Equine Herpesvirus Type 1: Filling Gaps Toward Improved Outbreak Management

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Academic Abstract

Equine herpesvirus type 1 (EHV-1) is a common pathogen of horses that typically causes upper respiratory disease, however is also associated with late-term abortion, neonatal foal death and neurologic disease. Once a horse is infected, the virus concentrates to local lymphoid tissue, where it becomes latent. The virus can recrudesce during times of stress, which can lead to the initiation of devastating outbreaks. Some variants of EHV-1 have been associated with more severe disease outcomes. Appropriate outbreak management focuses on minimizing the movement of potentially exposed horses. This approach lacks a strategy for prevention at the level of latency largely due to a knowledge paucity in regards to carriage rate of latent EHV-1. Biosecurity decisions are also dependent on awaiting currently-available diagnostic testing that often take several days for results. Thus, our work has been focused on understanding the carriage rate of the latent virus in different geographic regions as well as improving diagnostic efficiency, both of which are essential for improving the management of EHV-1 disease. Loop mediated isothermal amplification (LAMP) is a method that amplifies nucleic acid rapidly at a constant temperature and is minimally affected by inhibitors that are often found in clinical samples. This procedure

can be followed by multiple detection methods. A new, efficient sequencing method, called nanopore sequencing, has been developed in a handheld device, called MinION, that provides thorough output in a timely manner. When combined with LAMP, it has been referred to as LAMPore. The first objective of our work was to estimate the prevalence of latent EHV-1 and compare the frequency of each variant in the submandibular lymph nodes from horses in Virginia. Our second objective was to perform direct DNA sequencing of EHV-1 using the mobile MinION sequencer in combination with LAMP viral enrichment. Our findings demonstrated a low apparent prevalence of latent EHV-1 DNA in submandibular lymph nodes in this population of horses in Virginia as well as successful detection and identification of EHV-1 in equine nasal swab samples using LAMPore sequencing.

General Public Abstract

Horses can develop disease from a virus called equine herpesvirus type 1 (EHV-1). Symptoms can vary from mild respiratory signs to the inability to rise leading to death or euthanasia. Horses transmit this virus to other nearby horses; however, the virus also becomes dormant once a horse is infected, meaning the virus is not infectious but is present within the animal. When the horse undergoes stress, such as during travel or competition, the virus can become active again, leading to the spread to other horses. This results in outbreaks, many of which are devastating to the equine industry. In order to minimize the risks of this virus spreading and causing disease, management is currently focused on minimizing the movement of horses that may have been exposed to the virus. There is little information regarding the number of horses that harbor the dormant virus and the current methods to detect the infectious virus can take multiple days for results. These limit decision-making during the management of an outbreak. Our work seeks to determine the number of horses in a region that harbor EHV-1 and also to test a new, efficient diagnostic method to identify the virus in samples from horses. Our findings showed a low number of horses in Virginia harbor dormant EHV-1 in the lymph nodes under their mandible, a common site of dormancy. Further, we found that our new method of detection was effective in identifying the virus in samples from nasal secretions from horse.

Dedication

I dedicate the entirety of my efforts in research to animals and those whom love them. This includes, fortunately, my husband, my friends and my colleagues. May we all be connected through our love for animals.

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I owe a special thank you to a substantial number of people who have helped me become a clinician scientist. These include every single veterinarian, researcher, technician, colleague and mentor that have given me their shoulders to stand on since I started on this rewarding journey almost two decades ago. Thank you, from the bottom of my heart.

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List of Abbreviations

CVA	Central Virginia
DNA	Deoxyribonucleic acid
EHM	Equine herpesvirus myeloencephalopathy
EHV-1	Equine herpesvirus type 1
LAMP	Loop mediated isothermal amplification
NAHLN	National Animal Health Laboratory Network
NVA	Northern Virginia
OVA	Outskirts of Virginia
PCR	Polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RNA	Ribonucleic acid
SARS-CoV-2	Severe acute respiratory syndrome, coronavirus 2
SDA	Strand displacement assay
SEVA	Southeast Virginia
SMLN	Submandibular lymph nodes
SWVA	Southwest Virginia
TMA	Transcription-mediated assay
USDA	United States Department of Agriculture

Chapter 1: Introduction

EHV-1 Latency and Outbreaks

Equine herpesvirus type 1 (EHV-1) is an alphaherpesvirus that is ubiquitous in the equine population – affecting horses of every breed and discipline. While the majority of horses have been exposed by one year of age and typically experience mild respiratory disease, infections are also associated with late term abortion, neonatal foal death, and neurologic disease (Equine Herpes Myeloencephalopathy or EHM).[1–3] Additionally, infected horses develop a latent infection which has the potential to recrudesce in periods of stress. Once a horse is infected, the virus can travel retrograde and via cell-associated viremia to respiratory lymphoid tissue and neural tissue including the retropharyngeal lymph nodes, pharyngeal roof, submandibular lymph nodes and trigeminal ganglia.[4] It is a defining feature of herpesviruses that viral shedding and transmission occur from recrudescence, or reactivation, of the latent virus. Instances of EHV-1 recrudescence are likely what contribute to the occurrence of large scale outbreaks such as was seen in 2021 in Valencia, Spain at the Show Jumping tour. This event was attended by over 750 horses from 9 different countries with a profound number of horses affected.[5] Similarly, in 2011 at the National Cutting Horse Association Western National Championships in Ogden, Utah significant losses were seen. This event was attended by over 400 horses from 19 different states. These occurrences adversely impact horse owners as well as the local, national and international equine industry.[6] Such outbreaks are not uncommon. United States Department of Agriculture (USDA) classified EHM as a 'potentially emerging disease in 2007 .[7] The continued occurrence of EHV-1 outbreaks worldwide maintains the significance of the disease.[8]

Our current understanding of the epidemiology of EHV-1 and EHM surrounds duration of shedding and risk factors. It has been shown that nasal shedding of EHV-1, in naturally occurring EHM, occurs for upwards of 9 days and tends to be intermittent in nature.[8]

Additionally, based on a case-control study, risk factors that are associated with a higher risk for developing EHM in the face of an outbreak included vaccination within 5 weeks prior to a competition where an outbreak occurred. And when compared to non-neurologic EHV-1 infected animals, being a mare and having increased number of biosecurity risks (i.e., sharing water, using wash rack, grazing on premise, being tied in the barn, having veterinary or farrier work done) and number of classes competed in increased the risk of developing EHM. In this same outbreak, supplementations with zinc decreased the risk for developing the disease.[9] This epidemiological insight is helpful for determining which animals may offer a threat to other susceptible animals.

However, latency poses another challenge in the management of EHV-1, and specifically EHM. Because the initial infection with EHV-1 could have happened months or years prior to a recrudescence event, it is difficult to determine the incubation period of the clinical manifestations of EHM. Identifying the source of an outbreak and containing the disease before it is spread thus becomes minimally feasible. As a result, we tend to rely on quarantine once an active infection is recognized.[9]Currently, the American Association of Equine Practitioners recommends a 28-day quarantine after the resolution of clinical signs in all horses,[10] and the American College of Veterinary Internal Medicine suggests that this can be reduced to 14-days with nasal swab testing via PCR for 2-4 consecutive days.[3] Extended quarantine periods and the necessity for multiple tests per horse come with significant cost and delays in horse movement.

Detection of EHV-1

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Considering that recrudescent EHV-1 is associated with not only sporadic cases but also with lateral transmission, targeting latency to minimize the risk of clinical disease seems reasonable. [3]Developing approaches to managing latency and recrudescence requires a more thorough understanding of the pathogenicity as well as the epidemiology of latent EHV-1 in horses.

Typically, appropriate outbreak management focuses on minimizing the movement of potentially exposed horses and requires accurate and timely diagnostics. This approach lacks a strategy for prevention at the level of latency largely due to a knowledge paucity in regards to carriage rate of latent EHV-1. Currently, there are few studies regarding the prevalence of latent EHV-1 in horses. A study in Kentucky found 54% of thoroughbred broodmares had EHV-1 in their submandibular lymph nodes.[11] A similar study in California found the prevalence of EHV-1 was 15.6% in either the trigeminal ganglia or submandibular lymph nodes from 147 equids. In that same study 3.3% of the horses had EHV-1 in their submandibular lymph nodes.[12] Another similar study in New Zealand found the prevalence in retropharyngeal or submandibular lymph nodes from horses was 9.5%, and 4.7% from only submandibular lymph nodes. There are no studies regarding the prevalence of latent EHV-1 specifically in Virginia.

Further, new diagnostics are necessary and working toward a point-of-care solution is imperative to maximize the efficiency of diagnosing and managing EHV-1 outbreaks.

Biosecurity decisions are difficult to make when lacking objective, real-time diagnostic measures. Currently, clinicians rely on PCR and/or virus isolation for identifying EHV-1 in susceptible and clinically affected animals. Unfortunately, these methods require expensive equipment and expertise and they can take days (PCR) to weeks (virus isolation) to perform. Further, clinicians must rely upon diagnostic laboratories to perform these analyses, resulting in testing delays due to time associated with sample shipping and increased risk of sample-handling errors.

Although PCR is effective for post-mortem diagnosis and commonly relied upon to manage active EHV-1 in equine populations, and is generally considered the gold standard diagnostic, new genetic sequencing tools are now available that may allow for real-time detection of EHV-1 in veterinary relevant samples collected in the field (e.g., nasal swabs during an outbreak). DNA sequencing has evolved over the past 50 years from the initial expensive, arduous "first generation" techniques that were used to successfully map the entire human genome, to faster, less expensive "second generation" and "next generation" techniques allowing for simultaneous sequencing reactions, which can complete a genome within days. Oxford Nanopore Technologies (Oxford, UK) has recently developed a new sequencing method based on an ionic current change across small protein pores embedded in a membrane, rather than sequencing by synthesis as is typical of next generation sequencing technologies. In this new nanopore technology, DNA or RNA is fed through the pores causing a change in ionic current, referred to as a 'squiggle', which corresponds to the nucleotide sequence of the DNA or RNA molecules passing through the pore.[13]

Recent advances in nanopore sequencing allows for 95-99% accuracy, read speeds of >450 bases per second per pore, and real-time availability of data.[13] As data is available in real time, rather than after a complete run as with other techniques, new samples can be tested once the desired depth or genotyping is reached for the previous sample, resulting in more efficient use of flow cells. This technology is housed in a small, portable and inexpensive device, called the MinION, allowing for diagnostic accessibility in the field. This process has advantages over PCR, such as effectively enriching sequences of interest using less expensive equipment. This is particularly beneficial when testing complex samples such as nasal secretions, and reduces the time from testing to data inference, an advantage when making real-time decisions in the field. In fact, it has been successfully used in the field for on-site sequencing of Ebola virus in Africa[14] and Zika virus in South America[15] and more recently, SARS-CoV2.[16] Excitingly, NAHLNs has provided over $400,000 in funding for the development of point of care testing for animal diseases. Agencies are investing in applications such as MinION for high impact diseases and thus, should be included in impending EHV-1 diagnostic improvements.[17]

Sequencing results are largely dependent upon the quantity and quality of DNA input. Inhibitors from clinical samples, such as nasal swabs, can block polymerase activity and thus prevent target amplification. Improving DNA quantity is, thus, necessary to provide appropriate input. DNA amplification is a process of increasing the number of copies of a portion of target DNA. There are three methods of amplification that can improve nucleic acid detection: PCR, strand displacement assay (SDA) and transcription-mediated assay (TMA). While PCR is affected by inhibitors and TMA is specifically for RNA amplification, loop mediated isothermal amplification (LAMP) is an SDA method that amplifies nucleic acid rapidly at a constant temperature and is minimally affected by inhibitors that are often found in clinical samples (i.e. nasal secretions).[18] This method can

be followed by multiple detection methods. When combined with nanopore sequencing, it has been referred to as LAMPore, which combines barcoded multi-target amplification, a 15-minute library preparation and real-time nanopore sequencing.

Filling in Gaps Toward Improved Outbreak Management

Management of EHV-1 disease depends on a more thorough understanding of the latent state of the virus as well as more efficient diagnosis during outbreak scenarios.

The first objective of our work was to estimate the prevalence of latent EHV-1 and compare the frequency of each variant in the submandibular lymph nodes from horses in Virginia. This is discussed in Chapter 3: Prevalence of latent equid herpesvirus type 1 in submandibular lymph nodes of horses in Virginia.

Our second objective was to perform direct DNA sequencing of EHV-1 using the mobile MinION sequencer in combination with LAMP viral enrichment. This is discussed in Chapter 4: LAMPore sequencing as a diagnostic tool for equine herpesvirus type 1.

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Communication

Prevalence of Latent Equid Herpesvirus Type 1 in Submandibular Lymph Nodes of Horses in Virginia

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Abstract: Equine Herpesvirus type 1 (EHV-1) typically causes mild respiratory disease, but can also cause late-term abortion, neonatal foal death and neurologic disease. Once a horse is infected, the virus concentrates to local lymphoid tissue, where it becomes latent. The virus can be reactivated during times of stress, which can lead to the initiation of devastating outbreaks. Understanding the carriage rate of latent EHV-1 in different geographic regions is essential for managing the disease. The objective of the current study was to estimate the prevalence of latent EHV-1 and compare the frequency of each variant in the submandibular lymph nodes from horses in Virginia. Submandibular lymph nodes were collected 63 post-partem from horses submitted to regional labs for necropsy and qPCR was performed. All samples were negative for gB gene of EHV-1. The results demonstrated a low apparent prevalence of latent EHV-1 DNA in submandibular lymph nodes in this population of horses in Virginia. Despite this, the mainstay for outbreak prevention and mitigation continues to focus on minimizing risks and using appropriate and diligent biosecurity.

Keywords: horse; equine herpesvirus-1; herpesvirus; latency; submandibular lymph nodes; PCR

1. Introduction

Equine Herpesvirus type 1 (EHV-1) is a double stranded DNA virus that typically causes mild respiratory disease. However, viremia can lead to late-term abortion, neonatal foal death and neurologic disease (equine herpesvirus myeloencephalopathy or EHM)[1-3]. The virus is ubiquitous in equine populations and affects horses of every breed and discipline. Once a horse is infected, the virus localizes to trigeminal ganglia or lymphoid tissues for the latent genome maintenance[4-5].

While the virus is latent, the animals are not infectious and are clinically normal. However, the virus can be reactivated during times of stress, such as transport or competition. Recrudescence of EHV-1 infections is sporadic but has also been attributed to the initiation of outbreaks, which have proven to be damaging for multiple facets of the equine industry. Recrudescence is likely responsible for the spread of EHV-1 to susceptible horses resulting in outbreaks [6].

One of the most devastating EHV-1 outbreaks occurred in Europe, starting in February 2021 during an international show jumping competition. Ten countries were involved with at least 118 horses infected, 18 of which died. Equestrian events across Europe were halted until the middle of April 2021, having profound economic effects across the industry [7].

Considering the role that latency, and reactivation thereof, plays with maintaining the virus within a population and initiating outbreaks, respectively, the mechanisms of recrudescence, including its environmental triggers, are of key importance for disease control. These facets of EHV-1 are currently poorly understood. Combined with a lack of feasibility of ante-mortem detection of latently infected horses there is a profound paucity in well-rounded EHV-1 disease management strategies.

Understanding the carriage rate of latent EHV-1 in different regions is essential for management of the disease. Currently, there are few studies regarding the prevalence of latent EHV-1 in horses, and none specifically in Virginia. The objective of the current study was to estimate the prevalence of latent EHV-1 and compare the frequency of each variant in the submandibular lymph nodes from horses in Virginia.

2. Materials and Methods

The study sample consisted of a total of 63 equids submitted for necropsy to diagnostic labs in 5 different pre-determined regions of Virginia from January 2020 to March 2021. The pre-determined regions of Virginia were Northern Virginia (NVA), Southwest Virginia (SWVA), Central Virginia, Southeast Virginia (SEVA) and the Outskirts (OVA) (see image 1). Inclusion criteria included horses and mules over the age of 2 years that did not have signs of acute respiratory or neurologic disease that could be due to EHV-1.

2.1. SMLN Collection

Submandibular lymph nodes (SMLN) were collected from each study animal during routine postmortem examination at each of 5 regional diagnostic laboratories in Virginia. The entirety of the SMLNs were retrieved from each study animal using sterile, disposable instruments. The samples were placed into sterile whirlpacks and immediately stored at -80°C until further processing. Fresh, disposable instruments were used for sample collections from each animal to avoid cross-contamination. Sample collection procedures were approved by the Institutional Animal Care and Use Committee of Virginia-Maryland College of Veterinary Medicine prior to collection.

2.2. DNA Isolation

DNA extraction was performed under a biosafety cabinet with sterile labware to reduce environmental contamination and DNA carryover between samples. DNA was extracted from all samples using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Based on manufacturer recommendations, a 25mg piece of SMLN from each horse was placed in individual petri dishes and minced. The minced tissue was transferred to a clean microcentrifuge tube containing Buffer ATL (Qiagen) and 600 mAU/ml proteinase K, then digested overnight at 56°C. A mixture of the tissue lysate, buffer AL and ethanol was pipetted into a DNeasy Mini spin column (Qiagen), then centrifuged for 1 min at 13,300 rpm. The supernatant was discarded and Buffer AW1 (Qiagen) was added to the spin column then centrifuged again. The spin column was then washed with Buffer AW2 (Qiagen), which was centrifuged for 3 min. The DNA was eluted in 100ul Buffer AE (Qiagen)

by incubating at room temperature for 1 min before centrifugation for 1 min at 13,300 rpm. The DNA was stored at -20°C until downstream processing.

2.3. Polymerase Chain Reaction

Each sample of purified nucleic acid underwent a PCR preamplification step as previously described [7]. Briefly, a reaction was performed using Advantage 2 Polymerase Mix (Takara Bio USA, San Jose, CA) containing the target primers for the gB gene and the ORF 30 gene of EHV-1 in 50ul total volume. The primer mix was amplified in a thermocycler (Biometra, Gottingen, Germany) under the following conditions: 1 min at 94C, followed by 25 cycles of 15 sec at 94C, 15 sec at 55C and 45 sec at 70c, followed by 5 min at 70C.

All samples were tested for the presence of the gB and 2 variants (A & G) of the DNA polymerase (ORF 30) gene of EHV-1 as previously described [9]. Nine of the 63 samples were also tested for the C variant of the ORF 30 gene as previously described [10]. Nine samples were also tested for the presence of the C variant of EHV-1.

Four positive controls were used in this study as follows:

A SMLN from a horse with confirmed natural latent infection with the G_{2254} strain of EHV-1 was used as a positive control of SMLN, DNA from a horse with confirmed, natural infection with C_{2254} strain of EHV-1, DNA from wild-type EHV-1 (National Veterinary Services Laboratory, Ames, IA) as a control for $A_{2254,}$ and DNA from EHV-1 vaccine (Rhinomune, Boehringer Ingelheim Vetmedica, MO) as a control for $G_{2254.}$

3. Results

Samples were received from a total of 62 horses and one mule from five pre-determined regions of Virginia. 17 from NVA, 30 from SWVA, 11 central, 1 SEVA, 4 OVA and the rest of the samples were from an unknown region of Virginia. There were 35 geldings, 18 mares and the remainder were undefined. The age of the horses and mule ranged from age 3 years to 25 years, (11) were 5-7 years, (8) were over 20 years (median 12.0 years).

Several breeds were represented most of which were quarter horse (17 horses), thoroughbred (14), warmblood (5), unlisted (14) but also included, American paint horse (2), standardbred (2) saddlebred (1), percheron (1), morgan (1), mustang (1), Tennessee walking horse (1), pony (2), gypsy vanner (1), Norwegian fjord (1), Arabian (1), haflinger (1), spotted saddle horse (1) and mule (1).

The control positive SMLN consistently tested PCR positive for the gB gene of EHV-1 and G variant of the ORF30 with a Ct of 25.54 and 25.92, respectively. The A_{2254} and C_{2254} controls had a Ct of 26.08 and 25.90, respectively. All of the 63 SMLN samples were qPCR negative for the gB gene of EHV-1. 63/63 were negative for the Orf30 gene for the A, G and C variants with a 95% confidence interval of 0-5.75 [11].

4. Discussion

This study represents the first evaluation of the prevalence of EHV-1 latency in the Virginia with an attempt to evaluate throughout the region. We were anticipating a frequency of detection somewhere between 3.3 percent and 54 percent based on studies by Pusterla and others [8] and Allen and others [12], respectively. In this current study, we found a low estimated prevalence. The

California study, which was in a sample population of 147 horses, 4 mules and 3 donkeys, had low prevalence in submandibular lymph nodes as well[8]. Our findings were dissimilar to those of the Kentucky study where they assessed the prevalence of EHV-1 in SMLN of 132 thoroughbred mares in Kentucky[12]. Although we did not find any positives in this population of horses, we were able to detect EHV-1 in samples that were confirmed positive. Furthermore, the confidence interval of our current study indicates a similar estimated prevalence in submandibular lymph nodes to the California study. This does not equate to zero prevalence of EHV-1 in horses, as our findings are an estimate of the prevalence in horses in the study area. For instance, the confidence interval indicates the range that the prevalence might be given that we had zero detections out of 63 equids sampled. Also, we cannot rule out latency in other tissues, such as the retropharyngeal lymph nodes or trigeminal ganglia, that have been shown to carry EHV-1 in latency [13]. It is plausible that the overall prevalence of EHV-1 of this population of horses in Virginia is low, however, follow-up studies in a larger population of horses and multiple sampling sites is likely warranted to make a more accurate prevalence conclusion in this region. Based on a recent affinity study, samples from retropharyngeal lymph nodes, pharyngeal roof and the trigeminal ganglia should be included with submandibular lymph node samples for future prevalence studies [13]. Thus, the estimated prevalence of EHV-1 in, specifically, the SMLN of horses in Virginia is low.

Differences in prevalence in this sample type, could be related to the variability in disciplines or horse use across the country. For instance, in 2003, 39% of

California's horse population was used for showing or racing [14]. This is compared to a report from 2001 indicating that 24.5% of Virginia horses were used for showing or racing [15]. The population of horses used in the Allen paper were specifically thoroughbred broodmares. These horses are required to endure live cover breeding, thus are typically transported frequently and interact with horses from a wide geographic area. These discipline differences should be considered when looking at the variable prevalences reported.

While the lymph node sample size in this current study (25mg) is small compared to the entire organ, the PCR results from the Pusterla 2010 study showed that the EHV-1 is evenly distributed in the tissue during latency. Our detection method consisted of PCR pre-amplification followed by traditional qPCR, which appropriately detected EHV-1 in our positive control lymph node. This is also true considering our sample size of 25mg compared to previous studies using 500mg [8,12].

The low estimated prevalence in this population of horses indicates that the focus of outbreak mitigation should be on preventing exogenous infection with consistent vaccination, prompt diagnosis and diligent biosecurity. Current vaccine recommendations for EHV-1 are bi-annual intramuscular inoculations [16]. It is also prudent to encourage horse care-takers to be meticulous in their monitoring and timely in their outreach for veterinary intervention when early clinical signs are noted. In horses that develop EHV-1 infection and recover, care should be taken to abate the likelihood of recrudescence. This can be done by minimizing stress and providing prudent biosecurity, particularly with those horses; continuing every 6

month vaccinations; and remaining diligent with monitoring and prompt diagnosis and appropriate treatment.

In conclusion, the results of the current study demonstrated a low estimated prevalence of latent EHV-1 DNA in, specifically, submandibular lymph nodes in this population of horses in Virginia. Despite this, the mainstay for outbreak prevention and mitigation continues to focus on minimizing risks and using appropriate and diligent biosecurity.

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Chapter 3: LAMPore sequencing as a diagnostic tool for equine herpesvirus type 1

Introduction

Equine herpesvirus-1 (EHV-1) is an enveloped, double stranded DNA virus that is associated with primary respiratory disease, abortions, neonatal foal death and myeloencephalopathy (EHM).[1,2] EHV-1 is widely distributed with high morbidity and mortality rates, spreading through a group of horses quickly, thus often having devastating effects on large populations of horses.

Infected horses develop a latent infection which has the potential to recrudesce in periods of stress, likely contributing to the occurrence of large scale outbreaks such as was seen in 2021 in the Show Jumping Tour hosted in Valencia, Spain – attended by over 750 horses from 9 different countries; and 2011 at the National Cutting Horse Association Western National Championships in Ogden, Utah – attended by over 400 horses from 19 different states. These occurrences adversely impact horse owners as well as the local, national and international equine industry[5,6]. These are but two of the many outbreaks reported annually worldwide, suggesting an apparent increase in prevalence of EHM. Such outbreaks had prompted the United States Department of Agriculture (USDA) to classify EHM as a 'potentially emerging disease in 2007[7] .

Appropriate biosecurity decisions for managing EHV-1 outbreaks are generally dependent on appropriate diagnosis. The most commonly used diagnostic test for EHV-1 is

polymerase chain reaction (PCR), which requires expensive equipment and can take days to perform due to sample shipping and preparation. The typical region targeted by PCR for EHV-1 is the ORF30 which codes for DNA polymerase.[19] Since this method relies on primers specific to the target, this limits the ability to identify strains that may be different than those included in the reaction mixture. Another target frequently used and recommended to be included is the glycoprotein B (gB) gene, which is conserved in all strains of EHV-1. Although PCR is commonly relied upon to manage EHV-1 in equine populations and is generally considered the gold standard, new genetic sequencing tools are now available that may allow for real-time detection of EHV-1 in veterinary relevant samples in the field (e.g., nasal secretions during an outbreak).

Although DNA sequencing as a means of virus detection has been limited due to cost, equipment size and processing, this approach has evolved over the past 50 years. Techniques have developed to be faster, less expensive and allow for simultaneous sequencing reactions, which can complete a genome within days. Despite these advances, the instruments are expensive and the process requires synthesis of a complementary strand of DNA with detection of individual nucleotides via color, light or pH

Oxford Nanopore Technologies (Oxford, UK) has recently developed a new sequencing method, nanopore technology, where DNA or RNA is fed through membrane pores causing a change in ionic current, which corresponds to the nucleotide sequence of the DNA or RNA molecules passing through the pore.[13] This technique should allow for 95-99% accuracy and increased read speeds of >450 basepairs per second per pore. The data output is available in real time, rather than after complete runs, thus samples can be switched when

the desired depth or genotype is reached. The unit is small enough to be portable and is affordable for practices, thus can be a feasible diagnostic resource in the face of an outbreak.[13,16] Excitingly, NAHLMs has provided over $400,000 in funding for the development of point of care testing for animal diseases. Thus, agencies are investing in applications such as MinION for high impact diseases, showing promise for this utility.[17]

Sequencing results are largely dependent upon the quantity and quality of DNA input. Clinical samples, such as nasal swabs, can contain inhibitors that can impede DNA recovery by blocking polymerase activity and thus prevent target amplification. Amplification methods that are resistant to inhibitor effects can be helpful with improving yields post-extraction. There are three methods of amplification that can improve viral DNA detection: PCR, strand displacement assay (SDA) and transcription-mediated assay (TMA). While PCR is affected by inhibitors and TMA is specifically for RNA amplification, loop mediated isothermal amplification (LAMP) is an SDA method that amplifies nucleic acid rapidly at a constant temperature and is minimally affected by inhibitors that are often found in clinical samples (i.e. nasal secretions)[18] This thereby negates the need for a nucleic acid purification and a thermocycler, allowing the technique to be used in field applications. The method uses four basic primers and two additional primers, called loop primers, to accelerate the reaction.[20] When combined with nanopore sequencing, it has been referred to as LAMPore, which combines barcoded multi-target amplification, a 15-minute library preparation and real-time nanopore sequencing.[21]

Advancing diagnostic approaches to EHV-1 detection is imperative for improving real-time decision making for disease management. The objective of this study was to perform direct DNA sequencing of EHV-1 using the mobile MinION sequencer in combination with LAMP viral enrichment. We hypothesized that these combined techniques will have a similar sensitivity and specificity to qPCR for the detection and strain differentiation of EHV-1 in equine nasal secretions.

Materials and methods

Samples

Forty-three banked equine nasal swab samples were used in this study. Thirty-seven nasal swabs were from horses with EHV-1 infections confirmed with qPCR prior to storage at -80C. Six nasal swabs were from horses that did not have clinical signs consistent with EHV-1 and were confirmed EHV-1 negative via allelic discrimination quantitative (q) PCR for the ORF30 DNA polymerase gene as previously described[22], to serve as negative controls.

Two positive control samples were used in this study: DNA from wild-type EHV-1 (National Veterinary Services Laboratory, Ames, IA) as a control for $A_{2254,}$ and DNA from EHV-1 vaccine (Rhinomune, Boehringer Ingelheim Vetmedica, MO) as a control for $G_{2254.}$ Nuclease free water was used for a non-template control.

DNA Extraction

DNA extraction was performed under a biosafety cabinet with sterile labware to reduce environmental contamination and DNA carryover between samples. DNA was extracted from all samples using High-Pure PCR Template Preparation Kit (Roche). A 200 μL sample of nasal secretions from each horse was added to individual microcentrifuge tubes. Binding buffer and proteinase K were added to the sample, mixed immediately and incubated at 70°C for 10 minutes. Isopropanol was added prior to the contents being transferred to the upper buffer reservoir of a filter tube. The sample was washed with inhibitor removal buffer, followed twice by wash buffer. Thereafter, the sample was eluted in 200 μL of pre-warmed elution buffer.

LAMP

Equid herpesvirus 1 ORF30-specific primer and probe sets were used as previously described.[18] LAMP reactions were prepared on ice. A reaction mixture was prepared containing final concentrations of 1X of 10X Isothermal Amplification buffer, 6 mM $MgSO_4$, 1.4 mM dNTP, 1.6 μM FIP and BIP primers, 0.2 μM F3 and B3 primers, 0.4 μM Loop F and B primers, 320 μ/ml *Bst* 3.0 DNA polymerase, 5ul of DNA and 10ul of nuclease free water for a total reaction volume of 25 μl. The reactions were incubated at 65C for 35 minutes.

Library preparation & Nanopore Sequencing

Library preparation was performed using the Rapid Barcoding Kit, SQK-LSK004 (Oxford Nanopore Technologies, ONT). Briefly, 200 ng of DNA was combined with appropriate

rapid barcode that was specific for each sample within the batch, then incubated at 30C for 1 minute, then 80C for 1 minute. After cooling on ice, the barcoded samples were combined and concentrated with AMPure XP beads in a 1:1 ratio. The mixture was washed with 70% ethanol and eluted in 6 ul of 10 mM Tris-HCl pH 7.5-8.0 with 50 mM NaCl. The eluted samples were then mixed with 0.5 ul of RAP and incubated for 5 minutes at room temperature.

Flongle Flow Cell Priming Kit, EXP-FSE001 (ONT) was used for loading the barcoded library into a Flongle flow cell within the MinION Mk1C (ONT). Briefly, the flow cell was primed with 117 ul of Flush Buffer and 3 ul of Flush Tether. A sequencing mix was prepared by adding the DNA library to 15ul sequencing buffer and 10 ul of loading beads. The sequencing mix was loaded through the sample port into the Flongle flow cell.

Nanopore sequencing without LAMP was performed on five samples and one control for comparison.

Bioinformatics

Real-time analysis was performed with MinKNOW software v. 22.12.5 on the Mk1C. The Fast5 files were basecalled and the barcodes were demultiplexed using Guppy v. 6.4.6.

The reads were mapped with Geneious at medium sensitivity to GenBank reference NC_001491, EHV-1 whole genome, using Geneious Prime v. 2022.2.1.

Results

LAMPore sequencing was performed on a total of 43 samples and 3 controls. Quantitative PCR was used as the gold standard.

All five clinical samples and one positive control sequenced without LAMP recovered less than 0.1% of the total reads mapped to EHV-1. One hundred percent (38/38) of the clinical samples that were positive for EHV-1 via qPCR were also positive via LAMPore. Five of these were also sequenced without enrichment resulting in low yield of mapped sequences (see Table 1, column 6). Of the nasal swab samples that were negative via qPCR, 70% (7/10) were negative for EHV-1 via LAMPore, with the three false positives having fewer than 0.1% reads that mapped to the reference. Both positive control samples, A_{2254} and G_{2254}, appropriately tested positive for EHV-1 using LAMPore.

Compared to allelic discrimination qPCR, the calculated sensitivity and specificity of LAMPore detection of EHV-1 from equine nasal swabs was 100% and 70%, respectively.

Genotypes were reported for 36 of the sequenced samples. Results of 31/36 (86.1%) of the sequenced genotypes matched the genotype from qPCR.
See Table 1 for data.

Table 1: Table of PCR cycle threshold (Ct) values and DNA sequencing reads with strain variations. The first column (sample) refers to the sample or control ID. The second column (PCR Ct) are the Ct values for the reference qPCR, followed by the strains identified by

qPCR in the third column (PCR Strain). The fourth column (LAMPore) are the number of reads that mapped to EHV-1 out of total number of reads followed by the strain identified with bioinformatics in the fifth column (LAMPore Strain). The final column (Nanopore) is the number of reads that mapped to EHV-1 out of the total number of reads when sequencing was performed without LAMP. * While reads mapped to the EHV-1 the single nucleotide polymorphism was not included.

Sample	PCR Ct	PCR Genotype	LAMPore (EHV-1 reads/total reads)	LAMPore Genotype	Nanopore (EHV-1 reads/total reads)
1	29.63	A	82/3000	A	3/3980
2	27.77	A	686/6000	G	4/5882
3	32.53	G	6/6000	A	4/6255
4	35.54	G	681/371322	G	74/182,350
5	18.76	A	426/185192	A	22/123,420
6	30.95	G	500/5000	A	
7	35.54	G	599/5307	G	
8	25.44	A	321/3101	A	
9	37.59	A	427/1847	A	
10	32.69	A	343/11895		
11	27.38	A	539/3908	A	
12	30.44	A	122/2000		
13	27.73	A	203/7738	A	
14	18.47	A	366/3810	A	

15	38.37	NA	132/314	A	
16	28.58	A	15/4102		
17	35.95	A	13/2170	A	
18	20.89	A	5/5000	A	
19	30.06	A	371/3751	A	
20	28.95	A	122/200,000	A	
21	34.27	G	350/5026	G	
22	26.88	G	586/5956	G	
23	29.29	A	74/1000	A	
24	31.78	A	142/2195	A	
25	23.67	A	335/2128	A	
26	26.99	A	450/2531	A	
27	26.16	A	447/4000	A	
28	33.01	A	364/3616	A	
29	27.05	G	408/4283	G	
30	34.11	G	76/2392	A	
31	35.58	G	7040/91012	G	
32	30.73	A	1/1036	A	
33	24.51	A	371/3751	A	
34	24.92	A	271/598	A	
35	31.42	A	850/6690	A	
36	35.75	G	289/1218	A	
37	28.58	A	317/2576	A	
Controls					
38-Negative	0		0		
39-Negative	0		0		

40-Negative	0		3/3016	*	
41-Negative	0		0		
42-Negative	0		1/7074	*	
43-Negative	0		3/5652	*	
44-G Positive	22.05	G	1316/2610	G	
45-A Positive	22.96	A	4670/11,900	A	121/152,450
46- NTC	0		534/101,000		0

Discussion

LAMP sequencing appropriately identified EHV-1 in all the positive samples. The specificity of this method for this sample population indicates that false positives can be expected. When determining whether a horse should be quarantined and/or transportation should be restricted, it is important that false negatives are minimized. Thus, the high sensitivity of this test allows for more efficient decision-making during outbreak situations. Considering the false positives reported in our results, it is necessary to consider follow-up testing with qPCR with any positives for further management and epidemiological evaluation.

Results of shotgun sequencing of 5 clinical samples and one positive control indicated low EHV-1 DNA output. The low yield can be caused by inhibitors throughout the sampling and preparation process including biological inhibitors from the nasal swab samples, PCR inhibitors, and/or handling errors. The LAMP enrichment improved the output in all 6 of those samples and thus was used for the remainder of the study.

The proportion of samples with viral strains reported in the sequencing analysis that matched the qPCR results were 86.1%. Mismatches can be caused by multiple instances. Horses shedding the virus can be shedding multiple strains of the virus[3]. When amplifying DNA for improved sequencing input, there may be an amplification bias of the strain that is less represented in the sample, causing the sequencing to identify more of that strain in the sample[23]. Likewise, qPCR may perform similarly and fail to identify a region that had not amplified as readily. Further, it would be remiss to ignore that although the sensitivity of the MinION is 95-99%, some point mutations may be mis-identified.

The results of this study indicate that LAMPore sequencing is an effective method of identification of equine herpesvirus-1 in equine nasal swabs. With improved library preparation, this could become an efficient stall-side diagnostic tool to improve management decisions during an outbreak.

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Chapter 4: Conclusion & Future Directives

The work within this dissertation informs the efforts of EHV-1 disease management in horse populations from the aspects of regional prevalence and a proof of concept for a novel diagnostic method. To accomplish this, two main objectives were outlined. The first objective of our work was to estimate the prevalence of latent EHV-1 and compare the frequency of each variant in the submandibular lymph nodes from horses in Virginia. The prevalence in the study population of horses was found to be low with 95% confidence interval of 0-5.75, indicating that the focus of outbreak mitigation should be on preventing exogenous infection with consistent vaccination, prompt diagnosis and diligent biosecurity. Our second objective was to perform direct DNA sequencing of EHV-1 using the mobile MinION sequencer in combination with LAMP viral enrichment. LAMPore reliably detected EHV-1 and differentiated between strains of the virus with a sensitivity of 100% and specificity of 70%, indicating this could lead to an effective point-of-care test for EHV-1.

While current detection methods for EHV-1 have been useful in diagnosing EHV-1 disease and allowing for outbreak management, more efficient methods are being sought to provide prompt and affordable decision-making. EHV-1 has been detected using fluorescent loop

primer LAMP with good agreement with qPCR for genotype differentiation. [18] The need for a thermocycler makes both of these methods unfeasible in field applications. However an isothermal PCR, POCKIT Central PCR, has been developed for field use and specifically for EHV-1, which provides an option for stall-side detection.[24] LAMPore has been shown to detect SARS-CoV-2 with diagnostic sensitivity and specificity of 99.1% and 99.6%, respectively. LAMP has been successfully used in the field for rapid point-of-care (POC) detection of SARS-CoV-2.[25,26] [27] Traditional lamp could be used in field applications for EHV-1, despite not being able to distinguish between genotypes, ensuring that the tubes are not opened after processing to prevent environmental contamination. The major limitation with that is DNA extraction in the field, however LAMP has been shown to identify pathogens without nucleic acid purification.[28] Considering that nanopore sequencing has been used for POC detection of SARS-CoV-2[29], Zika[14,15] and Ebola[14], combining these POC techniques for these pathogens, as well as for EHV-1, would be a reasonable goal. The combination of in-device and laptop applications for bioinformatic analyses, the main impediment to in-field LAMPore sequencing of EHV-1 is the library preparation. Automated nucleic acid technologies, such as Oxford Nanopore Technologies VolTRAX, are being developed and will allow LAMPore sequencing to become closer to a field accessible detection method for EHV-1.[30]

Performing limit-of-detection and determining analytic sensitivity and specificity are reasonable next steps for developing LAMPore as a diagnostic tool for EHV-1.

Current literature regarding the prevalence of latent EHV-1 in equine populations is quite limited. Our current findings parallel studies in California and New Zealand, where the

prevalence of EHV-1 in submandibular lymph nodes of horses were 3.3% and 4.8%, respectively.[12,31] In both of these studies, additional tissues were sampled; trigeminal ganglia and retropharyngeal lymph nodes, respectively. Recently, latent EHV-1 affinity for trigeminal ganglia, retropharyngeal lymph nodes, pharyngeal roof and submandibular lymph nodes of horses has been reported.[4] The identification of the virus in one of these tissues did not necessitate identification of the virus in the other tissues within the same horse. Because true prevalence would require examining multiple tissues, the current prevalence estimate will be lower than true prevalence. Thus, the inference of low prevalence in our study population could be challenged further by performing a similar study with a larger population of horses, and sampling multiple samples from each of several tissues including trigeminal ganglia, submandibular lymph nodes, retropharyngeal lymph nodes and pharyngeal roof.

Overall, each study regarding EHV-1 brings us one step closer to improved management of clinical manifestations.

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