et al.* 2012, Badenhorst *et al.* 2014). One of the environmental risk factors that has not been addressed is overall herd size and the influence herd size has on management style. In addition to host and environmental effects, pathologies of the umbilicus or placenta causing insufficient nutrition of the fetus, and infectious agents have been implicated when analysing aborted tissues (Smith *et al.* 2003, Laugier *et al.* 2011). In consideration that many of these infectious agents are contagious, herd management recommendations have also evolved to include improved mare housing, sanitation, and infection control to limit pathogen spread (Arthur 2011, Rosanowski *et al.* 2013). Herd management recommendations are made based on regional risks and diseases. Both large and small breeding operations have been affected by abortion stemming from any of the risks listed above, despite intensive management.

One of the main infectious agents involved in reproductive loss is equine herpesvirus 1 (EHV-1), which has been implicated in as many as 8.9 % of abortions in Germany (Weber *et al.* 2018) and

18 % of abortions submitted for analysis in 2003-2004 in Ontario, Canada (McEwen & Carman 2004). Of all the infectious causes of abortion, EHV-1 is the only pathogen for which we have a vaccine labeled for protection during gestation. EHV-1 can lead to reproductive, neurological, and respiratory disease in horses worldwide, and affects any age group. EHV-1 can be transmitted to foals by their dams and can later recrudesce in response to unknown stimuli (Gilkerson *et al*. 1999a & b). Widespread vaccine use is assumed, as EHV-1 vaccination is considered a core vaccine by the American Association of Equine Practitioners (AAEP) and is recommended during gestation as well (AAEP 2018). However, despite vaccination, outbreaks have continued to occur. For example, in 2017, a farm in Peterborough, Ontario with a small breeding operation was affected by all 3 forms of EHV-1, neurologic and respiratory disease, as well as late-term abortions. Larger farms have also experienced EHV-1-associated abortion storms in Ontario as recently as 2019, and in Germany (Walter *et al*. 2013). Most recently, the MHC class 1B2 allele was implicated as a risk factor in mares which abort due to EHV-1 when controlling for environmental and host factors (Kydd *et al*. 2016), adding a new facet of mare genetics to the risk factors of EHV-associated reproductive loss.

At this time, there are no publications on the health of the Ontario breeding industry outside of what has been published by the Thoroughbred and Standardbred breeding associations. Despite the high periodic incidence of EHV-1-associated abortion in Ontario, the proportion of farms using the EHV-1 vaccine is unknown, as is the level of concern regarding EHV-1 among breeders. The objectives of this study were to use survey data to describe the current state of the Ontario broodmare industry in terms of sources of income, herd sizes, and abortion incidence and to investigate how various farm-level management factors influence the reporting of vaccine use, respiratory disease and abortions.

2.3 Materials and methods

2.3.1. Database of breeders

A database of Ontario horse breeders was created by identifying breeders through internet searches and public online forums. Keyword searches included: “Ontario horse breeder”, “equine breeder Ontario”, “foal sale Ontario”, “Ontario foals”, “Ontario breeder+breed”, “breed+” “association Ontario”, and their variations. Social media, specifically Facebook®, was also utilized to contact breeders, as many farm webpages identified by internet searches had Facebook® links on their homepage. A recruitment script was approved by the University of Guelph Research and Ethics Board (REB, Approval #16FE021) and included an anonymized link to the survey.

A farm was added to the database if: foal sales were advertised, broodmare management was identified explicitly, horses under the age of 2 y were sold, or images of foals were on the website. Once a farm was identified by name, searches were conducted using the internet, publicly accessible social media pages and public membership registry lists to create the database of breeds, farm addresses, city, owner or manager name, any associated phone numbers, e-mail addresses, and primary veterinarian (if available). E-mail was used for ease of survey dissemination. All farms that were identified, but did not have an associated e-mail address, were contacted via telephone to complete the survey.

From the initial database of 664 businesses, 80 had no valid contact information, 5 were not interested in participating, 33 farms reported no current breeding activity and were excluded, and 67 could not receive e-mails and had no public phone number. Consequently, 479 businesses were contacted via e-mail or telephone to complete the survey.

2.3.2. Survey

An online questionnaire titled “Surveillance of Ontario broodmares and abortion occurrences” was created using Qualtrics Survey Software (Seattle, Washington) (Appendix 3). The survey was approved by the University of Guelph REB (Approval #16FE021).

The questionnaire was disseminated via e-mail directly to the breeders identified in our database, through social media, and via a uniform resource locator (URL) from February to May 2016. The questionnaire, containing 23 questions, was designed to take on average less than 10 min to complete. Questions were primarily in multiple selection format, but a text box for further comment was available when "Other" was selected. Questions regarding herd demographics and abortions were numeric short answer. Medical records, veterinary treatments, and sample submission were investigated using a 5-point ordinal scale of potential responses: "never", "rarely", "sometimes", "often", and "always". Short answer space was also made available for identifying the geographic location of the farm.

Additional information was gathered concerning the number of horses, broodmares, and foals on the property per year, as well as abortions if they occurred. Farm management questions asked about medical records management, veterinary involvement, EHV-1 vaccination, and the submission of diagnostic samples from ill horses and abortive tissues to investigate etiologic agents. EHV-1 vaccination was further investigated by identifying which vaccination protocols were most commonly used. The most common options were included for selection in the questionnaire: "once" and "twice" annual vaccination are self-evident, and the "pre-foaling protocol" was used to describe the AAEP recommendation of vaccination at 5, 7, and 9 months of gestation using vaccines labelled for EHV-1 protection against abortion.

Lastly, permission to contact breeders concerning future studies relating to EHV-1 and abortion investigations was requested, and a short answer space for contact information was provided. After final survey dissemination, at least 3 attempts at contact were made to ensure maximum participation. Of those who wished to participate, 7 requested to complete the survey over the phone rather than online.

2.3.3. Statistical analysis

Data were downloaded from the Qualtrics software and stored in a spreadsheet using Microsoft Office Excel (2010, Microsoft Corp., Redmond, Washington, USA). Descriptive statistics including medians, ranges, and interquartile ranges were reported for continuous variables, and proportions with their 95 % confidence intervals were generated for categorical variables.

Exact logistic regression was used to examine the association between herd size and the likelihood of being a business, using the farm revenue as a primary source of income, and medical record management. Exact logistic regression was also used to examine the association between the respondents' demographic and management styles and the following dependent variables: respiratory illness, and vaccination. To examine the associations between these factors and the odds of an abortion, we used multi-level logistic regression models with a random intercept for herd/facility. For all these models, the results of univariable models were compared to models in which the effect of herd size (broodmare herd size for abortions) was controlled. All measures of herd size were categorized into tertiles, and only the measure of population size most relevant to the outcome of interest were explored in our analyses. For each model, the odds ratio and 95 % confidence interval of each variable were reported. For the multi-level logistic regression models, the intra-class correlation coefficient (ICC) was also reported. In addition, for these multi-level models, model fit was assessed by examining the assumptions of homogeneity of variance and normality of the best linear unbiased predictors (BLUPS) graphically; Pearson residuals were also examined to identify outlying observations/covariate patterns. If the assumptions concerning the BLUPS were not met, we compared the fit of the multi-level model to an ordinary logistic regression model to determine if model fit was improved by the inclusion of the random effect using Akaike's information criterion (AIC). No statistical analyses were conducted on the EHV-1 incidence in samples submitted from sick or aborted tissues due to the rarity of the outcome being reported. All statistical tests were conducted using STATA (STATA Intercooled 15.0; StataCorp, College Station, Texas, USA).

2.4 Results

2.4.1. Respondents

Surveys were collected from 101 farms, but only 88 met the inclusion criteria and were complete. Most of the respondents, were from Southern Ontario (94.3 %, 83/88), with 39.8 % (33/83) in Southwestern Ontario, 30.1 % (25/83) specifically from the Golden Horseshoe region of Southwestern Ontario (the most densely populated region of Ontario that stretches from the western end of Lake Ontario, south to Lake Erie and north to Lake Scugog), 19.3 % (16/83) from Eastern Ontario, and 10.8 % (9/83) from the Central Ontario region. Five respondents were from Northern Ontario.

For 78.4 % (69/88) of respondents, breeding was for business purposes, but only 35.2 % (31/88) of respondents indicated their operations to be strictly breeding facilities. Most farms (58.0 %; 51/88) gained income from other sources, such as farming, training, or boarding (Table 2.1). When "other" was selected, crop farming, off-site jobs, retirement funds and veterinary services were included as additional sources of income (Table 2.1).

The size of the farms varied widely, from 3 to 200 horses (Table 2.2). The median herd size was similar for the 2 years investigated: total herd size median was 19.5 in 2015 and 20.5 in 2014, broodmares and foal numbers remained stable with medians of 5 and 3, respectively, for both years. Median total herd size was divided into small (< 13.5), medium (13.5 to 30.0), and large (> 30.0). Similarly, median broodmare herd size was divided into small (< 4), medium (4 to 8), and large (> 8) for subsequent analyses using exact logistic regression.

Farms that reported their breeding operations as businesses included 63.3 % (19/30) of the small, 79.3 % (23/29) of the medium, and 93.1 % (27/29) of the largest herd sizes. Only farms run as businesses reported their breeding operation as a primary source of income. The largest herds were more likely to be a primary source of income compared to the small (OR = 5.67, 95 % CI 1.78 to 18.08, p = 0.003) and medium (OR = 3.72, 95 % CI 1.24 to 11.17, p = 0.019) herd sizes.

2.4.2. Veterinary involvement

Based on the sources of income for the respondents, 2 veterinarians participated in the survey as they identified “veterinary services” as other sources of income to the breeding operation. Medical records were most often kept on farm (89.8 %, [79/88]), although veterinary records were also popular (56.8 %, [50/88]). Only 1 respondent indicated no medical records were kept (Table 2.1). Most respondents had farm records regardless of herd size or function, however when total herd size was large, farms were less likely to rely on their veterinarian’s records compared to small (OR = 0.31, 95 % CI 0.11 to 0.89, p = 0.029), and medium sized herds (OR = 0.32, 95 % CI 0.11 to 0.94, p = 0.038).

A veterinarian was only “sometimes” requested to examine upper respiratory illness on 69.4 % (25/36) of farms, and at least “about half” of the time in 19.44 % (7/36) of farms. In contrast, when abortions occurred, a veterinarian would be involved “always” on 72.73 % (64/88) of farms.

2.4.3. Abortions

The farms reported 56 abortions having occurred in 2015, 28 of which were early embryonic losses, 6 occurred during mid-gestation, 20 during late gestation, and 2 perinatal deaths (Table 2.2). Similar values were reported for 2014, when 35 abortions occurred, 13 embryonic losses, 8 mid-gestation and 10 late-term abortions, and 4 perinatal deaths (Table 2.2). The overall incidence of abortion per reported pregnancy was 8.5 % and 6.1 % in 2015 and 2014, respectively. Of the 39.8 % (35/88) of farms which had experienced abortions in the past 2 y, 48.6 % (17/35) had submitted fetal or placental tissues for pathogen testing. Only 1 was attributed to EHV-1 infection in 2015, and 4 in 2014.

Based on unadjusted multi-level models and those adjusted for broodmare herd size, the odds of an abortion were significantly higher if the facility used veterinary records, and they were significantly lower for facilities for which the reason for breeding was classified as “business” compared to “other” (Table 2.3). The intra-class correlation coefficient for abortion status for mares within a herd were relatively high and exceeded 30 % in our all models (Table 2.3). The assumption of constant variance of the BLUPS was met, but the BLUPS did not meet the

assumption of normality in most of the models. However, the fit of the model, based on lower AICs, were always improved with the inclusion of the random effect for farm.

2.4.4. Illness

Respiratory disease, defined as nasal discharge, cough and/or fever, was present on 40.9 % (36/88) of farms. Only 25.0 % (9/36) of farms with respiratory illness were submitting samples for viral pathogens testing. Of those farms with illness reported, 52.8 % (19/36) had experienced abortions in the previous 2 y.

When exploring the unadjusted univariable models, total herd size and farms used as primary sources of income were significantly associated with the presence of respiratory illness on farm (Table 2.4). The odds of respiratory illness were significantly greater in the larger herds compared to smaller ones (Table 2.4). Operations that were primary sources of income had increased odds of reporting illness than those that had additional sources of income (Table 2.4). When controlling for herd size, the significant association between primary income source and increased odds of reporting respiratory disease persisted, and no significant difference was identified between herd sizes (Table 2.4). In contrast to abortions, the use of veterinary medical records was not significantly associated with increased odds of having had respiratory illness on the farm when herd size was controlled (Table 2.4). Neither strict breeding facilities nor farms considered businesses were associated with increased odds of illness, and vaccination did not significantly affect the odds of reporting illness (Table 2.4).

2.4.5. Vaccination

In total, 72.7 % (64/88) of respondents were vaccinating for EHV-1. The most popular vaccination protocol was the “twice annual” and “pre-foaling” series. There was no significant association between the herd size and the odds of vaccination (Table 2.5). Furthermore, neither using the farm as a primary source of income nor as a business was associated with vaccination (Table 2.5). However, strict breeding operations had 5.35 times greater odds of vaccinating than farms that had other uses (95 % CI 1.39 to 30.83, p-value = 0.011), and this association persisted after controlling for total herd size (Table 2.5). No significant differences between herd sizes was identified. As seen with the presence of abortion and respiratory illness, the odds of vaccination

were greater in farms which used veterinary medical records, once we controlled for total herd size (Table 2.5). After controlling for use of veterinary records, farms with large herds had significantly greater odds of vaccinating for EHV-1 compared to medium herds (OR = 3.78, 95 % CI 0.96 to 17.07, p = 0.041), but there was no significant difference in the odds of vaccine use when compared to small herds (OR = 1.31, 95 % CI 0.28 to 6.18, p = 0.745).

2.5 Discussion

Since the census performed by Equine Canada in 2011 (Equine Canada 2011), no industry-wide questionnaires have been completed to assess the current status of horse breeding in Ontario. The participation of 88 farm owners in this survey provided a small window of information on the current state of the industry in terms of operation characteristics, health management, and disease and abortion experiences.

In the Equine Canada census of 2011 (Equine Canada 2011), 5225 participants reported owning mares of breeding age (average 6.61 mares/owner), approximately 34,538 broodmares in Ontario, but only 30,175 horses in total were reported as "breeding stock" (including stallions), and only 3779 foals were reportedly born in 2006. At the end of the census, participants had anticipated a reduction in foal production of approximately 15 % for 2011. A 2016 Census for Agriculture identified that consolidation is also occurring across Canada: 193,492 farms were counted in 2016, down 5.9 % from the previous census in 2011 (Statistics Canada 2016).

The database for this project identified only 664 Ontario breeding operations in 2016. A total of 88 farm owners completed the survey and reported a total of 857 broodmares. By providing equal access to the survey to all identified farms, this small subset potentially provided a representative sample of the industry in Ontario. In total, 589 foals were born on the properties which participated in the survey over the 2-year period of study. The risk of abortion in our 2015 population was 8.5 %, like the total gestational loss values of other countries, such as France, with 9.1 % (Langlois *et al.* 2012), and Japan, with 8.7 % (Miyakoshi *et al.* 2012). Only 1 of the abortions in 2015 was positive for EHV-1, whereas 4 were identified in 2014. Based on the results, it appeared that the odds of a mare having experienced an abortion were significantly lower if the primary reason for breeding was business compared to those in which the primary reason was other than "breeding" and "business". While we are cautious based on the small number of farms given the "other" classification, we suspect biosecurity measures may differ among farms based on their reasons for breeding.

Of those who participated, 72.7 % of farms reported vaccinating for EHV-1. Interestingly, 85.7 % of abortions (30/35), and 80.6 % of respiratory illness (29/36) occurred in these vaccinated herds. Without more consistent submission of both respiratory and abortive tissue

samples, it would be impossible to distinguish the underlying causes, but farms which confirmed EHV-1-associated abortions in this survey were also vaccinating. In other studies, foals continued to be infected with EHV-1 at a similar rate despite adoption of widespread vaccination (Foote *et al.* 2004). Even after repeated vaccination for EHV, 9/55 abortions were caused by EHV-1 in Germany (Bresgen *et al.* 2012). Similarly, it has been reported that "winter pyrexia", blamed partially on EHV-1, was reduced when 95 % of the population was vaccinated, but outbreaks continued to occur (Bannai *et al.* 2014). More likely, incomplete protection by vaccination, as seen by repeated outbreaks within vaccinated herds, may be involved (Barrandeguy *et al.* 2002, Kydd *et al.* 2006). It is possible that farms which experienced abortion or respiratory disease began vaccinating to control for EHV-1 empirically. It is of interest to note that the use of vaccination was not associated with the odds of abortions nor the odds of illness occurring on the farm once we controlled for the effect of herd size. The outcomes reviewed in this study were non-specific (i.e. abortion vs. abortion due to EHV-1), which inherently could lead to non-differentiated misclassification of disease and result in bias towards the null.

Furthermore, herd size was not associated with the use of vaccination against EHV-1; only primary breeding facilities had increased odds of reporting vaccine use compare to farms which had other sources of revenue, no matter their size. To improve the efficacy of vaccines in broodmares, vaccination every 2 mo during the higher risk period of pregnancy is recommended (AAEP 2018). As EHV-1 is only 1 of the many causes of abortion, it would make most sense that facilities focused on breeding would expend as much effort as possible, including vaccinating, to reduce any risk to the foal crop. However, monthly vaccination for 3 mo in 2 racetrack populations in Japan provided no additional increase in viral neutralization titers, and only an additional 2 to 3 % of horses seroconverted with additional vaccinations (Bannai 2014). More recently, serum titers were followed in mares after each vaccination, and titers even declined despite repeated vaccination (Wagner *et al.* 2015). Previous groups found that only 33 to 35 % of vaccinated horses seroconverted after vaccination (Mumford *et al.* 2003, Bannai *et al.* 2014), which is consistent with the findings of up to 69.8 % "non-responders" in the study by Foote *et al.*(2002). Despite this evidence to the contrary, vaccination is still considered as a key factor in mitigating the effect of EHV-1-associated disease in horses by the industry.

In Canada, vaccines are only available through a veterinarian, therefore the increased association between farms which vaccinate, and veterinary medical record use is expected. Veterinarians were often involved when abortions occurred but less frequently when respiratory illness was reported, therefore the increased odds of reporting an abortion in farms with veterinary records was also expected. Few of the participating farms elected to submit respiratory samples or aborted tissues for testing during the 2 years of the survey, limiting the ability to confirm the threat of EHV-1 on the property and the association with future illness or abortions. The lack of sample submissions also limits the quality of our laboratory surveillance programs and may influence the significance of reports of EHV-1 in Ontario, as only samples with higher suspicion may be submitted.

A major limitation of this database was the use of the internet as a primary source for identifying potential breeders, eliminating those who do not use e-mail or publish their business information online. In addition, organizations occasionally require fees from their members, limiting those who register their business on the organization's website. Telephone recruitment was utilized to contact those with no e-mail address, and as a follow-up to improve participation. As with any survey with voluntary participation, farms had control of their responses (misclassification bias), and the option to not participate at all (non-response bias). As with any other retrospective survey, misclassification in the form of a recall bias may have skewed any specific numbers provided in the survey. A limitation of using cross-sectional study designs is being unable to decipher the relative timing of exposures, in this case vaccination, and disease or abortion. Similarly, only farms with interest in research, or those which have had issues with abortions or disease may have wanted to participate in the survey, and if this difference in participation were also linked to different exposures it could lead to a selection bias. The wording of the survey title and description attempted to limit this possibility by emphasizing the general management practice interest, rather than abortion or vaccination specifically. Furthermore, the accessibility of the survey was maximized using e-mail and internet links, and telephone follow-up in case of limited access.

In summary, this study provides information about a subset of the equine breeding industry in Ontario; its herd sizes and management styles. Abortions occurred on 39.8 % of the properties, most of which were in herds vaccinated for EHV-1, but the overall abortion risk of 8.5 % for

individual mares per reported pregnancy in this survey was similar to that in other countries. Vaccination was not associated with the odds of reporting abortions or respiratory illness, once we controlled for herd size. Only strict breeding operations were significantly more likely to vaccinate for EHV-1, regardless of herd size. Veterinarians were more often involved when abortions occurred compared to respiratory disease, despite there being an increased odds of abortions on farms which reported illness, especially in large herds. Few respiratory samples and abortive tissues were submitted for diagnostic testing, limiting the surveillance ability of our regional laboratories. From the 2011 Equine Canada census, only 31 % of participants said they "would expect to learn about a contagious disease from their veterinarian", and 41 % said they would learn about it "from word-of-mouth". There remains a significant opportunity for veterinarians to educate their clients about infectious disease and the mitigation of abortions on breeding farms. Further investigations into the protective effect of EHV-1 vaccination and the underlying EHV-1 prevalence in Ontario are warranted.

Figure 2.1: Map of Ontario broodmare survey (2014-2015) respondents' geographical farm locations.

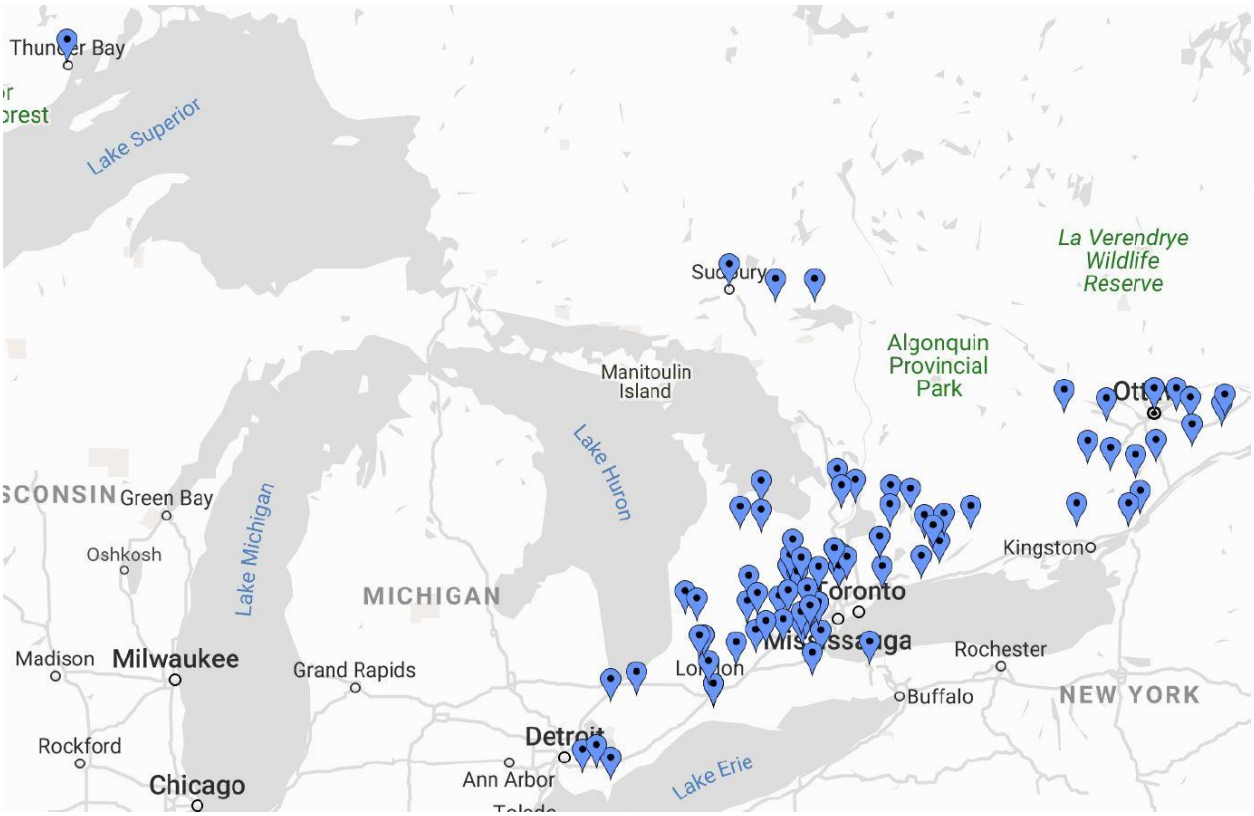

Table 2.1: Summary of Ontario broodmare farm survey (2014-2015) results – categorical variables.

Questions	Options	N	Percent	95 % CI
Reason for breeding	**Hobby**	12	13.6	7.8 – 22.7
	Business	69	78.4	68.4 – 85.9
	Other	7	8.0	2.8 – 16.0
Primary income	**Yes**	31	35.2	25.8 – 45.9
	No	57	64.8	54.1 – 74.2
Sources of income	**Strictly breeding**	31	35.2	25.8 – 45.9
	Boarding	24	27.3	18.9 – 37.7
	Training	14	15.9	9.6 – 25.3
	Farming	11	12.5	7.00 – 21.4
	Other	30	34.1	24.3 – 45.0
	Unknown	3	3.4	0.7 – 9.6
Medical records	**By vet**	50	56.8	46.1 – 66.9
	On farm	79	89.8	81.3 – 94.7
	None	1	1.1	0.2 – 7.9
Vaccinate EHV-1	**Yes**	64	72.7	62.3 – 81.1
	No	24	27.3	18.9 – 37.7
Vaccine Protocols used on farm	**Once per year**	40	62.5	49.8 – 73.7
	Twice per year	15	23.4	14.5 – 35.7
	Pre-foaling	50	78.1	66.0 – 86.8
Respiratory Illness	**Yes**	36	40.9	33.3 – 57.9
	No	49	55.7	42.1 – 66.7
	Unknown	3	3.4	0.7 – 9.6
Treated by veterinarian	**Never**	4	11.1	3.1 – 26.1
	Sometimes	25	69.4	51.9 – 83.7
	About half	4	11.1	3.1 – 26.1
	Most of the time	1	2.8	0.1 – 14.5
	Always	2	5.6	0.7 – 18.7
Viral testing pursued	**Yes**	9	25.0	12.1 – 42.2

Questions	Options	N	Percent (%)	95 % CI
	No	25	69.4	51.9 – 83.7
	Unknown	2	5.6	0.7 – 18.7
EHV-1 positive	**None**	8	88.9	51.8 – 99.7
	1	0	0.0	0.0 – 33.6
	2	1	11.1	0.3 – 48.3
Veterinary assistance for abortions	**Never**	9	10.2	4.8 – 18.5
	Sometimes	7	8.0	3.3 – 15.7
	About half	0	0.0	0.0 – 4.1
	Most of the time	5	5.7	1.9 – 12.8
	Always	64	72.7	62.2 – 81.7
	Unknown	3	3.4	0.7 – 9.6
Abortions in 2 years	**Yes**	35	39.8	29.9 – 50.5
	No	53	60.2	49.5 – 70.1
Submission of abortive tissues	**Yes**	17	48.6	31.4 – 66.0
	No	18	51.4	34.0 – 68.6
EHV-1 positive	**2015**	1	9.1	0.2 – 41.3
	2014	4	40.0	12.2 – 73.8
	None	7	63.6	30.8 – 89.1
	Unknown	1	9.1	0.2 – 41.3

Table 2.2: Summary of Ontario broodmare farm survey (2014-2015) results – continuous variables.

Variable	Year	N	Median	Range (IQR)
Total herd size [a]	**2015**	2468	19.5	3 – 200 (4 – 80)
	2014	2449	20.5	0 – 200 (6 – 80)
Broodmares [a]	**2015**	857	5.0	0 – 58 (2 – 35)
	2014	827	5.0	0 – 75 (2 – 35)
Foals [a]	**2015**	589	3.0	0 – 60 (0 – 30)
	2014	540	3.0	0 – 50 (0 – 26)
Total number of abortions	**2015**	55	1.0	0 – 8 (0 – 3)
	2014	30	0.5	0 – 5 (0 – 3)
Early embryonic failures	**2015**	28	0.0	0 – 8 (0 – 2)
	2014	13	0.0	0 – 3 (0 – 2)
Midgestation	**2015**	6	0.0	0 – 2 (0 – 1)
	2014	8	0.0	0 – 4 (0 – 1)
Late-gestation	**2015**	20	0.0	0 – 5 (0 – 1)
	2014	10	0.0	0 – 5 (0 – 1)
Perinatal deaths	**2015**	2	0.0	0 – 1 (0 – 0)
	2014	4	0.0	0 – 3 (0 – 0)

[a] Continuous variables were described using median, range and interquartile range (IQR) due to skewed data.

Table 2.3: Results[a] of multi-level logistic regression models examining the associations between a mare aborting and farm demographics and management based on the 2014-2015 survey.

		Unadjusted				Adjusted for Broodmare herd size			
Variable	**Categories**	**OR**	**95 % CI**	**p-value**	**ICC (95 % CI)**	**OR**	**95 % CI**	**p-value**	**ICC (95 % CI)**
Broodmare herd size[b]	Referent = Small herd (< 4)				0.41 (0.25 – 0.59)				
	Medium herd (4-8)	1.58	0.39 – 6.46	0.524					
	Large herd (> 8)	1.19	0.31 – 4.56	0.801					
Reason for breeding[c]	Referent = Other				0.34 (0.19 – 0.54)				0.35 (0.19 – 0.54)
	Hobby	0.23	0.03 – 1.96	0.178		0.25	0.03–2.28	0.220	
	Business	0.11	0.02 – 0.64	0.014		0.07	0.01–0.51	0.008	
Primary income	Yes vs. No	0.58	0.23 – 1.46	0.244	0.39 (0.23 – 0.57)	0.51	0.18–1.46	0.209	0.39 (0.23 – 0.58)
Primarily breeding	Yes vs. No	1.23	0.47 – 3.22	0.677	0.41 (0.25 – 0.59)	1.25	0.47–3.32	0.649	0.41 (0.25 – 0.59)
Use of veterinary records	Yes vs. No	2.77	1.06 – 7.27	0.038	0.39 (0.24 – 0.57)	2.98	1.03–8.57	0.043	0.40 (0.24 – 0.58)
Use of farm records	Yes vs. No	0.65	0.11 – 3.63	0.619	0.41 (0.25 – 0.59)	0.57	0.09–3.52	0.542	0.41 (0.25 – 0.59)
Use of EHV-1 vaccine	Yes vs. No	2.46	0.68 – 8.93	0.171	0.42 (0.26 – 0.59)	2.55	0.70–9.28	0.154	0.41 (0.25 – 0.59)
Respiratory illness	Yes vs. No	1.10	0.42 – 2.97	0.848	0.41 (0.26 – 0.59)	1.10	0.42–2.86	0.852	0.41 (0.25 – 0.59)

[a] Results in the table include unadjusted and adjusted models for broodmare herd size. All models include a random intercept for herd and the intra-class correlation coefficient (ICC) concerning the correlation in abortion status among mares that were pregnant in the same herd.
[b] Medium vs. Large: OR = 1.33; 95 % CI = 0.46-3.86; p = 0.600
[c] Hobby vs. Business: i. Unadjusted: OR = 2.06; 95 % CI = 0.51-8.41; p = 0.312; ii. Adjusted: OR = 3.51; 95 % CI = 0.65-18.88; p = 0.143

Table 2.4: Results[a] of exact logistic regression models examining the associations between farm demographics and management and presence of respiratory illness on farm based on the 2014-2015 survey.

			Unadjusted			Adjusted for Farm Size		
Variable	**Categories**	**% Positive (n/n)**	**OR**	**95 % CI**	**p-value**	**OR**	**95 % CI**	**p-value**
Total herd size	Referent = Small Herd (< 13.5)	30.0 (9/30)						
	Medium herd (13.5-30)	31.0 (9/29)	1.05	0.30 – 3.68	> 0.99			
	Large herd (> 30)	62.1 (18/29)	**3.73**	**1.14 – 13.02**	**0.019**			
Reason for breeding	Referent = Other	28.6 (2/7)						
	Hobby	41.7 (5/12)	1.73	0.18 – 25.41	0.656	1.37	0.13 – 20.29	> 0.99
	Business	42.0 (29/69)	1.80	0.27 – 20.14	0.694	1.03	0.14 – 12.24	> 0.99 [b]
Primary income	Yes vs. No	64.5 (20/31)	**4.57**	**1.66 – 13.25**	**0.001**	**3.43**	**1.18 – 10.26**	**0.013**
Primarily breeding	Yes vs. No	48.4 (15/31)	1.60	0.60 – 4.27	0.365	1.57	0.57 – 4.39	0.323 [b]
Use of veterinary records	Yes vs. No	46.0 (23/50)	1.63	0.63 – 4.32	0.284	2.79	0.93 – 9.60	0.060 [b]
Use of EHV-1 vaccine	Yes vs. No	45.3 (29/64)	2.00	0.67 – 6.52	0.225	1.85	0.58 – 6.37	0.304 [b]

[a] Results in the table include unadjusted univariable analyses and analyses adjusted for farm size.
[b] The odds of reporting respiratory disease on a farm were significantly greater in large herds compared to medium and small herds in these models.

Table 2.5: Results[a] of exact logistic regression models examining the associations between farm demographics and management and presence of EHV-1 vaccination on farm based on the 2014-2015 survey.

			Unadjusted			Adjusted for Farm Size		
Variable	**Categories**	**% Positive (n/n)**	**OR**	**95 % CI**	**p-value**	**OR**	**95 % CI**	**p-value**
Total herd size	Referent = Small herd (< 13.5)	80.0 (24/30)						
	Medium herd (13.5-30)	58.6 (17/29)	0.36	0.09 – 1.29	0.095			
	Large herd (> 30)	79.3 (23/29)	0.96	0.22 – 4.17	> 0.99			
Reason for breeding	Referent = Other	85.7 (6/7)						
	Hobby	66.7 (8/12)	0.35	0.01 – 4.93	0.603	0.36	0.01 – 5.27	0.597
	Business	72.5 (50/69)	0.44	0.01 – 4.04	0.668	0.46	0.01 – 4.71	0.656
Primary income	Yes vs. No	83.9 (26/31)	2.57	0.79 – 9.95	0.132	2.57	0.73 – 10.61	0.114
Primarily breeding	Yes vs. No	90.3 (28/31)	**5.35**	**1.39 – 30.83**	**0.011**	**5.19**	**1.33 – 30.25**	**0.010**
Use of veterinary records	Yes vs. No	80.0 (40/50)	2.31	0.81 – 6.84	0.095	**2.94**	**0.95 – 9.82**	**0.044[b]**

[a] Results in the table include unadjusted univariable analyses and analyses adjusted for farm size.
[b] The odds of a farm vaccinating for EHV-1 were significantly greater in large herds compared to medium herds (OR 3.78, 95 % CI 0.96 – 17.07, p-value = 0.041), but not significantly different than small herds (OR 1.31, 95 % CI 0.28 – 6.18, p-value = 0.745) in this model.

Chapter 3 Factors that Influence the Molecular Prevalence of Equid Alphaherpesvirus 1 in Healthy Ontario Broodmares

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Carina J Cooper[1], Luis G Arroyo[2], Jutta D Hammermueller[1], Michaela M Botts[1], David L Pearl[3], Sarah K Wootton[1], Brandon N Lillie[1].

Department of Pathobiology[1], Department of Clinical Studies[2], and Department of Population Medicine[3], Ontario Veterinary College, University of Guelph, 50 Stone Rd W, Guelph, Ontario, Canada, N1G 2W1.

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Corresponding author: Brandon N Lillie, Department of Pathobiology, Ontario Veterinary College, University of Guelph, 50 Stone Rd W, Guelph, Ontario, Canada, N1G 2W1. blillie@uoguelph.ca

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Conflicts of interest: None.

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3.1 Abstract

Background: Equid alphaherpesvirus 1 (EHV-1) has been considered ubiquitous in the horse population, but actual prevalence estimates have ranged from 3-88 % depending on the population and method of sampling. No prevalence studies have been performed in Ontario, Canada.

Hypothesis: Repeated sampling of healthy broodmares will confirm that most horses are harbouring EHV-1, and some will have viral DNA in the nose and/or vagina.

Animals: A total of 381 mares from 42 farms in Ontario were sampled, including pregnant and barren broodmares.

Methods: Samples were collected using a cross-sectional study design from the nose, vagina, and blood from each mare up to six times from December 2016 through October 2017. EHV-1 gB copy number were measured using droplet digital PCR. A survey was completed at time of sampling regarding signalment, pregnancy status, and vaccination status.

Results: Overall, 85 % of the mares sampled were positive for EHV-1 from at least one site, on at least one occasion. Positive samples were 8.1 %, 15.8 %, and 17.2 % from the nose, vagina, and white blood cells, respectively. Pregnant mares had increased odds of shedding virus from the nose (OR = 1.50, 95 % CI 1.03 – 2.18, p = .037). Vaccination only reduced the odds of virus presence in blood (OR = .70, 95 %CI .49 – .99, p = .043). Increasing months of gestation also reduced the odds of virus presence in blood (OR = .89, 95 %CI .85 –.93, p < .001).

Conclusions: Most mares in Ontario were positive for EHV-1 despite being healthy and vaccinated. Additionally, the vagina was identified as a source of viral shedding.

3.2 Introduction

Equid alphaherpesvirus 1 (EHV)-1, an Alphaherpesvirus, causes disease in horses of all ages. Foals are suspected to get exposed to, and are frequently infected with EHV-1 from their dams (Higgins *et al.* 1987, Gilkerson *et al.* 1999a), after which the virus may live latently within the bronchial lymph nodes, trigeminal ganglia, and peripheral blood mononuclear cells (PBMC) (Edington *et al.* 1994, Chesters *et al.* 1997). The clinical signs produced by EHV-1 most commonly include cough and nasal discharge, distal limb edema, late-term abortion, and myeloencephalopathy (Van Maanen *et al.* 2001, Khusro *et al.* 2020, Oladunni *et al.* 2019). Throughout their lives, horses are at risk of repeated exposure to EHV-1 from other horses, via direct contact or fomites, or recrudescence of a latent virus.

Recrudescence of latent EHV-1 is possibly the most important factor in precipitating natural outbreaks of EHV-1 neurological and abortive disease rather than novel infection (Allen *et al.* 2008), therefore a better understanding of the population prevalence of EHV-1 would be beneficial for understanding individual risk. The reported prevalence of EHV-1 carriage ranges from 3 % to 88 % depending on the sampled population and method of detection (Edington *et al.* 1994, Carvalho *et al.* 2000, Allen 2006, Carlson *et al.* 2013). In an attempt to increase the sensitivity of conventional PCR, Allen *et al*. (2008), for example, reported a prevalence of 54 % using a magnetic bead-based, sequence-capture, nested qPCR on postmortem samples from Thoroughbreds in Kentucky, USA. A prevalence of 88 % was estimated from horses sampled at slaughter over a 6-year period (Carvalho *et al*. 2000).

Due to the threat to horses of all ages, vaccines for EHV-1 have been distributed since the 1960s. Although these vaccines were initially lauded for the reduction in abortion frequency (Frymus *et al*. 1986), outbreaks of disease and EHV-1-associated abortions continue to occur across the world. No studies have directly assessed the prevalence of the EHV-1 within a population prior to and after vaccination. As commercial vaccines have been unsuccessful at preventing infection, they have been labeled as an aid in the reduction of clinical signs only. Commercial vaccines available in Canada to aid in preventing EHV-1-associated abortion are Prodigy™ from Merck and Pneumabort K + 1b™ from Zoetis. However, a study from Australia confirmed that despite aggressive vaccination, abortion rates remained largely unchanged (Foote *et al*. 2004). Similarly, outbreaks of both myeloencephalitis and abortion associated with EHV-1 have occurred in

vaccinated herds in Ontario. Vaccination, therefore, has also been providing incomplete protection in the Ontario equine population (Kydd *et al*. 2006, Bresgen *et al*. 2012). Shedding and reactivation is thought to occur intermittently in response to external stimuli, such as stress, and other unknown factors (Schulman *et al*. 2014). During the reactivation period, humoral immunity is rendered essentially ineffective as viral spread can occur from cell-to-cell, partially explaining the lack of protection from vaccination. Two in vitro models found evidence of direct cell-to-cell transfer of virus allowing infection of uterine and nervous endothelial cells by infected PBMC, as the presence of virus neutralizing (VN) antibodies did not alter the infection rate significantly (Smith *et al*. 2002, Goehring *et al*. 2011, Spiesschaert *et al*. 2015). Furthermore, virus shedding has been identified without clinical signs and recrudescence of disease (Allen *et al*. 2008).

The objectives of this study included the following: 1) estimating the prevalence of EHV-1 carriage in healthy broodmares of Ontario as measured in blood, the nose and vagina using ultrasensitive droplet digital PCR (ddPCR); and 2) examine the associations between testing positive from these sources and vaccination status, signalment and breeding status.

3.3 Material and methods

3.3.1. Farm participation

Based on a suspected 80 % population prevalence of EHV-1 from previous data (Carvalho *et al*. 2000), and a total active breeding population of 17 000 mares in Ontario (Equine Canada 2011), a sample size of at least 243 mares was required to achieve 5 % precision (95 % CI) in estimating the expected prevalence.

Thirty-eight participating farms were identified using a survey distributed to Ontario horse breeders. Survey results and data were published previously (see Cooper *et al*. 2020 in press). Four additional farms were recruited after this survey, via communication with farms. A broodmare survey was completed at the time of each sample collection. Data collected included: age, breed, pregnancy status, breeding and foaling dates, EHV-1 vaccination status and most recent vaccination date, EHV-1 vaccine product used, and any history of abortion. The institutional Animal Care Committee at the University of Guelph approved the study (AUP#3257) which conformed to the standard of the Canadian Council on Animal Care.

3.3.2. Sample collection

To assess the impact of sampling site on the prevalence of EHV-1 and association with demographic and management variables, research team members sampled from the nose, vagina, and blood. Sample collection was performed bimonthly from December 2016 through October 2017. Samples were collected by farm personnel or the authors (CJC, MMB and LGA). The authors wore disposable gloves between farms and followed each farm's biosecurity protocols, including boot covers and protective frocks. A minimum of 10 ml of whole blood was collected in an evacuated ethylenediaminetetraacetic acid (EDTA) vacutainer tube and an additive-free serum vacutainer tube (Monoject, Tyco HealthCare Group, Mansfield, USA) by direct venepuncture with a 20-gauge needle. Nasal and vaginal secretions were collected using two 15 cm rayon-tipped swabs (CultureSwab Liquid Stuart, Becton Dickinson, Sparks, USA). The swabs were advanced into the ventral meatus of either the right or the left nostril, or into the vagina, and rotated to improve mucus yield. Samples were kept refrigerated and transported overnight to the laboratory at the University of Guelph, Ontario. Serum was obtained by

centrifugation (2000 x g, 10 min). Swab and serum samples were stored at -20 °C until processing and labelled with an anonymous number the researchers could decode for subsequent analyses.

3.3.3. White blood cell DNA isolation

For white blood cell (WBC) nucleic acid extraction, the buffy coat was aspirated using a single-use transfer pipette after centrifugation (2000 x g, 10 min), and placed in a 2 ml conical Eppendorf tube. Any remaining red blood cells (RBCs) in the buffy coat aspirate were lysed with 1.5 ml of sterile MilliQ water. The samples were centrifuged (3,000 x g, 10 min) to form a pellet. The supernatant of lysed RBC debris was discarded, and the pellet stored at -20 °C.

3.3.4. DNA isolation

Genomic deoxyribonucleic acid (DNA) was extracted using an E.Z.N.A. tissue DNA extraction kit (Omega Biotek, Norcross, USA). For WBCs, 200 µL of TL buffer was mixed with approximately 30 mg of WBC material. A 5mm sterile stainless-steel bead (QIAGEN, Valencia, USA) was added and the sample was placed in a TissueLyzer (QIAGEN, Valencia, USA) for 3 min at 30 Hz to homogenize the sample and disrupt the WBC membranes. This step was crucial to extract the maximal yield of DNA from WBC since without this step the cellular debris would occasionally congeal and clog the columns provided. A protease enzyme (Protease K solution, Omega Biotek, Norcross, USA) enzyme was added, and the samples were incubated at 55 °C with shaking for at least 3 hours. Thereafter, 220 µL of BL buffer were added, and the sample was incubated at 70 °C for 10 min. Once 220 µL of ethanol was added, the entire volume was applied to the E.Z.N.A. DNA isolation columns provided. The samples and their columns were spun at 14,000 x g for 1.5 min and the columns were washed with HBC buffer followed by DNA wash buffers twice, as per the kit protocol. DNA was eluted into 100 µL of elution buffer and frozen at -20 °C.

Nasal and vaginal swab tips were placed in conical Eppendorf tubes and covered with TL buffer and protease enzyme prior to incubation as described above. As the volume of reagents had to cover the entire rayon tip, the volumes of TL buffer and protease enzyme were doubled to maintain the final solution concentration. Volumes of BL buffer and ethanol were also doubled

in compensation to maintain the effective ratios described by the manufacturer. Once the samples were centrifuged through the DNA isolation columns, the protocol was the same as described above. The final DNA concentration was determined by a spectrophotometric method with a NanoDrop 1000 instrument (Wilmington, USA).

3.3.5. ddPCR

Using ddPCR with a Taq polymerase and glycoprotein B (gB)-specific primer (Diallo *et al.*, 2006) viral pathogen load was quantified in each sample by the Laboratory Services of the Animal Health Laboratory of the University of Guelph. The ddPCR reaction mixture consisted of 1x ddPCR Supermix for Probe (Bio-Rad, Mississauga, ON), with primers at a concentration of 96 nM [EHV-1F1F (5′ CATGTCAACGCACTCCCA 3′), EHV-1R1R (5′ GGGTCGGGCGTTTCTGT 3′)], 64 nM concentration of the EHV-11 probe (5′ FAM-CCCTACGCTGCTCC-MGB-NFQ 3′) (Diallo *et al*. 2006), and 4 µL of sample DNA in a final volume of 25 µL. From each PCR reaction mixture, 20 µL were mixed with 70 µL of Droplet Generation oil for Probes (Bio-Rad) in a DG8 Cartridge (Bio-Rad). PCR droplets were then generated using a QX200TM Droplet Generator (Bio-Rad). From each droplet mix, 20 µL were transferred to a 96-well PCR plate (Bio-Rad). The plate was sealed with a foil heat seal using a PX1TM PCR plate Sealer (Bio-Rad). Each plate included 92 samples, 2 positive laboratory controls with known EHV-1 concentrations, 1 positive control (synthetic gene fragment) and 1 negative control (water). PCR amplification was carried out on a GeneAmpTM PCR System 9700 (Applied Biosystems, Foster City, CA) under the following settings: 95 °C for 10 min, followed by 48 cycles of: 95 °C for 20 s and 62 °C for 40 s, followed by 1 final cycle at 98 °C for 10 min (Shehata *et al*. 2017). After amplification, droplets from each well were read automatically on a QX200TM droplet reader (Bio-Rad). ddPCR data were acquired and analyzed with QuantaSoft software (Bio-Rad) and recorded as copies/µL. The final viral load (copies/mL) of each sample was determined by multiplying the average copy number per µL of PCR mixture in each well by the sample volume of 2 µL.

3.3.6. Statistics

A cross-sectional study design was used to estimate the prevalence of EHV-1 within the Ontario broodmare population, and the associations between EHV-1 status and the following independent variables: month of sampling, age, breed, pregnancy status at time of sampling, whether or not the mare had been vaccinated for EHV-1 within the previous 2 years or previous 2 months, which EHV-1 containing vaccine was used most recently, whether the mare had a history of abortion, month of gestation (if applicable), and the number of months since most recent foaling (if applicable). Descriptive statistics including medians, ranges, and interquartile ranges were reported for continuous variables, and proportions with their 95 % confidence intervals were generated for categorical variables.

Multi-level logistic regression models were used to examine the associations between demographic and management characteristics of broodmares and their EHV-1 ddPCR results at each sampling site and overall status based on a parallel interpretation of multiple sampling sites (i.e., considered positive if any site tests positive). The assumption of linearity between continuous independent variables and the log odds of the outcome was examined using lowess curves (i.e., locally weighted regression). If the assumption was not met, the independent variable was categorized, or if appropriate, a quadrative relationship was modeled. Random intercepts were included to account for autocorrelation within individual animals and within a farm. If a variance component was less than 10-10 and if the model fit was not improved based on changes in Akaike's Information Criterion (AIC), that random effect was excluded from the final model. In addition, for these multi-level models, model fit was assessed by examining the assumptions of homogeneity of variance and normality of the best linear unbiased predictions (BLUPs) graphically; Pearson residuals were also examined to identify outlying observations/covariate patterns. If the assumptions concerning the BLUPs were not met, we compared the fit of the multi-level model to an ordinary logistic regression model to determine if model fit was improved by the inclusion of the random effect(s) using AIC. All statistical tests were conducted using STATA (STATA Intercooled 16.0; StataCorp, College Station, Texas, USA).

3.4 Results

3.4.1. Descriptive statistics

In total, 381 broodmares on 42 farms were sampled. Forty-one of the farms had at least one mare test positive. Using ddPCR of the EHV-1 gB gene, the presence of EHV-1 DNA in nasal and vaginal secretions and WBC was quantified. The numbers of EHV-1 gB copies per µL of original sample DNA extract identified using ddPCR ranged from 0 – 55,625.00 in nasal samples, 0 – 23.75 in vaginal samples, and 0 – 618.13 in WBC. Of the 5504 samples collected, 4748 (86.3 %) were negative for EHV-1 DNA. The cumulative period prevalence of EHV-1 in broodmares sampled was 85.0 % (95 % CI 81.1 – 88.5 %) (Table 3.1, Figure 3.1). The largest number of mares sampled occurred in February 2017, but the largest percent of positive samples occurred in December 2016 (Table 3.1, Figure 3.1). EHV-1 prevalence was lowest in nasal swabs (149/1832, 8.1 %, 95 % CI 6.9 – 9.4 %) and highest in WBC (316/1828, 17.3 %, 95 % CI 15.6 – 19.1 %).

3.4.2. Risk factors analysed

3.4.2.1. Month

A notable variation in positive sample prevalence was observed in the different months sampling occurred, with February having a lower percentage of positive samples overall (Table 3.1 and Figure 3.1). The odds of broodmares testing positive based on overall status was significantly lower in February compared to all other months (Table 3.2). The odds of positive nasal and vaginal swab samples were significantly higher in April compared to February, but WBC positivity was not significantly different (Table 3.2). Viral presence in nasal swabs was significant lower in June compared to February (Table 3.2). Except for April, all months sampled had higher odds of WBC samples being positive compared to February (Table 3.2).

3.4.2.2. Age

The ages of broodmares sampled varied from 2 – 27 years (median 10, interquartile range 7 – 14). The presence of EHV-1 particles in the nose, vagina, and blood was not associated with age (Table 3.2).

3.4.2.3. Breed

There was a large variety of breeds in the sampled population, representative of those present in Ontario: Thoroughbreds, Standardbreds, Dutch and Hanoverian Warmbloods, Belgian, Clydesdale, Friesian, Shire, Percheron, and Canadian draft horses, Rocky Mountain and Quarterhorse, Fjord, and pony breeds. Breeds were reduced to five categories for analysis: Thoroughbreds (589/1853, 31.8 %, 95 % CI 29.7 – 34.0 %), Standardbreds (565/1853, 30.5 %, 95 % CI 28.4 – 32.6 %), Warmbloods (Dutch and Hanoverian; 446/1853, 24.1 %, 95 % CI 22.1 – 26.1 %), Drafts (Belgian, Clydesdale, Friesian, Shire, Percheron, and Canadian; 154/1853, 8.3 %, 95 % CI 7.1–9.7 %), and Other (Rocky Mountain and Quarterhorse, Fjord, and pony breeds; 80/1853, 4.3 %, 95 % CI 3.4 – 5.3 %). No breed was associated with viral presence (Table 3.2).

3.4.2.4. Pregnancy and foaling

Over the sampling period, 64.2 % (1189/1853, 95 % CI 61.9 – 66.4 %) of the samples were collected from pregnant mares. Pregnancy had no significant effect on the odds of testing positive for EHV-1 overall, in vaginal, or in WBC samples (Table 3.2). However, in nasal swabs, the odds of testing positive were significantly greater in pregnant mares (Table 3.2). During gestation, the odds of testing positive significantly decreased based on WBC sample testing and overall status (Table 3.2).

Finally, neither the number of months since foaling nor a history of abortions was associated with the odds of testing positive for EHV-1 at any sampling site or based on overall status (Table 3.2).

3.4.2.5. Vaccination

Most mares sampled had been vaccinated at the time of sampling (85.6 %, 1586/1853, 95 % CI 83.9 – 87.2 %). The vaccines used included multivalent inactivated vaccines from the Prestige™ (Merck), Innovator™ (Zoetis), and Vetera™ (Boehringer Ingelheim) product lines, Calvenza™ (Boehringer Ingelheim), as well as monovalent inactivated whole vaccines labeled to aid in preventing EHV-1-associated abortion (Prodigy™ from Merck, Pneumabort K + 1b™ from Zoetis). The vaccines used were reduced to 6 categories Pneumabort K + 1b™ (572/1589, 36.0 %, 95 % CI 33.6 – 38.4 %), Prodigy™ (381/1589, 23.9 %, 95 % CI 21.9 – 26.2 %), Vetera™ (351/1589, 22.1 %, 95 % CI 20.1 – 24.2 %), Prestige™ (182/1589, 11.5 %, 95 % CI 9.9 – 13.1 %), and Innovator™ (38/1589, 2.39 %, 95 % CI 1.7 – 3.3 %). An "Other" category (65/1589, 4.1 %, 95 % CI 3.2 – 5.2 %) included "unknown combination vaccine" and Calvenza™. The odds of having EHV-1 DNA present in the WBC were lower in vaccinated mares (Table 3.2). Similarly, recent vaccination (within two months of sampling) was associated with lower WBC positive samples (Table 3.2).

Individual vaccine products were investigated for their role in EHV-1 particle presence, and all vaccine products had lower odds than the referent, "Innovator™", to be positive on WBC samples (Table 3.2).

3.4.2.6. Random effects and diagnostics

Random intercepts were included in all models to control for clustering by farm. The variance component at the mare level was very small (i.e., < 10-10) and therefore removed. The best linear unbiased predictions (BLUPs) generally met the assumptions of homoscedasticity and normality. For the majority of models, the fit was improved with the inclusion of the random intercept for farm based on the AIC. All potential outlying observations were investigated, but their removal did not impact the final models.

3.5 Discussion

Presence of EHV-1 viral DNA particles was essentially ubiquitous in the study population of Ontario horses. Using highly sensitive diagnostic ddPCR, EHV-1 was found in 97.6 % of Ontario herds sampled and in 85.0 % of broodmares. The ddPCR period prevalence is comparable to the 86 % prevalence of seroconversion described by Matumoto *et al*. (1965). Interestingly, the differences in sampling techniques and demographics appear to play a role in the prevalence estimates reported in the literature, where prevalence has ranged from 0-88 % (Edington *et al*. 1994, Gilkerson *et al*. 1999b, Carvalho *et al*. 2000, Foote *et al*. 2004, Allen *et al*. 2008, Schulman *et al*. 2014, Badenhorst *et al*. 2015). In addition to the well-described nose and blood sources, to the authors' knowledge, this is the first report describing the suitability of vaginal swabs for the detection of EHV-1 DNA. Due to the high sensitivity of ddPCR, the biological significance of the EHV-1 DNA presence is unclear, as this assay may be reporting latent and inactive virus as well as active shedding and replication.

Droplet digital PCR (ddPCR) is a relatively new technology that has been commercially available since 2011 (Hindson *et al*. 2011). The sensitivity and specificity of conventional PCR is poorly defined and requires modification such as "nesting" to improve detection limits (Wang *et al*. 2007). As with qPCR, ddPCR technology utilizes Taq polymerase in a standard PCR reaction to amplify a target DNA fragment, in this case gB. However, ddPCR partitions the PCR reaction into thousands of individual reaction vessels (droplets) prior to amplification and acquires raw data at the reaction end point in the form of a positive-negative result for every droplet. The advantage of quantification of DNA without standard curves or conversions is improved precision and reproducibility of data compared to qPCR, especially in the presence of sample contaminants. Therefore, ddPCR technology can be used for extremely low-target quantitation or in variably contaminated samples, where the sample dilution requirements would likely generate undetectable target levels using qPCR (Taylor *et al*. 2017).

In our population, the majority of mares were positive for EHV-1 and were so in multiple sampling sites at various timepoints, despite being healthy and vaccinated. Only one other study, to the best of our knowledge, investigated the presence of EHV-1 in a clinically healthy, non-experimental cross-sectional study, but this was done in a postmortem setting (Allen *et al*. 2008). Shedding of EHV-2 and EHV-5 has been identified in otherwise asymptomatic horses (Dall

Agnol *et al*. 2019, Lee & Lee 2019), so it would follow that EHV-1 would be found in this otherwise healthy population. A study from Australia also described EHV-1 silently circulating throughout the equine population (Foote *et al*. 2004). Virus transmission has been described through nose-to-nose contact and fomites, as large numbers of viral particles have been found in nasal secretions, with and without the presence of viremia (Burgess *et al*. 2012). This is also consistent with our findings where virus presence at each sampling site appeared to be relatively independent based on differences in months of peak prevalence among sample sites and the miniscule variability constant associated with the horse. In contrast to *Carlson et al.* (2013), there was no age association with viral DNA presence in the nose or blood. Although stress can induce viral recrudescence, shedding was increased from nasal samples in pregnant mares, but not higher among horses that recently foaled. It is also of note that the odds of testing positive declined as the months of gestation increased (Badenhorst *et al*. 2015).

The presence of viral particles in the vagina is a novel finding but is unsurprising as the virus is known to affect the uterus and reproductive organs of male and female equids. Neither pregnancy, nor foaling increased the odds of finding EHV-1 in vaginal samples, even though virus can be collected from vaginal secretions after an EHV-1-induced abortion. Lee & Lee (2019) also identified EHV-2 and EHV-5 in genital swabs of healthy mares in South Korea. For foals, their exposure to EHV-1 may therefore not only come from the nose but also from the mare's vagina and contamination of the tail region. As with the nose, vaginal shedding was not affected by vaccination, indicating additional biosecurity measures may be required to limit spread between mares (e.g., antiviral sanitation during breeding examinations).

Consistent with other studies, vaccination appeared to lower the odds of virus presence in blood, especially when vaccinated within 2 months of sample collection (Goehring *et al*. 2010). Vaccine studies typically test for viral presence and seroconversion within days or weeks after vaccination (Bresgen *et al*. 2012, Wagner *et al*. 2017, Khusro *et al*. 2020) as evidence of protection. The Innovator™ vaccine (Zoetis) was most recently used in the spring prior to the study by only three farms, limiting the significance of the increased odds of a positive WBC test compared to the other vaccine product categories as none of these horses were vaccinated recently. Currently available vaccine strains of EHV-1 appear to stimulate an IgG1 and IgG4/IgG7 humoral response (Goodman *et al*. 2006, Goehring *et al*. 2010), which may reduce

viral shedding at the nose (Perkins *et al*. 2019) in addition to lowered levels of viremia. Vaccines that are labeled to aid in the prevention of abortion associated with EHV-1 tend to have higher antigen loads, compared to vaccines containing multiple antigens (Holmes *et al*. 2006). To take advantage of this protective effect, there is a strong recommendation to vaccinate mares at 5, 7, and 9 months of gestation as well as using a combination vaccine that includes EHV-1 and EHV-4 antigens 30 days prior to foaling (AAEP 2018). As would be expected with breeders following vaccination recommendations and the timing of the North American breeding season, associations between month of gestation, vaccination within two months of sample collection, and the month of sampling were identified in this study for WBC samples.

A sparing effect in WBC samples was identified in this study as month of gestation increased, suggestive of a protective host response to viremia in these healthy broodmares. In contrast to these healthy broodmares, when broodmares suffer from EHV-1-associated abortion, increasing amounts of virus are present in the uterus during the last trimester of gestation leading to endothelial damage, and potential infection of the fetus (Gardiner *et al*. 2012). Therefore, despite most of these mares harbouring the virus, there may be a host factor that is associated with the reduced virus presence at the end of gestation in this study population. In vitro, susceptibility to EHV-1 infection is higher in cells with the MHCI-B2 genotype (Azab *et al*. 2014). Kydd *et al*. (2016) reported that mares carrying the B2 allele may have higher rates of pregnancy loss in Thoroughbreds but could not confirm increased EHV-1 susceptibility. Similarly, Dunuwille *et al*. (2020) could not identify any genetic association to cases with equine herpesvirus myeloencephalopathy, thus there is evidently more work to be done in the host-factor realm. Interestingly, different immune responses have been associated with the different equine leukocyte antigen (ELA) haplotypes as well, where the ELA-A10 haplotype produced lower titers (Bodo *et al.* 1994). Further investigation on the response of the host to vaccination, IgG response and virus shedding is recommended, especially in healthy versus broodmares suffering from EHV-1 abortion.

Because the limit of detection with ddPCR is very low, the clinical significance of finding EHV-1 in our study is unclear. It was interesting that the random intercept for horse contributed so little to our models that it could be removed, suggesting that the presence of EHV-1 at any particular time as identified by ddPCR is independent of a horse's status at another point in time.

Future work should compare the results of ddPCR detection with other PCR detection methods to determine the clinical applicability of this new assay. As with any PCR investigation, we are unable to differentiate virus DNA from infective virus. Based on these results, ddPCR would be of value in an experimental setting to compare shedding sites and perhaps better identify latency. Further investigation to determine if there is infectious virus shedding from the vaginal mucosa would be required, especially during an EHV-1 abortion event or outbreak. There is also a need for a comparative investigation of DNA variation of herpesviruses implicated in the clinically affected population and those present endogenously or endemically within this study population. Some viral strains have been more effective at producing higher amounts of shedding in experimental studies than others (Gardiner *et al*. 2012). Only the EHV-1 gB gene was investigated in this study, therefore we could not differentiate between the wildtype N752 or neuropathogenic D752 strains, or the unlikely possibility of a modified-live vaccine (MLV) strain (Schnabel *et al*. 2019). This study population was geographically and characteristically diverse which makes the study highly representative of horses in Ontario, however this possibly limited our power for studying certain risk factors. Some mares were lost to follow up due to sale, movement, or death, however over 80 % of the mares that enrolled in this study were sampled at four of six sampling periods. Although all mares in this study remained healthy during the sampling period, abortions and respiratory disease have sporadically occurred in these herds since the study was completed.

3.6 Conclusions

This study identified a period prevalence from December 2016 through October 2017 for EHV-1 of 85.0 % in a subset of the healthy Ontario broodmare population. Viral DNA was identified not only in the nose and peripheral blood, but also from vaginal secretions, from which it has not previously been reported. Because detectable virus levels were very low, ddPCR proved to be an effective tool for prevalence investigations, although clinical relevance of these low shedding levels is unknown. Known stressors, such as pregnancy, did increase virus presence in nasal samples, but foaling did not. This study was also in agreement with previous publications that virus levels in peripheral blood were lower after recent (< two months) vaccination. The odds of viral shedding from the vagina were not significantly reduced by vaccination, therefore further investigation of the vagina as a source of infection or environmental contamination is recommended. Lastly, the odds of having viral DNA present in WBC and overall status appeared to decrease as gestation progressed in this group of healthy broodmares, suggesting that there may be a host factor that is overcome during abortive-EHV-1 infection despite vaccination.

Table 3.1: Prevalence estimates of the EHV-1 status[a] of healthy Ontario broodmares and farms (overall[b] and stratified by sampling site) per sampling period from December 2016 to October 2017.

	Dec 2016		Feb 2017		Apr 2017		June 2017		Aug 2017		Oct 2017		Overall	
	n	**n (%) positive**	**n**	**n (%) positive**	**n**	**n (%) positive**	**n**	**n (%) positive**	**n**	**n (%) positive**	**n**	**n (%) positive**	**n**	**n (%) positive**
Mares sampled	352	147 (41.7 %)	356	90 (25.3 %)	311	116 (37.3 %)	307	127 (41.4 %)	262	94 (35.9 %)	266	92 (34.6 %)	381	324 (85.0 %)
Farms sampled	39	36 (92.3 %)	42	32 (76.2 %)	39	34 (87.2 %)	35	31 (88.6 %)	36	24 (66.7 %)	35	26 (74.3 %)	42	41 (97.6 %)
Nasal positive	351	28 (8.0 %)	353	29 (8.2 %)	308	41 (13.3 %)	293	12 (4.1 %)	261	11 (4.2 %)	266	28 (10.5 %)	1832	149 (8.1 %)
Vaginal positive	351	45 (33.5 %)	354	47 (13.3 %)	306	69 (22.6 %)	307	50 (16.3 %)	261	30 (15.3 %)	265	40 (15.1 %)	1844	291 (15.8 %)
WBC positive	351	100 (45.2 %)	354	19 (5.4 %)	305	19 (6.2 %)	292	83 (28.4 %)	261	57 (21.8 %)	265	38 (14.3 %)	1828	316 (17.2 %)

[a]EHV-1 status based on ddPCR

[b]Mare classified as positive if any sampling site during the testing period was positive

Table 3.2: The results of univariable multi-level[a] logistic regression models examining the associations between EHV-1[b] status for each sample type and sampling month, demographic characteristics, vaccination status and reproduction history.

		Nasal			Vaginal			WBC			Overall positivity		
Variables		**OR**	**95 % CI**	**p-value**	**OR**	**95 % CI**	**p-value**	**OR**	**95 % CI**	**p-value**	**OR**	**95 % CI**	**p-value**
Month	**REF = February**			**< .001**			**.012**			**< .001**			**< .001**
	December	.97	.56 – 1.66	.900	.96	.62 – 1.49	.851	**7.28**	**4.33 – 12.26**	**< .001**	**2.13**	**1.55 – 2.94**	**< .001**
	April	**1.72**	**1.04 – 2.85**	**.036**	**1.93**	**1.28 – 2.92**	**.002**	1.20	.62 – 2.31	.596	**1.77**	**1.27 – 2.48**	**.001**
	June	**.48**	**.24 – .96**	**.039**	1.27	.82 – 1.96	.288	**7.29**	**4.28 – 12.41**	**< .001**	**2.10**	**1.51 – 2.93**	**< .001**
	August	.49	.24 – 1.00	.051	1.21	.76 – 1.93	.415	**5.34**	**3.07 – 9.31**	**< .001**	**1.70**	**1.19 – 2.41**	**.003**
	October	1.31	.75 – 2.27	.338	1.19	.75 – 1.89	.454	**3.09**	**1.73 – 5.53**	**< .001**	**1.59**	**1.12 – 2.26**	**.010**
Age		.99	.95 – 1.03	.546	1.00	.97 – 1.03	.759	.99	.96 – 1.02	.410	.99	.97 – 1.01	.434
Breed	**REF = Other**			.277			.943			.087			.205
	Draft	1.60	.60 – 4.22	.346	.89	.38 – 2.08	.780	1.52	.73 – 3.19	.264	1.60	.88 – 2.90	.121
	STB	1.09	.44 – 2.71	.853	.94	.42 – 2.08	.877	.78	.40 – 1.53	.469	1.12	.65 – 1.92	.681
	TB	.88	.36 – 2.13	.776	.95	.45 – 2.03	.895	.76	.41 – 1.42	.388	.98	.59 – 1.64	.948
	WB	.79	.31 – 1.98	.610	.79	.36 – 1.73	.553	.91	.47 – 1.76	.787	1.05	.62 – 1.78	.853
Pregnant		**1.50**	**1.03 – 2.18**	**.037**	.89	.68 – 1.16	.377	1.05	.81 – 1.36	.710	1.06	.87 – 1.30	.562
Ever Vaccinated		1.00	.60 – 1.67	.991	1.04	.69 – 1.58	.850	**.70**	**.49 - .99**	**.043**	.84	.64 – 1.12	.230
Simple Product	**REF = Innovator**			.403			.445			**.019**			.775
	Other	1.11	.39 – 3.21	.841	5.71	.65 – 50.30	.117	**.29**	**.11 – .78**	**.015**	1.45	.61 – 3.47	.401
	Pneumabort	1.50	.85 – 2.64	.166	7.72	1.00 – 59.74	.050	**.30**	**.14 - .62**	**.001**	1.18	.67 – 2.10	.566
	Prestige	.92	.40 – 2.12	.839	6.44	.81 – 51.41	.079	**.32**	**.14 - .73**	**.006**	1.06	.55 – 2.05	.854
	Prodigy	1.55	.85 – 2.83	.157	6.70	.86 – 52.31	.070	**.30**	**.14 - .63**	**.002**	1.36	.75 – 2.47	.313
	Vetera	Omitted due to collinearity			6.37	.82 – 49.79	.077	**.41**	**.19 - .86**	**.019**	1.14	.63 – 2.07	.667
Abortions		.68	.36 – 1.27	.222	.97	.64 – 1.48	.887	.90	.59 – 1.36	.603	.93	.68 – 1.26	.625
Gestation (months)		1.04	.98 – 1.11	.228	.96	.92 – 1.01	.164	**.89**	**.85 - .93**	**< .001**	**.95**	**.91 – .98**	**.006**
Months since Foaling		0.98	0.85 – 1.12	.728	.93	.85 – 1.03	.158	1.04	.96 – 1.14	.345	.99	.92 – 1.06	.724

Months since Vaccination		.95	.89 – 1.01	.078	1.01	.97 – 1.04	.751	1.03	1.00 – 1.06	.098	1.00	.97 – 1.02	.840
Vaccinated <2months		1.37	.93 – 2.03	.112	.95	.71 – 1.27	.754	**.64**	**.49 - .85**	**.002**	.92	.74 – 1.14	.441

[a]Random intercept for farm.

[b]EHV-1 status based on ddPCR

*Within Nasal sampling models, the farm variance ranged between .11 and .25

* Within Vaginal sampling models, the farm variance ranged between .08 and .44.

* Within WBC sampling models, the farm variance ranged between .01 and .20.*Within "Overall" sampling models, the farm variance ranged between .01 and .09.

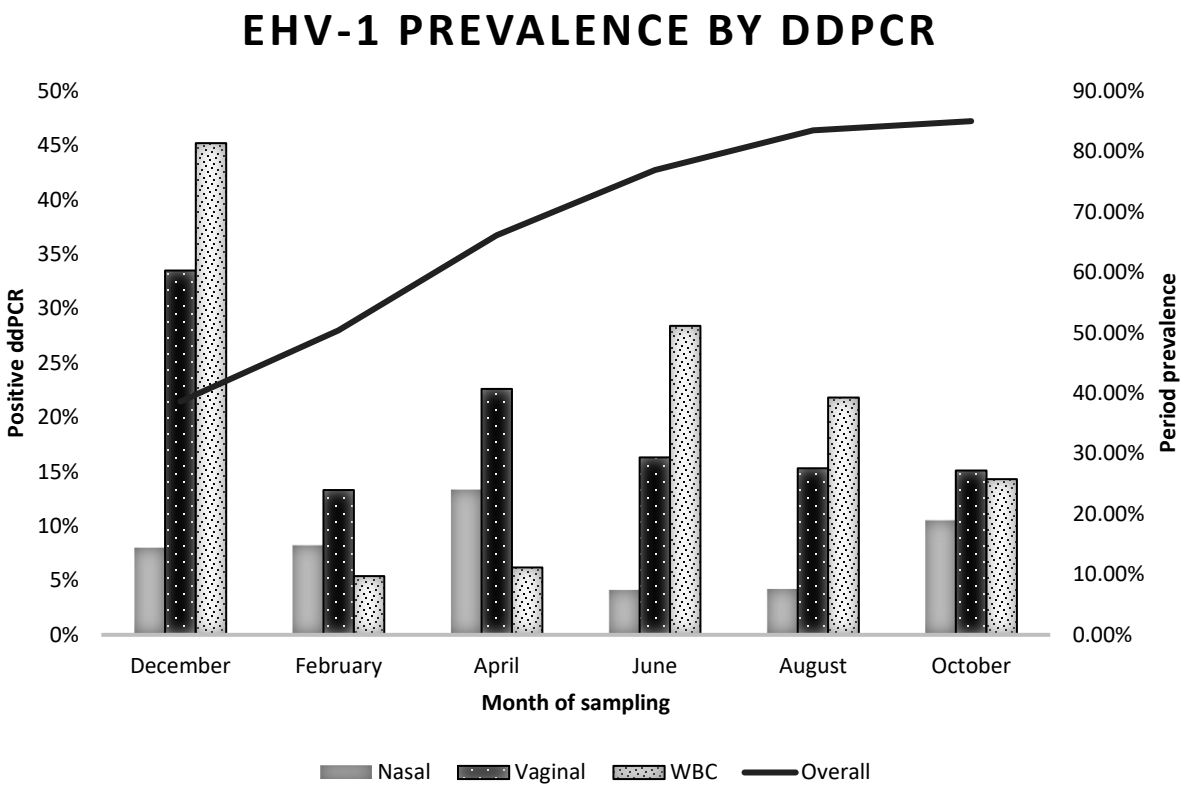

Figure 3.1: Graphic representation of EHV-1 prevalence by ddPCR from December 2016 to October 2017 with the linear curve depicting the cumulative period prevalence.

Chapter 4 Serological Evaluation of Healthy Ontario Broodmares for Equid Alphaherpesvirus 1 Exposure and Risk Factors

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Carina J Cooper[1], Gisela Soboll Hussey[2], Luis G Arroyo[3], Jutta D Hammermueller[1], David L Pearl[4], Brandon N Lillie[1].

Department of Pathobiology[1], Department of Clinical Studies[3], and Department of Population Medicine[4], Ontario Veterinary College, University of Guelph, 50 Stone Rd W, Guelph, Ontario, Canada, N1G 2W1.

Department of Pathobiology and Diagnostic Investigation[2], College of Veterinary Medicine, Michigan State University, 736 Wilson Rd, East Lansing, Michigan, USA, 48824.

Corresponding author: Brandon N Lillie, Department of Pathobiology, Ontario Veterinary College, University of Guelph, 50 Stone Rd W, Guelph, Ontario, Canada, N1G 2W1. blillie@uoguelph.ca

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4.1 Abstract

Equid alphaherpesvirus 1 is associated with abortion as well as respiratory disease and myeloencephalopathy. Vaccination is frequently used to mitigate the associated diseases and limit virus spread, but no studies have reported on the serological status of Ontario's broodmare population or the response to vaccination. We hypothesize that Ontario broodmares have a high anti-EHV-1 titer, likely due to vaccination and exposure, but that these titres are not associated with pregnancy status. Furthermore, we suspect that host factors such as age and breed are associated with different antibody responses. Sera were collected from 381 healthy broodmares from 42 Ontario farms bi-monthly from December 2016 through October 2017. Blood, nasal and vaginal swab samples for EHV-1 droplet digital PCR analysis were also collected bi-monthly alongside demographic surveys and vaccination information. Serology was assessed using virus neutralization and ELISA for IgGa, IgGb, and IgG(T). When looking at individual breeds/groups of breeds, titers were highest in vaccinated Standardbred and Thoroughbreds. Titers increased with age but were not affected by pregnancy status. Most mares had EHV-1 specific IgG and VN titers but 48.5 % of mares sampled did not have a biologically significant (more than 4-fold) change in their VN titers. Of vaccinated mares, 32.3 % did not have neutralizing titers above 1:512, and 42.1 % of had no biologically significant changes in their neutralizing titers during the study period. Each of the 5 vaccine products were associated with different IgG subisotype response patterns. Neither of the vaccines labeled for protection from abortion produced a statistically significant different IgG (4/7) response when compared to other vaccines administered. An increased IgG (3/5) titer during gestation paralleled a decrease of viremia, but no IgG subisotype was significantly associated with EHV-1 presence. In summary, vaccination was associated with increased titers regardless of type of vaccine used, however no vaccine product produced a consistent immune response in this population, and a proportion of mares did not respond to commercial vaccines despite regular boosting. Finally, titer levels were not significantly associated with EHV-1 shedding or viremia in the group of horses examined.

4.2 Introduction

Equid alphaherpesvirus 1 (EHV-1) has been reported in the horse population since at least the 19th century, as causing late-term abortion and respiratory disease, as well as neonatal death and myeloencephalopathy. Although all herpesviruses, including EHV-1, cause latency after infecting their host, vaccines are recommended to enhance the adaptive immune system, with the hopes of preventing infection or virus shedding upon reactivation. The effect of vaccination has been measured using virus neutralization (VN) (Thomson *et al*. 1979, Holmes *et al*. 2006) and, more recently, immunoglobulin G (IgG) subisotype analysis (Soboll Hussey *et al*. 2011). An increase in VN titer was suggestive of protection against EHV-1 in early publications of vaccine trials (Gerber *et al*. 1977, Thomson *et al*. 1979). However, this protection appeared to be incomplete as outbreaks occurred in the face of high titers (Thomson *et al*. 1979). The inadequacy of a high antibody titer against a persistent virus such as EHV-1 is unsurprising as the herpesvirus enters respiratory epithelium and lymphocytes quickly after exposure and can spread from cell to cell (Goehring *et al*. 2011, Spiesschaert *et al*. 2015). Once the virus has replicated within the cell, it lyses the cell and sheds from mucosal surfaces or spreads to neighbouring cells. Upon rupturing the infected cell, the virus is in the environment, but the antibodies have only a brief opportunity to neutralize it. Although the antibody's direct effectiveness at preventing disease is limited, antibody measurements are still crucial in vaccine research since they have been indicative of the cellular immune response as well as representative of the humoral response, and may be useful by proxy (Soboll Hussey *et al*. 2011, Goodman *et al*. 2012, Wagner *et al*. 2015, Wimer *et al*. 2018, Perkins *et al*. 2019)**.** The VN titer has been correlated with the most prevalent immunoglobulin in the body, IgG, and found to have the highest correlation with IgGb (also known as IgG (4/7)) (Soboll Hussey *et al*. 2011, Goodman *et al*. 2012). After vaccination, some studies have found that increases in certain IgG subisotypes have been protective against infection and have limited virus shedding (Wimer *et al*. 2018, Perkins *et al*. 2019)**.** In fact, an IgG4/7^{high}/IgG1^{low} pattern was present in horses that were protected from infection (Perkins *et al*. 2019), as were horses with a high IgGb (4/7): IgG(T) (3/5) ratio (Soboll Hussey *et al*. 2011, Goodman *et al*. 2012). Interestingly, the IgG 4 and 7 subisotypes have been found to be increased when cytotoxic T lymphocytes are active and producing interferon gamma (IFNγ) (Wimer *et al*. 2018, Perkins *et al*. 2019). This study supports the idea that IgG subisotypes are associated with T helper 1 or 2 activity and therefore may

indicate which arm of the immune system is activated. This association is useful as direct measuring of cytotoxic lymphocyte activity is cumbersome, therefore using IFNγ and IgG subisotyping may simplify the analysis of vaccine candidates and host responses.

These patterns suggest vaccines should stimulate a high VN titer, IgG (4/7) and IFNγ response to ensure protection from infection. However, commercial EHV-1 vaccines have been unable to consistently stimulate a protective immune response, as seen by continued infection and outbreak occurrence, and EHV-1-associated late-term abortions (Frymus *et al*. 1986, Holmes *et al*. 2006). Prodigy™ (Merck) was released in 1996 with a similar label claim to Pneumabort K™ (Zoetis) to aid in the prevention of EHV-1-associated abortion, but since then no new vaccines have been licensed to protect against EHV-1. Furthermore, most vaccine strains are from isolates collected before 1970. In order to maintain as much immunity as possible using currently available vaccines, vaccination of pregnant mares in their 5th, 7th and 9th month of gestation is recommended to reduce the incidence of EHV-1-associated abortion (Bürki *et al*.1991). After vaccinating a small group of pregnant ponies, 1 out of 4 aborted despite increased VN titers when challenged with EHV-1, but all had reduced shedding duration (Heldens *et al*.2001). Another vaccine challenge study found reduced nasal virus shedding from 18 days to 8 and 12 days, using Rhinomune™ (Boehringer Ingelheim) and Pneumabort K™ (Zoetis), respectively, but neither produced significant VN increases after each vaccine booster (Goehring *et al*. 2010). Those with a lack of antibody titer response after vaccination have been dubbed "non-responders" and have included up to 69.8 % of a vaccinated horse population (Foote *et al*. 2002).

To date, all investigations into the association between VN and IgG subisotypes and their role in EHV-1 shedding or protection from abortion in broodmares have only been in experimental models. This study aimed to examine the serological status of the Ontario broodmare population, comparing mares that have been vaccinated to those who have not, and assessing various risk factors such as breed, age, and gestation on titer response. A previous study investigating the molecular prevalence of EHV-1 in the same population identified subclinical shedding using droplet digital PCR (ddPCR) (Chapter 3). The authors also aim to explore the association between serological status and the subclinical presence of EHV-1.

4.3 Materials and methods

Forty-two farms from across Ontario participated in broodmare sampling. Farm recruitment was performed as previously described (Chapter 3). Data collected from each broodmare included: age, breed, pregnancy status, breeding and foaling dates, EHV-1 vaccination status, most recent vaccination date, EHV-1 vaccine product used, and any history of abortion. The institutional Animal Care Committee at the University of Guelph approved the study (AUP#3257) which conformed to the standard of the Canadian Council on Animal Care.

4.3.1. Sample collection

Serum was collected for analysis, as well as samples from the nose, vagina, and blood to determine molecular prevalence of EHV-1 using ddPCR as described previously (Chapter 3). Briefly, bimonthly sampling from mares occurred from December 2016 through October 2017. Personal protective equipment was worn and farm biosecurity protocols, including wearing boot covers and protective frocks, were followed. Whole blood was collected into an evacuated ethylenediaminetetraacetic acid (EDTA) vacutainer tube and an additive-free serum vacutainer tube (Monoject, Tyco HealthCare Group, Mansfield, USA) by direct venepuncture with a 20-gauge needle. Using 15 cm rayon-tipped swabs (CultureSwab Liquid Stuart, Becton Dickinson, Sparks, USA), nasal and vaginal secretions were collected, refrigerated, and transported overnight to the laboratory at the University of Guelph, Ontario. Serum was obtained by centrifugation (5000 x g, 10 min). All samples were stored frozen at -20 °C.

4.3.2. Droplet digital PCR

Details regarding DNA isolation and droplet digital protocol can be found in Chapter 3. Briefly, after the buffy coat was isolated, remaining red blood cells were lysed using MilliQ water, and the pellet was stored at -20 °C. DNA was isolated using the E.Z.N.A. viral DNA extraction kit (Omega Biotek, Norcross, USA). DNA was eluted into 100 uL of elution buffer and frozen at -20 °C. Taq polymerase and glycoprotein B (gB)-specific primers (Diallo *et al*. 2006) were used to quantify the viral pathogen in each sample after PCR amplification was carried out on a GeneAmpTM PCR System 9700 (Applied Biosystems, Foster City, CA). The final viral load

(copies/mL) of each sample was determined by multiplying the average copy number per µL of PCR mixture in each well by the sample dilution factor.

4.3.3. Virus neutralization

The most complete sampling sets, meaning that at least 5 of 6 sampling periods were collected from a mare, were selected for virus neutralization (n = 99). The subset included both vaccinated (n = 76) and unvaccinated mares (n = 23).

Type-specific virus neutralizing antibody titers in serum samples, which differentiates between antibodies induced by EHV-1 or EHV-4, were determined at the Michigan State University Veterinary Diagnostic Laboratory, Diagnostic Center for Population and Animal Health. Briefly, heat-inactivated serum samples were serially diluted from 1:4 to 1:4096 in 96-well tissue culture plates (Corning Inc.). Positive and negative controls were included on the first plate of each run, then incubated with EHV-1 at 37.8 °C, 5 % CO_2 for 1 h. Subsequently, 100 µL of 3.0x10^5/mL Equine Dermal (ED) cells were added and plates were incubated for an additional 4 days at 37.8 °C, 5 % CO_2. Wells were inspected for cytopathic effect and rated either positive or negative.

4.3.4. IgG subisotype ELISA

Rabbit kidney cells (RK13) (ATCC, Manassas, VA, USA) cells were cultured in T150 flasks in M199 medium (both Thermo Fisher Scientific Waltham, MA, USA) with 6 % heat inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and 1 % penicillin/streptomycin (Thermo Fisher Scientific, USA). The RK13 cells were used to cultivate EHV-1 Ab4 virus protein supernatant in sufficient quantities to perform all ELISA studies of interest.

Each 96-well polystyrene plate (ImmulonII, Dynatech Laboratories Inc., Chantilly, VA) was coated with 10 µg/ml of gradient-purified, concentrated EHV-1 in carbonate-coating buffer and incubated overnight at 4 ºC. Plates were then washed three times using PBS/0.5 % Tween (Thermo Fisher Scientific, USA) and blocked at room temperature for at least 1 hour using cold-water fish gelatin (1 % in PBS, Sigma-Aldrich, St. Louis, MO, USA) to inhibit non-specific binding. After washing the plates, sera were diluted at 1:1000 for IgGa, 1:8000 for IgG(T) and 1:50000 for IgGb analysis using the 1 % fish gelatin (Sigma, USA) blocking buffer, loaded in triplicate, and incubated at 37 ºC for 2 hours. A serially diluted positive control serum was used

to create a 4-parameter standard curve against which each sample was compared, as described in Soboll *et al*. (2006). Individual antibody subisotype responses to EHV-1 were measured using 1:10 diluted monoclonal antibodies (mAbs) specific for equine IgGa (CVS 45), IgGb (CVS 39), and IgG(T) (CVS 38), by indirect ELISA (Lunn *et al*. 1995, Sheoran *et al*. 1998). Plates were washed again prior to incubation with peroxidase conjugated Affinipure^{TM} goat anti-mouse IgG & IgM (H + L) (Jackson Immuno Research Laboratories Inc., West Grove, PA) at 1:2000 dilution. To develop the color, 3,30,5,50-tetramethybenzidine substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) was added, the reaction was stopped using 1 M phosphoric acid, and the plate was measured spectrophotometrically at 450 nm using an ELISA reader (Bio Tek Instruments, Winooski, VT).

4.3.5. Statistical analysis and study design

A cross-sectional study design was used to estimate the mean values of serological response (using IgG subisotypes and VN) to EHV-1 within the Ontario broodmare population, and the associations between the serological response and the following independent variables: month of sampling, age, breed, pregnancy status at time of sampling, whether or not the mare had been vaccinated for EHV-1 within the previous 2 years or previous 2 months, which EHV-1 containing vaccine was used most recently, whether the mare had a history of abortion, month of gestation (if applicable), and the number of months since most recent foaling (if applicable). The association between serology and the presence of EHV-1 DNA, as measured by ddPCR from the nose, vagina, WBC, and overall status (whether one mare was positive from any site at any time during the sampling period) was also explored. Descriptive statistics including medians, ranges, and interquartile ranges were reported for continuous variables, and proportions with their 95 % confidence intervals were generated for categorical variables. The assumption of linearity between continuous independent variables and the various outcomes of interest was examined using lowess curves (i.e., locally weighted regression). If the assumption was not met, then a quadrative relationship was modeled, or the independent variable was categorized.

4.3.5.1. Tobit

Multi-level tobit (censored regression) models, as described by Ekstrand and Carpenter (1998) with random intercepts for farm and horse were fitted to assess the association between the independent variables and the reciprocal of the VN titer and the various $\log_{10}$-transformed IgG subisotype level. The tobit model is similar to a linear regression model, but accounts for observations being censored by the limit of detection (McDonald & Moffit1980). Data were censored to account for all values that were beyond the range of detection using the lowest and highest recorded values as the lower and upper limits, respectively. The lower limit for the VN titer was 4, and the upper limit was 4096. For the $\log_{10}$ of the IgG subisotypes, the lower limit for IgG (1) was 2.30, the upper limit was 4.51; for IgG (4/7), the lower limit was 3.90 and the upper limit was 5.63; for IgG (3/5), the lower limit was 1.30, and the upper limit was 5.63. The fit of the mixed tobit models were assessed by examining the assumptions of homoscedasticity and normality of the raw residuals and best linear unbiased predictions (BLUPs) of the random effects by plotting the BLUPs and residuals against the predicted outcome and using normal quantile plots, respectively. The plots with the raw residuals were used to identify outliers. All statistical tests were conducted using STATA (STATA Intercooled 16.0; StataCorp, College Station, Texas, USA).

4.4 Results

4.4.1. Descriptive statistics

In total, 381 broodmares on 42 farms were sampled, and generated 1853 samples for analysis (Table 4.1). A total of 1832 serum samples were analyzed for each EHV-1 specific IgG subisotype. For IgGa (1), the log_{10}-titers ranged from 2.35 to 4.51, with 43 samples below the limit of detection and 231 above (median 4.16, IQR: 3.90 – 4.36). For IgG(T) (3/5), the log-titers ranged from 1.33 to 5.62, with 227 samples below the limit of detection and 468 above (median: 5.01, IQR: 4.50 – 5.62). Lastly, the log_{10}-titers for IgGb (4/7) ranged from 3.94 to 6.49, with 13 samples falling below the limit of detection and 157 above (median: 5.75, IQR: 5.39 – 6.11).

A subset of 99 horses generated 588 samples for analysis using VN and results ranged from 4 to 2048. Four samples fell below the lowest detection limit, and 17 were above, with a median of 512 (IQR: 256 – 1024).

4.4.2.Risk factors analyzed

4.4.2.1. Month

There was a notable month-to-month variation in serological titers outcomes. Log_{10}IgGa (1) values varied from a median of 4.06 (IQR 3.76 to 4.27) in December to 4.23 (IQR 3.99 to 4.39) in June, log_{10}IgGb (4/7) varied from 5.65 (IQR 5.27 to 5.99) in December and 5.85 (IQR 5.51 to 6.29) in October, and log_{10}IgG(T) (3/5) varied from 4.83 (IQR 4.28 to 5.29) in December to 5.11 (IQR 4.61 to 5.63) in October. The median of the VN titer remained constant at 512 for each month with variation in the IQR.

When examining the values for log_{10}IgGa (1), December had a significantly lower mean titer value compared to all other months (Table 4.2). In contrast, June samples had significantly higher titers compared to all other months (vs. February: $\beta = 0.07$, 95 %CI 0.03, 0.11, $p < 0.001$; April: $\beta = 0.06$, 95 % CI 0.02, 0.10, $p = 0.003$; October: $\beta = 0.06$, 95 %CI 0.02, 0.10, $p = 0.006$), except August. With log_{10}IgGb (4/7), horses had significantly lower titer levels in December (Table 4.2) and higher in October (vs. February: $\beta = 0.19$, 95 % CI 0.15, 0.23, $p < 0.001$; April: β

= 0.07, 95 % CI 0.03, 0.12, p = 0.001; June: β = 0.13, 95 %CI 0.08, 0.17, p < 0.001; August: β = 0.10, 95 % CI 0.06, 0.15, p < 0.001).

Although log_{10}IgG(T) (3/5) and VN titers were significantly lower in horses in December as well (Table 4.2), the highest titers for log_{10}IgG(T) (3/5) were in April (vs. February: β = 0.74, 95 % CI 0.56, 0.92, p < 0.001), as they were also for VN (vs. October: β = 179.54, 95 % CI 37.58, 321.49, p = 0.013).

4.4.2.2. Age

The ages of broodmares sampled varied from 2 – 27 years (median 10, interquartile range (IQR) 7 – 14). There was a significant positive association between age and all serological assays (Table 4.2).

4.4.2.3. Breed

Breeds were reduced to five categories for analysis: Thoroughbreds (31.8 %), Standardbreds (30.5 %), Warmbloods (Dutch and Hanoverian; 24.1 %), Drafts (Belgian, Clydesdale, Friesian, Shire, Percheron, and Canadian; 8.3 %), and "Other" (Rocky Mountain and Quarterhorse, Fjord, and pony breeds; 4.3 %).

Standardbred and Thoroughbred and Warmblood mares were significantly higher than the "Other" category for log_{10}IgGa (1) and log_{10}IgG(T) (3/5) (Table 4.2). In addition to being higher than the "other" category, Standardbreds and Thoroughbreds also had higher log_{10}IgGb (4/7) titers compared to Draft mares (β = 0.40, 95 % CI 0.15, 0.65, p = 0.002 and β = 0.27, 95 % CI 0.04, 0.49, p = 0.022, respectively). Thoroughbred and Warmblood mares had significantly higher log_{10}IgG(T) (3/5) titers compared to the Drafts (β = 1.31, 95 % CI 0.45, 2.17, p = 0.003 and β = 0.97, 95 % CI 0.20, 1.73, p < 0.013, respectively).

Standardbreds had significantly higher VN titers than the "Other" breed category (Table 4.2), as well as Draft (β = 1011.49, 95 % CI 414.16, 1608.03, p = 0.001) and Warmblood mares (β = 462.06, 95 % CI 13.38, 910.73, p = 0.044), but were not significantly different from

Thoroughbred mares (β = 304.09, 95 % CI -81.84, 690.02, p = 0.123). The difference in VN titer was significantly higher in Thoroughbred compared to Draft mares (β = 707.40, 95 % CI 73.15, 1341.65, p = 0.029).

4.4.2.4. Pregnancy and foaling

Over the sampling period, 64.2 % (1189/1853) of the samples were collected from pregnant mares (Table 4.1). Pregnancy was not significantly associated with any titers, and neither was a history of abortion (Table 4.2).

Unique to $\log_{10}$IgG(T) (3/5), the $\log_{10}$ titer significantly decreased as month of gestation increased (Table 4.2). The number of months since foaling was not significantly associated with either $\log_{10}$ IgG(a) (1) or the $\log_{10}$ IgG(T) (3/5) but was positively associated with $\log_{10}$IgGb (4/7) titer and negatively associated with VN titers (Table 4.2).

4.4.2.5. Vaccination

Most mares sampled (85.8 %, 1590/1853) had been vaccinated at the time of sampling (Table 4.1). The vaccines used included multivalent vaccines from the Prestige™ (Merck), Innovator™ (Zoetis), and Vetera™ (Boehringer Ingelheim) product lines, the divalent vaccine Calvenza™ (Boehringer Ingelheim), as well as monovalent vaccines labeled for the aid in preventing EHV-1-associated abortion (Prodigy™ from Merck, Pneumabort K + 1b™ from Zoetis). The vaccines used were reduced to 6 categories: Pneumabort K + 1b™ (36.0 %), Prodigy™ (23.9 %), Vetera™ (22.1 %), Prestige™ (11.5 %), and Innovator™ (2.39 %). An “Other” category (4.1 %) included “unknown combination vaccine” and Calvenza™.

Vaccinated animals had significantly higher $\log_{10}$IgG titers, and the $\log_{10}$ IgGa (1) and IgGb (4/7) titers significantly decreased as the months since vaccination increased (Table 4.2). Unlike $\log_{10}$ IgGa (1) and IgGb (4/7), $\log_{10}$ IgG(T) (3/5) titer was not associated with recent vaccination and did not decrease significantly as months since vaccination increased (Table 4.2).

Vaccine products Pneumabort™, Prestige™, Prodigy™, and Vetera™ had significantly higher log_{10}IgGa (1) titers compared to "Other" (Table 4.2). Prestige™ was also significantly greater than Innovator™ (β = -0.17, 95 % CI -0.32, -0.03, p =0.021).

All vaccine products had significantly higher log_{10}IgGb (4/7) titers than the "Other" category (Table 4.2). Innovator™ had significantly higher titers when compared to Pneumabort K™ (β = 0.23, 95 % CI 0.05, 0.41, p = 0.014), Prodigy™ (β =0.18, 95 % CI 0.01, 0.36, p = 0.042) and Vetera™ (β = 0.24, 95 % CI 0.06, 0.42, p = 0.011). Prestige™ had higher log_{10}IgGa (1) titers compared to Pneumabort K™ (β = 0.12, 95 % CI 0.04, 0.21, p = 0.006) and Vetera™ (β = 0.14, 95 % CI 0.03, 0.24, p = 0.010).

Compared to the "Other" vaccine category, only Innovator™, Prestige™, and Prodigy™ had significantly higher log_{10}IgG(T) (3/5) titers (Table 4.2). Pneumabort K™ had a significantly lower log_{10}IgG(T) (3/5) titer than Prodigy™ (β = -0.46, 95 % CI -0.83, -0.10, p = 0.013), Innovator™ (β = -0.74, 95 % CI -1.41, -0.07, p = 0.030) and Vetera™ products (β = -0.29, 95 % CI -0.53, -0.04, p = 0.020).

Vaccination and the different vaccine products were not associated with the VN titer (Table 4.2). During the study period, 48.5 % of horses (95 % CI 38.3 – 58.7 %) did not have a four-fold or greater change in their VN titer (Table 4.3). In addition, 42.1 % (95 % CI 30.9 – 54.0 %) of vaccinated horses did not have a four-fold or greater change in VN titers (Table 4.3). In fact, 32/99 (32.3 %, 95 % CI 23.3 – 42.5 %) of horses tested did not develop titers above 1:512 despite vaccination during the sampling period (Table 4.3).

4.4.2.6. EHV-1 molecular prevalence using ddPCR

When molecular presence of EHV, as measured by ddPCR, was compared to serological data, no significant association between the presence of EHV-1 and IgG subisotype or VN titer was identified (Table 4.2).

4.4.2.7. Intraclass correlation and model diagnostics

IgGa (1), IgG(T) (3/5), and IgGb (4/7) were not significantly associated with VN when the effect of farm and horse were controlled.

To control for repeated measures per mare and clustering by farm, random intercepts were included in all models, which also improved the model fit based on the AIC. The intra-class correlation (ICC) for farm ranged from 0.06 – 0.35, and for broodmare from 0.58 – 0.90 among our tobit models (Table 4.2). The best linear unbiased predictions (BLUPs) generally met the assumptions of homoscedasticity and normality. All potential outlying observations were investigated, but their removal did not impact the final models.

4.5 Discussion

Using a population of Ontario broodmares, the host risk factors associated with the serological response to EHV-1 was investigated. A tobit analysis allowed the results to be more completely described as the information within the censored data were incorporated into the final models. This cross-sectional investigation confirmed that most broodmares in Ontario had anti-EHV-1 IgG and VN antibodies, regardless of vaccination status, and that a portion did not significantly alter their VN titers with vaccination.

Of the six sampling periods collected from our population of broodmares, there was a large variability in the IgG subisotype levels depending on the month, with all IgG subisotypes being lowest in December. The $\log_{10}$IgGa (1) was highest in June (4.23), but the $\log_{10}$IgGb (4/7) was highest in October (5.83). There was no significant difference among months in terms of $\log_{10}$IgG(T) (3/5) value or VN titer.

Host risk factors were associated with the IgG and VN titers level including breed and age. Of the farms who participated, the largest herds belonged to Thoroughbreds, Warmbloods, and Standardbreds, and these large farms tended to be closed dedicated breeding operations that vaccinated frequently. Typically, Standardbred and Thoroughbred broodmares had the highest antibody titers of the breeds assessed. Older mares were also significantly associated with higher IgG and VN titers measured in this study. There have been some investigations into genetics associated with vaccine response based on Old Kladruber and Thoroughbred horses where microsatellites appeared to be strongly associated with an individual's responsiveness to *Rhodococcus equi* and *Lawsonia intracellularis* infection (Horín *et al*. 2004). Microsatellites are simple tandem repeats of DNA base pairs used to identify alleles as part of "DNA fingerprinting" and paternity tests (Tozaki *et al*. 2001). Exploring Duvaxyn 1, 4™ vaccination (a divalent inactivated vaccine against EHV-1 and -4 from Zoetis) in the same population revealed associations between the TKY343 microsatellite and poor vaccine response in grey Old Kladruber horses, whereas the TKY325 was significantly associated with high or average responders with black coats, when horses were divided based on coat colour (Rusek *et al*. 2013). Further research in other breeds would be necessary but this parallels our study's findings of breed differences in immune response to infection and vaccine exposure.

Despite all vaccines used during this study being inactivated, their associated serological response was very different as described in Table 4.2; relative to our "Other" category, animals vaccinated with Prestige™ had significantly higher log_{10}IgGa (1) and log_{10}IgGb (4/7) responses, and Innovator™ had significantly higher log_{10}IgG(T) (3/5) and log_{10}IgGb (4/7) response. Pneumabort K™ and products from the multivalent Vetera™ category had significantly higher log_{10}IgGa (1) response, while Prodigy™ was associated with a log_{10}IgGa (1) and log_{10}IgG(T) (3/5) response. The fact that 32.3 % did not develop a VN titer above 1:512 despite vaccination is consistent with other reports where 30.0 – 48.5 % of horses repeatedly vaccinated did not increase their titers (Foote *et al*. 2002, Rusek *et al*. 2013). Bannai *et al.* (2014 and 2019), using a new inactivated vaccine, only reported that 22 % of horses responded with at least a four-fold titer increase. In a study of influenza vaccination, younger age and a lower initial titer were predictors of larger antibody response, as younger horses typically had lower initial antibody levels (Muirhead *et al*. 2008, Muirhead *et al*. 2010). To the knowledge of the authors, there have been no similar studies with herpesvirus vaccination. The lack of VN response may be due to the broodmare population studied, but pregnant mares should have the same humoral response to vaccination and EHV-1 exposure when challenged, as demonstrated by an increase in VN titer, as non-pregnant mares (Gerber *et al*. 1977). Based on our models, it should be noted that the correlation of observations of two samples collected from the same horse was high, ranging from 0.58 – 0.90, and that most of the variance was explained at the horse level.

In addition to the quality of the immune response generated, vaccines are tested for their capacity to limit disease and virus shedding. Virus shedding from the nose was significantly reduced to 5.2 days versus 10 days in unvaccinated weanlings but required both boosters in order for a VN to develop (Heldens *et al*. 2001). Previous reports indicate that VN titers should be associated with IgGa (1) and IgGb (4/7), but not IgG(T) (3/5) (Wagner *et al*. 2015, Wimer *et al*. 2018, Schnabel *et al*. 2019). However, this study did not identify any significant associations between IgG subisotype and VN titer values. Interestingly, there was no association with EHV-1 DNA presence at the mucosal surfaces or in blood and the various IgG or VN titers, either. In this study, we did observe that IgG(T) (3/5) titer had a significant negative association with months of gestation. A similar negative association was noted in our analysis of EHV-1 viremia with ddPCR in this same study population (Chapter 3). Although there was no significant association between IgG(T) (3/5) and EHV-1 virus presence in this study, this parallel may indicate a

biologically important association that may not have reached statistical significance due to type II error. Previous analysis of the ddPCR results in this population found no difference between the vaccines used and EHV-1 shedding but did report a decrease in EHV-1 DNA in blood after vaccination (Chapter 3) which would coincide with increased $\log_{10}$-IgG(T) (3/5) titers reported here.

A limitation of this study was the use of an unknown serum as a standard and large sample dilutions required, limiting the utility of the calculated ELISA IgG result and increasing the risk of test variability. A quantification method for IgG subisotypes has been described by Wimer *et al* (2018)**,** but its utility in non-experimental situations has not been determined. In Wimer *et al*. (2018)'s study, very few mares had EHV-1 DNA present, limiting the interpretation of the titers and their potential impact on virus shedding. Furthermore, the ELISA may be identifying antibodies to EHV-4 which is antigenically similar to EHV-1 and therefore included in multivalent vaccine products used during this study, and which occurs much more commonly in horses (Pusterla *et al.* 2011, Smith *et al.* 2018).

In summary, most broodmares in Ontario had elevated VN titers to EHV-1, indicative of vaccination or previous EHV-1 exposure. Standardbreds and Thoroughbreds appeared to have the highest $\log_{10}$IgG titers, and Standardbreds had the highest VN titers. Despite repeated vaccination, 42.1 % of broodmares did not appear to have biologically significant differences in their titers across sampling periods. The VN titer increased with increasing age but was not influenced by vaccination or pregnancy in this population and was not significantly associated with a subisotype of IgG, unlike previous reports. This finding was unexpected as VN titers are used to measure vaccine response and exposure to viral pathogens. It is possible that the population in this study was sampled at a frequency that did not capture the variation in titer values adequately, or that the individual horse variability was too high to produce statistical significance. There was no association with IgG or VN titers and the presence of EHV-1, preventing any conclusions to be drawn regarding protective immunity or vaccine efficacy in this population.

Table 4.1: Number of blood samples collected and ddPCR status for EHV-1 from healthy Ontario broodmares and farms per sampling period from December 2016 to October 2017.*

	Dec 2016		Feb 2017		Apr 2017		June 2017		Aug 2017		Oct 2017		Overall	
	n sampled	**n (%) positive**	**n sampled**	**n (%) positive**	**n sampled**	**n (%) positive**	**n sampled**	**n (%) positive**	**n sampled**	**n (%) positive**	**n sampled**	**n (%) positive**	**n sampled**	**n (%) positive**
EHV-1 PCR positive – broodmares	352	147 (41.7 %)	356	90 (25.3 %)	311	116 (37.3 %)	307	127 (41.4 %)	262	94 (35.9 %)	266	92 (34.6 %)	381[a]	324 (85.0 %)
EHV-1 PCR positive – farms	39	36 (92.3 %)	42	32 (76.2 %)	39	34 (87.2 %)	35	31 (88.6 %)	36	24 (66.7 %)	35	26 (74.3 %)	42[a]	41 (97.6 %)
Survey results														
Pregnant	352	262 (74.4 %)	356	252 (70.8 %)	311	164 (52.7 %)	307	171 (55.7 %)	262	167 (63.7 %)	265	173 (65.3 %)	1853	1189 (64.2 %)
Vaccinated	352	292 (83.0 %)	355	297 (83.7 %)	311	274 (88.1 %)	307	266 (86.6 %)	262	226 (86.3 %)	266	231 (86.8 %)	1853	1590 (85.8 %)

*Modified from results in Cooper *et al.* 2020 – submitted for peer review

[a] – number of unique mares or farms that were included in the study.

Table 4.2: The results of the mixed effects tobit models of the log_{10}IgG and VN measures of EHV 1 with random intercepts for farm and mare.

		Log_{10} IgGa (1)			**log_{10} IgGb (4/7)**			**log_{10} IgG(T) (3/5)**			**VN**		
Variables		**Coeff**	**95 % CI**	**p-value**	**Coeff**	**95 % CI**	**p-value**	**Coeff**	**95 % CI**	**p-value**	**Coeff**	**95 % CI**	**p-value**
Month	**REF = December**			**< 0.001**			**< 0.001**			**< 0.001**			**0.001**
	February	**0.12**	**0.09, 0.16**	**< 0.001**	**0.07**	**0.03, 0.11**	**< 0.001**	0.10	-0.07, 0.27	0.267	**250.97**	**109.44, 392.49**	**0.001**
	April	**0.14**	**0.10, 0.18**	**< 0.001**	**0.19**	**0.15, 0.23**	**< 0.001**	**0.84**	**0.66, 1.02**	**< 0.001**	**256.32**	**114.84, 397.80**	**< 0.001**
	June	**0.20**	**0.16, 0.23**	**< 0.001**	**0.14**	**0.09, 0.18**	**< 0.001**	**0.73**	**0.54, 0.92**	**< 0.001**	**237.46**	**94.87, 380.05**	**0.001**
	August	**0.20**	**0.16, 0.24**	**< 0.001**	**0.16**	**0.12, 0.20**	**< 0.001**	**0.67**	**0.48, 0.86**	**< 0.001**	135.62	-6.72, 277.96	0.062
	October	**0.14**	**0.10, 0.18**	**< 0.001**	**0.26**	**0.22, 0.31**	**< 0.001**	**0.69**	**0.49, 0.88**	**< 0.001**	76.79	-65.09, 218.66	0.289
Age		**0.02**	**0.01, 0.03**	**< 0.001**	**0.03**	**0.02, 0.04**	**< 0.001**	**0.13**	**0.10, 0.17**	**< 0.001**	**34.56**	**3.95, 65.17**	**0.027**
Breed	**REF = Other**			**< 0.001**			**0.004**			**< 0.001**			**0.005**
	Draft	0.22	-0.05, 0.50	0.115	0.05	-0.25, 0.35	0.745	0.39	-0.67, 1.46	0.471	-340.68	-1133.60, 452.23	0.400
	STB	**0.42**	**0.13, 0.72**	**0.005**	**0.45**	**0.15, 0.76**	**0.004**	**1.30**	**0.17, 2.42**	**0.024**	**670.81**	**61.70, 1279.91**	**0.031**
	TB	**0.43**	**0.20, 0.66**	**< 0.001**	**0.32**	**0.06, 0.57**	**0.016**	**1.70**	**0.80, 2.61**	**< 0.001**	366.72	-226.61, 960.04	0.226
	WB	**0.42**	**0.16, 0.68**	**0.001**	0.24	-0.03, 0.51	0.087	**1.36**	**0.37, 2.35**	**0.007**	208.75	-457.28, 874.78	0.539
Pregnant		0.00	-0.03, 0.04	0.833	0.01	-0.03, 0.04	0.697	-0.08	-0.24, 0.09	0.349	13.09	-107.50, 133.68	0.832
Ever Vaccinated		**0.36**	**0.37, 0.45**	**< 0.001**	**0.27**	**0.17, 0.36**	**< 0.001**	**1.09**	**0.69, 1.49**	**< 0.001**	66.10	-233.36, 365.56	0.665
Simple Prod	**REF = Other**			**0.001**			**0.001**			**0.001**			0.238
	Innovator	0.10	-0.08, 0.28	0.276	**0.44**	**0.21, 0.67**	**< 0.001**	**1.06**	**0.23, 1.89**	**0.012**	248.28	-806.82, 1303.34	0.645
	Pneumabort	**0.20**	**0.08, 0.32**	**0.001**	**0.21**	**0.06, 0.36**	**0.006**	0.32	-0.24, 0.87	0.261	448.31	-109.05, 1005.73	0.115
	Prestige	**0.27**	**0.14, 0.40**	**< 0.001**	**0.34**	**0.17, 0.51**	**< 0.001**	**0.63**	**0.01, 1.24**	**0.046**	710.32	95.15, 1325.47	0.024
	Prodigy	**0.23**	**0.10, 0.35**	**0.001**	**0.26**	**0.09, 0.42**	**0.002**	**0.78**	**0.18, 1.38**	**0.011**	545.32	-30.95, 1121.71	0.064
	Vetera	**0.19**	**0.08, 0.31**	**0.001**	**0.20**	**0.06, 0.35**	**0.007**	0.60	0.07, 1.13	0.26	427.48	-104.82, 959.74	0.115
Abortions		0.04	-0.06, 0.15	0.410	0.02	-0.09, 0.14	0.699	0.36	-0.08, 0.80	0.111	268.68	-94.98, 632.35	0.148
Gestation		-0.00	-0.01, 0.00	0.702	-0.00	-0.01, 0.01	0.879	**-0.02**	**-0.05, 0.00**	**0.038**	16.44	-1.63, 34.50	0.075

Months since foaling		-0.00	-0.01, 0.00	0.524	**0.02**	**0.01, 0.03**	**< 0.001**	0.01	-0.02 , 0.04	0.579	**-31.56**	**-57.04, -6.09**	**0.015**
Month since Vaccination		**-0.03**	**-0.03, 0.02**	**< 0.001**	**-0.02**	**-0.03, 0.01**	**< 0.001**	-0.01	-0.03, 0.01	0.379	-12.45	-29.88, 4.99	0.162
Vaccinated < 2 months		**0.04**	**0.01, 0.06**	**0.004**	**0.05**	**0.02, 0.09**	**0.001**	-0.11	-0.24, 0.01	0.083	28.77	-82.56, 140.09	0.613
VN titers		-0.00	-0.01, 0.00	0.313	-0.00	-0.01, 0.00	0.502	0.00	-0.00, 0.00	0.142	-	-	-
PCR Positivity	**Nasal**	0.00	-0.04, 0.05	0.918	0.03	-0.02, 0.08	0.231	0.01	-0.22, 0.24	0.939	19.97	-137.78, 177.72	0.804
	Vaginal	-0.02	-0.05, 0.02	0.329	-0.03	-0.07, 0.00	0.087	-0.05	-0.22, 0.12	0.573	-136.73	-276.86, 3.40	0.056
	WBC	-0.02	-0.05, 0.01	0.272	0.02	-0.02, 0.06	0.313	0.06	-0.10, 0.23	0.452	-60.41	-190.35, 69.53	0.362
	Overall	-0.01	-0.04, 0.01	0.330	0.00	-0.03, 0.03	0.915	0.06	-0.07, 0.19	0.364	-61.05	-163.96, 41.86	0.245

The variance for the farm effect ranged from 0.02 to 0.11 for log_{10}IgGa, from 0.03 to 0.09 for log_{10}IgGb, from 0.34 to 1.61 for log_{10}IgG(T), and from 36832.29 to 104235.40 for VN.

The variance for the mare effect ranged from 0.12 to 0.22 for log_{10}IgGa, from 0.17 to 0.20 for log_{10}IgGb, from 1.70 to 2.09 for log_{10}IgG(T), and from 334203.40 to 412853.10 for VN.

The variance at the sample level ranged from 0.02 to 0.06 for log_{10}IgGa, from 0.05 to 0.08 for log_{10}IgGb, from 0.43 to 1.49 from log_{10}IgG(T), and from 255899.60 to 314597.70.

The intraclass correlation coefficient for the farm effect ranged from 0.06 to 0.35 for log_{10}IgGa, from 0.10 to 0.25 for log_{10}IgGb, from 0.11 to 0.31 for log_{10}IgG(T), and from 0.06 to 0.14 for VN

The intraclass correlation coefficient for the mare effect ranged from 0.76 to 0.90 for log_{10}IgGa, from 0.74 to 0.81 for log_{10}IgGb, from 0.68 to 0.87 for log_{10}IgG(T), and from 0.58 to 0.73 for VN.

Table 4.3: The summary of VN titer change, either increase or a decrease, in a subset of 99 healthy Ontario broodmares collected during the sampling period from December 2016 through October 2017.

		Immune change (no.) in horses with December 2016 VN titer of:									
		< 4	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	**Total**
Overall	Biologically Significant* change	1	0	4	3	5	16	9	4	9	51
	No change	0	2	1	2	5	9	13	12	4	48
	Total	**1**	**2**	**5**	**5**	**10**	**25**	**22**	**16**	**13**	**99**
Vaccinated	Biologically Significant* change	1	-	2	2	4	15	9	4	7	44
	No change	0	-	0	0	2	7	9	11	3	32
	Total	**1**	**-**	**2**	**2**	**6**	**22**	**18**	**15**	**10**	**76**
Un-vaccinated	Biologically Significant* change	-	0	2	1	1	1	0	0	2	7
	No change	-	2	1	2	3	2	4	1	1	16
	Total	**-**	**2**	**3**	**3**	**4**	**3**	**4**	**1**	**3**	**23**

* A four-fold change (either increase or decrease) in titer or higher during the study period was considered significant.

Chapter 5 General Discussion

The aims of the thesis were to better understand the risk EHV-1 has on the horse breeding industry in Ontario, by measuring the prevalence of breeder behaviours in terms of the perceived risk and potential host risk factors. To determine if the breeder behaviours are justified based on EHV-1 risk, we estimated the prevalence of EHV-1 using ddPCR on samples from the nose, vagina, and blood. Exploration of this population's antibody levels to EHV-1 was also undertaken to better understand the response to vaccination as well as the level of immunity in a natural population.

To address the first objective, we surveyed 88 breeders from across Ontario. While this is only a fraction of the total number of breeding farms (approximately 1% based on inferences made from the Equine Canada census data in 2011), the hope was that this sample would provide unbiased estimates of the opinions and behaviours of horse breeders in the region. The resulting data confirmed that most farms are aware enough about EHV-1 that they are vaccinating regularly. Also, it was interesting to note that the use of vaccines was not associated with herd size or breeding for business, rather that facilities identifying as "strictly for breeding" were most likely to vaccinate. Moreover, the vaccination protocols used were varied, with twice annual and the "pre-foaling protocol" being most commonly selected. Veterinarians were involved most often when abortions occurred but were not typically contacted for respiratory disease. Furthermore, the number of respiratory or abortive samples that were submitted for the diagnostic testing were very low.

After instituting vaccination at racetracks in Japan, Bannai et al. (2014, 2019) recommended that a vaccination rate of 79.3-85.3 % should be sufficient to control outbreaks. Considering that only 72.7 % (64/88) of breeders surveyed in Ontario in these studies were vaccinating at the herd level, this population would probably be at risk for EHV-1 outbreaks since a relatively large proportion of farms are not vaccinating their animals. Additionally, when sampling individual broodmares for the subsequent prevalence studies, many breeders indicated that they vaccinated pregnant mares differently than barren mares, and that both were treated differently than non-breeding horses on the same property. Most of the mares we sampled were vaccinated (85.8 %,

1590/1853). However, the definition of vaccination used in these studies was “administered within the previous 2 years” which means that some mares had not actually been vaccinated as frequently as recommended by current guidelines. Furthermore, breeders admitted that barren mares were sometimes not vaccinated at all until they were in foal, despite being housed with pregnant mares or on the same property as riding horses. Outbreaks in Ontario have been occurring infrequently with individual farm outbreaks producing all forms of EHV-1-associated disease every year from 2017-2020. Some farms have also reported recurring abortion storms every few years, despite vaccination and segregation of at-risk groups. In 2020, a large outbreak of EHM in horses at a local racetrack, as well as the annual outbreaks from 2017-2020 in Ontario, generated concerns that the neurologic form of EHV-1 is emerging and becoming endemic in the population. The most recent outbreak showed a rapid spread and large number of horses affected with neurologic signs. Thankfully, EHM is a notifiable disease in the province of Ontario, as is the reporting of samples submitted to diagnostic laboratories harboring the ORF30 neuropathogenic mutant strain. This surveillance program provides some information and guidance to local veterinarians on how to mitigate disease and outbreaks as they occur. Thankfully, no multi-site outbreaks have occurred in Ontario recently.

It would be interesting to repeat the survey again with the hopes of attracting more respondents and to compare results to see if there has been a change in behaviour with the news of recent outbreaks. Certain breeders stated that they felt that EHV-1-associated disease was uncommon or that the commercially available EHV-1 vaccines were ineffective. In the same conversation, many did not understand the pathogenesis of the disease or that latency and recrudescence can occur. This survey was disseminated primarily via email and online, but perhaps one administered by the farm veterinarian, or by telephone may generate a better response rate and different information. It would also be useful to question the various veterinary practices and better understand veterinarian’s perceptions of EHV-1 risk and vaccination, since the decision to submit samples in respiratory and abortion cases may be influenced by the veterinarian’s opinion. Similarly, veterinarians may provide insight into vaccination patterns and recommendations, identifying if their opinions differ from current guidelines, and if there are vaccine product preferences.

The skepticism of some breeders was unsurprising as there has been a growing trend of anti-vaccination for both humans and pets. Therefore, the second part of the thesis would hopefully add information about individual risk. This study is the first in Canada to determine population-based prevalence estimates for EHV-1. After sampling broodmares six times, 85.0 % were found positive for EHV-1 DNA particles from at least one sampling site using ddPCR. The nose did contain the highest number of viral copies, but the vagina was actually more often positive. To the author's knowledge, this is the first report indicating that subclinical viral shedding may occur from the vaginal mucosa.

Highly sensitive assays have been sought to diagnose EHV-1 infection and shedding early and thereby prevent outbreaks and the spread of disease (Smith 2013). Because qPCR is commonly used in research and diagnostic facilities, a more sensitive and allele-specific PCR was developed by Smith *et al*. (2012) and confirmed to be 10 times more sensitive than the original qPCR by Allen (2006), having a lower detection limit of just 10 virus particles. This was achieved by generating new primers targeting the ORF30 region where the neuropathogenic-associated mutation occurs. With the goal of proper diagnosis and better understanding of the pathogenesis of EHV-1 in horses, we utilized the ultrasensitive ddPCR to identify as many infected horses within our population as possible, but with the original Taq-Man® primer as described by Diallo *et al*. (2006). Where qPCR has been suggested to have a threshold of approximately 100 copies (Allen & Breathnach 2006), ddPCR in theory is able to identify the presence of even just one DNA particle within the sample. Despite the ultra-low detection limit of ddPCR, most of the samples we collected were negative (64.1 %). Those that were positive were mostly below 1 copy/ uL of original DNA. As with any PCR, the assay assumes that the target DNA is diffuse within the sample, but if there are so few virus particles present, it is possible that the aliquot submitted for analysis did not contain the virus. Furthermore, perhaps the reason for the very low viral copy numbers in our study is due to the fact that we targeted the gB gene, a late protein. The presence of few gB gene copies may suggest that the virus is present but latent or inactive. It was reassuring that although most horses were identified as carriers, almost all were only shedding very low levels of virus, well below the suggested viral load associated with disease spread (Pusterla *et al.* 2009b). It was unexpected that the intra-correlation coefficient of ddPCR results within mares was exceptionally low, meaning that having viral particles present in one sample did not predict the likelihood of being present in any other

samples or at any other time-point. However, it has been reported that reactivated latent virus can migrate and be shed from the nasal mucosa or induce a viremia even without clinical signs (Edington *et al*. 1985, Slater *et al*. 1994). And yet abortion does not have to follow viremia because infected leukocytes in the uterus allow spread of the virus to the local endothelium (Smith 2007, Smith *et al*. 2010). That virus presence differs at each sampling site at any given time may suggest more complexity about the pathogenesis of the virus: perhaps this indicates that the virus particles were from latently infected cells rather than active shedding, or that reactivation occurs independently throughout the body until a threshold is met and recrudescence begins.

Ideally, a follow-up investigation would differentiate the strains present within this population as either the neuropathogenic (D752, mutant) or non-neuropathogenic (N752, wildtype) subtypes of the virus. Inoculation of foals with the neuropathogenic strain produced an earlier-onset, longer lasting viremia than the non-neuropathogenic strain (Allen & Breathnach 2006). Also, the neuropathogenic strain has been associated with more virus shedding, especially when neurological signs are present (Pusterla *et al.* 2008b). Although not as high, subclinical and febrile horses can also shed a significant amount of virus (Pusterla *et al.* 2008b). It follows then, that the neuropathogenic and non-neuropathogenic strains may also have differing rates of disease spread or transmission parameters. Using the new probes developed by Smith *et al*. (2012) may alter the sensitivity and utility of the ddPCR assay as well by allowing differentiation of the EHV-1 strains. EHV-1 associated EHM and the neuropathogenic strain are reportable in Ontario, as mentioned above, but there is no national reporting system established. Without consistent information of cases and outbreak investigations, it is not possible to identify trends in disease occurrence, or formally indicate whether disease incidence is changing or not, much less whether vaccination has proven effective at reducing disease. When samples were compared to the diagnostic laboratory's qPCR, only samples with a copy number above 5 were associated with a positive qPCR result ($Ct < 37$) (data not shown). Only 20 horses in our study had a sample that had a particle count above 5. We recommend that ddPCR be used for prevalence assays and latency investigations, but further investigation into the clinical significance of ddPCR values is needed.

To further investigate the question of risk amongst this broodmare population, we investigated the immune response using EHV-1-specific ELISA and related responses to EHV-1 DNA presence (as determined by ddPCR). Unfortunately, our serological investigation of the vaccinated broodmare population demonstrated a large number that were not responsive to current commercial vaccination, despite being vaccinated multiple times in a given year. In all, 55 of 99 mares did not alter their neutralization titers in response to vaccination. None of the serology assays were associated with ddPCR positivity, but overall vaccinated mares did have higher $\log_{10}$IgG titers, but not VN titers. No mares had EHV-1-associated illness during the time of our study, however three vaccinated farms suffered abortions caused by EHV-1 in subsequent years. Although VN titers were not generated consistently in our study, in line with previous vaccine comparisons (Frymus *et al*. 1986, Bürki *et al*. 1990, Bürki 1991, Holmes *et al*. 2006), perhaps investigating the development of mucosal IgA may be more predictive of immunity as had been suggested by Breathnach *et al.* (2001). A recent novel vaccine study found that mucosal IgG (4/7) and IgA were associated with reduced respiratory epithelium infection (Wimer *et al*. 2018, Schnabel *et al*. 2019, Perkins *et al*. 2019). It would be interesting to see if vaginal IgA is also present and associated with protection from uterine virus infection.

The IgG subisotype assay in our study used a reference serum sample serially diluted to generate a 4-parameter concentration curve against which the optical densities of the samples could be measured. A direct quantitative approach was developed by Wagner *et al*. (2015) using a fluorescent bead-based multiplex assay and purified gC and gD antigens. This assay used the same biotinylated monoclonal antibodies for IgG (1) (CVS45) and for IgG (4/7) (CVS39) as we did but a different IgG (3/5) antibody, clone 586 (Wagner *et al*. 2015). Results were reported as median fluorescent intensities (MFIs) without conversion or adaptation between runs. However, the subisotypes found in the fluorescent bead assay were only the IgG (1) and IgG (4/7) depending on the target antigen used possibly because of a lack of IgG (3/5) stimulation or because of a failure to detect it. Our samples required very high dilutions, up to 1:1,280,000 to dilute out IgG (4/7) completely, risking dilution errors and variability between assays. The sample sera were diluted from 1:1000 to 1:50,000, again lending to potential dilution error potentially. Furthermore, the serum used as the standard often had much lower IgG content than our samples, limiting the interpretation of the test results and requiring a censoring of the data at the lower and higher limits of detection for analysis. During analysis, we did not find a

significant association between an IgG subisotype and the VN titers, which is in contrast to previous publications (Soboll-Hussey *et al*. 2011, Goodman *et al*. 2012, Wagner *et al*. 2015). It would be interesting to repeat a subset of samples using the ELISA protocol with either a different antigen or a different serum as standard to see if more information can be teased out and a pattern of response formed. Validation of the ELISA assay used with a quantitative method would also be important to reduce the inter-assay variability and minimize the potential dilution error.

To overcome the assay limitations, data were dichotomized, as had been done by Burgess *et al*. (2012) and Perkins *et al*. (2019). Dichotomization of continuous data are controversial as it reduces the information within the data and the ability to understand associations. However, in this instance, it did allow for general comparison between high and low titer groups and various risk factors. Unfortunately, unlike other studies, dichotomization of the data did not lead to the identification of the associations between VN levels and the various IgG subisotypes or association between serology and ddPCR positivity and therefore the data was not included in the study results.

In summary, this thesis was able to confirm that most breeders surveyed in Ontario are concerned about EHV-1 affecting their broodmares enough to vaccinate and involve veterinarians during abortions. However, with only 72.7 % of breeders vaccinating their horses, and the vaccination protocol varying dramatically between pregnant and non-pregnant mares, Ontario is at risk of continued EHV-1-associated outbreaks. Furthermore, these concerns are founded in that 85 % of broodmares did test positive for EHV-1 from either the nasal or vaginal mucosae or the blood at least once over the one-year sampling period. Lastly, in response to the question regarding vaccine efficacy, it appears that 32.3 % of the population did not generate a titer above 1:512 despite vaccination, and the 48.5 % of the entire population did not have a biologically significant change in VN titers at all over the course of the study. There were no associations between IgG subisotype or VN titers and ddPCR results either.

A survey of the breeders should be repeated to elucidate whether news of recent outbreaks has changed their opinions or behaviour. Involvement of the veterinarians would also be informative as it would indicate how much their professional opinions can influence the farm behaviours of the breeders and the uptake of vaccination recommendations. The results of ddPCR need to be

compared to those from qPCR or virus isolation to determine the validity of the assay and clinical relevance of the results, especially in relation to mucosal shedding. This is the first report to show that vaginal shedding appears to occur in pregnant and barren mares, and is not associated with foaling, providing a novel source of potential environmental contamination. The clinical significance of the number of viral particles identified by ddPCR needs to be further investigated, but the technique has proven useful in situations where viral copies are low, and contamination is high. A challenge study would be useful in this regard to determine what viral loads can be measured using ddPCR versus the widely utilized qPCR. From this baseline, the protection by vaccination can then be more accurately quantified. The implications of poor response to vaccination should be further investigated in natural populations to confirm this result and verify if any available vaccine is more effective at generating appropriate immunity. Utilizing the IgG subisotype ELISA along with ddPCR would provide more objective understanding of how vaccines are affecting EHV-1 presence in future research and vaccine development.

Chapter 6 Summary and Conclusions

In summary, my research confirmed that breeders are still concerned about the possible infection and diseases associated with EHV-1 enough to vaccinate. However, the type of vaccine used, and the frequency administered are not consistent. Furthermore, veterinarians are not always involved when respiratory disease occurs on the farms, leaving a gap in the potential education of owners and EHV-1 disease mitigation. Veterinarians are involved, unsurprisingly, when abortions occurred, but even then, samples are often not submitted leaving the etiology of the abortion unknown.

When looking into the individual mares on Ontario, 85 % were found to have EHV-1 DNA in either their nasal or vaginal mucosae, or in the blood with ddPCR. There was no significant difference in the presence of the virus in mucosae whether the mare was vaccinated or not, but vaccination had a protective effect on DNA presence in whole blood. Also, interestingly, as the months of gestation increased, the odds of being EHV-1 positive decreased, especially in blood samples.

Serology was then explored to see if any mare risk factors played a role with the immune response mounted to vaccination and the susceptibility of EHV-1 shedding. Although vaccination did increase IgG and VN titers overall, some vaccines were more effective than others, and the IgG subisotype levels varied widely. Of note, 30 % of mares did not respond to vaccination, as demonstrated by an increase in VN titer.

Altogether, the results show that all horses likely harbor the virus and that despite vaccination, a subset will still be at risk for reinfection or disease development. Vaccines, although helpful, still remain only a component of protection from EHV-1 disease until more immunogenic vaccines become available.

Appendices

Appendix 1: Chronological summary of key EHV-1 prevalence studies in horses around the world, including outbreaks and neuropathogenic strain identification.

Author	Country	Population	Method	Samples	Conclusion for EHV-1
Giles *et al*. 1993	USA	Abortions	Indirect fluorescent antibody test (IFAT)	Postmortem: fetus, placenta	4 % positive
Edington *et al*. 1994	UK	40 random abattoirs	Nested PCR and restriction endonuclease digestion	Postmortem: Submandibular, retropharyngeal and bronchial lymph nodes, tonsillar area, spleen, alveolar macrophages and trigeminal ganglion	87.5 % α-herpesviruses in bronchial lymph nodes: 62.5 % was EHV-1, 57.5 % was EHV-4 (32.5 % contained both).
Gilkerson *et al*. 1999b	Australia	2 large breeding farms	gG specific ELISA	Serum	Seropositive: 24.0 % of newborns (from colostrum), 81.8 % by 90-days old, 23 % of weanlings
Carvalho *et al*. 2000	Brazil	70 abattoir horses, 2 breeding farms, 4 archived fetuses. Samples collected over 6 years.	Virus isolation; virus neutralization; PCR for gC or thymidine kinase and restriction endonuclease digestion	Postmortem: various tissues. Nasal swabs, whole blood and serum, placenta	PCR: 88 % in abattoir animals; VN: 20/30 positive in pregnant mares from abattoir, 9/9 positive VN from breeding farm but foals were negative.
Van Maanen *et al*. 2000	Netherlands	Outbreak of trotting horse abortion in 1991	Immunofluorescence, virus isolation, PCR for gG, restriction endonuclease digestion, virus neutralization, complement fixation, gG specific ELISA	Serum, whole blood, nasopharyngeal swab, abortive tissues	7/12 abortions (4 positive), 1 foal died. 0 % positive from nasopharynx or blood. 1 mare seroconverted, 16 had high titers
Van Maanen *et al*. 2001	Netherlands	Outbreak of EHM in 1995	Virus isolation, gG specific ELISA	Serum, nasal swab, postmortem tissues	28/41 seroconverted, 12/41 had high VN at onset.

Barrangeguy *et al*. 2002	Argentina	Outbreak in 2 polo barns and 1 adjacent center	Virus isolation of abortive tissues; gG specific ELISA	Abortive tissues, serum from mares	50/173 mares aborted, 8/8 fetuses positive, mare VN ranged 1:32 – 1:512
Smith *et al*. 2003	UK	Abortions	IFAT	Abortive tissues	6.2 % positive for EHV-1
Foote *et al*. 2003	Australia	1 large breeding farm – Follow up to Gilkerson *et al*. 1999b. Pre- and post-vaccine introduction	gG specific ELISA	Serum	Positive mares: 31.8 % in 2001, 22.5 % in 2000 vs 26.2 % in 1995. Positive foals: 7.6 % in 2001, 3.8 % in 2000 vs 11.4 % in 1995
Foote *et al*. 2004	Australia	1 large breeding farm – Follow up to Gilkerson *et al*. 1999b/Foote *et al*. 2003	PCR for gC, gG specific ELISA	Nasal swabs, serum	Nasal PCR: 6 foals, 1 mare Seropositive: 30.4 % in mares, 27.0 % in foals
Allen 2006	Kentucky, USA	24 previously infected with EHV-1, 12 donated aged mares	Magnetic bead, sequence capture, nested PCR vs conventional nested vs real-time PCR	Mandibular lymph node biopsy	75 % of previously infected and 67 % of donated mares
Holmes *et al*. 2006	USA	55 adult mares 2-7 years old	Nested PCR	Blood	47 % positive
Hussey *et al*. 2006	Wisconsin, USA	19 ponies, 1-2	Real-time PCR	Blood and nasal swabs	
Brown *et al*. 2007	California, USA	1 endemic farm, Thoroughbred	Real-time PCR/ ELISA	Nasopharyngeal, blood, placenta, colostrum	Blood = 0/590, nasopharyngeal = 3/590, 1 positive placenta, seroconversion = 3/30 foals
Wang *et al*. 2007	Australia	Group 1: 64 5-8-month-old foals collected twice. Group 2: 141 weanlings. Group 3: 92 racehorses	Multiplex PCR EHV 1-5 and conventional PCR	PBMC, nasal swabs	PBMC: 0 in weanlings using multiplex but 12.5 % using direct PCR in group 1. 4.6 % in group 2. 14.1 % in racehorses. Nasal: None.
Allen *et al*. 2008	Kentucky USA	132 Thoroughbred broodmares	Magnetic bead, sequence capture, nested PCR vs conventional nested vs real-time PCR	Postmortem: submandibular lymph nodes,	Magnetic bead: 54 % positive. 18 % of which had

					neuropathogenic strain vs. conventional nested: 28 % positive.
Léon *et al*. 2008	France	407 fetuses, stillbirths and premature foals collected over 2.5 years	Multiplex consensus PCR for *DNApol* gene (EHV 1-5)	Abortive tissues	15 % (59/407) positive. 45 % positive in lung and placenta, 16 % positive in placenta.
Pusterla *et al*. 2009c	California, USA	302 imported horses to California, 2007-2008	ELISA (Svanovir), real-time PCR	Blood and nasopharyngeal swabs	1 % PCR positive. 15.2 % seropositive on admission. 9 seroconverted (4 were vaccinated during quarantine) Overall: 2.6 % evidence of EHV-1 infection
Pusterla *et al*. 2009b	California, USA	EHM-associated outbreak at racetrack, 39 Thoroughbreds and 35 other breeds	Real-time PCR	Blood and nasopharyngeal swabs	Over 22 days: 25 % to 14 %. Only index and neighbour were neuropathogenic.
Hebia-Fellah *et al*. 2009	France	390 stallion semen samples	Real-time PCR	semen	13 % (51/390) positive
Pusterla *et al*. 2010b	California, USA	147 horses, 4 mules, 2 donkeys	Real-time PCR	Postmortem; 68 antemortem nasal and blood	Antemortem samples: 0 positive. Trigeminal ganglia: 19 horses positive. SMLN: 5 horses positive.
Carr *et al*. 2011	Michigan and California, USA	124 horses with acute colic	Real-time PCR	Blood and nasal swab. 34 submandibular lymph nodes	0 % positive on all samples.
Nemoto *et al*. 2011	Japan	100 febrile Thoroughbred racehorses	LAMP with conventional PCR against gC	Nasal swabs	39/100 positive
Pusterla *et al*. 2011	USA	761 horses, mules, donkeys with acute respiratory or neurological signs (2008-2010)	Real-time PCR	Blood and nasal swabs	23/761 positive. Nasal only: 15 Blood only: 2 Both: 6 Neuropathogenic: 5 Both neuro and non: 7

Gryspeert *et al*. 2011	Belgium	Abortion outbreak in 13 premises (6 included in study)	Virus isolation, seroconversion	Serum, whole blood	All clinical were EHV-1-positive with neuropathogenic strain
Burgess *et al*. 2012	Saskatchewan, Canada	EHM outbreak in 3 facilities, 104 horses	Real-time PCR	Nasal swabs. Central nervous tissue from postmortem	75 % of horses with EHM + 1 febrile horse.
Pusterla *et al*. 2012	California, USA	70 racing Thoroughbreds	Real-time PCR	Postmortem: submandibular and bronchial lymph nodes, trigeminal ganglia	25.7 % positive. Trigeminal ganglia more often positive vs. lymph nodes
Carlson *et al*. 2013	USA	Young horses at horse shows and sales	Duplex real-time PCR (EHV 1,4)	Nasal swabs	3.9 % positive overall
Marenzoni *et al*. 2013	Italy	67 abortions and 22 stillbirths, 14 neonatal death	Nested PCR	Postmortem, abortive tissues	22/27 (81.5 %) positive: 16 neuropathogenic and 6 non-neuropathogenic
Walter *et al*. 2013	Germany	Outbreak of abortion and EHM	Virus isolation and PCR	Abortive tissues, blood, serum, nasal swabs, semen (3 samples)	29 % seroconversion. PCR: 17/49 horses positive Semen: all positive Abortion: 6/6
Sonis& Goehring 2013	Colorado, USA	Febrile and afebrile hospitalized horses	Real-time PCR	Nasal swabs	0 % positive in all samples
Damiani *et al*. 2014	Germany	Outbreak of abortion in 25 Standardbred broodmares	Virus isolation and PCR	Abortive tissues, blood, nasal swabs	16 aborted, 2 neonatal deaths. 14/14 tested positive
Schulman *et al*. 2014	South Africa	8 Thoroughbred broodmares for sale, 6 control	Real-time PCR	Nasal swab	0 % positive
Bażanów *et al*. 2014	Poland	34-year retrospective, 347 aborted fetuses, 105 neonatal deaths	Cell cytopathy, IFAT	Abortive tissues, postmortem	25.6 % (116/452) positive
Van Galen *et al*. 2015	France	Retrospective on neurological disease 2008-2011	Real-time PCR	Nasal swabs, blood and/or cerebrospinal fluid, postmortem tissues	26/219 neurological were EHM, with positive PCR

Schulman *et al*. 2015	South Africa	2 Abortion outbreaks in unvaccinated Thoroughbred broodmares	Immunostaining, real-time PCR	Abortive tissues	7.8 % positive in abortions (30 % Farm 1, 5.7 % Farm 2)
Badenhorst *et al*. 2015	South Africa	Thoroughbreds at auction	Real-time PCR	Nasal swabs	0 %
Pusterla *et al*. 2016	USA	4228 acute respiratory or neurologic cases (2008-2014)	Real-time PCR	Blood and nasal swabs	2.7 % (117/4228) positive Blood: 47 Nasal: 100 Both: 33
Smith *et al*. 2018	California, USA	167 imported horses to California	qPCR	Nasal swab	1.2 % positive
Brown *et al*. 2019	South Africa	1 endemic farm, 71 Thoroughbred foals	qPCR	Placenta	0 % positive from all samples

Appendix 2: Summary of studies exploring archived EHV-1 isolates and strain differentiation.

Author	Country	Population	Method	Samples	Conclusion for EHV-1
Perkins *et al*. 2009	New York, USA	176 archived EHV-1 isolates collected (1984-2007)	Real-time Taq-Man((R)) allelic discrimination PCR	Various	Neuropathogenic strain: 11 % (most from EHM cases, 76 %)
Smith *et al*. 2010	California, USA	426 archived EHV-1 isolates collected (1951-2006)	Real-time Taq-Man((R)) allelic discrimination PCR	Abortive tissues	Neuropathogenic strain: 3.3 % in 1960s, 14.4 % in 1990s, 19.4 % between 2000 -2006.
Carman *et al.* 2012	Ontario, Canada	15 EHV-1 isolates collected (2003-2007, and 2007-2012)	Real-time PCR	Abortive tissues, neonatal death	Neuropathogenic: 2003 - 2007 = 1/12 2007 – 2012 = 4/15
Vaz *et al. 2016*	Australia	11 Australian EHV 1 (1977-2007)	DNA sequencing	Various	Little recombination in EHV-1
Bryant *et al*. 2018	UK	78 archived EHV-1 isolates collected (1982-2016)	DNA sequencing and phylogeny	Various	Sequence conserved: > 99 %

Appendix 3: Breeder survey for Chapter 3.

Survey of the equine broodmare industry, abortion, and equine herpesvirus 1 vaccination in Ontario (2014–2015).

Q1 Letter of Information
❍ Proceed to questions (1)

Answer If Letter of Information Proceed to questions Is Selected

Q2 Do you breed horses?
❍ Yes (1)
❍ No (2)

If No Is Selected, Then Skip To End of Survey

Q3 Do you have at least 2 broodmares?
❍ Yes (1)
❍ No (2)

If No Is Selected, Then Skip To End of Survey

Q4 Please select primary reason for breeding:
❍ Hobby (1)
❍ Business/Professional (2)
❍ Other (3) _____
❍ Unknown (4)

Q5 Is the breeding operation a primary source of income?
❍ Yes (1)
❍ No (2)
❍ Unknown (3)

Q6 Select other sources of income for your property (Select all that apply)
❑ Strictly Breeding Facility (1)
❑ Boarding/ Lessons (2)
❑ Training (Racehorse) (3)
❑ Farming (sheep, goats, llama, alpaca, cattle, etc) (4)
❑ Other (5) _____
❑ Unknown (6)

Q7 Average number on premises

	January to December 2015 (1)	January to December 2014 (2)
Horses Total (1)		
Broodmares (2)		
Foals born (3)		

Q8 Medical records for broodmares are maintained (Select all that apply):
- ❑ By the veterinarian (1)
- ❑ On the farm (2)
- ❑ No medical records kept (3)
- ❑ Don't know (4)

Q9 Do you vaccinate for equine herpesvirus 1 (e.g. Pneumabort K, Prodigy, Calvenza, Fluvac Innovator, Rhinommune, combination vaccines, etc) ?
- ❍ Yes (1)
- ❍ No (2)
- ❍ Don't know (3)

Answer If Do you vaccinate for equine herpesvirus 1 (e.g. Pneumabort K, Prodigy, Calvenza, Fluvac Innovator, Rhinommune, combination vaccines, etc)? Yes Is Selected

Q10 Select all vaccination protocols that apply to the horses (including broodmares) on your property.
- ❑ Once per year (1)
- ❑ Twice per year (2)
- ❑ Pre-foaling protocol (e.g. 5, 7, and 9 months gestation) (3)
- ❑ Other (4) _____
- ❑ Don't know (5)

Q11 Have any horses on the property been affected with a runny nose, cough, or fever between January to 2014 and December2015?
- ❍ Yes (1)
- ❍ No (2)
- ❍ Don't know (3)

Answer If Have you had any horses on the property affected with runny nose, cough, or fever from January 2014 to December 2015? Yes Is Selected

Q12 Was medical treatment by a veterinarian required (e.g. anti-inflammatory, antibiotics, hospitalization)?
- ❍ Never (1)
- ❍ Sometimes (10-40 %) (2)
- ❍ About half the time (40-60 %) (3)
- ❍ Most of the time (60-90 %) (4)
- ❍ Always (5)
- ❍ Don't know (6)

Answer If Have you had any horses on the property affected with respiratory disease (runny nose, cough, snorting, fever, etc) from January 2014 to December 2015? Yes Is Selected

Q13 Were any cases tested for viral pathogens (e.g. influenza, herpesvirus, rhinovirus, etc)?
- ❍ Yes (1)
- ❍ No (2)
- ❍ Don't know (3)

Answer If Were any respiratory cases tested for viral pathogens (e.g. influenza, herpesvirus, rhinovirus, etc)? Yes Is Selected

Q14 How many tested positive for equine herpesvirus 1, also known as Rhinopneumonitis ("Rhino")?
- ❍ None (1)
- ❍ 1 (2)
- ❍ 2 (3)
- ❍ > 2 (4)
- ❍ Don't know (5)

Q15 If an abortion occurs, do you contact your veterinarian:
- ❍ Never (1)
- ❍ Sometimes (10-40 %) (2)
- ❍ About half the time (40-60 %) (3)
- ❍ Most of the time (60-90 %) (4)
- ❍ Always (5)
- ❍ Don't know (6)

Q16 Have you experienced abortion(s) in the past 2 years (January 2014 - December 2015)?
- ❍ Yes (1)
- ❍ No (2)

If No Is Selected, Then Skip To Please provide the City in which your...

Q17 How many abortions total (include: early fetal loss, active abortion, stillbirths) occurred?

	January to December 2015 (1)	January to December 2014 (2)
Abortion (1)		

Q18 Optional: Categorize the abortions from (Select all that apply), as appropriate

	January to December 2015 (1)	January to December 2014 (2)
Early Fetal Loss (1)		
Mid Gestation (4-6 months) (2)		
Late Gestation (7-10 months) (3)		
Peri-natal (4)		

Q19 Have fetus(es) and/or placenta ever been submitted for diagnostic testing to your veterinarian or a laboratory (e.g. IDEXX, Animal Health Laboratory of the University of Guelph)?
- ❍ Yes (1)
- ❍ No (2)
- ❍ Don't know (3)

If No Is Selected, Then Skip To Please provide the City in which your...If Don't know Is Selected, Then Skip To Please provide the City in which your...

Q20 How many abortions were submitted for testing from January to December 2015?
- ❍ None (1)
- ❍ Few (10-40 %) (2)
- ❍ Some (40-60 %) (3)
- ❍ Most (60-90 %) (4)
- ❍ All (5)
- ❍ Unknown (6)

Q21 How many abortions were submitted for testing from January to December 2014?
- ❍ None (1)
- ❍ Few (10-40 %) (2)
- ❍ Some (40-60 %) (3)
- ❍ Most (60-90 %) (4)
- ❍ All (5)
- ❍ Unknown (6)

Q22 How many tested positive for equine herpesvirus 1, also known as Rhinopneumonitis ("Rhino")?
- ❑ 2015 (1) _____
- ❑ 2014 (2) _____
- ❑ None (3)
- ❑ Unknown (4)

Q23 Please provide the town in which your property is located

Q24 Are you interested in being contacted regarding future studies on equine herpesvirus 1 and abortions?
- ❍ Yes (1)
- ❍ No (2)
- ❍ Maybe (3)

Q25 Would you be interested in having your broodmares tested for equine herpesvirus 1, at no cost to you?
- ❍ Yes (1)
- ❍ No (2)
- ❍ Maybe (3)

If No Is Selected, Then Skip To End of Survey

Optional: Please provide your name (Last, First)
_____

Optional: Please provide your phone number
_____

Optional: Please provide the Farm or Business name
_____

References

Aizawa Y, Koyama A, Ishihara T, Onodera O, Saitoh A. 2016. "Performance of a real-time PCR–based approach and droplet digital PCR in detecting human parechovirus type 3 RNA." *J Clin Virol* 84: 27-31.

Allen GP, Bryans JT. 1986. "Molecular epizootiology, pathogenesis and prophylaxis of equine herpesvirus 1 infections." *Vet Microbiol lmmunol* 2: 78-144.

Allen G, Yeargan M, Costa LR, Cross R. 1995. "Major histocompatibility complex class I-restricted cytotoxic T-lymphocyte responses in horses infected with equine herpesvirus 1." *J Virol* 69 (1): 606-612.

Allen GP, Breathnach CC. 2006. " Quantification by real-time PCR of the magnitude and duration of leucocyte-associated viraemia in horses infected with neuropathogenic vs. non-neuropathogenic strains of EHV-1." *Equine Vet J* 38 (3): 252-257.

Allen GP. 2006. "Antemortem detection of latent infection with neuropathogenic strains of equine herpesvirus-1 in horses." *Am J Vet Res* 67 (8): 1401-1405.

Allen GP, Bolin DC, Bryant U, Carter CN, Giles RC, Harrison LR, Hong CB, Jackson CB, Poonacha K, Wharton R, Williams NM. 2008. "Prevalence of latent, neuropathogenic equine herpesvirus-1 in the Thoroughbred broodmare population of central Kentucky." *Equine Vet J* 40 (2): 105-110.

Allen GP. 2008. "Risk factors for development of neurologic disease after experimental exposure to equine herpesvirus-1 in horses." *Am J Vet Res* 69 (12): 1595-1600.American Associate of Equine Practitioners (AAEP) 2018. "*Vaccinations for Adult Horses."* Accessed May 15, 2018. https://aaep.org/sites/default/files/Guidelines/AdultVaccinationChart81216.pdf.

Aranyos AM, Roff SR, Pu R, Owen JL, Coleman JK, Yamamoto JK. 2016. "An initial examination of the potential role of T-cell immunity in protection against feline immunodeficiency virus (FIV) infection." *Vaccine* 34 (12): 1480-1488.

Arthur, R. 2011. "Biosecurity and vaccination strategies to minimise the effect of an equine influenza outbreak on racing and breeding." *Aust Vet J* 89 (1): 109-113.

Azab, W, Harmann, R., Miller, D., Tallmadge, R., Frampton, A.R., Antczak, D.F., Osterrieder, N. 2014. "Equid herpesvirus type 4 uses a restricted set of equine major histocompatibility complex class I proteins as entry receptors." *J. Gen. Virol.* 95: 1554–1563.

Badenhorst M, Page PC, Ganswindt A, Guthrie AJ, Schulman ML. 2014. "Sales Consignment and Nasal Shedding of Equine Herpesvirus-1 (EHV-1) and 4 (EHV-4) in Young Thoroughbred Horses in South Africa." *Equine Vet J* 46 (Suppl 47): 13.

Badenhorst M, Page P, Ganswindt A, Laver P, Guthrie A, Schulman M. 2015. "Detection of equine herpesvirus-4 and physiological stress patterns in young Thoroughbreds consigned to a South African auction sale." *BMC* 11: 126.

Balasuriya UB, Crossley BM, Timoney PJ. 2015. " A review of traditional and contemporary assays for direct and indirect detection of Equid herpesvirus 1 in clinical samples." *J Vet Diagn Invest* 27 (6): 673-687.

Ballagi-Pordany A, Klingeborn B, Flensburg J, Belak S. 1990. "Equine herpesvirus type 1: detection of viral DNA sequences in aborted fetuses with the polymerase chain reaction." *Vet Microbiol* 22 (4): 373-381.

Bannai H, Mae N, Ode H, Nemoto M, Tsujimura K, Yamanaka T, Kondo T, Matsumura T. 2014. "Successful control of winter pyrexias caused by equine herpesvirus type 1 in Japanese training centers by achieving high vaccination coverage." *Clin Vaccine Immunol* 21 (8): 1070-1076.

Bannai H, Tsujimura K, Nemoto M, Ohta M, Yamanaka T, Kokado H, Matsumura T. 2019. "Epizootiological investigation of equine herpesvirus type 1 infection among Japanese racehorses before and after the replacement of an inactivated vaccine with a modified live vaccine." *BMC Vet Res* 15 (1): 280. doi:10.1186/s12917-019-2036-0.

Barrandeguy, M. E., F. Lascombes, J. Llorente, H. Houssay, and F. Fernandez. 2002. "High case-rate Equine herpesvirus-1 abortion outbreak in vaccinated polo mares in Argentina." *Equine Veterinary Education* 14 (3): 132-135.

Baxi MK, Efstathiou S, Lawrence G, Whalley JM, Slater JD, Field HJ. 1995. "The detection of latency-associated transcripts of equine herpesvirus 1 in ganglionic neurons." *J Gen Virol* 76 (12): 3113-3118.

Bażanów BA, Frącka AB, Jackulak NA, Staroniewicz ZM, Ploch SM. 2014. "A 34-year retrospective study of equine viral abortion in Poland." *Pol J Vet Sci* 17 (4): 607-612.

Bodo G, Marti E, Gaillard C, Weiss M, Burckner L, Gerber H, Lazary S. 1994. "Association of the immune response with the major histocompatibility complex in the horse." In *Equine Infectious Diseases* , by H., W.P (Eds.) Nakajima, edited by H., W.P (Eds.) Nakajima, 143–151. Tokyo: R&W Publications.

Borchers K, Wolfinger U, Ludwig H. 1999. "Latency-associated transcripts of equine herpesvirus type 4 in trigeminal ganglia of naturally infected horses." *J Gen Virol* 80 (8): 2165-2171.

Borchers K, Thein P, Sterner-Kock A. 2006. "Pathogenesis of equine herpesvirus-associated neurological disease: a revised explanation." *Equine Vet J* 38 (3): 283-287.

Bosh KA, Powell D, Neibergs JS, Shelton B, Zent W. 2009. "Impact of reproductive efficiency over time and mare financial value on economic returns among Thoroughbred mares in central Kentucky." *Equine Vet J* 41 (9): 889-894.

Breathnach CC, Yeargan MR, Sheoran AS, Allen GP. 2001. " The mucosal humoral immune response of the horse to infective challenge and vaccination with equine herpesvirus-1 antigens." *Equine Vet J* 33: 651-657.

Breathnach CC, Soboll G, Suresh M, Lunn DP. 2005. "Equine herpesvirus-1 infection induces IFN-gamma production by equine T lymphocyte subsets." *Vet Immunol Immunopathol* 103 (3-4): 207-215.

Breathnach CC, Sturgill-Wright T, Stiltner JL, Adams AA, Lunn DP, Horohov DW. 2006. "Foals are interferon gamma-deficient at birth." *Vet Immunol Immunopathol* 112 (3-4): 199-209.

Bresgen C, Lämmer M, Wagner B, Osterrieder N, Damiani AM. 2012. "Serological responses and clinical outcome after vaccination of mares and foals with equine herpesvirus type 1 and 4 (EHV-1 and EHV-4) vaccines." *Vet Microbiol* 160 (1-2): 9-16.

Brown JA, Mapes S, Ball BA, Hodder AD, Liu IK, Pusterla N. 2007. "Prevalence of equine herpesvirus-1 infection among Thoroughbreds residing on a farm on which the virus was endemic." *J Am Vet Med Assoc* 231 (4): 577-580.

Brown LJ, Brown G, Kydd J, Stout TAE, Schulman ML. 2019. "Failure to detect equid herpesvirus types 1 and 4 DNA in placentae and healthy new-born Thoroughbred foals." *J S Afr Vet Assoc* 90 (0): e1-e5. doi:10.4102/jsava.v90i0.1736.

Bryans JT, Allen GP. 1982. "Application of a chemically inactivated, adjuvanted vaccine to control abortigenic infection of mares by equine herpesvirus 1." *Dev Biol Stand* 52: 493-498.

Bryans, JT. 1969. "On immunity to disease caused by equine herpesvirus I." *J Am Vet Med Assoc* 155: 294-300.

Bryant NA, Wilkie GS, Russell CA, Compston L, Grafham D, Clissold L, McLay K, et al. 2018. "Genetic diversity of equine herpesvirus 1 isolated from neurological, abortigenic and respiratory disease outbreaks." *Transbound Emerg Dis* 65 (3): 817-832.

Burgess BA, Tokateloff N, Manning S, Lohmann K, Lunn DP, Hussey SB, Morley PS. 2012. "Nasal shedding of equine herpesvirus-1 from horses in an outbreak of equine herpes myeloencephalopathy in Western Canada." *J Vet Intern Med* 26 (2): 384-392.

Bürki F. 1988. "Equine rhinopneumonitis vaccination - an unsettled problem." *J Eq Vet Sci* 8 (1): 65-69.

Bürki F, Rossmanith W, Nowotny N, Pallan C, Möstl K, Lussy H. 1990. "Viraemia and abortions are not prevented by two commercial Equine Herpesvirus-1 vaccines after experimental challenge of horses." *Vet Quart* 12 (2): 80-86.

Bürki F, Nowotny N, Oulehla J, Schmehlik O, Möstl K, Pallan C, Rossmanith E. 1991. "Attempts to immunoprotect adult horses, specifically pregnant mares, with commercial vaccines against clinical disease induced by equine herpesvirus-1." *Zentralbl Veterinarmed B* 38 (6): 432-440.

Burns, TA. 2016. "Effects of Common Equine Endocrine Diseases on Reproduction." *Vet Clin N Am- Equine* 32 (3): 435-449.

Burrows R, Goodridge D, Denyer MS. 1984. "Trials of an inactivated equid herpesvirus I vaccine:challenge with a subtype 1 virus." *Vet Rec* 114: 369-374.

Carlson JK, Traub-Dargatz JL, Lunn DP, Morley PS, Kohler A, Kasper K, Landolt GA, Barnett DC, Lunn KF. 2013. "Equine viral respiratory pathogen surveillance at horse shows and sales." *J Equine Vet Sci* 33 (4): 229-237.

Carman S, McEwen B, DeLay J, Hazlett M, Stalker M. 2012. *Update on EHV-1 in Ontario.* Guelph: Animal Health Laboratory.

Carman S, Rosendal S, Hube L, Gyles C, Mckee S, Willoughby RA, Dubovi E, Thorsen J, Lein D. 1997. "Infectious agents in acute respiratory disease in horses in Ontario." *J Vet Diagn Invest* 9 (1): 17-23.

Carr E, Schott H, Pusterla N. 2011. "Absence of Equid Herpesvirus-1 Reactivation and Viremia in Hospitalized Critically Ill Horses." *J Vet Int Med* 25 (5): 1190-1193.

Carvalho R, Oliveira AM, Souza AM, Passos LMF, Martins AS. 2000. "Prevalence of equine herpesvirus type 1 latency detected by polymerase chain reaction." *Arch Virol* 145 (9): 1773-1787.

Charan S, Palmer K, Chester P, Mire-Sluis AR, Meager A, Edington N. 1997. "Transforming growth factor-beta induced by live or ultraviolet-inactivated equid herpes virus type-1 mediates immunosuppression in the horse." *Immunology* 90 (4): 586-591.

Charvat RA, Breitenbach JE, Ahn BC, Zhang Y, O'Callaghan DJ. 2011. "The UL4 protein of equine herpesvirus 1 is not essential for replication or pathogenesis and inhibits gene expression controlled by viral and heterologous promoters." *Virol J* 412 (2): 366-377.

Chesters PM, Allsop R, Purewal A, Edington N. 1997. "Detection of latency-associated transcripts of equid herpesvirus 1 in equine leukocytes but not in trigeminal ganglia." *Virol J* 71 (5): 3437-3443.

Chevalier-Clement F. 1989. "Pregnancy loss in the mare." *Animal Reprod* 20 (3): 552-557.

Chowdury SI, Kubin G, Ludwig H. 1986. "Equine Herpesvirs -1 (EHV-1) induced abortions and paralysis in a Lipizzaner stud: a contribution to the classification of equine herpesviruses." *Arch Virol* 90: 273-288.

Cliffe AR, Knipe DM. 2008. "Herpes simplex virus ICP0 promotes both histone removal and acetylation on viral DNA during lytic infection." *J Virol* 82 (24): 12030-12038.

Cliffe AR, Arbuckle JH, Vogel JL, Geden MJ, Rothbart SB, Cusack CL, Strahl BD, Kristie TM, Deshmukh M. 2015. "Neuronal stress pathway mediating a histone methyl/phospho switch is required for herpes simplex virus reactivation." *Cell Host Microbe* 18 (6): 649-658.

Cliffe AR, Wilson AC. 2017. "Restarting Lytic Gene Transcription at the Onset of Herpes Simplex Virus Reactivation." *J Virol* 91 (2): pii: e01419-16. doi:10.1128/JVI.01419-16.

Coombs DK, Patton T, Kohler AK, Soboll G, Breathnach C, Townsend HG, Lunn DP. 2006. "Cytokine responses to EHV-1 infection in immune and non-immune ponies." *Vet Immunol Immunopath* 111 (1-2): 109-116.

Corrales-Aguilar E, Trilling M, Reinhard H, Falcone V, Zimmermann A, Adams O, Santibanez S, Hengel H. 2016. "Highly individual patterns of virus-immune IgG effector responses in humans." *Med Microbiol Immunol* 205 (5): 409-424.

Crabb BS, MacPherson CM, Reubel GH, Browning GF, Studdert MJ, Drummer HE. 1995. "A type-specific serological test to distinguish antibodies to equine herpesviruses 4 and 1." *Arch Virol* 140: 245-258.

Dall Agnol AM, Beuttemmuller EA, Pilz D, Leme RA, Saporiti V, Headley SA, *et al*. 2019. "Detection of Equid gammaherpesvirus 2 and 5 DNA in the upper respiratory tract of asymptomatic horses from Southern Brazil." *Braz J Microbiol* 50 (3): 875-878. doi:10.1007/s42770-019-00100-7.

Damiani AM, de Vries M, Reimers G, Winkler S, Osterrieder N. 2014. "A severe equine herpesvirus type 1 (EHV-1) abortion outbreak caused by a neuropathogenic strain at a breeding farm in northern Germany." *Vet Microbiol* 172 (3-4): 555-562.

Davis EG, Bello NM, Bryan AJ, Hankins K, Wilkerson M. 2014. "Characterisation of immune responses in healthy foals when a multivalent vaccine protocol was initiated at age 90 or 180 days." *Equine Vet J.* 47 (6): 667-674.

Diallo IS, Hewitson G, Wright L, Rodwell BJ, Corney BG. 2006. "Detection of equine herpesvirus type 1 using a real-time polymerase chain reaction." *J Virol Methods* 131 (1): 92-98.

Diallo IS, Hewitson G, Wright LL, Kelly MA, Rodwell BJ, Corney BG. 2007. "Multiplex real-time PCR for the detection and differentiation of equid herpesvirus 1 (EHV-1) and equid herpesvirus 4 (EHV-4)." *Vet Microbiol* 123 (1-3): 93-103.

Dunuwille WM, Mashouf NY, Balasuriya UB, Pusterla N, Bailey E. 2020. "Genome-wide association study for host genetic factors associated with equine herpesvirus type-1 induced myeloencephalopathy." *Equine Vet J. Accepted Author Manuscript* doi:10.1111/evj.13261.

Edington N, Bridges CG, Huckle A. 1985. "Experimental reactivation of equid herpesvirus 1 (EHV 1) following the administration of corticosteroids." *Equine Vet J* 17 (5): 369-372.

Edington N, Smyth B, Griffiths L. 1991. "The role of endothelial cell infection in the endometrium, placenta and foetus of equid herpesvirus 1 (EHV-1) abortions." *J Comp Pathol* 104 (4): 379-387.

Edington N, Welch HM, Griffiths L. 1994. "The prevalence of latent equid herpesviruses in the tissues of 40 abattoir horses." *Equine Vet J* 26 (2): 140-142.

Ekstrand C, Carpenter TE. 1998. "Using a tobit regression model to analyse risk factors for foot-pad dermatitis in commercially grown broilers." *Prev Vet Med* 37 (1-4): 219-228.

Elia G, Decaro N, Martella V, Campolo M, Desario C, Lorusso E, Cirone F, Buonavoglia C. 2006. "Detection of equine herpesvirus type 1 by real time PCR." *J Virol Methods* 133: 70-75.

Equine Canada. 2011. *2010 Canadian Horse Industry Profile Study*. Census, Ottawa: equinecanada.ca. Accessed 10 01, 2016. https://www.equestrian.ca/cfs/files/resources/wf9c32LH4uErLanMs/IN_2011_cnd_horse_study_chapter_2-e.pdf?token=eyJhdXRoVG9rZW4iOiIifQ %3D %3D

Estell KE, Dawson DR, Magdesian KG, Swain E, Laing ST, Siso S, Mapes S, Pusterla N. 2015. "Quantitative molecular viral loads in 7 horses with naturally occurring equine herpesvirus-1 infection." *Equine Vet J* 689-693.

Foote CE, Love DN, Gilkerson JR, Whalley JM 2002. "Serological responses of mares and weanlings following vaccination with an inactivated whole virus equine herpesvirus 1 and equine herpesvirus 4 vaccine." *Vet Microbiol* 88 (1): 13-25.

Foote CE, Gilkerson JR, Whalley JM, Love DN. 2003. "Seroprevalence of equine herpesvirus 1 in mares and foals on a large Hunter Valley stud farm in years pre-and postvaccination." *Aust Vet J* 81 (5): 283-288.

Foote CE, Love DN, Gilkerson JR, Whalley JM. 2004. "Detection of EHV-1 and EHV-4 DNA in unweaned Thoroughbred foals from vaccinated mares on a large stud farm." *Equine Vet J* 36 (4): 341-345.

Fradinho MJ, Correia MJ, Grácio V, Bliebernicht M, Farrim A, *et al*. 2014. "Effects of body condition and leptin on the reproductive performance of Lusitano mares on extensive systems." *Theriogenology* 81 (9): 1214-1222.

Franz M, Goodman LB, Van de Walle GR, Osterrieder N, Greenwood AD. 2017. "A point mutation in a herpesvirus co-determines neuropathogenicity and viral shedding." *Viruses* 9 (1): 6.

Frymus T, Kita J, Woyciechowska S, Ganowicz M. 1986. "Foetal and neonatal foal losses on equine herpesvirus type 1(EHV-1) infected farms before and after EHV-1 vaccination was introduced." *Pol Arch Weter* 26 (3-4): 7-14.

Gardiner DW, Lunn DP, Goehring LS, Chiang YW, Cook C, *et al*. 2012. "Strain impact on equine herpesvirus type 1 (EHV-1) abortion models: viral loads in fetal and placental tissues and foals." *Vaccine* 30 (46): 6564-6572.

Garré B, Gryspeerdt A, Croubels S, De Backer P, Nauwynck H. 2009. "Evaluation of orally administered valacyclovir in experimentally EHV1-infected ponies." *Vet Microbiol* 135 (3-4): 214-221.

Gerber JD, Marron AE, Bass EP, Beckenhauer WH. 1977. "Effect of age and pregnancy on the antibody and cell-mediated immune responses of horses to equine herpesvirus 1." *Can J Comp Med* 41 (4): 471-478.

Gibson JS, Slater JD, Awan AR, Field HJ. 1992. "Pathogenesis of equine herpesvirus-1 in specific pathogen-free foals: primary and secondary infections and reactivation." *Arch Virol.* 123 (3-4): 351-366.

Giles RC, Donahue JM, Hong CB, et al. 1993. "Causes of abortion, stillbirth, and perinatal death in horses: 3,527 cases. 1986-1991." *J Am Vet Med Assoc* 203 (8): 1170-1175.

Gilkerson JR, Whalley JM, Drummer HE, Studdert MJ, Love DN. 1999a. "Epidemiology of EHV-1 and EHV-4 in the mare and foal populations on a Hunter Valley stud farm: are mares the source of EHV-1 for unweaned foals." *Vet Microbiol* 68 (1-2): 27-34.

Gilkerson JR, Whalley JM, Drummer HE, Studdert MJ, Love DN. 1999b. "Epidemiological studies of equine herpesvirus 1 (EHV-1) in Thoroughbred foals: a review of studies conducted in the Hunter Valley of New South Wales between 1995 and 1997." *Vet Microbiol* 68 (1-2): 15-25.

Goehring LS, Wagner B, Bigbie R, Hussey SB, Rao S, Morley PS, Lunn DP. 2010. "Control of EHV-1 viremia and nasal shedding by commercial vaccines." *Vaccine* 28 (32): 5203-5211.

Goehring LS, Hussey GS, Ashton LV, Schenkel AR, Lunn DP. 2011. "Infection of central nervous system endothelial cells by cell-associated EHV-1." *Vet Microbiol* 148 (2-4): 389-395.

Goodman LB, Wagner B, Flaminio MJ, Sussman KH, Metzger SM, Holland R, Osterrieder N. 2006. "Comparison of the efficacy of inactivated combination and modified-live virus vaccines against challenge infection with neuropathogenic equine herpesvirus type 1 (EHV-1)." *Vaccine* 24 (17): 3636-3645.

Goodman LB, Wimer C, Dubovi EJ, Gold C, Wagner B. 2012. "Immunological correlates of vaccination and infection for equine herpesvirus 1." *Clin Vaccine Immunol* 19 (2): 235-241.

Granzow H, Klupp BG, Fuchs W, Veits J, Osterrieder N, Mettenleiter TC. 2001. "Egress of alphaherpesviruses: comparative ultrastructural study." *J Virol* 76 (6): 3675-3684.

Gryspeerdt A, Vandekerckhove A, Van Doorsselaere J, Van de Walle G, Nauwynck H. 2011. "Description of an unusually large outbreak of nervous system disorders caused by equine herpesvirus 1(EHV1) in 2009 in Belgium." *Vlaams Diergeneeskundig Tijdschrift* 80 (2): 147-153.

Guidotti LG, Chisari FV. 2001. "Noncytolytic control of viral infections by the innate and adaptive immune response." *Annu Rev Immunol* 19: 65-91.

Gulati BR, Sharma H, Riyesh T, Khurana SK, Kapoor S. 2015. "Viral and Host Strategies for Regulation of Latency and Reactivation in Equid Herpesviruses." *Asian J Anim Vet Adv* 10: 669-689.

Guo H, Shen S, Wang L, Deng H. 2010. "Role of tegument proteins in herpesvirus assembly and egress." *Protein Cell* 1 (11): 987-998.

Hannant D, O' Neill T, Jessett, DM, Mumford, JA. 1991. "Evidence for non-specific immunosuppression during the development of immune responses to Equid Herpesvirus-1." *Eq Vet J* 23 (S12): 41-45.

Hayden RT, Gu Z, Ingersoll J, Abdul-Ali D, Shi L, *et al.* 2013. "Comparison of Droplet Digital PCR to Real-Time PCR for Quantitative Detection of Cytomegalovirus ." *J Clin Microbiol* 51 (2): 540-546.

Hebia-Fellah I, Léauté A, Fiéni F, Zientara S, Imbert-Marcille BM, *et al.* 2009. "Evaluation of the presence of equine viral herpesvirus 1 (EHV-1) and equine viral herpesvirus 4 (EHV-4) DNA in stallion semen using polymerase chain reaction (PCR)." *Theriogenology* 71 (9): 1381-1389.

Hegazy AN, Peine M, Helmstetter C, Panse I, Fröhlich A, *et al.* 2010. "Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions." *Immunity* 32 (1): 116-128.

Heldens, JGM, Hannant D, Cullinane AA, Prendergast MJ, Mumford JA, *et al.* 2001. "Clinical and virological evaluation of the efficacy of an inactivated EHV1 and EHV4 whole virus vaccine (Duvaxyn EHV1, 4). Vaccination/challenge experiments in foals and pregnant mares." *Vaccine* 19 (30): 4307-4317.

Henneke DR, Potter GD, Kreider JL. 1984. "Body condition during pregnancy and lactation and reproductive efficiency of mares." *Theriogenology* 21 (6): 897-909.

Higgins WP, Gillespie JH, Robson DS. 1987. "Studies of maternally-acquired antibodies in the foal to equine influenza a 1 and a 2, and equine rhinopneumonitis." *J Equine Vet Sci* 7 (4): 207-210.

Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, *et al.* 2011. "High-throughput droplet digital PCR system for absolute quantitation of DNA copy number." *Analytical Chemistry* 83 (22): 8604-8610.

Holmes MA, Townsend HGG, Sussey S, Breathnach C, Barnett C, *et al.* 2003. "Immune responses to commercial equine vaccines." *49th Annual Convention of the American Association of Equine Practitioners.* New Orleans.

Holmes MA, Townsend HG, Kohler AK, Hussey S, Breathnach C, *et al.* 2006. "Immune responses to commercial equine vaccines against equine herpesvirus-1, equine influenza virus, eastern equine encephalomyelitis, and tetanus." *Vet Immunol Immunopathol* 111 (1-2): 67-80.

Holz CL, Nelli RK, Wilson ME, Zarski LM, Azab W, *et al.* 2017. "Viral genes and cellular markers associated with neurological complications during herpesvirus infections." *J Gen Virol* 98 (6): 1439-1454.

Holz CL, Sledge DG, Kiupel M, Nelli RK, Goehring LS, Soboll Hussey G. 2019. "Histopathologic Findings Following Experimental Equine Herpesvirus 1 Infection of Horses." *Front. Vet. Sci.* 6: 59. Accessed 03 21, 2019. doi: 10.3389/fvets.2019.00059.

Holznagel DL, Hussey S, Mihalyi JE, Wilson WD, Lunn DP. 2003. "Onset of immunoglobulin production in foals." *Equine Vet J* 35 (6): 620-622.

Hooper-McGrevy KE, Wilkie BN, Prescott JF. 2003. "Immunoglobulin G subisotype responses of pneumonic and healthy, exposed foals and adult horses to Rhodococcus equi virulence-associated proteins." *Clin Diagn Lab Immunol* 10 (3): 345-351.

Horín P, Smola J, Matiasovic J, Vyskocil M, Lukeszová L, Tomanová K, Králík P, Glasnák V, Schröffelová D, Knoll A, Sedlinská M, Krenková L, Jahn P. 2004. "Polymorphisms in equine immune response genes and their associations with infections." *Mamm Genome* 15 (10): 843-850.

Horohov DW, Crim JA, Smith PL, Siegel JP. 1988. "IL-4 (B cell-stimulatory factor 1) regulates multiple aspects of influenza virus-specific cell-mediated immunity." *J Immunol* 141 (12): 4217-4223.

Horohov, DW. 2000. "Equine T-cell cytokines. Protection and pathology." *Vet Clin North Am-Equine* 16 (1): 1-14.

Hu Z, Zhu C, Chang H, Guo W, Liu D, *et al.* 2014. "Development of a single-tube duplex EvaGreen real-time PCR for the detection and identification of EHV-1 and EHV-4." *Appl Microbiol Biotechnol* 4179–4186.

Huggett JF, Cowen S, Foy CA. 2015. "Considerations for Digital PCR as an Accurate Molecular Diagnostic Tool." *Clin Chem* 79-88.

Hussey SB, Clark R, Lunn KF, Breathnach C, Soboll G, Whalley JM, Lunn DP. 2006. "Detection and quantification of equine herpesvirus-1 viremia and nasal shedding by real-time polymerase chain reaction." *J Vet Diagn Invest* 18 (4): 335-342.

Ibáñez FJ, Farías MA, Gonzalez-Troncoso MP, Corrales N, Duarte LF, Retamal-Díaz A, González PA. 2018. "Experimental Dissection of the Lytic Replication Cycles of Herpes Simplex Viruses in vitro." *Front Microbiol* 9: 2406.

ICTV, International Commitee of Taxonomy of Viruses. 2017. *International Commitee of Taxonomy of Viruses - Herpesviruses.* Accessed 9 15, 2016. www.ICTVonline.org.

Intervet. 2018. *Summary of Studies Supporting USDA Product Licensure.* Feb 6. Accessed Apr 22, 2020. https://www.aphis.usda.gov/wcm/connect/ba428bea-9ec6-42bf-96d3-e0548f5406fd/165A-152521.pdf?MOD=AJPERES&CONVERT_TO=url&CACHEID=ROOTWORKSPACE-ba428bea-9ec6-42bf-96d3-e0548f5406fd-m8B1NaR.

Johnson DC, Baines JD. 2011. "Herpesviruses remodel host membranes for virus egress." *Nat Rev Microbiol* 9 (5): 382-394.

Khusro A, Aarti C, Rivas-Caceres RR, Barbabosa -Pliego A. 2020. Equine Herpesvirus-I Infection in Horses: Recent Updates on its Pathogenicity, Vaccination, and Preventive Management Strategies. *J Equine Vet Sci* 87: 102923. doi: 10.1016/j.jevs.2020.102923.

Kim S, Ahn BC, O'Callaghan DJ, Kim SK. 2012. "The early UL31 gene of equine herpesvirus 1 encodes a single-stranded DNA-binding protein that has a nuclear localization signal sequence at the C-terminus." *Virology* 432 (2): 306-315.

Kydd JH, Smith KC, Hannant D, Livesay GJ, Mumford JA. 1994. "Distribution of equid herpesvirus-1 (EHV-1) in the respiratory tract of ponies: implication for vaccination strategies." *Equine Vet J* 26 (6): 466-469.

Kydd JH, Wattrang E, Hannant D. 2003. "Pre-infection frequencies of equine herpesvirus-1 specific, cytotoxic T lymphocytes correlate with protection against abortion following experimental infection of pregnant mares." *Vet Immunol Immunopathol* 96 (3-4): 207-217.

Kydd JH, Townsend HG, Hannant D. 2006. "The equine immune response to equine herpesvirus-1: the virus and its vaccines." *Vet Immunol Immunopathol* 111 (1-2): 15-30.

Kydd JH, Case R, Winton C, MacRae S, Sharp E, *et al*. 2016. "Polarisation of equine pregnancy outcome associated with a maternal MHC class I allele: Preliminary evidence." *Vet Microbiol* 188 (1): 34-40.

Langlois B, Blouin C. 2004. "Statistical analysis of some factors affecting the number of horse births in France." *Reprod Nutr Dev* 44 (6): 585-595.

Langlois B, Blouin C, Chaffaux S. 2012. "Analysis of several factors of variation of gestation loss in breeding mares." A*nimal* 6 (12): 1925-1930.

Laugier C, Foucher N, Sevin C, Leon V, Tapprest J. 2011. "24-Year Retrospective Study of Equine Abortion in Normandy (France)." *J Equine Vet Res* 31 (3): 116-123.

Lee SK, Lee I. 2019. "The Molecular Detection of Equine Herpesviruses 2 and 5 in Genital Swabs From Clinically Normal Thoroughbred Mares in South Korea." *J Equine Vet Sci* 79: 68-72. doi:10.1016/j.jevs.2019.05.013.

Léon A, Fortier G, Fortier C, Freymuth F, Tapprest J, *et al.* 2008. "Detection of equine herpesviruses in aborted foetuses by consensus PCR." *Vet Microbiol* 126 (1-3): 20-29.

Lewis MJ, Wagner B, Woof JM. 2008. "The different effector function capabilities of the seven equine IgG subclasses have implications for vaccine strategies." *Mol Immunol* 45 (3): 818-827.

Lillsunde Larsson G, Helenius G. 2017. "Digital droplet PCR (ddPCR) for the detection and quantification of HPV 16, 18, 33 and 45 - a short report." *Cell Oncol* 521-527.

Luckheeram RV, Zhou R, Verma AD, Xia B. 2012. "CD4$^+$T cells: differentiation and functions." *Clin Dev Immunol* e925135.

Lunn DP, Holmes MA, Gibson J, Field J. 1991. "Haematological changes and equine lymphocyte subpopulation kinetics during primary infection and attempted re-infection of specific pathogen free foals with EHV-1." *Equine Vet J* 23 (S12): 35-40.

Lunn DP, Holmes MA, Schram B, Duffus WP. 1995. "Monoclonal antibodies specific for equine IgG sub-isotypes including an antibody which recognizes B lymphocytes." *Vet Immunol Immunopathol* 47 (3-4): 239-251.

Lunn DP, Soboll G, Schram BR, Quass J, McGregor MW, *et al*. 1999. "Antibody responses to DNA vaccination of horses using the influenza virus hemagglutinin gene." *Vaccine* 17 (18): 2245-2258.

Lunn DP, Davis-Poynter N, Flaminio MJBF, Horohov DW, Osterrieder K, *et al*. 2009. "Equine herpesvirus-1 consensus statement." *J Vet Int Med* 23 (3): 450-461.

Lv Y, Zhou S, Gao S, Deng H. 2019. "Remodeling of host membranes during herpesvirus assembly and egress." *Protein Cell* 10 (5): 315-326.

Ma G, Feineis S, Osterrieder N, Van de Walle GR. 2012. "Identification and characterization of equine herpesvirus type 1 pUL56 and its role in virus-induced downregulation of major histocompatibility complex class I." *J Virol* 86 (7): 3554-3563.

Ma G, Azab W, Osterrieder N. 2013. "Equine herpesviruses type 1 (EHV-1) and 4 (EHV-4)—masters of co-evolution and a constant threat to equids and beyond." *Vet Microbiol* 167 (1-2): 123-134.

Maclachlan NJ, Dubovi EJ, eds. 2010. *Fenner's veterinary virology.* Academic press.

Mador N, Panet A, Steiner I. 2002. "The latency-associated gene of herpes simplex virus type 1 (HSV-1) interferes with superinfection by HSV-1." *J Neurovirol* 8 (suppl.2): 97-102.

Marenzoni ML, Bietta A, Lepri E, Casagrande Proietti P, Cordioli P, Canelli E, Stefanetti V, Coletti M, Timoney PJ, Passamonti F. 2013. "Role of equine herpesviruses as co-

infecting agents in cases of abortion, placental disease and neonatal foal mortality." *Vet Res Commun.* 37 (4): 311-317.

Matsumura T, Sugiura T, Imagawa H, Fukunaga Y, Kamada M. 1992. " Epizootiological aspects of type 1 and type 4 equine herpesvirus infections among horse populations." *J Vet Med Sci* 54 (2): 207-211.

Matsumura T, Kondo T, Sugita S, Damiani AM, O'Callaghan DJ, Imagawa H. 1998. "An equine herpesvirus Type 1 recombinant with a deletion in the gE and gI genes is avirulent in young horses." *Virology* 242 (1): 68-79.Matumoto M, Isehizaki R, Shimizu T. 1965. "Serological survey of equine rhinolmeumonitis vires infection among horses in various countries." *Arch ges Virusforsch* 50: 609-623.

Maxwell LK, Bentz BG, Gilliam LL, Ritchey JW, Pusterla N, *et al*. 2017. "Efficacy of the early administration of valacyclovir hydrochloride for the treatment of neuropathogenic equine herpesvirus type-1 infection in horses." *Am J Vet Res* 78 (10): 1126-1139.

Mayr A, Pette J. 1968. "1st experiments concerning the vaccination of horses against rhino-pneumonia (viral abortion of mares) with a live vaccine from cell cultures." *Bull Off Int Epizoot* 70: 133-140.

McDonald JF, Moffitt RA. 1980. "The uses of tobit analysis." *Rev Econ Stat* 62 (2): 318-321.

McEwen B, Carman S. 2004. *Equine abortion, 2003-2004.* Guelph: Animal Health Laboratory.

McEwen B, Carman S. 2013. *Equine Abortion submissions declining.* Guelph: AHL Diagnostic Laboratory.

Meier WA, Husmann RJ, Schnitzlein WM, Osorio FA, Lunney JK, Zuckermann FA. 2004. "Cytokines and synthetic double-stranded RNA augment the T helper 1 immune response of swine to porcine reproductive and respiratory syndrome virus." *Vet Immunol Immunopathol* 102 (3): 299-314.

Mellerick DM, Fraser NW. 1987. "Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state." *Virology* 158 (2): 265-275.

Meyer H, Thein P, Hübert P. 1987. "Characterization of two equine herpesvirus (EHV) isolates associated with neurological disorders in horses." *Zentralbl Veterinarmed B* 34 (1-10): 545-548.

Miyakoshi D, Shikichi M, Ito K, Iwata K, Okai K, *et al*. 2012. "Factors influencing the frequency of pregnancy loss among thoroughbred mares in Hidaka, Japan." *J Equine Vet Sci* 32 (9): 552-557.

Morris LH, Allen WR. 2002. "Reproductive efficiency of intensively managed Thoroughbred mares in Newmarket." *Equine Vet Jl* 34 (1): 51-60.

Muirhead TL, McClure JT, Wichtel JJ, Stryhn H, Frederick Markham RJ, *et al*. 2008. "The effect of age on serum antibody titers after rabies and influenza vaccination in healthy horses." *J Vet Intern Med* 22 (3): 654-661.

Muirhead TL, McClure JT, Wichtel JJ, Stryhn H, Markham RJ, *et al*. 2010. "The effect of age on the immune response of horses to vaccination." *J Comp Pathol* 142 (Suppl 1): S85-90.

Mumford EL, Traub-Dargatz JL, Carman J, Callan RJ, Collins JK, *et al*. 2003. "Occurrence of infectious upper respiratory tract disease and response to vaccination in horses on six sentinel premises in northern Colorado." *Equine Vet J* 35 (1): 72-77.

Nelson KM, Schram BR, McGregor MW, Sheoran AS, Olsen CW, Lunn DP. 1998. "Local and systemic isotype-specific antibody responses to equine influenza virus infection versus conventional vaccination." *Vaccine* 16 (13): 1306-1313.

Nemoto M, Ohta M, Tsujimura K, Bannai H, Yamanaka T, *et al.* 2011. "Direct Detection of Equine Herpesvirus Type 1 DNA in Nasal Swabs by Loop-Mediated Isothermal Amplification (LAMP)." *J Vet Med Sci* 73 (9): 1225-1227.

Notkins, AL. 1974. "Immune mechanisms by which the spread of viral infections is stopped." *Cell Immun* 11: 478-483.

Nugent J, Birch-Machin I, Smith KC, Mumford JA, Swann Z, *et al*. 2006. "Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus nonneuropathogenic disease outbreaks." *J Virol* 80 (8): 4047-4060.

Oladunni FS, Horohov DW, Chambers TM. 2019. "EHV-1: A Constant Threat to the Horse Industry." *Front Microbiol* 10: 2668. doi:10.3389/fmicb.2019.02668.

Oliveira LJ, Barreto RS, Perecin F, Mansouri-Attia N, Pereira FT, Meirelles FV. 2012. "Modulation of maternal immune system during pregnancy in the cow." *Reprod Domest Anim* 47 (Suppl 4): 384-393.

O'Neill T, Kydd JH, Allen GP, Wattrang E, Mumford JA, Hannant D. 1999. "Determination of equid herpesvirus 1-specific, CD8+, cytotoxic T lymphocyte precursor frequencies in ponies." *Vet Immunol Immunopathol* 70 (1-2): 43-54.

Owen DJ, Crump CM, Graham SC. 2015. "Tegument Assembly and Secondary Envelopment of Alphaherpesviruses." *Viruses* 7 (9): 5084-5114.

Paillot R, Hannant D, Kydd JH, Daly JM. 2006. "Vaccination against equine influenza: quid novi?" *Vaccine* 24 (19): 4047-4061.

Paillot R, Case R, Ross J, Newton R, Nugent J. 2008. "Equine herpes virus-1: virus, immunity and vaccines." *Open Vet J* 2 (1): 68-91.

Pan D, Flores O, Umbach JL, Pesola JM, Bentley P, *et al*. 2014. "A neuron-specific host microRNA targets herpes simplex virus-1 ICP0 expression and promotes latency." *Cell Host Microbe* 15 (4): 446-456.

Patel JR, Heldens J. 2005. "Equine herpesviruses 1 (EHV-1) and 4 (EHV-4)–epidemiology, disease and immunoprophylaxis: a brief review." *Vet J* 170 (1): 14-23.

Perkins, GA, Goodman LB, Tsujimura K, Van de Walle GR, Kim SG, *et al*. 2009. "Investigation of the prevalence of neurologic equine herpes virus type 1 (EHV-1) in a 23-year retrospective analysis (1984–2007)." *Vet Microbiol* 139 (3-4): 375-378.

Perkins G, Babasyan S, Stout AE, Freer H, Rollins A, *et al.* 2019. "Intranasal IgG4/7 antibody responses protect horses against equid herpesvirus-1 (EHV-1) infection including nasal virus shedding and cell-associated viremia." *Virology* 531: 219-232.

Poelaert KCK, Van Cleemput J, Laval K, Favoreel HW, Couck L, Van den Broeck W, Azab W, Nauwynck HJ. 2019. "Equine Herpesvirus 1 Bridles T Lymphocytes To Reach Its Target Organs." *J Virol* 93 (7): e02098-18.

Poland GA, Ovsyannikova IG, Jacobson RM, Smith D. 2007. "Heterogeneity in vaccine immune response: the role of immunogenetics and the emerging field of vaccinomics." *Clin Pharmacol Ther* 82 (6): 653-64.

Preston CM, Efstathiou S. 2007. ""Molecular basis of HSV latency and reactivation." Chap. Chapter 33 in *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis.*, by Campadelli-Fiume G, Mocarski E, et al., editors. Arvin A. Cambridge University Press;.

Proft A, Spiesschaert B, Izume S, Taferner S, Lehmann MJ, Azab W. 2016. "The Role of the Equine Herpesvirus Type 1 (EHV-1) US3-Encoded Protein Kinase in Actin Reorganization and Nuclear Egress." *Viruses* 8 (10): E275.

Pusterla N, Mapes S, Wilson WD. 2008a. "Diagnostic sensitivity of nasopharyngeal and nasal swabs for the molecular detection of EHV-1." *Vet Rec* 162 (16): 520-521.

Pusterla N, Mapes S, Wilson WD. 2008b. "Use of viral loads in blood and nasopharyngeal secretions for the diagnosis of EHV-1 infection in field cases." *Vet Rec* 162 (22): 728-729.

Pusterla N, Hussey SB, Mapes S, Leutenegger CM, Madigan JE, *et al*. 2009a. "Comparison of four methods to quantify Equid herpesvirus 1 load by real-time polymerase chain reaction in nasal secretions of experimentally and naturally infected horses." *J Vet Diagn Invest* 21 (6): 836-840.

Pusterla N, Wilson WD, Mapes S, Finno C, Isbell D, *et al*. 2009b. "Characterization of viral loads, strain and state of equine herpesvirus-1 using real-time PCR in horses following natural exposure at a racetrack in California." *Vet J* 179 (2): 230-239.

Pusterla N, Mapes S, Madigan JE, MacLachlan NJ, Ferraro GL, *et al*. 2009c. "Prevalence of EHV-1 in adult horses transported over long distances." *Vet Rec* 165 (16): 473.

Pusterla N, Hussey SB, Mapes S, Johnson C, Collier JR, *et al*. 2010a. "Molecular Investigation of the Viral Kinetics of Equine Herpesvirus-1 in Blood and Nasal Secretions of Horses after Corticosteroid-Induced Recrudescence of Latent Infection." *J Vet Intern Med* 24 (5): 1153-1157.

Pusterla N, Mapes S, Wilson WD. 2010b. "Prevalence of equine herpesvirus type 1 in trigeminal ganglia and submandibular lymph nodes of equids examined postmortem." *Vet Rec* 167 (10): 376-378.

Pusterla N, Kass PH, Mapes S, Johnson C, Barnett DC, *et al*. 2011. "Surveillance programme for important equine infectious respiratory pathogens in the USA." *Vet Rec* 169 (1): 12.

Pusterla N, Mapes S, Wilson WD. 2012. "Prevalence of latent alpha-herpesviruses in Thoroughbred racing horses." *Vet J* 193 (2): 579-582.

Pusterla N, Mapes S, Akana N, Barnett C, MacKenzie C, *et al*. 2016. "Prevalence factors associated with equine herpesvirus type 1 infection in equids with upper respiratory tract infection and/or acute onset of neurological signs from 2008 to 2014." *Vet Rec* 178 (3): 70.

Riaz A, Dry I, Levy CS, Hopkins J, Grey F, *et al*. 2014. "Ovine herpesvirus-2-encoded microRNAs target virus genes involved in virus latency." *J Gen Virol* 95 (2): 472-480.

Riddle WT. 2003. "Clinical observations associated with early fetal loss in mare reproductive loss syndrome during the 2001 and 2002 breeding seasons." *First Workshop on Mare Reproductive Loss Syndrome, 2002*:12.

Roizman, B. 1983. *The Herpesviruses.* Chicago: University of Chicago. ISBN 978-1-4684-7012-3.

Roizmann B, Desrosiers RC, Fleckenstein B, Lopez C, Minson AC, Studdert MJ. 1992. "The family Herpesviridae: an update." *Arch Virol* 123 (3-4): 425-449.

Rosanowski, SM, Cogger N, Rogers CW. 2013. "An investigation of the movement patterns and biosecurity practices on Thoroughbred and Standardbred stud farms in New Zealand." *Prev Vet Med* 108 (2-3): 178-187.

Rosas CT, Goodman LB, von Einem J, Osterrieder N. 2006. "Equine herpesvirus type 1 modified live virus vaccines: quo vaditis?" *Expert Rev Vaccines* 5 (1): 119-131.

Rusek J, Klumplerova M, Molinkova D, Sedlinska M, Dusek L, *et al*. 2013. "Genetics of anti-EHV antibody responses in a horse population." *Res Vet Sci* 95 (1): 137-142.

Rusli ND, Mat KB, Harun HC. 2014. "A review: interactions of equine herpesvirus-1 with immune system and equine lymphocyte." *Open J Vet Med* 4 (12): 294.

Sairanen J, Katila T, Virtala AM, Ojala M. 2011. "Effects of racing on equine fertility." *Anim Reprod Sci* 124 (1-2): 73-84.

Sarkar, S. 2015. "Modulation of type-i interferon mediated immune response: a novel innate immune evasion strategy of equine herpesvirus 1." Lexington, Kentucky: ProQuest LLC. Accessed 05 15, 2020.

Schnabel CL, Babasyan S, Rollins A, Freer H, Wimer CL, *et al*. 2019. "An Equine Herpesvirus Type 1 (EHV-1) Ab4 Open Reading Frame 2 Deletion Mutant Provides Immunity and Protection from EHV-1 Infection and Disease." *J Virol* 93 (22): e01011-19. doi: 10.1128/JVI.01011-19.

Schnobrich MR, Riddle WT, Stromberg AJ, Leblanc MM. 2013. "Factors affecting live foal rates of Thoroughbred mares that undergo manual twin elimination." *Equine Vet J* 45 (6): 676-680.

Schulman M, Becker A, Ganswindt S, Guthrie A, Stout T, Ganswindt A. 2014. "The effect of consignment to broodmare sales on physiological stress measured by faecal glucocorticoid metabolites in pregnant Thoroughbred mares." *BMC Vet Res* 10: 25.

Schulman ML, Becker A, van der Merwe BD, Guthrie AJ, Stout TA. 2015. "Epidemiology and reproductive outcomes of EHV-1 abortion epizootics in unvaccinated Thoroughbred mares in South Africa." *Equine Vet J.* 47 (2): 155-159.

Shakya AK, O'Callaghan DJ, Kim SK. 2017. "Comparative Genomic Sequencing and Pathogenic Properties of Equine Herpesvirus 1 KyA and RacL11." *Front Vet Sci* 4: 211.

Shehata HR, Li J, Chen S, Redda H, Cheng S, Tabujara N, Li H, Warriner K, Hanner R. 2017. "Droplet digital polymerase chain reaction (ddPCR) assays integrated with an internal control for quantification of bovine, porcine, chicken and turkey species in food and feed." *PLoS One* 12 (8): e0182872. doi:10.1371/journal.pone.0182872.

Sheoran AS, Lunn DP, Holmes MA. 1998. "Monoclonal antibodies to subclass-specific antigenic determinants on equine immunoglobulin gamma chains and their characterization." *Vet Immunol Immunopathol* 62 (2): 153-165.

Slater JD, Borchers K, Thackray AM, Field HJ. 1994. "The trigeminal ganglion is a location for equine herpesvirus 1 latency and reactivation in the horse." *J Gen Virol* 75 (Pt 8): 2007-2016.

Smith DJ, Hamblin A, Edington N. 2002. "Equid herpesvirus 1 infection of endothelial cells requires activation of putative adhesion molecules: an in vitro model." *Clin Exp Immunol* 129 (2): 281-287.

Smith, KC, Blunden AS, Whitwell KE, Dunn KA, Wales AD. 2003. "A survey of equine abortion, stillbirth and neonatal death in the UK from 1988 to 1997." *Equine Vet J* 35 (5): 496-501.

Smith, KC, Whitwell KE, Blunden AS, Bestbier ME, Scase TJ, *et al*. 2004. "Equine herpesvirus-1 abortion: atypical cases with lesions largely or wholly restricted to the placenta." *Equine veterinary journal* 36 (1): 79-82.

Smith, KL. 2007. "Tracing the origin of the recent rise in neuropathogenic EHV-1." Vol. 496. University of Kentucky Master's Theses. Accessed 03 20, 2020. https://uknowledge.uky.edu/gradschool_theses/496.

Smith KL, Allen GP, Branscum AJ, Frank Cook R, Vickers ML, *et al*. 2010. "The increased prevalence of neuropathogenic strains of EHV-1 in equine abortions." *Vet Microbiol.* 141 (1-2): 5-1.

Smith KL, Li Y, Breheny P, Cook RF, Henney PJ, *et al*. 2012. "New real-time PCR assay using allelic discrimination for detection and differentiation of equine herpesvirus-1 strains with A2254 and G2254 polymorphisms." *J Clin Microbiol* 50 (6): 1981-1988.

Smith, KL. 2013. "Development of a new allelic discrimination real-time PCR assay for the diagnosis of Equine Herpesvirus-1 and characterization of the virulence determinants of the virus." Kentucky: University of Kentucky. https://uknowledge.uky.edu/gluck_etds/12.

Smith FL, Watson JL, Spier SJ, Kilcoyne I, Mapes S, *et al*. 2018. "Frequency of shedding of respiratory pathogens in horses recently imported to the United States." *J Vet Intern Med* 32 (4): 1436-1441.

Soboll G, Horohov DW, Aldridge BM, Olsen CW, McGregor MW, *et al*. 2003. "Regional antibody and cellular immune responses to equine influenza virus infection, and particle mediated DNA vaccination." *Vet Immunol and Immunopathology* 94 (1-2): 47-62.

Soboll G, Hussey SB, Whalley JM, Allen GP, Koen MT, *et al*. 2006. "Antibody and cellular immune responses following DNA vaccination and EHV-1 infection of ponies." *Vet Immunol Immunopathol* 111 (1-2): 81-95.

Soboll Hussey G, Hussey SB, Wagner B, Horohov DW, Van de Walle G, *et al.* 2011. "Evaluation of immune responses following infection of ponies with an EHV-1 ORF1/2 deletion mutant." *Vet Research* 42: 23-41.

Soboll Hussey G, Goehring LS, Lunn DP, Hussey SB, Huang T, *et al.* 2013. "Experimental infection with equine herpesvirus type 1 (EHV-1) induces chorioretinal lesions." *Vet Res* 44 (1): 118.

Sonis JM, Goehring LS. 2013. "Nasal Shedding of Equid Herpesvirus Type 1 and Type 4 in Hospitalized, Febrile Horses." *J Equine Vet Sci* 33 (9): 756-759.

Spiesschaert B, Osterrieder N, Azab W. 2015. "Comparative analysis of glycoprotein B (gB) of equine herpesvirus type 1 and type 4 (EHV-1 and EHV-4) in cellular tropism and cell-to-cell transmission." *Viruses* 7 (2): 522-542.

Stasiak, K, Rola J, Ploszay G, Socha W, Zmudzinski JF. 2015. "Detection of the neuropathogenic variant of equine herpesvirus 1 associated with abortions in mares in Poland." *BMC Vet Res* 11 (1): 105.

Statistics Canada. 2016. *Census of Agriculture.* Census, Ottawa: Statistics Canada. Accessed 09 15, 2018. https://www150.statcan.gc.ca/.

Steinbach F, Borchers K, Ricciardi-Castagnoli P, Ludwig H, Stingl G, Elbe-Bürger A. 1998. "Dendritic cells presenting equine herpesvirus-1 antigens induce protective anti-viral immunity." *J Gen Virol* 79 (12): 3005-3014.

Stokes A, Wardley RC. 1988. "ADCC and complement-dependent lysis as immune mechanisms against EHV-1 infection in the horse." *Res Vet Sci.* 44 (3): 295-302.

Strang BL, Stow ND. 2007. "Blocks to herpes simplex virus type 1 replication in a cell line, tsBN2, encoding a temperature-sensitive RCC1 protein." *J Gen Virol* 88 (Pt 2): 376-383.

Studdert MJ, Simpson T, Roizman B. 1981. "Differentiation of respiratory and abortigenic isolates of equine herpesvirus 1 by restriction endonucleases." *Science* 214 (4520): 562-564.

Studdert MJ. 1983. "Restriction endonuclease DNA fingerprinting of respiratory, foetal and perinatal foal isolates of equine herpesvirus type 1." *Arch Virol* 77 (2-4): 249-258.

Su C, Zhan G, Zheng C. 2016. "Evasion of host antiviral innate immunity by HSV-1, an update." *Virol J* 13 (1): 38-47.

Tandon R, von Einem J. 2020. "Editorial: Herpesvirus Maturation." *Front Microbiol* 11: 657. doi:10.3389/fmicb.2020.00657.

Taylor SC, Laperriere G, Germain H. 2017. "Droplet Digital PCR versus qPCR for gene expression analysis with low abundant targets: from variable nonsense to publication quality data." *Sci Rep* 1-8.

Tearle JP, Smith KC, Boyle MS, Binns MM, Livesay GJ, Mumford JA. 1996. "Replication of equid herpesvirus-1 (EHV-1) in the testes and epididymides of ponies and venereal shedding of infectious virus." *J Comp Pathol* 115 (4): 385-397.

Telford E, Watson M, McBride K, Davison A. 1992. " The DNA sequence of equine herpesvirus-1." *Virology* 189 (1): 304-316.

Thomson, GR, JA Mumford, and IM. Smith. 1979. "Exerimental immunization against respiratory disease due to Equid Herpesvirus 1 infection (Rhinopneumonitis) using formalin-inactivated virus with various adjuvants." *Vet Microbiol* 4: 209-222.

Tizard, Ian. 2017. "Veterinary Immunology: An Introduction." Chap. 15 in *Veterinary Immunology*, by Ian Tizard, 552. Elsevier.

Tozaki T, Kakoi H, Mashima S, Hirota K, Hasegawa T, *et al*. 2001. "Population study and validation of paternity testing for Thoroughbred horses by 15 microsatellite loci." *J Vet Med Sci* 63 (11): 1191-1197.

Turcotte S, Letellier J, Lippe R. 2005. "Herpes simplex virus type 1 capsids transit by the trans-Golgi network, where viral glycoproteins accumulate independently of capsid egress." *J Virol* 79 (14): 8847–8860.

Turtinen, LW, Allen, GP, Darlington, RW, Bryans, JT. 1981. " Serological and molecular comparisons of several equine herpesvirus type 1 strains." *Am J Vet Res* 42: 2099-2104.

Van de Walle GR, Goupil R, Wishon C, Damiani A, Perkins GA, Osterrieder N. 2009. "A single-nucleotide polymorphism in a herpesvirus DNA polymerase is sufficient to cause lethal neurological disease." *J Infect Dis* 200 (1): 20-25.

van Galen G, Leblond A, Tritz P, Martinelle L, Pronost S, Saegerman C. 2015. "A retrospective study on equine herpesvirus type-1 associated myeloencephalopathy in France (2008–2011)." *Vet Microbiol* 179 (3-4): 304-309.

van Lint AL, Murawski MR, Goodbody RE, Severa M, Fitzgerald KA, *et al*. 2010. "Herpes simplex virus immediate-early ICP0 protein inhibits Toll-like receptor 2-dependent inflammatory responses and NF-κB signaling." *J Virol* 84 (20): 10802-10811.

van Maanen C, Willink DL, Smeenk LA, Brinkhof J, Terpstra C. 2000. "An equine herpesvirus 1 (EHV1) abortion storm at a riding school." *Vet Quart.* 22 (2): 83-87.

van Maanen C, Sloet van Oldruitenborgh-Oosterbaan MM, Damen EA, Derksen AG. 2001. "Neurological disease associated with EHV-1-infection in a riding school: clinical and virological characteristics." *Equine Vet J* 33 (2): 191-196.

Vandekerckhove AP, Glorieux S, Gryspeerdt AC, Steukers L, Van Doorsselaere J, *et al*. 2011. "Equine alphaherpesviruses (EHV-1 and EHV-4) differ in their efficiency to infect mononuclear cells during early steps of infection in nasal mucosal explants." *Vet Microbiol* 152 (1-2): 21-28.

Vargas-Bermudez D, Corredor AF, Ramírez-Nieto G, Vera VA, Jaime JC. 2018. "Detection of equine herpesvirus 1 and 4 and its association with latency-associated transcripts in naturally infected horses in Colombia." *Revista MVZ Córdoba* 23 (3): 6826-6837.

Vaz PK, Job N, Horsington J, Ficorilli N, Studdert MJ, Hartley CA, *et al.* 2006. "Low genetic diversity among historical and contemporary clinical isolates of felid herpesvirus 1." *BMC Genomics* 17 (1): 704.

Vynck M, Trypsteen W, Thas O, Vandekerckhove L, De Spiegelaere W. 2016. "The Future of Digital Polymerase Chain Reaction in Virology." *Mol Diagn Ther* 437-447.

Wagner, B. 2006. "Immunoglobulins and immunoglobulin genes of the horse." *Dev Comp Immunol* 30 (1-2): 155-164.

Wagner B, Goodman LB, Babasyan S, Freer H, Torsteinsdóttir S, *et al*. 2015. "Antibody and cellular immune responses of naïve mares to repeated vaccination with an inactivated equine herpesvirus vaccine." *Vaccine* 33 (42): 5588-5597.

Wagner B, Perkins G, Babasyan S, Freer H, Keggan A, *et al*. 2017. "Neonatal Immunization with a Single IL-4/Antigen Dose Induces Increased Antibody Responses after Challenge Infection with Equine Herpesvirus Type 1 (EHV-1) at Weanling Age." *PLoS One* 12 (1): e0169072. doi: 10.1371/journal.pone.0169072.

Walter J, Seeh C, Fey K, Bleul U, Osterrieder N. 2013. "Clinical observations and management of a severe equine herpesvirus type 1 outbreak with abortion and encephalomyelitis." *Acta Vet Scand* 55 (1): 19.

Wang L, Raidal SL, Pizzirani A, Wilcox GE. 2007. "Detection of respiratory herpesviruses in foals and adult horses determined by nested multiplex PCR." *Vet Micobiol* 121 (1-2): 18-28.

Weber R, Hospes R, Wehrend A. 2018. "Causes of abortion in horses - overview of the literature and own evaluations." *Tierarztl Prax Ausg G Grosstiere Nutztiere* 46 (1): 35-42.

Welch HM, Bridges CG, Lyon AM, Griffiths L, Edington N. 1992. "Latent equid herpesviruses 1 and 4: detection and distinction using the polymerase chain reaction and co-cultivation from lymphoid tissues." *J Gen Virol* 73 (2): 261-268.

Whitwell KE, Blunden AS. 1992. "Pathological findings in horses dying during an outbreak of the paralytic form of Equid herpesvirus type 1 (EHV-1) infection." *Equine Vet J* 24 (1): 13-19.

Wilsterman S, Soboll-Hussey G, Lunn DP, Ashton LV, Callan RJ, *et al*. 2011. "Equine herpesvirus-1 infected peripheral blood mononuclear cell subpopulations during viremia." *Vet Microbiol* 149 (1-2): 40-47.

Wimer CL, Schnabel CL, Perkins G, Babasyan S, Freer H, *et al*. 2018. "The deletion of the ORF1 and ORF71 genes reduces virulence of the neuropathogenic EHV-1 strain Ab4 without compromising host immunity in horses." *PLoS One* 13 (11): e0206679. doi:10.1371/journal.pone.0206679.

Witherspoon, DM. 1984. "Vaccination against equine herpesvirus 1 and equine influenza infection." *Vet Rec* 115 (14): 363.

Wohlsein P, Lehmbecker A, Spitzbarth I, Algermissen D, Baumgärtner W, *et al*. 2011. "Fatal epizootic equine herpesvirus 1 infections in new and unnatural hosts." *Vet Microbiol* 149 (3-4): 456-460.

Woolums AR, Siger L, Johnson S, Gallo G, Conlon J. 2003. "Rapid onset of protection following vaccination of calves with multivalent vaccines containing modified-live or modified-live and killed BHV-1 is associated with virus-specific interferon gamma production." *Vaccine* 21 (11-12): 1158-1164.

You Y, Cheng AC, Wang MS, Jia RY, Sun KF, *et al.* 2017. "The suppression of apoptosis by α-herpesvirus." *Cell Death Dis* 8 (4): e2749.

Zhang J, Wang K, Wang S, Zheng C. 2013. "Herpes simplex virus 1 E3 ubiquitin ligase ICP0 protein inhibits tumor necrosis factor alpha-induced NF-kappaB activation by interacting with p65/RelA and p50/NF-kappaB1." *J Virol* 87 (23): 12935–12948.