Epidemiology and Risk Factors of Equine Parvovirus-Hepatitis, Hepacivirus, *Pegivirus caballi*, and *Pegivirus equi* in U.S. Horses

by

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Abstract

Emerging equine viruses such as Equine parvovirus-hepatitis (EqPV-H), Equine hepacivirus (EqHV), *Pegivirus caballi* (*P. caballi*), and *Pegivirus equi* (*P. equi*) have recently gained recognition as significant contributors to equine infectious disease ecology and hepatic pathology. EqPV-H, in particular, has been implicated in Theiler's disease (TD), a potentially fatal form of acute hepatitis in adult horses. Despite growing global interest, limited data are available describing their molecular epidemiology in the United States. This study was designed to address that gap through a comprehensive molecular survey aimed at evaluating prevalence and potential host-associated risk factors for these viruses across multiple states.

A total of 1,195 equine serum samples were collected from diagnostic submissions and surveillance programs across Alabama, Georgia, and Texas. Using optimized quantitative PCR and RT-qPCR assays targeting conserved regions of each viral genome, the study quantified viral prevalence and examined associations with host demographics, including age, sex, breed, and geographic origin. Statistical analyses, performed in RStudio, employed both univariable and multivariable logistic regression models to identify significant predictors of viral positivity, supported by pairwise Fisher's exact and Wilcoxon rank-sum tests for prevalence and viral burden comparisons, respectively.

EqPV-H showed the highest prevalence (19.3%), followed by EqHV (5.6%) and *P. caballi* (1.7%), with only two cases of *P. equi* detected. EqPV-H infection was significantly associated with breed, age, and sex, whereas EqHV was primarily associated with breed, particularly Thoroughbreds and Quarter Horses. Demographic analysis revealed that EqPV-H-positive horses were significantly older, and male horses had 1.62 times the odds of infection compared to females. Breed-specific associations were also identified: Tennessee Walking Horses had higher odds of

EqPV-H positivity (OR = 2.46), while Quarter Horses (OR = 4.16) and Thoroughbreds (OR = 9.64) showed increased odds of testing positive for EqHV. EqPV-H-positive horses also exhibited significantly higher viral loads compared to animals positive for EqHV or pegiviruses. No host-related risk factors were identified for pegiviruses, though *P. caballi*-positive cases were only identified from horses in Georgia. Viruse sequences identified in this study were similar to the reported ones in the United States and other regions. This largest molecular survey highlights the widespread distribution of EqPV-H and EqHV in U.S. horses and underscore the importance of continued surveillance, particularly in high-risk breeds and settings. The data provides a foundation for developing preventive strategies and improving understanding of the epidemiology and potential clinical impact of these emerging equine viruses. These results expand the epidemiological landscape of equine hepatotropic viruses in in horses in the United States, offering valuable insights into their ecological niches and transmission patterns. EqPV-H's dominance in both prevalence and viral load reinforces its central role as a hepatotropic pathogen of concern. While EqHV remains largely subclinical, its occurrence in specific high-performance breeds raises questions regarding chronic infection.

In conclusion, this molecular survey represents one of the most extensive state-level assessments of EqPV-H, EqHV, *P. caballi*, and *P. equi* in the United States to date. The findings substantiate the endemic nature of EqPV-H and EqHV and delineate host and regional factors influencing their distribution. Collectively, these results underscore the urgency of integrated monitoring systems and molecular diagnostics to track the spread of these emerging equine viruses and mitigate their impact on equine health and industry sustainability.

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List of Abbreviations

BLASTn	Basic Local Alignment Search Tool, nucleotide search
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
CDS	Coding Sequence
CI	Confidence Interval
DNA	Deoxyribonucleic acid
EqHV	Equine hepacivirus
EPgV	Equine pegivirus
EqPV-H	Equine parvovirus-hepatitis
ER	Endoplasmic reticulum
FAM	Fluorescein amidite fluorescent dye
FRET	Förster resonance energy transfer
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
ICTV	International Committee on Taxonomy of Viruses
IQR	Interquartile range
IRES	Internal ribosome entry site
ISH	in situ hybridization
LIPS	Luciferase Immunoprecipitation System

MEGA	Molecular Evolutionary Genetics Analysis
NPHV	Nonprimate hepacivirus
NS	Nonstructural protein
OR	Odd's Ratio
ORF	Open reading frame
P. caballi	*Pegivirus caballi*
P. equi	*Pegivirus equi*
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RT-TaqMan	Reverse transcriptase TaqMan assay
TD	Theiler's disease
TDAV	Theiler's disease–associated virus
T. equi	*Theileria equi*
TE Buffer	Tris-EDTA buffer
USA	United States of America
USDA	United States Department of Agriculture
UTR	Untranslated region
VPs	Viral Proteins

WOAH	World Organization for Animal Health
μL	Microliter

Chapter 1. Literature Review

Theiler's disease (TD), or equine serum hepatitis encompasses a spectrum of viral infections affecting the liver of horses, several of which have been identified only within the past decade. They include equine hepacivirus (EqHV) (Burbelo, Dubovi et al. 2012), equine pegivirus (EPgV) (Kapoor, Simmonds et al. 2013), Theiler disease-associated virus (TDAV) (Chandriani, Skewes-Cox et al. 2013), and equine parvovirus-hepatitis (EqPV-H) (Divers, Tennant et al. 2018), all of which have demonstrated varying degrees of hepatotropism and associations with hepatic inflammation or liver disease in horses. This review aims to consolidate existing knowledge on these viruses, highlight areas that require further investigation, and discuss the potential clinical relevance of each infection. Equine parvovirus-hepatitis (EqPV-H) is a liver-targeting virus that likely contributes to subclinical hepatitis and is suspected of being a causative agent of TD in horses. Equine hepacivirus, also known as nonprimate hepacivirus (NPHV) (Ramsay, Evanoff et al. 2015, Walter, Rasche et al. 2017), is also hepatotropic but typically results in mild, asymptomatic hepatitis and is not linked to TD. Both EqPV-H and EqHV are widespread among horses and characterized by prolonged viremia; hence, their detection alone does not confirm causality of disease.

Equine pegiviruses belong to the genus *Pegivirus* within the family *Flaviviridae*. Over the past decade, their nomenclature has been updated to reflect the growing understanding of equine pegiviruses and their phylogenetic relationships to other flaviviruses (Postler, Beer et al. 2023). TDAV is now known as *Pegivirus equi* and EPgV is currently known as *Pegivirus caballi*. Although *P. equi* was initially suspected to contribute to TD pathogenesis, subsequent studies have demonstrated that both pegiviruses are non-hepatotropic. Instead, they exhibit lymphoid or bone marrow tropism, establishing persistent, non-cytolytic infections without biochemical or

histological evidence of liver disease (Tomlinson, Van de Walle et al. 2019, Tomlinson, Wolfisberg et al. 2020). These findings exclude *P. caballi* and *P. equi* as primary agents of equine hepatitis but suggest their possible roles as immunomodulatory or coinfecting viruses.

1.1 Equine parvovirus (EqPV)

Equine parvovirus (EqPV) was first identified in 2018 in a horse from Nebraska, USA, that died of TD (Theiler's disease) 65 days after receiving an equine-derived tetanus antitoxin, which was later found to contain EqPV DNA. The virus was initially identified through unbiased next-generation sequencing of liver tissue from a horse that succumbed to TD (Divers, Tennant et al. 2018). EqPV belongs to the Parvoviridae family, Parvovirinae subfamily, *Copiparvovirus* genus, and has been classified as *Ungulate copiparvovirus 6* (Divers, Tennant et al. 2018). Since its discovery, EqPV has consistently been detected in the serum and liver of horses with TD characterized by fulminant hepatic necrosis (Divers, Tennant et al. 2018, Tomlinson, Tennant et al. 2019, Tomlinson, Van de Walle et al. 2019, Baird, Tegtmeyer et al. 2020, Kopper, Schott et al. 2020, Vengust, Jager et al. 2020). The virus is hepatotropic and is believed to cause a spectrum of hepatic conditions, ranging from subclinical infections to severe hepatitis, positioning EqPV as the most likely etiological agent of TD, with only rare exceptions reported (Tomlinson, Tennant et al. 2019).

1.1.1 Morphology and Genomic Organization of EqPV

EqPV is a single-stranded DNA virus that persists in the liver as episomal DNA. Its genome is 5,308 nucleotides in length and contains two major open reading frames (ORFs) (Divers, Tennant et al. 2018). The first ORF, spanning nucleotides 1 to 1779, encodes a

nonstructural (NS) protein, whereas the second ORF, from nucleotides 1801 to 4722, encodes the structural viral proteins (VPs) (Divers, Tennant et al. 2018). These ORFs are separated by two intergenic regions: one short segment of 21 nucleotides and a longer region of 583 nucleotides. Computational analysis using DNA folding prediction software suggests that the longer intergenic region is capable of forming a large hairpin structure, along with several smaller, hairpin-like conformations (Divers, Tennant et al. 2018).

As with other parvoviruses, EqPV possesses a compact genome that does not encode the necessary proteins for independent replication (Berns 1990). Consequently, it is believed that these viruses depend either on co-infection with helper viruses or on the host's own replication machinery found in actively dividing cells (Berns 1990). However, studies have also indicated that parvoviruses can exploit host DNA damage-response pathways to support replication in non-dividing cells (Deng, Yan et al. 2016, Deng, Xu et al. 2017). Given that the liver is largely composed of quiescent, non-proliferating hepatocytes, the specific mechanisms through which EqPV achieve replication in hepatic tissue remain unknown. Elucidating these mechanisms will be an important area for future investigation (Tomlinson, Tennant et al. 2019).

1.1.2 Epidemiology of EqPV

EqPV appears to be globally distributed, with no clear geographic limitations. Surveillance studies conducted in clinically healthy horse populations across the United States, China, Germany, and Austria have reported the DNA prevalence of EqPV ranging from 7.1% to 17%, and seroprevalence rates between 15% and 34.7% (Divers, Tennant et al. 2018, Lu, Sun et al. 2018, Altan, Li et al. 2019, Meister, Tegtmeyer et al. 2019, Lu, Wu et al. 2020, Badenhorst, de Heus et al. 2022). Notably higher DNA prevalence has been documented on farms in the United

States and Canada where recent outbreaks of equine serum hepatitis occurred (Tomlinson, Tennant et al. 2019, Baird, Tegtmeyer et al. 2020, Tomlinson, Jager et al. 2020). The first confirmed case of EqPV-associated TD in Europe was reported in Slovenia, where four horses from the same farm developed fatal hepatitis and all tested PCR-positive for EqPV in liver tissue (Vengust, Jager et al. 2020).

Further supporting its global spread, analysis of commercial equine serum pools from New Zealand, the United States, Italy, Germany, and Canada revealed a high detection rate of EqPV, with 61.1% (11/18) testing PCR-positive and 77.8% (14/18) seropositive (Meister, Tegtmeyer et al. 2019). All PCR-positive samples were also antibody-positive, and an additional three samples showed antibody positivity in the absence of detectable viral DNA. These findings from both field cases and commercial biological products underscore the widespread presence of EqPV and support its international distribution (Meister, Tegtmeyer et al. 2019).

1.1.3 Phylogenetic Analyses of EqPV

Phylogenetic analyses of the nonstructural (NS) and structural (VP) proteins have shown that EqPV shares less than 50% amino acid identity with other members of the *Copiparvovirus* genus, which includes viruses infecting a broad range of hosts such as pigs, cattle, deer, sea lions, and horses (Li, Giannitti et al. 2015, Divers, Tennant et al. 2018, Lu, Sun et al. 2018, Altan, Li et al. 2019, Linden, Gilliaux et al. 2019). Alongside EqPV, two other equine-associated copiparvoviruses—equine parvovirus cerebrospinal fluid (CSF) and equine-copiparvovirus—have been sequenced and genetically characterized (Li, Giannitti et al. 2015, Altan, Li et al. 2019, Ou, Li et al. 2022). Additionally, two earlier reports describe equine parvoviruses of uncertain classification due to the absence of sequence data. One was isolated in 1985 from the liver of an

aborted fetus during an abortion outbreak on a Canadian farm (Wong, Spearman et al. 1985), and another was linked to equine synovitis in Australia in 2014 (Wang, Yu et al. 2014). Without genomic data, the relationship between these earlier viruses and known equine parvoviruses remains unclear.

Notably, phylogenetic comparisons indicate that EqPV shares greater genetic similarity with porcine and bovine copiparvoviruses than with other known equine parvoviruses, suggesting independent evolutionary origins within equine parvoviral lineages (Li, Giannitti et al. 2015, Divers, Tennant et al. 2018, Lu, Sun et al. 2018, Altan, Li et al. 2019). Based on its host specificity and distinct genomic features, EqPV has been formally assigned to the species *Ungulate copiparvovirus 6* under the updated taxonomic framework of the *Parvoviridae* family (Pénzes, Söderlund-Venermo et al. 2020). Recent comparative analyses of all 12 publicly available complete coding sequences (CDS) of *Ungulate copiparvovirus 6* from China and the United States, alongside four Austrian variants, revealed a high degree of genetic similarity, indicating limited diversity among global EqPV strains (Badenhorst, de Heus et al. 2022). Consistently low variability in the NS and VP genes has been observed across isolates from the United States, China, Canada, New Zealand, Italy, and Germany, suggesting strong conservation among circulating variants worldwide (Divers, Tennant et al. 2018, Lu, Sun et al. 2018, Meister, Tegtmeyer et al. 2019, Meister, Tegtmeyer et al. 2019, Baird, Tegtmeyer et al. 2020). Supporting this, identical partial NS1 sequences were detected in two horses that died of Theiler's disease on the same Canadian farm, 11 years apart, implying prolonged local circulation of genetically stable strains (Baird, Tegtmeyer et al. 2020).

Nonetheless, regional sequence variations have been noted, such as unique nucleotide substitutions in strains from China, indicating possible geographical clustering (Lu, Sun et al.

2018). Moreover, recent findings have identified genetic recombination within the VP1 protein between Chinese and American strains, highlighting emerging strain diversity (Lu, Wu et al. 2020). Despite these findings, comprehensive epidemiological studies are still needed to assess the extent of EqPV genetic diversity, the occurrence of recombination events, and their potential impact on viral pathogenicity and clinical outcomes.

Since its identification, EqPV DNA has been detected in the serum and/or liver of numerous horses presenting with acute, severe hepatitis—both as isolated cases and during larger outbreaks—in countries such as the United States, Canada, and Slovenia (Divers, Tennant et al. 2018, Tomlinson, Kapoor et al. 2019, Tomlinson, Tennant et al. 2019, Baird, Tegtmeyer et al. 2020, Kopper, Schott et al. 2020, Vengust, Jager et al. 2020). While the disease is frequently linked to the administration of equine-derived biological products (Divers, Tennant et al. 2018, Tomlinson, Kapoor et al. 2019, Kopper, Schott et al. 2020, Vengust, Jager et al. 2020), it has also been reported in horses without such exposure, suggesting alternate routes of transmission (Tomlinson, Tennant et al. 2019, Baird, Tegtmeyer et al. 2020, Vengust, Jager et al. 2020). In contact horses, those exposed to EqPV-positive individuals show a relatively high incidence of hepatitis (15%–27%), with infection significantly associated with hepatic pathology. Although many of these horses remain asymptomatic, some develop subclinical or overt clinical hepatitis (Baird, Tegtmeyer et al. 2020, Vengust, Jager et al. 2020).

Common clinical signs in affected horses include icterus, lethargy, reduced appetite, and neurological manifestations such as blindness, obtundation, head pressing, and ataxia (Chandriani, Skewes-Cox et al. 2013, Divers, Tennant et al. 2018, Tomlinson, Kapoor et al. 2019, Tomlinson, Tennant et al. 2019, Baird, Tegtmeyer et al. 2020, Kopper, Schott et al. 2020, Tomlinson, Jager et al. 2020, Vengust, Jager et al. 2020). Biochemical analysis frequently reveals elevated liver

enzymes and markers—including bilirubin, bile acids, and ammonia—while glucose levels may be decreased in some cases (Tomlinson, Kapoor et al. 2019, Tomlinson, Tennant et al. 2019, Baird, Tegtmeyer et al. 2020, Kopper, Schott et al. 2020, Vengust, Jager et al. 2020). In severe presentations, outcomes are often fatal, with death or euthanasia occurring rapidly following the onset of clinical signs. Gross pathological findings typically include atrophic, friable, discolored, or flattened livers, and histopathology commonly reveals hepatocellular necrosis, lobular collapse, and lymphocytic infiltration (Divers, Tennant et al. 2018, Tomlinson, Kapoor et al. 2019, Tomlinson, Tennant et al. 2019, Kopper, Schott et al. 2020, Vengust, Jager et al. 2020). Despite this, the majority of EqPV-infected horses identified through contact tracing or surveillance remain clinically normal, though some exhibit subclinical hepatitis accompanied by elevated liver biochemistry markers (Chandriani, Skewes-Cox et al. 2013, Tomlinson, Tennant et al. 2019, Baird, Tegtmeyer et al. 2020, Lu, Wu et al. 2020, Badenhorst, de Heus et al. 2022).

1.1.4 Pathogenesis of EqPV

To assess the pathogenic potential of EqPV, experimental infections have been conducted using EqPV-contaminated equine serum and biological products (Divers, Tennant et al. 2018, Tomlinson, Jager et al. 2020). In most cases, infected horses developed subclinical hepatitis, characterized by elevated liver enzyme levels without overt clinical signs (Tomlinson, Jager et al. 2020). However, one horse displayed mild clinical symptoms, including icterus, lethargy, and inappetence lasting six days. Peak viremia was observed around five weeks post-infection, with the highest viral loads detected in serum and liver tissue (Tomlinson, Jager et al. 2020). In addition to hepatic involvement, EqPV-DNA was detected in multiple organs and body fluids, including cerebrospinal fluid, synovial fluid, heart, kidney, colon, jejunum, salivary gland, synovium, bone marrow, lymph node, spleen, spinal cord, and lung (Tomlinson, Jager et al. 2020).

The virus was found to persist at low levels in many of these sites for at least 15 weeks following the infection. In both experimentally and naturally infected horses, viral replication was confirmed in hepatocytes via in situ hybridization (ISH), correlating with hepatocyte necrosis and periportal lymphocytic infiltration (Tomlinson, Jager et al. 2020, Vengust, Jager et al. 2020). The presence of lymphocytic infiltrates suggests an acute immune response, potentially involving cytotoxic T cells targeting infected hepatocytes (Divers, Tennant et al. 2018, Divers and Tomlinson 2020, Tomlinson, Jager et al. 2020). These findings confirm the hepatotropism of EqPV, though the clinical relevance of its presence in extrahepatic tissues warrants further investigation.

1.1.5 Transmission Dynamics of EqPV

EqPV transmission occurs through multiple routes, with iatrogenic horizontal transmission being the most well-documented. Experimental infections have confirmed that parenteral inoculation with virus-containing biological products—such as tetanus antitoxin, equine serum, and allogeneic mesenchymal stromal cell preparations—can lead to infection and hepatitis (Meister, Tegtmeyer et al. 2019, Tomlinson, Kapoor et al. 2019, Divers and Tomlinson 2020, Tomlinson, Jager et al. 2020, Vengust, Jager et al. 2020). Investigations have detected EqPV DNA in a majority of commercial equine serum samples, with viral loads reaching up to 10^5 copies/mL, and widespread use of pooled donor products like tetanus antitoxin has likely contributed to increased contamination risk (Divers, Tennant et al. 2018, Meister, Tegtmeyer et al. 2019, Tomlinson, Kapoor et al. 2019).

Noniatrogenic horizontal transmission is supported by cases of hepatitis in horses without any recent exposure to biological products, including in-contact horses, suggesting infectious and potentially contagious behavior of the virus (Tomlinson, Tennant et al. 2019, Baird, Tegtmeyer et

al. 2020, Lu, Wu et al. 2020). Although arthropod-mediated transmission remains a possibility, a recent study failed to confirm transmission via horse flies, indicating it may require a high number of bites (Tomlinson, Jager et al. 2020). Experimental studies have demonstrated intermittent nasal, oral, and fecal shedding, primarily around peak viremia, persisting up to 10 weeks post-infection. Oral inoculation with EqPV-positive serum resulted in infection in one horse, whereas intranasal exposure did not (Tomlinson, Jager et al. 2020). Vertical transmission has not been directly demonstrated in utero; however, most foals that were EqPV-negative at birth acquired infection within 7–10 months, likely due to horizontal transmission from infected dams or administration of contaminated anti-*Rhodococcus equi* plasma (Meister, Tegtmeyer et al. 2019, Tomlinson, Jager et al. 2020).

Unlike EqHV, there is currently no evidence of cross-species transmission of EqPV to other equids such as donkeys (Walter, Rasche et al. 2017, Badenhorst, de Heus et al. 2022). These findings emphasize the importance of implementing strict hygiene and surveillance measures, particularly given the potential for transmission from subclinically infected horses. In response to these risks, the USDA introduced mandatory EqPV testing and labeling requirements in 2019 for equine-derived products including antitoxins, antibodies, sera, and plasma (Tomlinson, Jager et al. 2020).

1.2 Equine Hepacivirus (EqHV)

EqHV is a member of the Flaviviridae family, which encompasses over 60 viral species (Simmonds, Becher et al. 2017) distributed across four genera: *Flavivirus*, *Pestivirus*, *Pegivirus*, and *Hepacivirus*. Among them, flavivirus represents the largest genus and comprises bloodborne arthropod-borne viruses transmitted primarily by mosquitoes and ticks, such as West Nile virus,

Usutu virus, and yellow fever virus. Flaviviruses are enveloped, single-stranded positive-sense RNA viruses. The *Pestivirus* genus primarily infects swine and ruminant species and includes pathogens like classical swine fever virus, border disease virus, and bovine viral diarrhea viruses (types 1 and 2). Pegiviruses are known to establish persistent infections in a broad range of mammalian hosts, although their pathogenic significance remains unclear. In contrast, hepacivirus members, most notably the human hepatitis C virus (HCV), are associated with chronic liver disease and significant global health burden (Kapoor, Simmonds et al. 2011).

1.2.1 EqHV Cell Tropism and Pathogenesis

Since its emergence in 2011, EqHV has been associated with variable liver enzyme elevations and the capacity to establish chronic infections in horses (Kapoor, Simmonds et al. 2011, Burbelo, Dubovi et al. 2012, Reuter, Maza et al. 2014, Pfaender, Cavalleri et al. 2015, Scheel, Kapoor et al. 2015, Elia, Lanave et al. 2017, Pfaender, Walter et al. 2017, Tegtmeyer, Echelmeyer et al. 2019, Tomlinson, Van de Walle et al. 2019, Tomlinson, Wolfisberg et al. 2021), a pattern that closely parallels HCV pathogenesis in humans (Reuter, Maza et al. 2014, Pfaender, Cavalleri et al. 2015, Ramsay, Evanoff et al. 2015, Scheel, Kapoor et al. 2015, Gather, Walter et al. 2016, Elia, Lanave et al. 2017, Tegtmeyer, Echelmeyer et al. 2019, Date, Sugiyama et al. 2020).

EqHV enters host cells via clathrin-mediated endocytosis, initiated by the binding of viral glycoproteins to receptors such as heparan sulfates. Following endosomal fusion and uncoating, viral RNA is released into the cytoplasm, where early viral proteins suppress host immune responses. Translation and replication occur in the ER, with non-structural proteins facilitating membrane remodeling and immune evasion. Virions assemble in the ER, acquire their envelope, and are released via the secretory pathway. The final composition of EqHV particles may vary

depending on the host cell type and subcellular environment (Gerold, Bruening et al. 2017). EqHV exhibits hepatotropism, as evidenced by findings from transfection assays (Scheel, Kapoor et al. 2015), tissue-specific PCR (Ramsay, Evanoff et al. 2015), and in situ hybridization techniques (Pfaender, Cavalleri et al. 2015). While experimental transmission through contaminated blood or serum has been demonstrated (Ramsay, Evanoff et al. 2015, Pfaender, Walter et al. 2017), the natural route of EqHV transmission remains unidentified. Vertical transmission is considered a possibility, supported by a case in which EqHV RNA was detected in the umbilical cord blood and postnatal serum of a foal born to one of four PCR-positive mares (Gather, Walter et al. 2016).

1.2.2 Epidemiology of EqHV

EqHV infections have been documented globally, with reports from countries including the United States, Great Britain, China, Japan, and Germany (Lyons, Kapoor et al. 2014, Reuter, Maza et al. 2014, Tanaka, Kasai et al. 2014, Matsuu, Hobo et al. 2015, Pfaender, Cavalleri et al. 2015, Scheel, Simmonds et al. 2015, Lu, Sun et al. 2016, Reichert, Campe et al. 2017). The virus is relatively prevalent; viremia has been identified in 2% to 18% of adult horses, and seropositivity rates range from 22% to 84% (Lyons, Kapoor et al. 2014, Tanaka, Kasai et al. 2014, Matsuu, Hobo et al. 2015, Lu, Sun et al. 2016, Reichert, Campe et al. 2017, Walter, Rasche et al. 2017). Notably, seroprevalence tends to increase with age (Pfaender, Cavalleri et al. 2015, Reichert, Campe et al. 2017).

Similar to the pattern observed in HCV infections, EqHV infection is characterized by a delayed humoral response, with antibody development typically occurring 3 to 8 weeks post-infection. Moreover, seroconversion frequently occurs several weeks prior to the complete clearance of the virus (Ramsay, Evanoff et al. 2015, Pfaender, Walter et al. 2017). Experimental

infections with EqHV have consistently resulted in subclinical hepatitis, with hepatic inflammation generally manifesting around the time of seroconversion (Pfaender, Cavalleri et al. 2015, Ramsay, Evanoff et al. 2015, Scheel, Kapoor et al. 2015).

1.2.3 Morphology and Genomic Organization of EqHV

EqHV is an enveloped virus with spherical virions measuring approximately 40–60 nm in diameter. Its structure includes a single, small capsid protein and two envelope glycoproteins, E1 and E2, which are arranged in icosahedral symmetry (Chambers, Mahy et al. 2010, Kapoor, Simmonds et al. 2011, Simmonds, Becher et al. 2017). While the virion architecture (Gerold, Bruening et al. 2017) of most Flavivirus species is well-characterized; typically comprising 90 envelope glycoprotein heterodimers that form a smooth, icosahedral surface; similar comprehensive structural data are lacking for other genera within the Flaviviridae family. In particular, proteomic analyses of non-Flavivirus members remain limited. Recent studies indicate that host cell type and tissue origin can influence the final composition of the viral envelope. However, the precise functional roles of any host-derived or accessory proteins potentially incorporated during the budding process remain to be elucidated (Gerold, Bruening et al. 2017).

EqHV is a single-stranded, positive-sense RNA virus with a genome of approximately 9500 nucleotides (Kapoor, Simmonds et al. 2011, Burbelo, Dubovi et al. 2012, Scheel, Kapoor et al. 2015). Of these, roughly 9200 nucleotides encode a single large open reading frame (ORF), which translates into a multifunctional polyprotein. This polyprotein undergoes cleavage by both host and viral proteases to yield ten distinct proteins: three structural proteins (core, E1, E2) and seven non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Hartlage, Cullen et al. 2016).

1.2.4 Transmission Dynamics of EqHV

EqHV transmission occurs through vertical, horizontal, and parenteral routes. Vertical transmission has been reported in two studies, with detection of EqHV RNA in foals, aborted fetuses, and allantochorion tissue, suggesting rare but possible in utero infection, potentially influenced by viral strain and placental barriers (Gather, Walter et al. 2016, Pronost, Fortier et al. 2019). Horizontal transmission is less defined; EqHV RNA has been found in nasopharyngeal swabs, and some foals and mares developed infection post-partum without known parenteral exposure, indicating potential oropharyngeal spread. However, fecal–oral transmission has been excluded (Gather, Walter et al. 2016, Pronost, Hue et al. 2016, Altan, Li et al. 2019, Yoon, Park et al. 2022). Parenteral transmission is well-established. Experimental infections and contaminated plasma transfusions have consistently resulted in subclinical hepatitis and persistent viremia (Lyons, Kapoor et al. 2014, Ramsay, Evanoff et al. 2015, Scheel, Kapoor et al. 2015, Gather, Walter et al. 2016, Pfaender, Walter et al. 2017, Altan, Li et al. 2019, Tomlinson, Wolfisberg et al. 2021).

EqHV contamination has also been frequently detected in commercial equine sera, posing risks for iatrogenic spread and cross-species transmission, particularly to dogs (Kapoor, Simmonds et al. 2011, Burbelo, Dubovi et al. 2012, Pfaender, Cavalleri et al. 2015, Scheel, Kapoor et al. 2015, Tomlinson, Wolfisberg et al. 2021). These findings highlight the critical need for screening and quality control of equine-derived biological products.

Although EqHV shares close genetic similarity with HCV, only two studies have explored its potential for cross-species transmission to humans (Pfaender, Walter et al. 2015, Date, Sugiyama et al. 2020). The historical proximity between humans and horses has raised speculation about possible zoonotic events, either direct or via intermediate hosts. However, no evidence

currently supports zoonosis, and horses appear to be the exclusive natural host for EqHV (Thézé, Lowes et al. 2015).

1.2.5 Diagnostic Challenges and Molecular Detection Techniques of EqHV

At present, EqHV is not included in routine diagnostic workflows, and existing protocols have primarily been applied in prevalence and genetic studies rather than validated for clinical diagnosis under international guidelines, such as those from the WOAH (World Organisation for Animal Health) (Pacchiarotti, Nardini et al. 2022). No commercial ELISA kits are currently available for detecting EqHV antibodies or antigens in equine sera. Serological detection methods described in the literature include the Luciferase Immunoprecipitation System (LIPS) (Burbelo, Dubovi et al. 2012, Lyons, Kapoor et al. 2014, Pfaender, Cavalleri et al. 2015, Reichert, Campe et al. 2017, Badenhorst, Tegtmeyer et al. 2018, Badenhorst, De Heus et al. 2019), which uses Renilla luciferase fused to the NS3 antigen; the Gaussia Luciferase Immunoprecipitation System (GLIPS) (Matsuu, Hobo et al. 2015, Abbadi, Lkhider et al. 2021), which employs a similar approach with Gaussia luciferase; and Western blotting (Tanaka, Kasai et al. 2014, Hayashi, Tanaka et al. 2018), targeting antibodies against recombinant core proteins. Molecular detection is typically performed using real-time PCR (Burbelo, Dubovi et al. 2012, Lyons, Kapoor et al. 2014, Elia, Lanave et al. 2017, Reichert, Campe et al. 2017, Badenhorst, Tegtmeyer et al. 2018, Schlottau, Fereidouni et al. 2019) or nested PCR (Burbelo, Dubovi et al. 2012, Gemaque, de Souza et al. 2014, Lyons, Kapoor et al. 2014, Matsuu, Hobo et al. 2015, Kim, Moon et al. 2017, Badenhorst, Tegtmeyer et al. 2018, Figueiredo, Lampe et al. 2018, Chen, Cai et al. 2021, Yoon, Park et al. 2022), targeting conserved genomic regions such as the 5′ UTR, NS3, and NS5B. These regions, along with E1/E2, are also

frequently used for EqHV phylogenetic analysis (Schmidt, Strimmer et al. 2002, Figueiredo, Lampe et al. 2015, Matsuu, Hobo et al. 2015, Pronost, Hue et al. 2017).

1.3 *Pegivirus caballi*

Pegivirus caballi is a member of the Flaviviridae family, genus *Pegivirus*, first identified in 2013 in horses in the United States. Unlike Equine Hepacivirus (EqHV) or Equine Parvovirus-Hepatitis (EqPV-H), *P. caballi* is not hepatotropic and has not been associated with clinical hepatitis. Instead, it establishes persistent, subclinical infections, mainly targeting bone marrow and lymphoid tissues, where its biological role remains poorly defined (Kapoor, Simmonds et al. 2013, Tomlinson, Wolfisberg et al. 2020).

Globally, *P. caballi* is widespread, with prevalence ranging from 6% to over 30% in equine populations across North America, Europe, and Asia (Kapoor, Simmonds et al. 2013, Tomlinson, Van de Walle et al. 2019). Although *P. caballi* is generally regarded as non-pathogenic and not directly linked to equine liver disease (Pfaender, Cavalleri et al. 2015, Tomlinson, Wolfisberg et al. 2020), its frequent co-detection alongside other hepatotropic viruses such as EqHV and EqPV-H has raised questions regarding its potential role in modulating host immune responses (Lu, Sun et al. 2016, Postel, Cavalleri et al. 2016).

Notably, a high rate of co-infection between EqHV and *P. caballi* has been reported in certain populations, including nearly 59% of horses tested in Brazil (Figueiredo, de Moraes et al. 2019). While definitive evidence of *P. caballi* -mediated immunological effects in horses is lacking, the possibility remains intriguing, particularly given parallels to human pegivirus (HPgV) infection, where co-infection in HIV-positive individuals has been associated with reduced immune activation, higher CD^4 counts, and prolonged survival (Chivero and Stapleton 2015,

Stapleton 2022). These observations highlight the need for further studies into whether *P. caballi*, though seemingly non-pathogenic, may exert subtle immunomodulatory influences during equine viral co-infections. Based on these research studies, current consensus is that *P. caballi* plays no direct role in equine hepatitis, but its high prevalence and immunomodulatory potential make it an important virus to monitor within the broader equine virome.

1.3.1 Morphology and Genomic Organization of *P. caballi*

Pegiviruses are small, enveloped, positive-sense RNA viruses within Flaviviridae. Unlike hepaciviruses, pegiviruses do not encode a canonical core (capsid) protein, and intact virions have rarely been visualized; for human pegivirus (HPgV-1) the particle size is estimated at ~50–100 nm from filtration studies. These properties indicate a virion architecture that is likely distinct from other Flaviviridae members (Kapoor, Simmonds et al. 2013).

P. caballi possesses a single-stranded, positive-sense RNA genome with one long open reading frame (ORF) flanked by structured 5′ and 3′ untranslated regions (UTRs)—the canonical organization across Flaviviridae. The prototype *P. caballi* genome is ~11,197 nt (nucleotides), among the largest reported for pegiviruses/hepaciviruses. Translation initiates within a structured 5′ UTR that contains internal ribosome entry site (IRES) motifs (e.g., a GNRA tetraloop and polypyrimidine tract). The 3′ UTR includes repeat and conserved sequence elements with predicted stem–loop structures that likely contribute to RNA stability/replication (Kapoor, Simmonds et al. 2013, Smith, Becher et al. 2016).

The single ORF encodes a ~3,300-aa polyprotein that is co-/post-translationally cleaved by host signal peptidase(s) in the structural region and by the viral NS3-4A serine protease in the nonstructural region—mirroring the gene order and proteolytic logic of hepaciviruses. In *P.*

caballi, the polyprotein yields structural proteins E1 and E2, plus a predicted small glycoprotein "X" downstream of E2 in longer pegivirus genomes, followed by NS2, NS3, NS4A, NS4B, NS5A, and NS5B. EPgV E1/E2 harbor multiple N-linked glycosylation sites, and *P. caballi* NS3 contains the catalytic triad for a chymotrypsin-like protease and Walker motifs of an RNA helicase; NS5B is the RNA-dependent RNA polymerase with the conserved RdRp motifs (Bailey, Lauck et al. 2015, Tomlinson, Wolfisberg et al. 2020).

In pegiviruses (including *P. caballi*), E1 and E2 are the only well-supported virion structural proteins; the absence of a core ORF is a defining feature of the genus. Recent comparative work across Flaviviridae indicates that the E1/E2 glycoproteins of pegiviruses, hepaciviruses, and pestiviruses form a structurally distinct class (relative to arthropod-borne flaviviruses), with implications for receptor use, fusion, and immune evasion. Although atomic-level structures for *P. caballi* E1/E2 are not yet available, their organization, heavy glycosylation, and predicted signal sequences fit this emerging paradigm (Tautz, Tews et al. 2015, Smith, Becher et al. 2016).

After polyprotein processing, NS2 (putative autoprotease/cofactor), NS3-4A (protease–helicase complex), NS4B (membranous web organizer), NS5A (phosphoprotein with roles in RNA replication and assembly), and NS5B (RdRp) coordinate cytoplasmic replication on remodeled intracellular membranes, as in other Flaviviridae. While these functions are best characterized in HCV, *P. caballi* conservation at key catalytic/functional motifs strongly supports analogous roles (Lindenbach and Rice 2013, Tomlinson, Wolfisberg et al. 2020).

The *P. caballi* E1/E2–NS2 junction includes signalase sites that, together with a downstream site at the start of NS2, create a predicted glycoprotein "X" (moderately glycosylated) in *P. caballi* —previously proposed in bat pegivirus and supported by sequence homology/signal

peptide predictions. Biologically, equine pegiviruses are bone-marrow tropic, establish persistent viremia, and are not associated with hepatitis, leading some authors to group TDAV-like sequences as EPgV-2 within the equine pegivirus clade (Thézé, Lowes et al. 2015, Tomlinson, Wolfisberg et al. 2020).

Whole-genome analyses place *P. caballi* within the diverse Pegivirus genus alongside human (HPgV), simian, and bat pegiviruses, with host- and geography-structured diversity typical of hepaci/pegiviruses. The gene order and protein functions of *P. caballi* parallel hepaciviruses, yet its lack of a core ORF and lymphoid/bone-marrow tropism underscore distinct evolutionary solutions to vertebrate infection within Flaviviridae (Kapoor, Simmonds et al. 2013, Tomlinson, Wolfisberg et al. 2020).

1.3.2 Pathogenesis of *P. caballi*

P. caballi exhibits unique genomic features and causes persistent viremia in horses, though its clinical significance remains undetermined (Chandriani, Skewes-Cox et al. 2013). Unlike other members of the Flaviviridae family, particularly hepatitis C virus and its equine homolog, *P. caballi* demonstrates remarkable tissue tropism characteristics that fundamentally distinguish it from hepatotropic viruses. Evidence indicates infection primarily involves bone marrow and spleen, with *P. caballi* showing no linkage to disease manifestations (Figueiredo, de Moraes et al. 2019). This non-hepatotropic nature has been definitively established through comprehensive tissue distribution studies, challenging early assumptions about pegivirus pathogenicity in equines and necessitating a complete reevaluation of its role in equine health. Pegiviruses frequently establish persistent infections lasting more than six months without causing apparent clinical disease, distinguishing them from most other Flaviviridae members.

1.4 *Pegivirus equi*

Pegivirus equi, previously known as Theiler's disease–associated virus (TDAV) was first discovered through deep sequencing of serum from a horse that had developed nonfatal serum hepatitis, following DNA digestion to enrich for RNA viruses. The virus was detected in both the affected horse and the botulism antitoxin it had received. Subsequent testing identified *P. equi* in other horses that had also been treated with the same antitoxin, but not in untreated horses on the same or neighboring farms. Notably, only animals that had received the *P. equi*-contaminated antitoxin developed hepatitis and tested positive for the virus. Furthermore, experimental inoculation of ponies with the implicated antitoxin resulted in virus transmission and hepatitis in two out of four animals, providing strong evidence for the role of *P. equi* in iatrogenic hepatitis and its transmissibility via contaminated biological products (Chandriani, Skewes-Cox et al. 2013).

1.4.1 Morphology and Genomic Organization of *P. equi*

P. equi is a member of the Flaviviridae family (Smith, Becher et al. 2016). Phylogenetic analysis clustered *P. equi* with GB virus–like pegiviruses; investigators have proposed grouping *P. equi* and close relatives within an equine pegivirus species (Chandriani, Skewes-Cox et al. 2013, Tomlinson, Wolfisberg et al. 2020). Pegiviruses are enveloped, single-stranded, positive-sense RNA viruses known to infect a wide range of host species. To date, no pegivirus has been conclusively demonstrated to be pathogenic. Interestingly, some evidence—though controversial—suggests that pegivirus infection may modulate or attenuate disease severity in co-infections with other lymphotropic viruses, such as HIV in humans (Bailey, Buechler et al. 2017,

N'Guessan, Anderson et al. 2017). Pegiviruses generally exhibit tropism for lymphoid tissues and bone marrow (Bailey, Lauck et al. 2015), and *P. equi* appears to follow this pattern, showing a primary affinity for bone marrow. Additionally, experimental intra-splenic transfection with *P. equi* RNA has been shown to induce infection, further supporting its lymphoid tissue tropism (Tomlinson, Van de Walle et al. 2019).

Pegiviruses encode a single polyprotein with conserved nonstructural domains (including an NS5B-like RNA-dependent RNA polymerase), and modeled polymerase structures from related equine hepaci/pegivirus sequences show conserved RdRp folds that are predicted to bind HCV polymerase inhibitors in silico. Structural modeling indicates the NS5B/RdRp fold is conserved enough that HCV nucleoside inhibitor binding modes (e.g., sofosbuvir) may be retained in related equine polymerases, supporting possible cross-reactive antiviral targeting in theory (de Albuquerque, Santos et al. 2020). *P. equi* strains exhibit significant genetic variability, with nucleotide similarities ranging from 90.3% to 93.6% among different isolates (Lu, Sun et al. 2018). This genetic diversity may influence the virus's epidemiological behavior and response to treatments.

1.4.2 Pathogenesis of *P. equi*

TD (Theiler's disease) was long suspected to have a viral etiology because of its frequent association with administration of equine-origin blood products. In 2013, Chandriani et al. used deep sequencing on the serum of affected horses to identify *P. equi* that seemed temporally associated with an outbreak of post-transfusion hepatitis, raising the possibility that *P. equi* might be the etiologic agent of Theiler's disease (Chandriani, Skewes-Cox et al. 2013). Based on this association, experimental inoculation of a *Theileria equi* (*T. equi*)-positive antiserum into naïve

horses was attempted; while viremia was induced and viral RNA detected, the evidence for resultant hepatic injury in those recipient horses was limited, and not all horses exposed developed overt hepatitis (Divers, Tomlinson et al. 2022). The virus is capable of efficient parenteral transmission, leading to both acute and chronic infections, similar to hepatitis C virus (Chandriani, Skewes-Cox et al. 2013). Despite its association with acute hepatitis, *P. equi* infections often remain subclinical, with no consistent biochemical alterations in infected horses (Figueiredo, de Moraes et al. 2019). The lack of consistent liver involvement (Lyons, Kapoor et al. 2014) and the presence of subclinical infections suggest that other factors, such as co-infections or host-specific responses, may influence disease manifestation. Further research is needed to clarify the exact role of *P. equi* in equine hepatitis and its molecular pathogenesis.

1.4.3 Epidemiology of *P. equi*

The epidemiology of *P. equi* reveals significant insights into its prevalence, genetic diversity, and potential risk factors affecting equine populations. Recent studies indicate that *P. equi* is present in various geographical regions, with notable findings from Brazil and China. In Brazil, *P. equi* was detected in 1.6% of 500 serum samples from horses, indicating its presence in multiple states, including Espirito Santo and Rio de Janeiro (Figueiredo, de Moraes et al. 2019). In China, genomic sequencing identified multiple *P. equi* strains, suggesting a broader distribution and genetic diversity of the virus (Lu, Sun et al. 2018). Younger horses (≤5 years) showed a higher likelihood of *P. equi* infection, with a 2.7 times increased risk compared to older horses (Figueiredo, de Moraes et al. 2019). Because *P. equi* is not widely recognized as a primary cause of severe liver disease in horses, knowledge of its epidemiology remains fragmentary and poorly characterized.

The aims for conducting this study arose from the growing recognition of newly identified equine viruses—Equine parvovirus-hepatitis (EqPV-H), Equine hepacivirus (EqHV), *P. caballi*, and *P. equi*—and the lack of comprehensive epidemiological data describing their prevalence, distribution, and host associations within the United States. Although these viruses have been detected worldwide and implicated in hepatic or persistent infections, their occurrence in U.S. equine populations, especially across southern states, had not been systematically examined. This study therefore sought to fill a critical gap by establishing a molecular and epidemiological overview of these viruses using field samples from multiple geographic locations.

Another reason for undertaking this research was the clinical uncertainty surrounding TD, which is the acute form of equine hepatitis. Despite increasing evidence implicating EqPV-H as a probable etiologic agent, the presence of other hepatotropic viruses—such as EqHV and the pegiviruses—complicates diagnostic interpretation. Understanding the relative prevalence and viral load of each virus was necessary to clarify their potential roles in hepatic pathology and to guide veterinarians in interpreting diagnostic outcomes in TD-like cases.

Moreover, molecular characterization of these viruses contributes to strengthening diagnostic and surveillance capacity in veterinary medicine. Reliable detection assays for EqPV-H, EqHV, and pegiviruses are essential not only for clinical diagnostics but also for the screening of biological products, donor sera, and breeding animals to prevent iatrogenic transmission. This study provides region-specific prevalence data and validates molecular tools that can inform these practices.

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Chapter 2. Epidemiology and Risk Factors of Equine Parvovirus-Hepatitis, Hepacivirus, *Pegivirus caballi*, and *Pegivirus equi* in U.S. Horses

2.1 Abstract

Emerging equine viruses, including equine parvovirus-hepatitis (EqPV-H), equine hepacivirus (EqHV), *Pegivirus* (*P.*) *caballi*, and *P. equi*, pose potential risks to equine health. However, comprehensive epidemiological data in the United States remain limited. This study analyzed 1,195 equine serum samples collected from university-owned horses and diagnostic submissions across Alabama, Georgia, and Texas. Quantitative PCR assays were conducted to detect EqPV-H, EqHV, *P. caballi*, and *P. equi*. EqPV-H was the most prevalent virus, detected in 19.3% (231/1,195) of samples, significantly higher than EqHV at 5.6% (67/1,195) and pegiviruses (*P. caballi* and *P. equi* combined) at 1.8% (22/1,195). EqPV-H-positive horses also exhibited significantly higher viral loads compared to animals positive for EqHV or pegiviruses. Demographic analysis revealed that EqPV-H-positive horses were significantly older, and male horses had 1.62 times the odds of infection compared to females. Breed-specific associations were also identified: Tennessee Walking Horses had higher odds of EqPV-H positivity (OR = 2.46), while Quarter Horses (OR = 4.16) and Thoroughbreds (OR = 9.64) showed increased odds of testing positive for EqHV. Viral sequences identified in this study were similar to the reported ones in the United States and other regions. This largest molecular survey highlights the widespread distribution of EqPV-H and EqHV in horses in the United States and underscores the importance of continued surveillance, particularly in high-risk breeds and settings. The data provides a foundation for developing preventive strategies and understanding of the epidemiology and potential clinical impact of these emerging equine viruses.

2.2 Introduction

Theiler's disease (TD), or equine serum hepatitis, is a major cause of acute liver failure in adult horses (Papapetrou, Arroyo et al. 2023). Emerging equine viruses pose significant challenges to equine health, particularly those associated with liver disease and subclinical infections. Among these, equine parvovirus-hepatitis (EqPV-H) has gained attention as a causative agent of hepatitis in horses. In 2018, EqPV-H was linked to a TD case following tetanus antitoxin treatment, suggesting an association (Divers, Tennant et al. 2018). EqPV-H-contaminated biologics are strongly associated with the two (Messer and Johnson 1994, Tomlinson, Kapoor et al. 2019, Vengust, Jager et al. 2020, Divers, Tomlinson et al. 2022). Cases also occur without biologic exposure (Tomlinson, Kapoor et al. 2019), indicating possible horizontal transmission. EqPV-H is hepatotropic and linked to both clinical and subclinical TD (Baird, Tegtmeyer et al. 2020, Tomlinson, Jager et al. 2020). The virus's environmental stability, asymptomatic carriage, and frequent iatrogenic exposure through biologics contribute to its widespread distribution and highlight its importance in equine health management.

Equine hepacivirus (EqHV) is a single-stranded RNA virus related to the human hepatitis C virus. It naturally infects horses and is primarily hepatotropic (Burbelo, Dubovi et al. 2012, Pacchiarotti, Nardini et al. 2022). A meta-analysis of biomolecular prevalence estimated a global average of 6.9% [95% confidence interval (CI): 5.4–8.5) (Pacchiarotti, Nardini et al. 2022). Its global prevalence, subclinical nature, and potential for transmission in breeding and racing populations underscore the need for continued epidemiological surveillance.

Two additional equine viruses, *Pegivirus caballi* (*P. caballi*) and *Pegivirus equi* (*P. equi*), belong to the genus *Pegivirus* in the family *Flaviviridae*. Over the past decade, their nomenclature has changed to reflect the growing understanding of equine pegiviruses and their relationship to

other flaviviruses (Postler, Beer et al. 2023). *P. caballi*, previously referred to as equine pegivirus type 1 (EPgV-1) (Kapoor, Simmonds et al. 2013), is detected in 1–4% of healthy equine blood donors, with an additional 5–13% showing seropositivity. In developing countries, the prevalence of viremia can reach up to 20% (Polgreen, Xiang et al. 2003, Mohr and Stapleton 2009, Stapleton, Foung et al. 2011), suggesting potential transmission risks through blood products or iatrogenic procedures.

In a study conducted in the United States, it was identified that *P. equi,* formerly known as Theiler's disease-associated virus (TDAV), was identified during an outbreak of acute serum hepatitis (Theiler's disease) in horses following equine biologic administration (Chandriani, Skewes-Cox et al. 2013).

Tomlinson et. al. (2020) developed full-length molecular clones of *P. caballi* and *P. equi* and successfully initiated long-term infections in horses using synthetic viral material. However, no signs of hepatitis were observed, raising doubts about the link between TDAV and liver disease in horses (Tomlinson, Wolfisberg et al. 2020). Regardless, continued monitoring and molecular characterization are critical to clarify its pathogenic potential and to ensure the safety of equine biologics.

Despite increasing recognition of these viruses, information about their prevalence, epidemiological patterns, and associated risk factors in the United States remains limited. Understanding the distribution and host factors associated with EqPV-H, EqHV, *P. caballi,* and *P. equi* is essential for assessing their impact on equine health and informing strategies for prevention and surveillance. This study aimed to provide a comprehensive molecular survey of these four emerging equine viruses, identify potential host-related risk factors, and characterize their prevalence across diverse horse populations in the United States.

2.3 Materials and methods

2.3.1 Equine serum samples

A total of 1,195 equine serum samples used in this study were collected from diagnostic submissions and surveillance programs in Alabama between 2022–2025 (n = 574), Georgia (n = 200), and Texas (n = 421). The samples from Texas included 234 samples provided from the Texas A&M Veterinary Medical Diagnostic Laboratory and 187 horses owned by Texas A&M University, from which serum samples were collected for annual testing for serological testing for equine infectious anemia virus infection. Each sample was assigned a unique identifier, including state of origin, age, sex, and breed, which were recorded in a standardized Excel file.

2.3.2 PCRs to Quantify EqPV-H, EqHV, *P. caballi*, and *P. equi*

Total nucleic acids were extracted from 200 µL of equine serum using an automated magnetic bead–based system (Indical Biosciences, Germany), yielding 100 µL of eluent.

Representative genome sequences of EqPV-H, EqHV, *P. caballi*, and *P. equi* were retrieved from GenBank and aligned using Vector NTI software v11.5 (Invitrogen, Carlsbad, CA, USA). Primers and probes were designed to target conserved regions of the non-structural protein gene for EqPV-H (amplicon size = 144 bp), the polyprotein gene for EqHV (amplicon size = 153 bp), the polyprotein gene for *P. caballi* (amplicon size = 187 bp), and the non-structural protein 3 gene for *P. equi* (amplicon size = 144 bp) (Table 1).

Table 1: **Oligonucleotides used in this study**

Targets		Oligonucleotide sequences (5'-3')	Gene target & amplicon size
EqPV-H	forward primer	ATCCTGGTGGGGAGGGAGTAA	Non-structural protein; 144 bp
	reverse primer	CATGGCGTGATGTGCACTACC	
	probe	6-FAM/CAAACACGTCGCTGCATTCTKAGTCC/IABkFQ	
EqHV	forward primer	GCAGGTCGAGGGAGCTGAAA	Polyprotein; 153 bp
	reverse primer	CAGACCGTGGGATAAAGGGG	
	probe	CY5/GA CCTTCGGAGCCGAAATTTGGG/IABRQSP	
P. caballi	forward primer	CACCCRCGGATCACRGAGATT	Polyprotein; 187 bp
	reverse primer	GACCAGAATAGTGGTAAAKTGCACG	
	probe	6-FAM/ACTGCTGAGGGGGACATTCCCTT/IABkFQ	
P. equi	forward primer	YGAGGTACACTCGGCTCTCTTTGT	Non-structural protein 3; 164 bp
	reverse primer	GCAGGGACAAGGGTGGACTTG	
	probe	/Cy5/TACGAGGAGMGATCCTTGTTYTTGCC/IAbRQSp	

Primers and hydrolysis probes were pre-combined into 5× oligonucleotide mixes to streamline reaction setup and ensure consistency across assays. For simplex assays (EqHV and EqPV-H), the final working concentrations of the forward and reverse primers were 5 µM each, and the probe was 1 µM. For the duplex assay (*P. caballi* and *P. equi*), the two primer pairs were each adjusted to 5 µM and both probes to 1 µM in the same mix. All oligonucleotide mixes were prepared in 1× TE buffer, vortexed thoroughly, centrifuged briefly, and maintained on ice until use. All reactions were performed in a total volume of 20 µL, consisting of 10 µL extracted nucleic acid template and 10 µL of master mix. Negative template controls (NTCs, nuclease-free water) and positive control templates (gBlock; DNA) were included on each run.

For RNA viruses (EqHV, *P. caballi* and *P. equi*), reactions were performed using an RT-TaqMan master mix, which incorporated ThermoScript reverse transcriptase for cDNA synthesis.

PCR assays were performed using a LightCycler® 96 real-time PCR system (Roche Diagnostics, Indianapolis, IN, USA), following protocols described previously for EqPV-H and EqHV as RNA viruses (Barua, Bai et al. 2022) and for *P. caballi* and *P. equi* as DNA viruses (Tarannum, Barua et al. 2025).

Here, we utilized a hydrolysis probe (TaqMan) detection format. For RNA templates, reactions were performed in a 10 µL final volume using a one-step RT-TaqMan (Reverse Transcriptase TaqMan) protocol. Each reaction contained 3.39 µL of nuclease-free water, 4.4 µL of 5× FRET buffer, 1.1 µL of 5× oligonucleotide mix (duplex oligos for *P. caballi* and *P. equi*, simplex oligos for EqHV), 0.44 µL of PCR nucleotide mix, 0.44 µL of DreamTaq DNA polymerase (high-fidelity), and 0.23 µL of ThermoScript reverse transcriptase (diluted 1:140).

For DNA templates (EqPV-H), a 10 µL TaqMan PCR master mix was prepared consisting of 3.62 µL of nuclease-free water, 4.4 µL of 5× FRET buffer, 1.1 µL of 5× oligonucleotide mix, 0.44 µL of PCR nucleotide mix, and 0.44 µL of DreamTaq DNA polymerase (high-fidelity).

Cycling conditions were optimized following the manufacturer's TaqMan assay guidelines, with modifications for the target viruses in this study. The PCR cycle condition was an initial reverse transcription at 55 °C for 10 min, followed by an initial denaturation at 95 °C for 2 min; 30 cycles of 95 °C for 10 s, 57 °C for 10 s, and 72 °C for 10 s; and a final cooling step at 40 °C for 30 s. The PCR cycle condition for EqPV-H was an initial denaturation at 95 °C for 2 min; 30 cycles of 95 °C for 10 s, 57 °C for 10 s, and 72 °C for 10 s; and a final cooling step at 40 °C for 30 s.

The sensitivity of the assays was verified using four gBlock gene fragments containing the PCR amplicons, synthesized by Integrated DNA Technologies (Coralville, IA, USA). Based on the molecular weight of each gBlock fragment, 10-fold serial dilutions ranging from 10^4 to 10^0 copies per 10 µL reaction were prepared in triplicate to determine the detection limit.

PCR products derived from both gBlock fragments and clinical samples were submitted to ELIM Biopharmaceuticals (Hayward, CA, USA) for bidirectional Sanger sequencing. The nucleotide sequences obtained from clinical samples were compared to existing genomes using BLASTn (https://blast.ncbi.nlm.nih.gov/).

2.3.3 Statistical Analysis

All statistical analyses were conducted using RStudio 2023.12.1+402 with the packages *tidyverse*, *gtsummary*, *emmeans*, and *broom*. Age was treated as a continuous variable, while sex, breed, and state were treated as categorical variables. Breeds with fewer than 20 observations were grouped into an "Other" category. Categorical variables were summarized as frequencies and percentages, while continuous variables were presented as means ± standard deviations. Between-group comparisons were performed using Chi-square or Fisher's exact test for categorical variables and Student's *t*-test for continuous variables.

Univariable and multivariable logistic regression models were used to assess associations between age, gender, and breed with viral detection for each virus. Models were fitted using generalized linear models (binomial family, logit link). All predictors were included in multivariable models. Odds ratios (ORs) and 95% confidence intervals (CIs) were reported. Model fit was evaluated using AIC and plotting the models.

Pairwise Fisher's exact tests with Bonferroni correction were used to compare viral prevalence, while pairwise Wilcoxon rank-sum tests with Benjamini–Hochberg adjustment were used to compare viral burdens. A p-value < 0.05 was considered statistically significant.

2.3 Results

The horses included in this study had an average age of 12.1 years (SD = 7.7), with ages ranging from 1 day to 40 years. Of the total population, 55.2% were male and 44.8% were female. Samples were submitted from horses representing 18 distinct breeds, most commonly the Quarter Horse (n = 563), followed by Thoroughbred (107), Warmblood (91), Tennessee Walking Horse (71), Paint Horse (61), Draft breeds (25), Miniature (16), Saddlebred (22), Arabian (14), mule (13), Appaloosa (12), Friesian (12), Mustang (11), Gypsy Vanner (9), Standardbred (8), Haflinger (7), and Andalusian (5). Additionally, there were samples from mixed-breed horses (n = 40), pony breeds (31), and other less common or unspecified breeds (22).

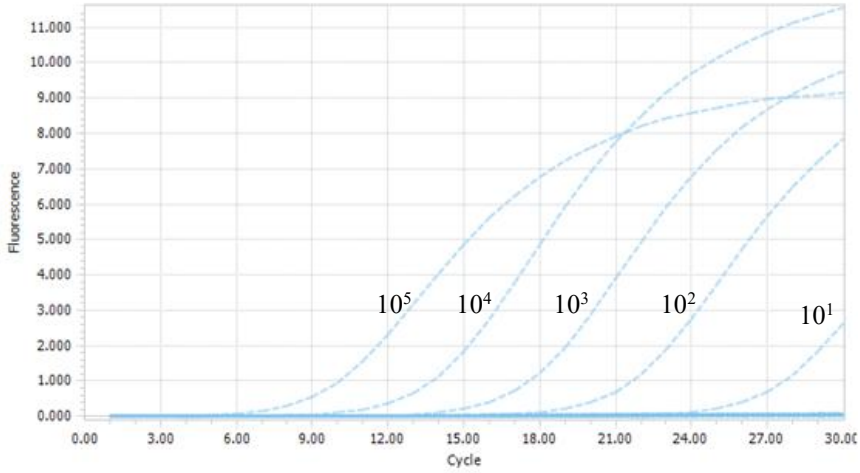

Figure 1: The sensitivity of qPCR to detect equine hepacivirus. The plasmid quantitative standard (10^5, 10^4, 10^3, 10^2, 10^1 / reaction) containing sequence of equine hepacivirus virus and the PCR negative control were detected by the one-step RT-TaqMan PCR established in this study. The detection limit was found to be ten copies per PCR reaction for EqHV and *P. equi* and one copy per PCR reaction for EqPV-H and *P. caballi*.

All four PCR assays used in this study demonstrated a detection limit of one copy per reaction for EqPV-H and *P. cabalii*; and 10 copies per reaction for EqHV and *P. equi*. The specificity of the PCRs for both DNA and RNA viruses was confirmed by DNA sequencing with the PCR product and BLASTn analysis of the obtained nucleotide sequences.

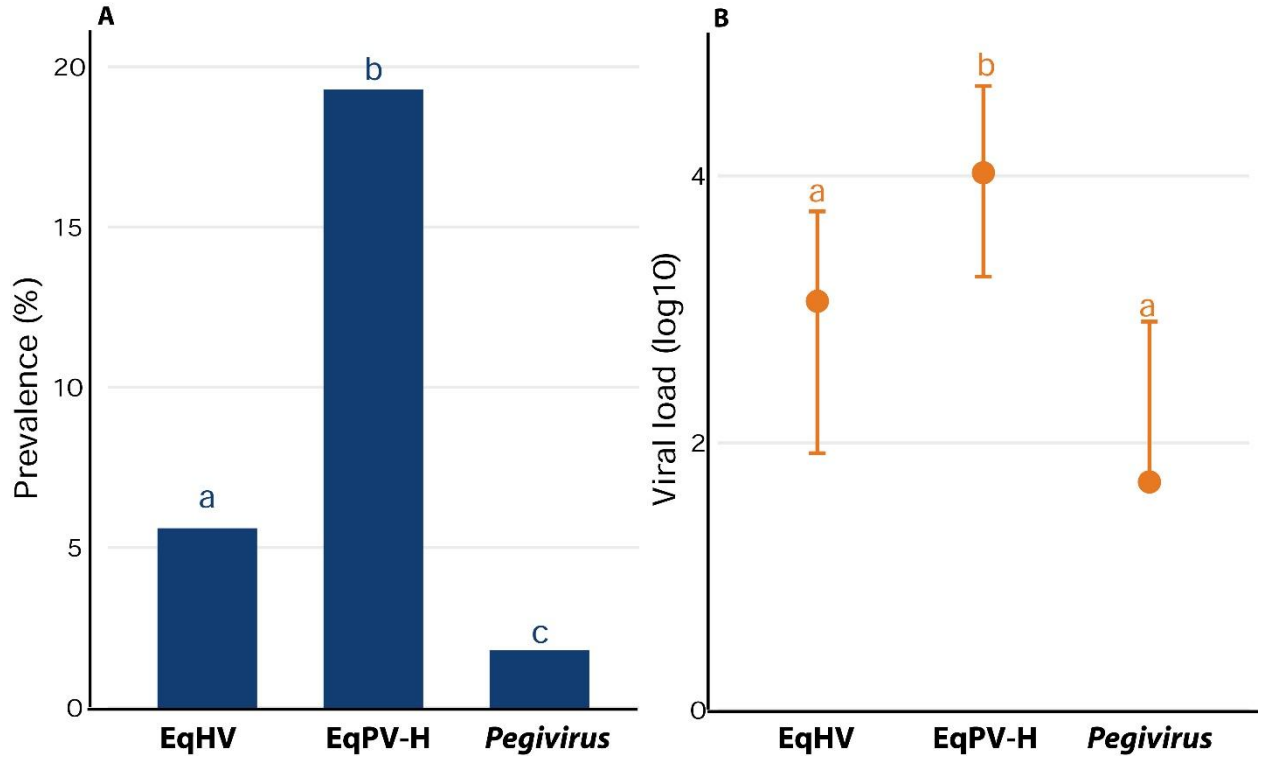

Figure 2: Viral prevalence and viral load among tested equine samples. Prevalence is shown as the percentage of positive samples among the total tested (blue bars). Viral load is shown as log10-transformed median viral load per mL (orange points) with interquartile range (IQR). Superscript letters above the bars indicate statistically significant differences in prevalence based on pairwise Fisher's exact tests with Bonferroni correction, and symbols above the points indicate statistically significant differences in viral load based on pairwise Wilcoxon rank-sum tests with Benjamini-Hochberg adjustment. Viruses sharing the same letter or symbol are not significantly different, whereas different letters or symbols indicate $p < 0.05$. (**A**). Global tests for overall differences were performed using the chi-square test for prevalence and the Kruskal-Wallis test for viral load (global p-values reported in the text). The overall prevalence differed significantly among viruses (χ^2 test, $p < 0.001$). EqPV-H was the most detected virus (19.3%), whereas EqHV and pegiviruses had lower prevalence (5.6% and1.8%, respectively). Pairwise comparisons indicated that EqPV-H prevalence was significantly higher than that of EqHV and pegivirus, while EqHV and pegiviruses did not differ significantly from each other. (**B**). Among positive samples, viral load also varied significantly between viruses (Kruskal-Wallis test, $p < 0.001$). Median viral loads were highest for EqPV-H [10,545 (1757.75-47110) copies/mL], being significantly higher than EqHV [(1,150 (82.8-5417.5)] and pegiviruses [50 (50-808)].

The prevalence of EqPV-H was 19.3% (231/1,195), which was significantly higher than the prevalence of EqHV at 5.6% (67/1,195) and pegiviruses (*P. caballi* 20/1,195; *P. equi* 2/1,195) at 1.8% (Figure 1). Similarly, viral loads (copies per ml of serum) were higher in EqPV-H-positive animals [10,545 (1,757.75–47,110)] compared to EqHV [1,150 (82.8–5,417.5)] and pegiviruses [50 (50–808)] (Figure 1). The prevalence of EqPV-H, EqHV, and *P. caballi* did not differ among samples from Alabama, Georgia, and Texas, while two *P. equi*-positive cases were identified in horses from Georgia.

EqPV-H-positive animals were significantly older than negative animals (15 ± 6 vs. 11 ± 8 years, $p < 0.001$). Each additional year of age was associated with a 7% increase in the odds of testing positive (OR = 1.07; 95% CI: 1.05–1.09; $p < 0.001$). In addition, a higher proportion of males tested positive compared to females (65% vs. 35%, $p < 0.001$). The odds of male horses testing positive were 1.6-fold higher compared to females (95% CI: 1.18–2.23; $p = 0.003$). Furthermore, Tennessee Walking Horses had significantly higher odds of EqPV-H positivity compared to other breeds, with an adjusted OR of 2.46 (95% CI: 1.26–4.8; $p = 0.008$). Thoroughbreds also showed increased odds (OR = 2.1; 95% CI: 1.1–3.9; $p = 0.019$).

There were no significant differences in sex distribution ($p = 0.8$) or age ($p = 0.6$) between EqHV-positive and -negative animals. Compared to other breeds, Quarter Horses had increased odds of testing positive for EqHV (OR = 4.2; 95% CI: 1.5–17.4; $p = 0.019$), and Thoroughbreds had significantly higher odds as well (OR = 9.6; 95% CI: 3.1–42.6; $p < 0.001$).

The prevalence of *P. caballi* was 1.7% (20/1,195). The median age of *P. caballi*-positive animals was slightly higher than that of negative animals (13 ± 9 vs. 12 ± 8 years), although this difference was not statistically significant ($p = 0.4$). There was no significant association between sex and *P. caballi* status, with similar distributions of males and females across groups ($p = 0.6$).

Only two horses were positive for *P. equi*, with a prevalence of 0.2% (2/1,195), and both originated from horses in Georgia.

NCBI BLASTn and MEGA (ClustalW) sequence alignment analysis was performed to compare nucleotide sequences obtained in this study with known viral genomes (Kumar S, Stecher G, Suleski M, Sanderford M, Sharma S, and Tamura K (2024) for MEGA12). The EqPV-H sequences showed 0 to 2 nucleotide differences (99–100% identity) in the targeted structural protein gene region when compared our study sequences to the reference EqPV-H genome from the United States (GenBank accession NC_076001.1). A similar analysis of EqHV nucleotide sequences from this study revealed 95–97% similarity to a sequence previously reported in the United States (GenBank accession KJ472766.1), with 6 to 13 nucleotide differences identified.

Similarly, *P. caballi* sequences from this study shared 95–97% nucleotide identity with sequences from the United States (GenBank accessions MT276217.1, MT276214.1), with 6 to 9 nucleotide differences across the aligned regions. Likewise, *P. equi* sequences demonstrated 95% similarity to a reference sequence (GenBank accession KY922942.1), with up to eight nucleotide differences observed across all available sequences.

2.5 Discussion

In this largest molecular survey to date of four equine viruses potentially associated with hepatitis, we found significantly higher prevalence and viral burdens of EqPV-H compared to EqHV, *P. caballi*, and *P. equi*, and identified potential risk factors—including age, sex, and breed—linked to these infections.

The prevalence of EqPV-H positivity in this study was 19.3% (231/1,195), being consistent with 13.0% (13/100) in New York, USA (Divers, Tennant et al. 2018). Other global reports in clinically healthy horses from Canada, China, Germany, Austria, Brazil, South Korea, France, and Australia have reported prevalence rates ranging from 3.2% to 19.8% (Lu, Sun et al. 2018, Meister, Tegtmeyer et al. 2019, Wu, Ou et al. 2020, Reinecke, Klohn et al. 2021, Yoon, Park et al. 2021, Badenhorst, de Heus et al. 2022, de Moraes, Salgado et al. 2022, Yoon, Park et al. 2022, Papapetrou, Arroyo et al. 2023). Together, these findings support the global distribution of EqPV-H and suggest that the virus is relatively widespread in equine populations. The relatively high prevalence may reflect its association with equine biologics, environmental resilience, and targeted surveillance efforts (Divers, Tennant et al. 2018). The comparable prevalences identified in the studied states provide initial evidence to suspect consistent spatial distribution at least in the Southern United States, and further studies are needed to confirm to estimate the prevalence in the rest of the country.

For EqPV-H, breed, age, and sex were all significantly associated with infection in this study. Tennessee Walking Horses ($p = 0.008$) and Thoroughbreds ($p = 0.019$) showed higher odds of infection compared to other breeds. In China, a high prevalence of EqPV-H was reported in imported Thoroughbreds, with 52.9% (9/17) testing positive (Lu, Sun et al. 2018). Similarly, in South Korea, the highest EqPV-H DNA prevalence was observed in breeding horses imported from

the United States, with 47.8% (11/23) testing positive (Yoon, Park et al. 2022). However, other studies did not identify breed as a significant risk factor (Yoon, Park et al. 2022, Papapetrou, Arroyo et al. 2023). Notably, Thoroughbreds have consistently shown higher EqPV-H viremia rates worldwide compared to other breeds (Pfaender, Cavalleri et al. 2015, Postel, Cavalleri et al. 2016). This trend may reflect genetic susceptibility or increased exposure associated with frequent international transport, intensive breeding practices, and medical interventions such as the use of orthobiologics and vaccinations (Tomlinson, Kapoor et al. 2019), all of which could contribute to elevated infection risk.

In this study, EqPV-H–positive animals were significantly older than negative animals ($p < 0.001$). Age has also been positively associated with EqPV-H detection in breeding horse populations in other studies (Badenhorst, de Heus et al. 2022, Yoon, Park et al. 2022, Papapetrou, Arroyo et al. 2023). Breeding horses may be at increased risk due to iatrogenic or natural transmission during the breeding process, which could expose them to contaminated instruments or semen as potential sources of infection. Furthermore, the persistence of EqPV-H viremia in the absence of clinical signs (Reinecke, Klohn et al. 2021) suggests cumulative exposure over time, particularly in older animals.

Our results showed that males had a significantly higher prevalence of EqPV-H infection than females ($p < 0.001$). A study in South Korea similarly reported a sex-related difference, with males showing higher infection rates (Lu, Wu et al. 2020, Lee, Park et al. 2021). However, the underlying reason for this pattern remains unclear, and the association between sex and EqPV-H infection warrants further investigation. In contrast, other studies, including those conducted in racehorses from New York racetracks, found no significant association between sex and EqPV-H infection (Badenhorst, de Heus et al. 2022, Jager, Tomlinson et al. 2022).

The estimated prevalence of EqHV was 5.6%. EqHV infections in this study have been reported worldwide, including in Europe (Lyons, Kapoor et al. 2012, Pfaender, Cavalleri et al. 2015, Postel, Cavalleri et al. 2016), Africa (Abbadi, Lkhider et al. 2021), the Americas (Gemaque, Junior Souza de Souza et al. 2014, Figueiredo, Lampe et al. 2015, Figueiredo, Lampe et al. 2018, Tomlinson, Kapoor et al. 2019), Asia (Matsuu, Hobo et al. 2015, Wu, Ou et al. 2020, Yoon, Park et al. 2022), and Oceania (Fortier, El-Hage et al. 2024). A meta-analysis of biomolecular prevalence (Pacchiarotti, Nardini et al. 2022) estimated a global average prevalence of 6.9% (95% CI: 5.4–8.5), though substantial heterogeneity was observed across regions, with Europe showing notably lower prevalence than other continents. A study in upstate New York, USA, utilizing a small number of samples from clinically affected horses, reported that 14.3% (2/14) of the serum samples were positive for EqHV (Tomlinson, Kapoor et al. 2019). However, a study in the same state utilizing a larger set of samples found EqHV RNA in 7.8% (8/103) of samples, which aligns with the prevalence estimated in the current study (Burbelo, Dubovi et al. 2012).

For EqHV, breed was the only significant risk factor, with Quarter Horses ($p = 0.019$) and Thoroughbreds ($p = 0.001$) showing higher odds of infection compared to other breeds, while age and sex were not significantly associated with infection in this cohort. Thoroughbreds, in particular, have consistently demonstrated higher EqHV viremia rates across multiple studies (Matsuu, Hobo et al. 2015, Postel, Cavalleri et al. 2016, Kim, Moon et al. 2017, Reichert, Campe et al. 2017, Badenhorst, Tegtmeyer et al. 2018). Interestingly, none of the 54 serum samples from indigenous Jeju horses tested positive for either EqPV-H or EqHV DNA (Yoon, Park et al. 2022). Racehorses, such as Thoroughbreds, may show higher EqHV positivity due to genetic predisposition or increased exposure associated with international transport, intensive breeding practices, and medical interventions (Pfaender, Cavalleri et al. 2015, Postel, Cavalleri et al. 2016).

In our study, age was not identified as a risk factor for EqHV infection. However, its role remains uncertain, as previous reports have shown conflicting results (Gather, Walter et al. 2016, Kim, Moon et al. 2017, Wu, Ou et al. 2020, Yoon, Park et al. 2022).Similarly, no significant difference in sex distribution was observed between EqHV-positive and -negative animals ($p = 0.8$). While some studies have reported higher viremia in females (Figueiredo, Lampe et al. 2018, Wu, Ou et al. 2020, Abbadi, Lkhider et al. 2021), others have found higher rates in males (Figueiredo, Lampe et al. 2015). These discrepancies may be influenced by factors such as transportation, breeding practices, and increased contact with other horses (Pacchiarotti, Nardini et al. 2022, Yoon, Park et al. 2022).

The overall prevalence of *P. caballi* in this study was 1.7%, while only two cases tested positive for *P. equi*. Globally, the molecular prevalence of *P. caballi* ranges from 1 to 32% (Chandriani, Skewes-Cox et al. 2013, Kapoor, Simmonds et al. 2013, Lyons, Kapoor et al. 2014, de Souza, Malheiros et al. 2015, Lu, Sun et al. 2016, Figueiredo, de Moraes et al. 2019). In contrast, *P. equi* has been reported much less frequently since its initial identification (Chandriani, Skewes-Cox et al. 2013, Figueiredo, de Moraes et al. 2019).

The higher prevalence of EqPV-H compared to EqHV and pegiviruses (*P. caballi* and *P. equi*) in this study likely reflects several factors. EqPV-H is often transmitted through contaminated equine-derived biologics, such as tetanus antitoxin and plasma, making iatrogenic spread a major route (Divers, Tennant et al. 2018, Meister, Tegtmeyer et al. 2019, Tomlinson, Kapoor et al. 2019, Tomlinson, Jager et al. 2020). As a non-enveloped virus, it is more environmentally stable, and infected horses frequently remain asymptomatic carriers, facilitating silent transmission. In contrast, EqHV and pegiviruses mainly cause subclinical infections and are less commonly associated with disease, which may reduce surveillance intensity. Additionally, many EqPV-H

studies focus on high-risk populations, potentially inflating prevalence estimates relative to broader studies of EqHV and pegiviruses (Jager, Tomlinson et al. 2022).

This study has limitations. First, to ensure high sensitivity, the PCR assays in this study were designed with short amplicon sizes. While this approach maximizes detection, it limits the ability to fully characterize the viral genomes. Future studies using whole genome sequencing and other advanced tools are needed to analyze longer genomic regions, which would allow comparison with global viral strains and a more comprehensive understanding of viral diversity.

Second, this study relied on convenience samples, which may not fully represent the broader equine population in the United States. Implementing a study design with geographically and demographically representative samples would provide more accurate prevalence estimates and allow for more reliable identification of risk factors associated with these infections.

Finally, this investigation was a molecular survey, and clinical data on the infected horses were limited. Correlating positive cases with health status—such as the presence or absence of hepatitis—would help clarify the clinical significance of these infections and improve understanding of the pathogenesis of these viruses.

In conclusion, EqPV-H is the most prevalent emerging equine virus in the United States, followed by EqHV and *P. caballi*, with *P. equi* detected only rarely. Breed, age, and sex might influence EqPV-H infection, while EqHV appears to be associated with breed, particularly Thoroughbreds and Quarter Horses. Pegiviruses showed no clear host-related risk factors, though geographic clustering was noted for *P. caballi*. These findings highlight the need for continued surveillance and targeted preventive measures, particularly for EqPV-H, which poses the greatest potential risk to equine health.

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