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Equine hepacivirus (EqHV) phylogeny, infectivity profile and entry in Thoroughbred racehorses

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Abstract

Hepatitis C virus (HCV) causes chronic and acute liver diseases in humans. This virus's origin is unknown, and many research works into the design of a prophylactic vaccine have been inhibited due to the lack of a tractable model, partly due to its narrow host range (humans and chimpanzees). Equine Hepacivirus (EqHV) in 2011 was discovered in respiratory samples of dogs and found to be the closest genetic relative to Hepatitis C virus (HCV). This virus naturally infects horses and has several similarities to HCV, ranging from delayed onset of seroconversion, persistent infection, and liver pathology, thereby making it a potential experimental model to study hepacivirus infections in their natural host.

This study investigated the prevalence of EqHV among Thoroughbred racehorses. Polymerase reaction (PCR) assays were designed to detect and quantify this virus in 66 Thoroughbred racehorses' serum samples. Approximately 38% of these horses were positive for the virus with a viral load range between $6.19 \times 10^2 - 1.26 \times 10^7$copies/mL. Using retrospective sera samples sampled at different time points, we further investigated this virus's infection profile among Thoroughbred racehorses. The results showed, similar to HCV that EqHV causes acute and chronic infections and that infected animals are susceptible to reinfection with varying seroreactivity degrees.

Further analysis of these sera showed diversification of these viral populations among Thoroughbred racehorses. Also, multiple signatures of vector-borne transmission and immune-mediated selection of viral variants were observed. Optimisation of an EqHV entry assay utilising retrovirus pseudotypes was performed. The stability of these virus particles was also investigated.

Publications

Barnabas King, **Terry Akagha**, C. Patrick McClure, Mascha Söhrmann, Mirelle Radley, Stuart Astbury, Bethany Edwards, Janet Daly, Julia Kydd, and Alexander Tarr. (Submitted) "Molecular analysis of equine hepacivirus in Thoroughbred racehorses suggests vector-borne horizontal transmission".

Patrick McClure, Gemma Clark, Akhil Chellapuri, Matthew Smitheman, Arwa Bagasi, **Terry Akagha**, Tasneem Khandaker, et al. (Accepted) Wombling surplus diagnostic nucleic acid for novel pathogenesis and genetic epidemiology of viral infections. Access Microbiology, 1(1A) 2019, 697.

Terry Akagha, Barnabas King, and Alexander Tarr. (In preparation) "Equine hepacivirus (EqHV): The story so far".

Presentation and Posters

School of Life Science Post-graduate Symposium oral presentation

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"Equine hepacivirus vs Hepatitis c virus: The story so far."

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"Equine hepacivirus vs Hepatitis c virus: The story so far."

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Prizes

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1st Runner up, Best Oral presentation prize

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Declarations

I hereby declare that this thesis is my work and effort and has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged. I was not involved in the collection of either serum samples or experiments with the Thoroughbred racehorses. Source of initial samples used for nuclei acid extractions and further analysis, presented in this thesis, are acknowledged in the text.

Signature: 

Date: 2nd November 2020

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Abbreviations

%GC - Percentage of GC content

(SH)-aLRT - Shimodaira-Hasegawa approximate likelihood ratio test

A - adenine

Ab - Antibody

aLRT - approximate likelihood ratio test

ALT - Alanine transaminase

ALT - Serum alanine transaminase

AST - Aspartate aminotransferase

B. Taurus: Bos Taurus

BLAST: Basic Alignment Search Tool

C: cytosine

C. jacchus - Callithrix jacchus

CD - Cluster of differentiation

cDNA - complementary DNA

C-E1 - Core-E1

CHIKV - chikungunya virus

CHV - Canine hepacivirus

DENV-1 - dengue virus genotype 1

DMEM - is a modification of Basal Medium Eagle (BME)

dN/dS - the ratio of the number of nonsynonymous substitutions per nonsynonymous site (pN) to the number of synonymous substitutions per synonymous site (pS)

DORs - Dinucleotide odds ratios

E1E2 - Envelope 1 Envelope 2 glycoprotein

EPgV - Equine Pegivirus

Eq. caballus - Equus caballus

EqHV - Equine hepacivirus

EqPV- H - Equine Parvovirus with hepatitis

F - forward

FASTA - Fast Alignment search tool

FBS - Fetal Bovine Serum

G - Guanine

GGT - Gamma-glutamyl transpeptidase

GLDH - Glutamate dehydrogenase

GNA - Galanthus nivalis lectin

H - Horse

H. sapiens - Homo sapiens

HBV - Hepatitis B virus

HCV - Hepatitis C virus

HEK293T or HEK cells - Human Embryonic Kidney cells

HIV - Human immunodeficiency virus

HVM - hepacivirus M

HVN - hepacivirus N

ICTV - International Committee on Taxonomy of Viruses

IRES - internal ribosomal entry site

Kb - Kilobase

LB - Luria-Bertani

LDH - lactate dehydrogenase

MAVS - Mitochondrial antiviral-signalling protein

MDA5 - Melanoma differentiation-associated protein 5

miR - microRNA

mL - maximum likelihood

n/s - not significant

NEAA - Non-Essential Amino Acids

NEH - Newmarket Equine Hospital

NKV - No known vector

NPHV - non-primate hepacivirus

NS - Non-structural

Nt - Nucleotide

OF - Outer forward

Opti-MEM - Reduced Serum Media (a modification of Eagle's Minimum Essential Media)

OR - Outer reverse

ORF - open reading frame

PBS - phosphate buffer saline

PCR - Polymerase chain reaction

PEI - polyethyleneimine

Pmol - picomole

pNPP - p-Nitro phenyl phosphate

qPCR - quantitative polymerase chain reaction

R - reverse

RACE - Rapid –amplification of cDNA ends

Real-time PCR - real-time polymerase chain reaction

RIG-I - Retinoic acid-inducible gene I

RLU - relative light units

RNA - Ribonucleic acid

RPM - Revolutions per minute

RT-PCR - Reverse transcription-polymerase chain reaction

Rubella - Rubella virus

SDH - sorbitol dehydrogenase

SIV - Simian immunodeficiency virus

SL - Stem-loop

TAE - Tris-Acetate-EDTA

TDAV - Theiler's disease-associated virus

TRIF - (TIR (Toll/IL-1 receptor) domain-containing adaptor protein)

U - uracil

UK - United Kingdom

USA - United States of America

UTR - untranslated region

VSV - Indiana vesiculovirus, formerly Vesicular stomatitis Indiana virus

µL - microliter

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1 Introduction

1.1 Viral hepatitis

Hepatitis, defined as inflammation of the liver, has several aetiologies, ranging from viral infections (hepatitis A-E, Epstein-Barr virus, Cytomegalovirus and herpes simplex virus), toxins (drugs (e.g. Isoniazid, amiodarone, halothane, and methyldopa)) and alcohol), to certain medical conditions which include essential mixed cryoglobulinemia, autoimmune hepatitis, Sjögren syndrome, α1 – antitrypsin deficiency, membranoproliferative glomerulonephritis, Wilson's disease, cutanea tarda, autoimmune thyroiditis and hemochromatosis (1).

Hepatitis can be further broadly classified into two clinical presentations: acute and chronic hepatitis. Acute hepatitis involves acute hepatic parenchyma damage. This cellular damage occurs throughout the liver, with the hepatocytes showing classical signs involving degenerative changes (vacuolation, cytoplasmic granulation and swelling), necrosis and regeneration. Very major damage can result in massive necrosis and destruction of a whole lobule, leading to acute liver failure. Chronic hepatitis, on the other hand, refers to any hepatitis lasting for six months or longer (using the definition for hepatitis C virus infection). This class of hepatitis is characterised by infiltration of mononuclear inflammatory cells via the portal tracts and damage of the periportal hepatocytes with continuous damage of the

parenchymal cells with fibrosis. The classical long term effect of hepatitis manifest as liver cirrhosis and hepatocellular carcinoma (2–7).

This disease has been one of the world's top burdens to the healthcare system, with virus infections being the most common cause (8). There are five major hepatitis viruses, with hepatitis C virus (HCV) and hepatitis B virus (HBV) responsible for more than 71 million and 248 chronic infections, respectively, hence making this disease a major global health issue (6,9–16). Recently, there have been breakthroughs with HCV therapy (17), but no vaccine is available. This lack of vaccine can be attributed mainly to the limited understanding of anti-hepacivirus immunity and no pre-clinical model for investigating potential vaccine candidates (4,17,18).

1.2 Proposed HCV origin, history of EqHV and Terminology changes over the years

When HCV was discovered in 1989 (19), several early attempts were made to identify suitable homologues to understand its origin further. Early initial findings suggested the possibility of a nonhuman primate origin (20). This suggestion was supported by the high diversity seen in endemic areas of Southeast Asia and sub-Saharan Africa, in regions where apes, humans and Old-World monkeys overlapped. Furthermore, corroborated by discovering the zoonotic origin of SIV/HIV from chimpanzees (21). Intensive screening of several apes, chimpanzees and Old World monkeys failed to prove this hypothesis or detect the presence of this HCV–like

551 viruses (2,22). In the absence of any empirical detection of viruses, the study of the
552 evolutionary origins of HCV and associated research was hindered.

553 Despite the great advances in understanding the pathogenesis of HCV, the
554 determination of its origin has eluded scientists for the past decades. Hence, vaccine
555 design still faces many challenges, partly because there is no reliable tractable
556 animal homologue (23,24) and, until the development of workable assays in cell
557 culture and transgenic mice, replication studies were restricted to only humans and
558 chimpanzees (25,26). Ideally, an HCV surrogate model should recapitulate the virus
559 hepatotropism, immune responses and pathogenesis, and persistent infections (27).
560 However, in addition to the challenge of narrow host tropism and the development
561 of small animal models, rodents have been documented to be naturally resistant to
562 HCV infection (28). Several approaches have been explored to study this virus in
563 mice and animal models' development over the years. These include Non-rodent
564 models (chimpanzee (29–32), and tree shrew (33,34)). Viral protein transgenic
565 mouse models (inducible transgene expression (35–37), and full HCV genome (38)),
566 immunocompromised human liver xenograft mouse models (Trimera mouse
567 (39,40), Alb-uPA-SCID mouse with humanized liver (41–43) and FRG mouse (44–46)),
568 immunocompetent xenograft mouse model (tolerized rat (47,48), AFC8-hu HSC/Hep
569 mouse (49) and HIL mouse (50,51)), viral adaptation (52,53), genetically humanized
570 mouse models (Rosa26-Fluc mouse (54,55) and ICR-C/OTg mouse (56)) and HCV

homologues in natural hosts (e.g. GB-virus in tamarins (57), EqHV in horses (58,59) and NrHV in rats (60,61)). Nevertheless, until 2011, GBV-B (named after the surgeon whose serum was shown to infect Tamarins and this patient later developed acute hepatitis) was the closest known HCV homologue (62,63).

In 2011 Kapoor et al. (64) discovered a virus in respiratory samples isolated from domestic dogs with respiratory illness in the United States. This study named the virus identified 'Canine hepacivirus' (CHV), which was approved by "The International Committee of Taxonomy of Viruses". Later, in 2012, this virus was tentatively called non-primate hepacivirus (NPHV) by Burbelo and his colleagues based on the detection of this virus in a different host (65). Presently, the virus is named equine hepacivirus (EqHV) (66). This virus will be referred to as EqHV throughout this thesis.

1.3 Hosts of EqHV

Hepacivirus RNA genomes have now been isolated from several other mammalian and non-mammalian host species. The mammalian hosts include dogs, horses, rodents, insectivorous bats, monkeys, cattle, donkeys and mules. To date, the catshark is the only known non-mammalian host of a hepacivirus (67,68,77,69–76).

589 Horses are a biological host of several flaviviruses (including vector-borne viruses)
590 responsible for several human and equine diseases (78,79). Presently, horses are
591 assumed to be the natural host for this virus, and this thesis will refer to it
592 accordingly (64–66,74). This virus's infection has been found in horses on all
593 continents (65,69,87,88,73,80–86) (Table 2).

594

595 ## 1.4 Hepacivirus classification

596 Recent new hepacivirus discoveries led to the reclassification of the genus into 14
597 species (A-N), with HCV denoted with species "C" and EqHV "A" (89). According to
598 the International Committee on Taxonomy of Viruses (ICTV), EqHV belongs to the
599 family Flaviviridae, genus Hepacivirus and species hepacivirus A (90). Other
600 members of this family include true Flaviviruses, Pestiviruses, and Pegiviruses (91).
601 Furthermore, Pegiviruses have been implicated in some liver diseases with some
602 mammalian hosts (92–94).

603

604 ## 1.5 Flaviviruses

605 All 73 viruses (95) belonging to the genus *Flavivirus* (family Flaviviridae) are positive
606 sensed single-stranded RNA genome of approximately 10-11kb that encodes a 3'
607 untranslated region (UTR), a long open reading frame (ORF) and a 3'UTR (96).
608 Although having a common genomic organisation, there are fundamental

differences in flaviviruses transmission cycles and host ranges (97–99). The majority of the known flaviviruses are transmitted horizontally between hematophagous arthropods (i.e. ticks and mosquitoes) and vertebrate hosts; for example, tick-borne encephalitis virus, Zika virus, dengue virus, Japanese encephalitis virus, yellow fever virus and West Nile virus (100–102). Other flaviviruses have been found in nature to be exclusively isolated from sandflies and mosquitoes and cannot replicate in sulking mice or cell lines of vertebrate (103,104). These groups of flaviviruses are assumed to be vertically transmitted between hosts and have insect-restricted host ranges (105,106). A group of flaviviruses, referred to as no known arthropod vector (NKV), have been almost always exclusively isolated from vertebrates (rodents, bats and sometimes humans) and never from arthropod cell cultures or wild-caught as well as laboratory-inoculated arthropods with some exceptions (e.g. Dakar bat virus and Sokoluk virus) (97,107).

1.6 Tissue tropism, EqHV isolation and disease association

A previous study hypothesised that EqHV causes respiratory illness in dogs with an apparent tropism for respiratory tracts (74), and several animal transmission studies were proposed (108). EqHV was found in canine liver samples of dogs that died due to gastrointestinal ailments, but it was not established if there was a correlation between hepatitis and persistent infection (65,108). Detection of EqHV in dogs'

hepatocytes could not be replicated in subsequent screening (74). Several reasons were suggested for this initial detection: the initially isolated EqHV from the positive dog having the respiratory tract disease might have ingested contaminated food and aid by its saliva into the digestive tract, after which it goes into the liver via the hepatic portal vein. This suggestion is possible if any part of the digestive tract integrity is compromised. Another possibility could be that EqHV was just found in the respiratory samples by chance, similarly to the case with GBV-C which was at first proposed as a unique hepatitis virus in humans (109,110).

EqHV was later isolated from canine hepatic and respiratory tissues, especially the lower respiratory tract (111). This isolation was attributed to the differences in dog populations screened. A study showed no significant association between EqHV and respiratory disease, with or without viral co-infection (65). Histological examination of respiratory tissues also showed similar findings of no significant association between the virus's presence and the observable changes in the tissues (65).

However, several studies could not validate this hypothesis, as no EqHV genomes (formerly referred to as canine hepacivirus) have been isolated from any canine sera or any respiratory samples after its initial documentation (65,69,74). Several studies have screened for the presence of EqHV in dog samples in the United Kingdom and United States of America (58,111), but neither its RNA nor anti-viral antibody could be isolated, thereby suggesting no prior exposure to the virus (65,110). Therefore,

649 present empirical data indicates no association between canine hepatitis and EqHV
650 (110). These results, however, cannot accurately depict the global ecology of the
651 virus in dogs. Also, the health status of all dogs in several studies was not known.
652 However, there is robust evidence that equines are the true natural hosts of EqHV
653 and the closest homologue to HCV and more closely identical to the initially isolated
654 CHV.

655 Blood biochemical analysis in EqHV- infected horses has revealed that significant
656 elevation in liver enzymes above the reference range does not occur during
657 infection, except in some cases with a mild increase in the upper end of the
658 reference range (66,74,83,112,113) is observed. Horses positive for EqHV RNA most
659 often show no signs of systemic disease or clinically significant symptoms (74).
660 Interestingly, Elia and colleagues (80) showed observable clinical chronic wasting
661 conditions with an altered level of some hepatic markers, including LDH (Lactate
662 dehydrogenase), AST (Aspartate aminotransferase) and ALT (Alanine transaminase).
663 It is currently unclear if this virus affects Thoroughbred racehorse performance on
664 the racetrack. This question remains a crucial question to be answered. Some
665 studies have shown an increase in sorbitol dehydrogenase (SDH), Glutamate
666 dehydrogenase (GLDH) and gamma-glutamyl transpeptidase (GGT) (up to 6 months)
667 with EqHV infection, as well as a short-term increase in serum liver enzymes during
668 the simultaneous clearance of the virus as well as seroconverting anti-NS3

669 antibodies. These are indications of liver disease (66,82,83,114). Conversely, some
670 studies showed no link between the observable rise in GGT with EqHV (65,74,83,85)
671 while others showed a slight increase in GGT in horses previously exposed to the
672 virus (82) or after an experimental inoculation (66).

673 The genetic sequence in the region of the virus genome encoding non-structural
674 protein 3 (NS3) from these dogs was found to be closely related to HCV, and that
675 pioneered the idea that hepaciviruses were not restricted to primates alone and led
676 to the possibility of the introduction of these viruses to the human population via
677 direct or indirect contacts (64). This discovery indirectly informed research into HCV,
678 which included the origin and evolution of HCV as well as a possible surrogate model
679 for HCV vaccine research (108). However, caution must be taken when analysing
680 such data because an observed association does not necessarily imply disease
681 causation -Koch's postulates- especially with respiratory and faeces which could
682 indicate ingested contaminants (65,115,116). Similarly, detection of a specific
683 adaptive immune response to viral proteins (structural and non-structural) infers a
684 particular past infection but does not necessarily define an active infection (65).

685 This vital question raised about Koch's postulates (115) for disease-causing agents
686 led to several schools of thought on hepaciviruses' origins. It could be suggested that
687 the EqHV infected dog stabled with horses, and the dog might have inhaled the viral
688 particle. Hence, its detection in the respiratory sample was evident in Lyons and

689 colleagues' experiment in 2014 (73), providing evidence for interspecies
690 transmission. The interspecies transmission hypothesis was further corroborated
691 with the study that detected an EqHV isolate in a commercial horse serum pool
692 called NZP-1, which had a very high level of similarity with that of a virus isolated
693 from a dog (65). Another possible hypothesis is that the initial isolation of EqHV from
694 dogs could suggest a close relationship between dogs and humans for hundreds to
695 thousands of years (64,108), thereby providing data supporting a possible spread to
696 humans (74). The isolated EqHV strain was distinct from the initially detected 'CHV'
697 infecting dogs, although some were identical. This data could serve as evidence to
698 show that EqHV could jump between host species.

699 In 2006, a study (117) carried out one of the most extensive serological screening
700 assays on old-world–nonhuman primates to date to determine the prevalence of
701 blood-borne hepatitis. The study showed no antibodies that cross-react with HCV
702 antigens could be detected of all wild-born primates screened. It could be argued
703 that the study used a purified antigen-specific commercial kit that would hamper its
704 ability to detect different variants of nonhuman origin (118).

705

706 1.7 EqHV infection – natural and experimental

707 In humans, an acute HCV infection is accompanied by changes in serum alanine
708 transaminase (ALT) concentrations and other hepatic markers predictive of a liver

problem (119,120). A Hungarian horse in 2014 showed a natural infection with EqHV and subsequent development of hepatitis and other associated clinical signs of liver damage which was supported with viral loads and liver function tests, and both showed a positive correlation with the clinical findings (86). Interestingly, this horse had no evidence of co-infection with other known viruses that may cause hepatitis in horses. It is plausible that other unidentified infections could cause liver disease. However, while the possibility that some other unknown virus causes this pathogenesis cannot be excluded, there was an association of EqHV with liver disease in the horse investigated.

Ramsay and colleagues (66) performed a more controlled study by conducting an experimental inoculation of horse and foals to study the natural course of viremia. They observed a striking similarity in the clinical manifestations of HCV infection observed in chimpanzees and humans. In some horses, observable hepatocellular necrosis was seen with an acute infection, usually with an acute episode of hepatitis (66). The study also showed a possibility of chronicity with EqHV viruses in the blood without NS3-specific antibodies; although, the specific reason for this is unknown (66). Furthermore, experimentally infected horses had similar hepatic membrane rearrangement as observed with HCV (113).

Therefore, analysing the above data together, it could be inferred that EqHV could be a potential causative agent of equine serum hepatitis (66,86).

1.8 A phylogenetic analysis including all other hepaciviruses

Based on phylogenetic analysis of the conserved RNA polymerase and RNA helicase genes, several Flaviviridae family members were found to cluster with HCV, including EqHV and GBV-B. Although virological and epidemiological data are lacking, phylogenetic analysis shows EqHV to be the closest genetically and from the evolutionary standpoint with HCV among all known non-primate hepacivirus strains (87,114,121).

Nucleotide distance analysis of EqHV isolates showed a high mean diversity (~26 %) and greater diversity based on distances (112). This study further suggested two subtypes based on the available diversity of sequences, requiring further validation. The first proposed subtype comprised sequences from the USA, Southeast Brazil and a single sequence from Scotland and Hungary. While, the second subtype was found in samples from Japan, the USA and Midwest Brazil. Another study postulated the possibility of two EqHV subtypes in the French horse population with about four-fold difference observed in viral loads between both groups (85). Also, these subtypes were concatenated sequences with about ~15 % variation in nucleotides, with ~12 to 14 % diversity within each group and ~17 % diversity between groups.

Several studies demonstrating the phylogenetic analysis of EqHV (using the NS3 gene sequences) showed no distinct correlation between geographical area and the

749 strain of virus detected (82). These results could be attributed to the fact that
750 Thoroughbred horses, being athletic animals, may have extensively travelled for
751 racing competitions/training.

752

753 ## 1.9 Course and possibilities of infection, Vertical and Horizontal transmission of
754 EqHV

755 The origin of HCV is unknown, and several schools of thought about the virus origin,
756 including the inter-species transmission of the virus from animal to man, similar to
757 that seen with coronavirus, human immune deficiency virus (HIV) and Ebola virus
758 (122–124). This hypothesis was supported by research showing EqHV protease were
759 able to cleave human TRIF (TIR (Toll/IL-1 receptor) domain-containing adaptor
760 protein) and MAVS (Mitochondrial antiviral-signalling protein); hence suggesting the
761 virus, like other hepaciviruses, can circumvent host immune responses even across
762 species barrier (125,126).

763 Presently, only a study conducted by Pfaender and colleagues (127) directly
764 investigated cross-species transmission between humans and horses. This study
765 aimed at answering this question by screening humans for EqHV RNA and antibody
766 (Ab) who were more likely to be exposed to the virus, primarily due to some
767 predisposing factors, including occupation in a horse clinic and frequent contact
768 with horses for at least 25 years. Several EqHV antigen constructs were designed to

769 determine exposure (using antibody targeting the structural envelope proteins 1
770 and 2 (anti-E1E2)) and replication/pathology (using antibody targeting the non-
771 structural three proteins anti-NS3). Variable levels of seroreactivity were observed
772 to several EqHV constructs irrespective of prior/no contact to horses. Furthermore,
773 all sera were negative for EqHV RNA, and no cross-reactive HCV antibodies were
774 observed in humans (127). This data supported other studies showing that it is
775 improbable that EqHV is cross-transmission to humans from horses. Therefore,
776 different possible transmission routes should be explored, including vector-borne
777 infection, contaminated medical instruments, blood products and non-direct blood
778 contacts (65,73,84,127,128), as well as airborne and smear infection (129). The
779 respiratory route as a route of transmission is likely to be ruled out as a route due
780 to the absence of cardinal signs of the route; for example, rapid spread, high rate of
781 acute infection, cluster in a phylogenetic tree due to highly similar amino acid
782 profile, and identical virus (130).

783 The high prevalence of EqHV RNA seen in horses in several studies conducted across
784 different continents indicates that this virus is enzootic with a wide range of
785 circulation (73). Compared with HCV, horses' EqHV seroprevalence rate is more
786 than double seen in humans, even in countries with a history of epidemic spread,
787 such as Egypt and Pakistan. This data provides evidence that there is a more efficient

788 way this virus is spreading and less probable to be only via a parenteral route
789 (73,131–133).

790 Commercially available horse sera were found to have a high incidence of RNA and
791 Ab (83). Contaminated blood or its products can transmit EqHV experimentally
792 among horses. However, commercial preparations are often heat-inactivated and
793 should be free from infectious material (66). Scheel and colleagues also validated
794 this high rate in 2015 (114), where ~93 % of sampled companies were contaminated
795 with a wide range of EqHV isolates from several infected animals. So contaminated
796 sera should be excluded from studies aiming to identify hepaciviruses. These blood
797 products should also be screened when used to develop vaccines to avoid the
798 possibility of inter-species cross-contamination to humans.

799 A study (64) suggested that zoonotic transfer of EqHV may have recently occurred
800 in light of its poor host adaptation, level of pathogenicity, ineffective non-parenteral
801 transfer, and no known HCV homologues in non-primates. There is evidence of
802 transmission of EqHV among the infected population aided by humans. However,
803 the actual mechanism is not yet known (77,82). There has also been evidence of
804 horizontal transmission among horses and infection of a naïve horse with an already
805 identified isolate in a study by Scheel and colleagues in 2015 (114). In Germany,
806 EqHV isolates from mares and foals recently infected after 3 and 6 months clustered
807 in their pasture herds; thereby, implying a horizontal route of transmission (129).

Furthermore, another study showed transmission of EqHV isolates within pasture herds, indicating a possibility of a direct horizontal transmission among these horses (129). Viral transmission via breeding should be explored, especially because natural breeding is compulsory among Thoroughbred horses. However, the mechanism of transmission is not well known.

Vertical transmission has been documented in several recent studies. A study in Japan showed that EqHV infects mares (4 - 6 months old) after weaning, although the mechanism is unknown (82). Another study in 2016 (129) showed that ~25 % of foals studied are infected at birth, and there is a transfer of maternal antibodies through the colostrum to the foal and not via the umbilical cord blood. These mares showed the acute and chronic infection course at six months without observable clinical signs and liver-specific enzymes within the reference ranges (129). Mares positive for EqHV had no detectable virus in the placental tissues (129). No relationship was seen between maternal EqHV infection and inflamed placenta at parturition (129). Maternal anti-EqHV NS3 antibodies decreased from the third month of age of the foal (129). This data could suggest the reason for the observed increased susceptibility of the foals to EqHV infection.

No specific risk factors in the yard presently have been identified to date. These include the sharing of used or unsterilised needles, risky operation practices, and systemic illness. Nevertheless, it is well-documented in humans that stress could

predispose a host to viral infection (134). Several suggestions have been put forward on possible ways this virus could be transmitted, and they include close contact from stable housing structure, iatrogenic transmission, age, breeding history, size of stud farms and international travel history or extensive travels (73,135). Other potential risk factors could be investigated. They include close contact on the racetrack, possibly through contaminated feed.

1.10 Prevalence, acute and chronic infection of EqHV:

Several studies have designed a range of polymerase chain reaction (PCR) screening assays (Table 1.1) to detect EqHV. Presently, this virus's prevalence rate across different studies since its discovery in 2011 till 2020 has ranged from 0.9 – 41 % and 22.6 – 83.7 % in RNA positivity and antibody positivity, respectively (Table 1.2). Generally, there has not been any significant correlation between viral load and age or gender (85). This correlation was generally not the case as a study showed a relatively slight increase among the male horses compared to the female horses (112).

Table 1.1: All primers used to date (2011-2019) in detecting EqHV in several hosts

844

EqHV region	Primer name	Round of PCR	Ref	Position	Forward / Reverse	Primer sequence	Ref
5' UTR	RU-O-17723	single	NZP1	F86	Forward	CACCATGTGTCACTCCCC	(58)
	RU-O-17724			R387	Reverse	CATGTCCTATGGTCTACGAG	
	EQ5→UTROS				Forward outer sense	ACA YYA CCA TGT GTC ACT CCC CCT	(74)
	EQ5→UTROAS				Reverse outer antisense	CYC ATG TCC TAT GGT CTA CGA GA	
	EQ5→UTRIS				Forward inner sense	ACA CGG AAA YGG GTT AAC CAY ACY C	
	EQ5→UTRIAS				Reverse inner antisense	GCC CTC GCA AGC ATC CTA TCA G	
Core-E2	RU-O-17951	Single	NZP1	F886	Forward	CTTGTRCGGTTTGTNGAGGACG	(58)
	RU-O-17953			R1844	Reverse	CCGAARCARGTNGGTTTGCCAC	
3'UTR	RU-O-17670	Single	NZP1	F8999	Forward	TTGTCGCGGACTACCTTTTC	(58)
	RU-O-17918			-		CCGCTGGAAGTGACTGACAC	
	RU-O-18357	First-round		F9006	Forward	GGACTACCTTTTCGGCTTCGC	
	RU-O-18354			R9349	Reverse	ACATGTTTTCGCCCCATAGG	
	RU-O-18328	First-round		F9265	Forward	ATTTCCTTTATTGGTTACTTCCTATG	
	RU-O-19847			R9498	Reverse	CCTTCAGAAGCTAGCCGTGAC	
	RU-O-19849	First-round		F9445	Forward	TTCTCTATTGATGGGTGGCTC	
	RU-O-19846			R9538	Reverse	ACATAGTCCCGGGATATCCCG	
	RU-O-18358	Second round		F9012	Forward	CCTTTTCGGCTTCGCTTCTGC	
	RU-O-18355			R9349	Reverse	ACATGTTTTCGCCCCATAGGG	
	RU-O-19136	Second round		F9273	Forward	TTTTGGTTACTTCCTATGGAAGAAC	
	RU-O-19848			R9472	Reverse	CTAAGGGGAGCCACCCATC	
	RU-O-19849	Second round		F9445	Forward	TTCTCTATTGATGGGTGGCTC	
	RUO-19846			R9538	Reverse	ACATAGTCCCGGGATATCCCG	
NS3	EQNS3OS				Forward outer sense	ATW TGT GAT GAR TGC CAY AGY AC	(74)
	EQNS3OAS				Reverse outer antisense	TAG TAG GTB ACA GCR TTA GCY CC	

	EQNS3IS				Forward inner sense	TCY AAR GGT GTD AAG CTT GTT GT	
	EQNS3IAS				Reverse inner antisense	TGG CAG AAG YTA AGR TGY CTY CC	
	Chcv-0F1 (helicase domain)	First-round			Forward outer sense	5′-TCCACCTATGGTAAGTTCTTAGC-3′	(110)
	Chcv-0R1 (helicase domain)	First-round			Reverse outer antisense	5′-ACCCTGTCATAAGGGCGTC-3	
	Chcv-0F2 (helicase domain)	Second round			Reverse inner sense	5′-CCTATGGTAAGTTCTTAGCTGAC-3′	
	Chcv-0R2 (helicase domain)	Second round			Reverse inner antisense	5′-CCTGTCATAAGGGCGTCCGT-3′	
	Chv-NS3F1	First-round			Forward outer sense	5′-GCCATAGCACAGACTCCACA-3′	
	Chv-NS3R1	First-round			Reverse outer antisense	5′-AAGGGTATGTCACCGCTCTG-3′	
	Chv-NS3F2	Second round			Reverse inner sense	5′-CCTATGGTAAGTTCTTAGCTGAC-3′	
	Chv-NS3R2	Second round			Reverse inner antisense	5′-CGATGTTAGGATGAGGGACAG-3′	
	HGLV-ak1	First-round			Forward outer sense	5′TACGCIACNGCIACNCCICC 3′	(71)
	HGLV-ak2	First-round			Reverse outer antisense	5′TCGAAGTTCCCIGTRTANCCIGT 3′	
	HGLV-ak3	Second round			Reverse inner sense	5′GACIGCGACICCICCIGG 3′	
	HGLV-ak4	Second round			Reverse inner antisense	5′TCGAAGTTCCCIGTRTAICCIGT 3′	
	AK4340F1	First-round			Forward outer sense	5′ GTACTTGCTACTGCNACNCC 3′	
	AK4630R1	First-round			Reverse outer antisense	5′ TACCCTGTCATAAGGGCRTC 3′	
	AK4340F2	Second round			Reverse inner sense	5' CTTGCTACTGCNACNCCWCC 3'	

	AK4630R2	Second round			Reverse inner antisense	5'TACCCTGTCATAAGGGCRTCNGT 3'	
NS3 contd	Hepaci-F3628				Forward	GCICCIACIGGIAGYGGIAA	(136)
	Hepaci-F3868a				Forward	TAYGAYGTIATIATITGYGAYGARTG	
	Hepaci-F3868b				Forward	TAYGAYGTIATIATITGYGAYGA	
	Hepaci-F3979				Forward	GCIACIGCIACICCICCIGG	
	Hepaci-R4268				Reverse	CCIGTCATIAGRGCRTCIGT	
	Hepaci-R4302				Reverse	CARTCIRTIACIGARTCRAARTYICC	
NS5	EQNS5BIS				Forward outer sense	AAR TGY TTT GAC TCY ACB GTC ACT C	(74)
	EQNS5BOIAS				Reverse outer antisense	ACT RTG ACT RAT YGT YTC CCA ACT CG	
	EQNS5BIS2				Forward inner sense	CAY GAT ATA GAH ACT GAG AGR GA	
	EQNS5BIAS2				Reverse inner antisense	TCR TCT TCC TCR ACG CCY TTR CTG G	

Table 1.2: Spread of EqHV around the world indicating animals screened and assay used

853

Continent	Country	Animals screened	Sample types	Screening PCR targets	Positive host	RNA prevalence (%)	Seroprevalence (%)	Reference
North America	United States of America	Dogs	Respiratory samples	5'UTR and 3'UTR	Dog (first discovery)	nil	nil	(58)
Asia	Japan	horses	Serum	NS3	Horse	35.5	22.6	(87)
North America	New York	Horses, dogs, rabbit, deer, and cows	Serum	5' UTR	Horse	7.8	35	(65)
Europe	United Kingdom	Horse, dogs, cats, mice, pigs and donkeys	Buccal swab, Bronchoalveolar lavage, venous blood	5'UTR, NS3, and NS5B	Horse	2.1	na	(74)
Europe	United Kingdom	Dogs	Liver and blood samples		None	0	0	(110)
North America	United States of America	Humans	Blood samples	NS3 and 5'UTR	None	0	0	(71)
Europe	Germany	Cats, dogs and horses	Sera	NS3	Horses	3.3	na	(136)
Europe	Hungary	Horse	Blood samples	NS5B	Horse	na		(86)
Africa and Europe	Cameroon (humans and nonhuman primates only), Scotland (horses, dogs, and cats), England	Humans, Nonhuman primates, horses, donkeys, dogs and cats	Serum samples	5'UTR, NS3, and NS5B	Horse	0.9	43.3 (0.9 in dogs because this dog was in regular contact with an infected horse)	(73)

	(horse and donkeys) and France (horse)							
Europe	Germany	Horses	sera	5'UTR (qPCR)	Horses	2.5	31.4	(137)
South America	Brazil	Horses, mules and donkeys	sera	NS3	Horses	8.3 %	na	(81)
Europe	Hungary	Horse (natural infection)	sera	NS5B	Horse	Not applicable (100 %)	Not applicable	(86)
North America	USA	Horses	sera	5'UTR or Core-E2	Horses	41 %	na	(76)
North America	USA	Horses	sera	5'UTR or Core-E2	Horses	3-5 %	na	(76)
Europe	United Kingdom	Dogs	Tissue samples from tracheal, lung and liver samples	NS3	Dogs	Trachea: 22.9-35 % Lungs: 15 % Liver: 40 %	na	(111)
Asia	Japan	Horses	Sera	5'UTR	Horses	13.68	33.55 (NS3)	(82)
Europe	Northern Germany	Human	Sera	NS3	None	0	0	(127)
South America	Brazil	Horses	Sera	NS5B	Horses	13.4	na	(112)
Europe	France	Horses	Sera	5'UTR	Horses	6.2	na	(85)
Europe, Asia, Africa, and Latin America	Germany. Spain, Italy, Bulgaria, France, Israel, Kenya, Costa Rica and Mexico	Donkeys	Sera	5'UTR and NS3	Donkeys	0.3	31.5	(77)
Asia	Korea	Horses	sera	NS3	Horses	18.9	na	(101)
Europe	Italy	Horses and donkeys	sera	5'UTR, NS3, and NS5B	Horses	4.7	na	(138)
Asia	China	Horses, mules, and donkeys	sera	NS5B and NS3	Horses	3.4	na	(139)
Europe	Northern and	Thoroughbred horses	sera	NS3 and 5'UTR	Horses	18.3	61.8	(135)

	Western Germany							
Asia	Japan	Horses	sera	Core	Horses	na	23.4	(91)
Asia	China	Horses	sera	NS3	Horses	3.4	na	(140)
Africa	South Africa	Horses	Sera	5'UTR, NS3 and NS5B	Horse	7.93	83.70	(88)
Europe	Germany	Alpaca, Badger, Beech marten, cat, cattle, dog, donkey, fox, goat, horses, marten, mink, pine marten, polecat, racoon, racoon dog, sheep and swine	sera	NS3	Donkey and horse	2.8 and 2.4, respectively	Na	(141)

854

855 Recently, descriptive statistics showed variation in prevalence related to age;
856 infection was significantly higher with horses less than two years old. These results
857 indicate that these young horses are more prone to EqHV, which could be attributed
858 to the absence of frequent reinfection or persistent humoral immunity associated
859 with advancing in age, hence the immature immune system of young horses
860 (82,135,142). Using a multivariate analysis, a study in Germany (135) showed that
861 young horses (< 8 years old) are more prone to EqHV infections after international
862 travels to a foreign country. These findings are consistent with the already
863 documented data that transportation (143–145), change in environment, and
864 disruption of social groups (134) in horses causes stress-induced
865 immunosuppression and a possible increase in exposure. Furthermore,
866 Thoroughbred horses are required to mate naturally, and this could also contribute
867 to stress-induced immunosuppression, particularly among maiden mares (135,146).
868 Horses in large farms were more prone to EqHV than smaller farm studs, probably
869 due to a combination of stress-induced immunosuppression and more frequent
870 travels and breeding, which could lead to more viral shedding and transmission
871 (135). Although the same study showed no correlation in prevalence between anti-
872 NS3 antibodies and ages of the horses, horses less than two years had the lowest
873 proportion of seroconversion (82). This slow seroconversion could result from the
874 horses being in the window period before a detectable amount of antibodies could

875 be seen. Furthermore, several studies have shown that horses can clear the
876 infection and simultaneously seroconvert with antibodies against NS3 (65,82,83).

877 Some studies have shown a surprisingly high frequency of infection recorded in
878 Thoroughbred horses, and as such, this population may either be more susceptible
879 or have greater exposure than any other breed (81,83,85,88,135). These could be
880 due to several factors, including genetic determinants associated with the breed,
881 more frequent exposure to the pathogen than any other breeds, transmission via
882 equine-derived veterinary products (e.g. immune sera, vaccines, or other
883 immunological and biological products), or body contacts during horse racing or
884 breeding (83,84,147).

885 EqHV is less likely to establish chronic infections in a host compared to HCV (~60-80
886 %); but similar to that seen in chimpanzees (~30 %), which could infer either a high
887 rate of clearance or higher acute infections in the majority of horses
888 (66,76,80,83,137). A different rate of clearance was also documented *in vivo*, but
889 the reason for this is not fully understood (66,113). Also, chronic infections in these
890 horses may last longer than a decade (114).

891 The viral loads detected in EqHV infections vary during acute and chronic infections.
892 However, horses do not exhibit any clinical symptoms with liver indices within the
893 reference range. Generally, horses with chronic infections were within the upper
894 end of the reference range (74). High viral loads have been documented in naturally

infected chronic young horses for up to 15 months (80) and some horses with detectable antibodies. Furthermore, some of the mares can eliminate the virus. Analysis of the data suggests that foals can progress from acute to chronic infection. These foals also showed no observable clinical signs of hepatic damage with liver-specific enzymes within the reference range. Although some horses showed mild elevation of enzyme activity (at 12-13 months), indicating a possibility of subclinical hepatitis as a result of persistent infection of EqHV (148); care must be taken when analysing data from changes in liver-specific enzymes because these changes could arise from a completely different and unrelated cause.

Comparing equid hosts of EqHV, rates of delayed clearance in donkeys were lower than horses (77). Available data also showed that hepaciviruses isolated from donkeys are genetically more divergent than isolates found in horses (141). Seroprevalence among donkeys increases with age, which was not the case in horses, with horses showing no significant relationship (77). In terms of sex, female donkeys were shown to be more likely to be infected than male donkeys. However, differences in gender distribution were not seen in infected horses (77). Similar to horses, liver-specific enzymes were all within the reference range, indicating a subclinical infection in these hosts (77). Furthermore, this study showed that there was no evidence supporting cross-species transmission between horses and donkeys.

It is hypothesised that the noticeable worldwide infection results from a single species' distribution (87). Several assays have been designed to detect this virus in host samples (Table 1.1). Serological testing of hepacivirus infections in several hosts suggests that horses' infection is more similar to EqHV than HCV in the helicase domain of NS3 protein (65). After the initial discovery of EqHV, several screening assays were carried out to determine the presence of antibodies specific for it (65), even in humans with indeterminate immunoblot patterns (149). Luciferase immunoprecipitation system (LIPS) assay (150,151) was used in the study, and 35 % of the total horses were immunoreactive, with 7.8% having the viral RNA (65).

1.11 Equine pegiviruses

Equine Pegivirus (EPgV) is similar to the human Pegivirus in its limited pathogenicity, inability to replicate in hepatocytes and infectivity restricted to mononuclear cells (152,153). Furthermore, this virus can display strain-dependent pathogenicity as well as long-term chronic infections (83). EPgV is not known to have an affinity for the liver or any hepatotropic effects at the writing time (121). However, this virus has been detected in horses infected with EqHV (co-infection) but not found in the liver (83). It cannot be ruled out that EPgV could contribute to the observed hepatocellular damage noticed in some EqHV-infected horses.

Nevertheless, some studies with horse samples showed that with a pre-existing infection of EPgV, there was no sign of liver damage or viral RNA in liver cells (66,83). These data raise doubts and investigate further its specificity for the liver or ability to cause any associated hepatocellular damage. Furthermore, TDAV (Theiler's disease-associated virus) has not been directly linked with hepatotropism but has been detected in serum hepatitis during an outbreak (154).

1.12 Molecular virology of hepaciviruses

Amongst the members of the genus Hepacivirus, HCV is the most intensively studied. As such, only the morphology of HCV has been investigated using filtration and electron microscopy. The structural properties and morphology of EqHV are yet to be determined. This difficulty is partly due to its recent discovery (64,74,155) and the lack of a robust cell culture system for EqHV.

1.13 Entry of EqHV

EqHV is a single-stranded positive sensed RNA virus, and it is classified in the genus *Hepacivirus A* (64,156). Similar to other viruses within this genus, EqHV is an enveloped virus. Hepaciviruses interactions with the host cellular membrane via attachment, internalisation, and fusion ensures delivery of viral genome into the host cell cytosol and hence start of its replication (157). The entry of EqHV in horses

954 or humans is yet to be determined or completely understood, with both having little
955 research. However, other genus Hepacivirus members' entry mechanisms,
956 especially HCV, have been characterised, and the replication of EqHV is likely similar
957 to HCV. These include EqHV possessing similar levels of E1E2 protein glycosylation
958 and a conserved seed-site for the miR-122 binding (137).

959 The initial stages of hepacivirus entry, modelled on HCV, is characterised by a
960 multistage and slow process. The initial binding of HCV particles by lipoproteins and
961 glycosaminoglycan receptors is accompanied by a complex interaction with
962 scavenger receptor class B type 1 (a crucial high-density lipoprotein receptor), tight
963 junction proteins (Claudin-1 and Occludin) and CD81 tetraspanin (158,159). These
964 receptor interactions eventually result in clathrin-dependent endocytosis via uptake
965 and cellular internalisation of viruses (160,161).

966 Hepaciviruses possess two type-1 transmembrane proteins, E1 and E2, that function
967 as receptor binding proteins. These highly glycosylated proteins form non-covalently
968 attached heterodimers on the surface of the viral envelope. These interactions lead
969 to large covalent complexes on the viral particles that are then stabilised by
970 disulphide bonds (162). E1 and E2 are crucial in mediating hepacivirus entry into
971 cells, especially in receptor binding. This binding eventually mediates the fusion
972 process involving the host endosomal cell membrane and the viral envelope.

Like other characterised hepacivirus infections, many assays and tools have been developed to overcome apparent molecular characterisation limitations. Two crucial and complementary infection assays are cell culture-grown genuine virus (163–165) and pseudoparticles possessing genuine E1 and E2 glycoproteins, which allow specific and rapid mutagenesis (166–168). While cell-cultured hepaciviruses, produced in human hepatoma cells (usually in HuH-7 or 7.5 cells) allow examination of the entire replication cycle, pseudoparticles (pp) provide a more flexible method to investigate the structure-function relationship of viral glycoproteins in cell culture as well as *in vitro* fusion (liposome membrane fusion assays) (169,170). Furthermore, pseudoparticles do not interact with lipoproteins because they are generated from 293T kidney cells (171), and hence permit the study of specific entry pathways into cells as a result of only the E1 and E2 glycoproteins (172). The lack of interaction of pseudoparticles with lipoproteins is a significant weakness of this assay because this interaction is essential during natural infection. The flexibility pseudoparticles provide allows many applications, including gene transfer, vaccine production and therapeutic agent screening (173).

1.14 Genome replication of EqHV

The method of replication of EqHV is not yet established. However, a study (83) demonstrated that the liver would be the significant replicative site of the virus as

993 shown by the presence of both positive and negative RNA strands in the liver,
994 possibly due to the sole presence of miR-122 binding sites in the liver like that seen
995 in humans. Some organs *in vivo* showed varied tissue tropism for this virus, including
996 the heart, lungs, and spleen. Nevertheless, the most prominent was the spleen (83),
997 which could be attributed to the high blood flow associated with the organ.
998 Conversely, this virus was present in the respiratory tissue but to a lower degree
999 when compared to the spleen (65).

1000 Furthermore, it is yet to be established if EqHV is responsible for the associated
1001 diseases in these organs (e.g. spleen and respiratory tissues). Detection of negative-
1002 stranded RNA in the hepatic tissues indicates replication of positive-strand
1003 (Baltimore class IV) viruses, and high levels of viral titre in these liver cells were also
1004 documented in later years by a study (114). This data was corroborated by detecting
1005 EqHV RNA, mainly in the hepatic cells in both chronic and acute horses (83).
1006 Although in both groups of detected RNA, the horses were usually asymptomatic.

1007 Presently, only one study (66) has attempted to describe the replication of EqHV
1008 completely *in vivo*. Replication kinetics and hepatic disease in an experimentally
1009 infected horse were determined after two horses were noted to be infected after
1010 blood transfusion. Furthermore, Scheel and colleagues (114) showed that EqHV full-
1011 length transcripts were infectious *in vivo* after inoculating the virus directly into the
1012 liver. The study revealed several associated signs of infection including, high viral

titres, detection of negative-stranded RNA, mild elevation in liver enzymes (with GLDH being the most prominent), and associated inflammation. However, this horse cleared the infection after elevating liver enzymes, similar to previous studies showing acute natural infection (83). The attempt to replicate EqHV *in vitro* has been unsuccessful. These failed attempts could be attributed to the lack of a suitable environment (usually in nonhepatic cell lines) appropriate for this virus, which could more likely be suited for equine liver cells (114). It is not surprising as similar results were observed during the early research days of other hepaciviruses (174). However, the consensus clone of EqHV can be translated, and viral proteins detected (114).

EqHV induces the vesicular formation and liver structures similar to those observed with HCV infected chimpanzees and cultured cells (113,175,176). Therefore, it can be inferred that EqHV and HCV could have a similar replication mechanism involving replication architectural frameworks called “replication factories”. These factories are also considered to have more functions beyond viral replication, such as immune evasion from the host innate immune cellular recognition apparatus like MDA5 and RIG-I (175). Based on available data, it can be hypothesised that EqHV contributes to a multifactorial disease in equines, leading to severe liver disease (147).

1.15 Genomic characterisation/architecture of EqHV

The first attempt at analysing the genomic sequences of EqHV was performed on isolates recovered from a dog with an acute respiratory disease (64). Several studies showed that EqHV and other hepaciviruses share comparable genome structural arrangement and ORF (64,137) (see Figure 1.1).

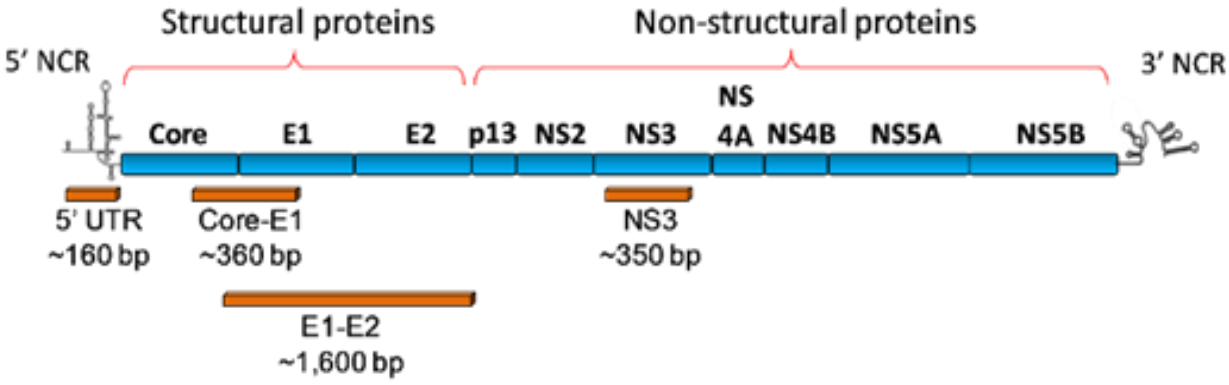

Figure 1.1: A Schematic of the EqHV genome (blue). The positions and lengths of genome regions amplified by the four PCR primer pairs used in this study are shown (orange) NCR, non-coding region; NS, non-structural.

The open reading frame of EqHV encodes about 3Kb amino acids and a short 5' UTR from at least 9kb nt (64). The high percentage of amino acid homology (about 95 %) seen between the initially detected CHV and EqHV suggests that they belong to the same species. At the genome level, a significant high similarity between other Hepaciviruses (using HCV as a case study) and non-primate hepacivirus nucleotides NS3 and NS5 (up to 65 %), although the reverse was observed with E1E2 glycoproteins amino acid identity (64). These similarities are significant as a lower

level of similarities are seen between HCV nucleotides (up to 33 %) and within lesser in subtypes (up to 25 %) (177).

Among all known EqHV strains, a very high amino acid homology (about 95 %) is seen, implying that all reported strains can be classified into one species (87). Furthermore, the observable high degree of homology seen between EqHV and CHV, given its absence in other hosts, infers a random infection of the dog by an equine isolate. It is also possible that this result came from contamination by horse-related products (65). Elucidating the functional properties of CHV will be aided by studying the conserved protein motifs between HCV and CHV.

A higher level of variation is seen within the genome's structural and non-structural region at the nucleotide level compared to the 5'UTR (65). Presently, most of the documented Intra-EqHV sequence diversity noted is within the coding region at synonymous sites with low dN/dS ratio values, and there is a high level of a consistent phylogenetic relationship between the strains (65). Furthermore, the observed sequence diversity in EqHV was greater than HCV (65).

Four stem-loops in the EqHV 5' UTR region closely resemble HCV structurally with the associated IRES site, facilitating translation from the main large polyprotein ORF. Compared to other hepaciviruses, the Stem-loop 1 of EqHV and HCV stem-loop three sidearms are longer (178). The most prominent structural differences and homology in sequences are usually seen in the 5' region of 5' UTR of HCV and EqHV. The first

stem-loop is different from HCV and the second stem is more homologous to HCV than GBV(64,179).

The functional domain prototypic type IV IRES (internal ribosomal entry site) found in HCV is similar to two of the identified stem-loops in EqHV (178). Furthermore, the translational control resulting from binding of microRNA miR-122 hypothesised for HCV is improbable to be the case for CHV (64,180,181). The 5'UTR of EqHV has been shown to have IRES activity in several cell lines, and the SL1 was shown not to be critical to translation but suppresses its activity to an extent (178).

The full length of the EqHV genome was unknown until 2014, when Tanaka et al. determined it and the 3' untranslated region (UTR) and 3' X tail (87). The difficulty in deciding this was primarily due to the interruption of the 3' rapid –amplification of cDNA ends (RACE) by the adenine-rich (A) sequence following the EqHV genome stop codon. Tanaka and colleagues also modified the RACE protocol and determined the RNA secondary structures of 5' and 3'UTR aided with minimum free energy predictions (87) using a poly (U) polymerase. These further showed that EqHV secondary structures could be conserved around both UTR's like HCV (87). Furthermore, the 3'UTR in both HCV and EqHV show a significant variation between the PolyU-rich/most 3' ends stem-loop and stop codon, with EqHV genome having stretches of an additional polyU/C and poly-A stretches inserted (87).

In 2015, a study's complete 3'UTR was determined to be ~328nt using empirical data (114). This region include: a short poly (A) tract (14-23nt), variable region (16-20nt), poly (U/C) tract (29-45nt), conserved intermediate region (70nt), long poly(U) tract (74-104nt) and a conserved 3'X region (88nt). This study further speculated that the conserved 3'X and intermediate region of this virus fold into three stem-loops. The extreme terminal of EqHV 3'UTR was also predicted to fold like HCV and hence support the fact that the EqHV 3' UTR has been determined (114). Furthermore, this study constructed a full-length consensus clone using NZP1 isolate (64,65,114). The 3'UTR and miR-122 stimulate IRES dependent translation in EqHV. Although miR-122 supplementation showed not to alter the rate of translation, its absence reduced translation significantly (114). More research is needed to establish EqHV miR-122 dependence; because the ectopic expression on several cell lines did not mediate the virus replication in them (114).

1.16 EqHV Innate and adaptive immune responses

Several studies showed that EqHV NS3-4A protease is similar to other hepaciviruses. This enzyme was capable of cleaving NS3-4A in mitochondrial antiviral-signalling (MAVS) protein in humans and can undergo *in vitro* translation in human hepatoma (HuH7) (114,182,183). Presently, we are yet to fully understand if this applies to equine MAVS, primarily due to observable differences in cleavage sites.

In horses, similar to HCV in humans (184), it was shown that the reported liver damage in acute infection could be attributed to the adaptive immune response, especially the T-cell mediated immunopathology and delayed seroconversion (66). The observable mild elevation can corroborate this suggestion, as seen with the corresponding liver enzymes profile changes during seroconversion in some infected horses (127).

Although the adaptive immune response regulates EqHV viral loads, the chronic infection was seen in horses that were immunocompetent with histological evidence of liver damage (66). Polyclonal antibodies specific for EqHV have been purified from commercial sera, which was previously shown to have a high prevalence in EqHV. Interestingly, these purified antibodies showed no cross-reactivity with HCV antibodies (114).

It is not clear if maternal antibodies transferred from mares to foals protect from infection. In 2016 a study (148) showed that foals who did not receive protection from maternal antibodies became infected at the same age as those with maternal antibodies. However, this is not conclusive evidence, as it is not yet shown if maternal antibodies are neutralising or protect mares from EqHV infection.

Another study (113) of experimentally infected horses showed an EqHV-independent immune reaction with no distinct immune cell frequencies, including EqHV-specific T cell proliferation in both infected and non-infected horses. The study

1124 further showed that these horses had moderate immune activation and serum
1125 cytokine levels which varies amongst horses (113).

1126 Reinfection by hepaciviruses is possible in primates and indicates secondary
1127 infections' potential (185–187). In experimentally reinfected horses, EqHV specific
1128 antibodies protected the horses against homologous or distinct EqHV isolates re-
1129 challenge; hence, no productive infection and observable hepatitis occurred after
1130 the histopathological and biochemical examination (113). During this re-challenge,
1131 the only noticeable adaptive immune cells was a weak T –cell proliferation. But, in
1132 all horses, no sterilising immunity due to trace amounts of detectable EqHV viral
1133 RNA (113).

1134

1135 ## 1.17 Similarities and differences between EqHV and HCV

1136 The polyprotein of EqHV is slightly shorter than HCV (3008 – 3033aa), although the
1137 genomic structural organisation of EqHV polyprotein cleavage is similar to HCV in
1138 producing ten viral proteins (85). Generally, the genomic structure has ten regions,
1139 which include: three structural regions (core, E1 and E2), viroporin region (p7) and
1140 six non-structural regions (NS) (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (64).

1141 The N-terminal end of the EqHV polyprotein, making up 25 % of the total protein, is
1142 cleaved by signal peptidase into mature structural protein (core, E1 and E2) and

viroporin (p7); while the C- terminus which accounts for the remaining 75 % is cleaved by viral proteases into non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (71). In HCV and GBV-B viruses, signal peptide peptidase (SPP) is involved during post-translational events in the structural protein. It is not yet known if SPP cleaves the C-terminal region of EqHV core proteins (87).

EqHV core protein has been shown to have a higher pH value, similarly with HCV pH profile. This data is congruous to the documented hepacivirus RNA binding and packaging function (64). As previously reported with HCV, the substitution of PHe^{191} and Ile^{190} with Leu and Ala, respectively, in the EqHV core protein was inferred to change the beta-sheet to an alpha-helix structure as HCV (87,188). This study further showed the relationship involving the cleavage of EqHV core protein and SPP and PHe^{191} and Ile^{190} as key to SPP-dependent cleavage (87). Furthermore, similarly to HCV (a possible underlining attributes of the Flaviviridae family), EqHV core protein is localised predominantly on lipid droplets (LD) as well as partially localised on the detergent-resistant membrane (DRM). It was also suggested that EqHV core protein partial localisation could be dependent on SPP-dependent processing (87,188,189). Furthermore, HCV Genotype 1b core protein shares about 50% amino acid homology with EqHV and a similar hydrophobic/hydrophilic profile with the core protein of HCV (87). Similarly, a high similarity (~65 %) was seen between the transmembrane region of EqHV and HCV core protein (87).

A high degree of amino acid similarity was observed in the E1E2 region of EqHV and HCV, especially half of the C-terminal of E2 (64). All 18 cysteine residues of the ectodomain of E2 are well documented to form stable disulphide bridges in all genotypes of HCV. These stable interactions are as well seen in EqHV E2 (14/18) but reverse with GBV-B. Hence making any tertiary structure could be more similar to HCV than any Flaviviridae member (64,190).

Tanaka and colleagues described a high level of conservation between EqHV and HCV in several secondary RNA structures, especially among the long-range RNA-RNA interaction and kissing loop. The study further showed complementary regions between the NS5B and 3'UTR of EqHV and HCV (87).

In HCV, it has been documented that the 3' region does not have a poly (A) tail, but a span of nucleotides (98nt) referred to as the X tail (191). This 3' X tail is preserved and seldom undergo genetic changes even in distantly related HCV types. This region of the UTR in HCV is believed to be implicated in HCV replication and detected in infectious sera (192). A slight difference is observed in the 3' region of EqHV, which has an adenine (A) rich sequence, but its 3' X tail sequence is relatively shorter when compared to HCV and predicted to have similar biological properties (65,87).

The upstream structural parts of the EqHV 3' region have no similarity to any known sequence, except the poly (U) and 3'X regions, which are similar structurally with HCV, although with some observable differences, especially in the stem-loop (114).

Direct comparison (pending experimental validation) of 3'UTR and 5'UTR of EqHV with HCV shows a high divergence level in these regions with about 66 % nucleotide identity (178).

The 5' terminus of EqHV (predicted using RACE procedure) and HCV are similar but not identical. EqHV 5'UTR has 366nt compared to HCV (341nt) compared to GBV-B (445nt) (64). It is predicted that, unlike HCV, the 5' terminus will have a large stem-loop (SL1) which is believed to be important in downstream processes involving microRNA-122(178). Furthermore, unlike HCV, EqHV lacks the domain IV region within the type IV IRES motif responsible for controlling translation (178,193). In 2018, Tanaka and colleagues (194) showed that the IRES element of EqHV is functionally exchangeable with the HCV IRES element.

EqHV G+C content is more similar to GBV-B but lower than that seen in HCV or pegiviruses and elevated in most other flaviviruses family members, especially pestiviruses (64). CpG (and UpA) dinucleotides are diminished in EqHV. This similar trend is seen in GBV-B and HCV but not in pestiviruses and flaviviruses (64).

The evolutionary patterns and genetic diversity of HCV have been elucidated using full-length genomes from infected humans, but that is not the case with EqHV. Studies have mainly relied on the analysis of partial sequences from several regions of the genome, including 5'UTR, NS3 and NS5B (65,73,112). The location of the hepacivirus hosts and the type of species has been postulated to affect these viruses'

diversities (195). Presently, phylogenetic analysis comparison showed EqHV to be the closest genetic relative to HCV; and GBV-B to be more related phylogenetically to rodent hepacivirus (195). For example, similar diversity profiles seen between HCV genotypes, especially in the E2 and 5' region of NS5A, are consistent between HCV and EqHV. However, variations still exist between other areas of the genome in terms of amino acid identity. The upper limit of amino acid identity is lies within NS3 and NS5B, while the lower limit lies with E1, N-terminal of E2, NS2 and C terminal of NS5A(64). Furthermore, among all other flaviviruses, the nucleotide identity based on sequence alignment using the Standard ClustalW-based approach between EqHV and HCV were higher (66 %) compared to GBV-B (57 %)(64).

Similar similarities are seen with p7 as well, and it includes liposome permeability features, comparable subcellular localization pattern in the ER membrane, ion channel activity, and its transmembrane domain. However, EqHV p7 could not directly replace HCV p7 during the assembly in the virus replication (196).

A notable difference between EqHV and HCV, which might be responsible for the observed differences in pathogenicity and biological significance, can be attributed to the possible absence of some specific micro(mi)RNA binding sequences in EqHV (64). Hepatitis C viruses have two highly conserved miR-122 binding sites among all genotype that supports its replication in liver cells (180,197). Nevertheless, dogs encode identical miRs-122, which is highly expressed in the liver but lacks the

needed binding sites; hence, implying that interaction might not be a route to infection (110).

Other similarities between HCV and EqHV include the ability to elicit a delayed host seroconversion, the ability to cause chronic infection and hepatotropic with a detectable level of viral RNA (66,83,113,137,198).

1.18 Model systems for the study of HCV

Very few closely related viruses infecting animals are known. HCV naturally infects only chimpanzees and humans, resulting in the sparsity of a small tractable animal model suitable for mechanistic analysis, immunogenicity, pathogenicity, treatment and vaccine development research (64,65,108,199,200). The chimpanzee model has its demerits, ranging from ethical considerations to economic reasons, making it not ideal for HCV research (200).

In 2015 (66), Ramsay and colleagues showed the kinetics associated with EqHV infection and hepatic pathology in horses with acute and chronic infection. The study further showed some merits for the use of the model and included the ability to perform long-term chronicity studies, large liver to perform several and consecutive liver biopsies, ability to study the closest genetic relative of HCV in its natural host and horses with SCID (Severe Combined Immunodeficiency) can aid

immunological studies. This data then validated the proposal for horses being used as a large animal model for HCV research. However, animal care cost, large size, specialized equine experience with vaccination and passive immunoprophylaxis through limited reagents availability could cause concern (66,76). These problems introduce a significant drawback to this model compared to conventional laboratory animal models, thereby making novel rodent hepaciviruses of particular interest (61,136,201).

Nevertheless, horses have advantages in their suitability as an animal model, especially in their striking similarity in natural history and genetic relatedness to HCV. The similarities shown by EqHV include a hepatotropic infecting virus capable of establishing chronic infections at a lower rate than HCV and having similar RNA transcripts in serum. Others include similar host immune responses with HCV, including delayed seroconversion, with a simultaneous rise in hepatic enzymes as well as inflammation necrosis (113,114).

1.19 Importance of EqHV research

1.19.1 HCV research:

Infectious disease research has been seen to be primarily dependent on small animal models. However, in HCV, this has been a problem, as well as its limited host range. These challenges, in turn, has hampered the complete understanding of its

pathogenesis and design of a suitable and effective prophylactic vaccine (202). Other models like chimpanzees have been explored as potential models, facilitating studies of infectivity and pathogenesis. However, they have significant drawbacks, including a lower rate of chronic infection, legality, ethical issues and finances in most countries (203,204). Another explored model was the human liver chimeric mouse model, which, like the chimpanzee, permits passive immunoprophylaxis studies. However, it does not permit studies in the adaptive immune arm of immunity, hence not aiding HCV vaccine research (205). In recent times, a range of different rodent models (see section 1.2) have shown to support HCV replication and proven very useful in evaluating the efficacy of several antiviral and neutralizing monoclonal antibodies (202,206).

Nevertheless, currently, genetically modified mice are used as a tractable small animal model for *in vivo* infection of HCV. These mice, homozygous for severe combined immune deficiency spontaneous mutation $Prkdc^{scid}$ (commonly known as SCID), are known to be deficient in adaptive cell-mediated immune responses, gamma globulins, lymphocytes and normal haematopoietic microenvironment (207–210). This mammal is not ideal for immunological and pathogenetic studies of HCV research, predominantly due to its immunodeficiency (211–213). Several surrogate models have been suggested (see section 1.2), and a typical example includes HIV studies in humans using simian immunodeficiency virus in Old World

monkeys and HCV research using GBV-B to infect Tamarins. Research of EqHV infection mechanism will elucidate and aid the studies into HCV pathogenicity and immunity.

1.19.2 Horse racing industries, horse performance and importance to equine health practitioners

When a companion animal gets liver disease, it is euthanized, and another horse is usually bought to replace it, partly because of its relatively low price and value. In contrast, some Thoroughbred racehorses can have values into millions of pounds. Thoroughbred racehorses with a particular pedigree and performance are usually inbred to preserve the desired genetic trait. As such, there is a market for interventions for liver disease in these high-value animals. In particular, active racehorses experiencing a hepacivirus infection may be treated with existing classes of HCV therapies. A rapid diagnostic test kit and potent therapeutics could be valuable tools to maintain racehorse performance.

The dynamics of the treatment of a viral infection that can infect both animals and humans raises new questions, including possible therapeutic interventions, the evolution of the virus into pathogenic strains, as well as possible pathological effects (108,214,215). Furthermore, EqHV research will help elucidate there if there are other diseases associated with EqHV.

1.19.3 Consequences on human health

Commercial horse sera are routinely used and licensed for treatment/prophylaxis of several human and animal ailments. These sera are usually heat-treated to make any viral particle non-infectious. Hence, the presence and inability to adequately detect and characterize EqHV in these commercial preparations could have significant consequences for the receiving host's wellbeing (animal or man), especially in poorly heated treated sera. Similarly, in cell culture research, several cell lines are used to cultivate viruses or other organisms grown under specific conditions using supplements derived directly or indirectly from horse sera. As virus components could still trigger activation of an innate response in some cell lines, this has a more significant impact. Furthermore, this also applies to other biologicals like live vaccines. Hence, screening for EqHV contaminants could prove crucial in several applications, thereby making it imperative for the virus to be researched and adequately characterized.

A study (84) showed that all commercially available horse single-cell culture sera batches used in cell culture experiments (except a foetal horse serum) from independent geographical locations had EqHV RNA and other hepaciviruses in it to different extents. This study research was corroborated with Lu and colleagues (139) research in China, which showed that commercial serum samples contained

multiple EqHV variants. Further analysis showed a certain level of biosecurity risk because of biologicals produced using equine sera and showed that ~69 % had EqHV genome with an average viral load of 9.2 x 10^5 copies/mL (84).

1.20 Aim of the project

The aim of this project was to interrogate the prevalence of EqHV infection in a Thoroughbred horse population and evaluate the consequences of infection. This knowledge will help design EqHV as an animal model for HCV infection and inform vaccine design for hepaciviruses.

Here we investigated the prevalence of EqHV in 66 Thoroughbred racehorses and their phylogenetic relationship, infection profile, and entry pathway into host cells. The main areas of research will be:

1. Understand the prevalence of EqHV among Thoroughbred racehorses. Sixty-six horses were screened for the virus's presence by designing primers targeting different structural and non-structural regions of the virus and included Core-E1 and E1E2.

2. Amplification of the envelope genes (E1 and E2) of infected animals. Perform Reverse transcription-polymerase chain reaction (RT-PCR) on 66 unique Thoroughbred horse samples using E1E2 assays.

3. Examine the quasispecies diversity in infected animals. Perform Sanger sequencing on any PCR positives generated, then analyse using maximum likelihood model.

4. Understanding the phylogenetic relationship as well as genetic relatedness between different strains of hepaciviruses. Phylogenetic trees of all amplified regions of the isolated viruses using appropriate software were produced.

5. Examine the entry properties of different virus isolates. Establish a pseudotype system that allows the generation of pseudoparticles that can be used to investigate EqHV entry.

2 Materials and methods

2.1 Study cohort

Newmarket Equine Hospital (NEH), located in the United Kingdom, is Europe's largest specialist equine veterinary centre, providing clinical diagnosis and referral services for horses throughout the UK and internationally. Samples used for this study were surplus diagnostic specimens collected for routine haematology and biochemistry tests. Samples were obtained for 66 Thoroughbred racehorses submitted to the NEH diagnostic laboratory between 14th and 27th August 2017. Ethical approval for this work was not needed as it was classified as an external diagnosis in collaboration with NEH.

The horses were actively training for flat horse racing in the UK and housed by 18 different trainers (47 unique owners). These were licensed and registered horse trainers with the National Trainers Federation (NTF), with jobs typically involving grooming, engage in daily feeding, and riding their horses. Usually, these trainers work with more than one horse per season for competition.

The study cohort consisted of 43 males and 23 female horses and ranged from 2-8 years of age at the time of sampling (Table 2.1). Thirty-five follow up samples for eight horses were obtained and used for retrospective analysis (Table 3.3). Samples were received at the NEH within 12 hours of collection and prepared by centrifugation (Universal 320®, Hettich) for 8 minutes at 2,800 RPM. Plasma (or

serum) was aspirated and stored at -80°C. All clinical sampling and analysis were performed following the UK stipulated guidelines and with the consent of the owner/trainer. No ethical approval needed as this study was classified as an extended form of diagnosis.

Table 2.1: Demographic parameters and demographics of the horses used in this study

Demographic parameters	**Number of horses**
Sex	
Male	43
Female	23
Date of birth	
2015-2013	41
2012-2010	7
Unknown	18
Trainers/Owners	
Trainers	18
Unique owners	47

2.2 EqHV RNA extraction

RNA extractions were performed on 100 µL of each sample using the QIAmp RNA MiniElute® Virus Spin RNA extraction kit (Qiagen, France) according to the manufacturer's instructions and eluted at a final volume of 50 µL based on a published method (216). Before extraction, serum and buffers were equilibrated to room temperature. Serum was mixed with 25 µL QIAGEN protease and 200 µL of buffer AL containing 28 µg/ml of carrier and guanidine hydrochloride. Cellular lysis was performed for 15 minutes at 56 °C. The resulting lysate was incubated with 250 µL of ethanol at room temperature to ensure adequate RNA binding onto the QIAmp MiniElute column.

Furthermore, RNA bound to the column after centrifugation at 6000 x g for 1 minute. Three separate washing steps were then carried out, involving first with 500 µL of AW1 (containing guanidine hydrochloride), then with 500 µL of buffer AW2 and finally with 500 µL of ethanol. An additional centrifugation step further eliminated residual contaminations and ethanol carryovers. RNA was then eluted in 20 – 50 µL of buffer AVE and stored at -80°C.

2.3 Complementary DNA (cDNA) synthesis

Viral complementary DNA (cDNA) was made from RNA extracts using a commercially available Thermoscript™ RT-PCR system with random hexamers primers (Life Technologies). Ten µL of viral RNA were used in a 20 µL reverse transcription reactions premix. The homogenized reaction was transferred to a thermal cycler; incubated at 42 °C for 60 minutes. The reaction was stopped by heating at 70 °C for 10 minutes.

2.4 Design of Polymerase Chain reaction (PCR) primer assays

All 15 full-length EqHV reference sequences on GenBank were downloaded based on high similarity with NZP1 (accession number NC_038425) using the BLAST (Basic Alignment Search Tool) search tool. These sequences were then aligned using the MUSCLE algorithm as implemented in Mega 7. Conserved sites within the Core-E1 and E1E2 were manually identified. Using an online PCR calculator (http://www.bioinformatics.org/sms2/pcr_products.html), the appropriate primer length (18 - 30 bases), product size, and degeneracy avoided in the 3' region, and appropriate primers were selected to amplify these genomes. The primer pair properties were then tested using an online software tool called Primer3 (http://primer3.ut.ee/). These included melting temperature (50 °C – 65 °C), %GC

content (40 to 60), absence of significant hairpin formation (>3bp), absence of dimerization capability and lack of secondary priming sites.

2.5 Amplification and quantification of the desired genetic sequence using Polymerase Chain reaction (PCR)

Three PCR based methods were used to amplify and quantify EqHV in selected samples. The first method used is called ''hot start PCR'', a modified form of the conventional PCR protocol that requires activation of the Hot Start Taq polymerase activity. This protocol involves reducing primer dimers and undesired products due to non-specific DNA amplification at ambient temperature (217,218). Hence, it required less handling and lowers the risk of contamination, although the enzyme activation step could denature and chemically modify the DNA and increase the PCR time due to the initial enzyme activation step (219,220).

A nested PCR method was used when generating E1E2 EqHV sequences due to it having a longer DNA fragment length and the need for higher sensitivity. This process involved two sets of primers, with the first set spanning a large region (892 to 2762 nucleotide position in alignment with NZP1 ref sequence) which included the target region and the second run with the second set of primers (937 to 2547 nucleotide position in alignment with NZP1 ref sequence) to target the desired area using the first amplicon as a template. Two sets of primers provide the necessary

specificity, preventing the amplification of non-specific products. Therefore, it mainly applied where small amounts of starting templates are available, theoretically as low as a single molecule of the template (221,222).

The amplified EqHV DNA sequence was monitored quantitatively using the real-time polymerase chain reaction (real-time PCR), also called quantitative polymerase chain reaction (qPCR) (223,224). A positive control qPCR template was generated by cloning (see section 2.11) a C-E1 PCR product (amplified using primers EqHV_C-E1f and r) into pGEM-T (Promega). Viral genome quantification was determined by qPCR with a standard curve generated using a serial dilution of the plasmid control from 3.57 x 107 to 357.14 copies/mL. cDNA samples (2 µL) were assayed with EqHV_C-E1f and EqHV_C-E1r primers using 2x qPCRBIO SyGreen Blue Mix (PCRBIO), as per the manufacturer's instructions, at an annealing temperature of 55°C and extension time of 30 seconds.

In all cases, an appropriate volume of PCR master mix was made using the appropriate primer set (Table 2.2) and aliquoted into individual strip reaction tubes to make a final volume of 20 µL with cDNA. Each reaction (amplifying either the 5'UTR, Core-E1, E1E2, NS3, E1E2, EpgV, EqPV-H and TDAV) was carried out at an appropriate as well as optimized PCR cycling conditions: a single round PCR (NS3, Core-E1, 5'UTR, EpgV, EqPV-H and TDAV) with hot start enzyme or nested PCR with Long-Amp (OF-OR (892 to 2762 nucleotide position in alignment with NZP1 ref

sequence), and f-r (937 to 2547 nucleotide position in alignment with NZP1 ref sequence)). For the single-round PCR, the optimized PCR parameters used were 55 cycles at 95 °C for 15 minutes, 95 °C for 20 seconds, 55 °C for 20 seconds and 72 °C for 30 seconds, then a final extension step at 72 °C for 60 seconds extension time. In contrast, the optimized Long-Amp PCR parameters for both rounds were 45 cycles at 98 °C for 10 seconds, 55 °C for 15 seconds and 72 °C for 60 seconds.

1488

Table 2.2. Primers used in this study. Nucleotide numberings relative to reference sequences: EqHV, NC038425; TDAV, MH063521; EPgV, KC410872; EqPV-H, MG13672. [1] primers designed by members of Virus research group.

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Primer name	Primer sequence (5' – 3')	Notes
Equine hepacivirus (EqHV)		
AK4340F1 (NS3f)	GTACTTGCTACTGCNACNCC	nt 4357-4376 of EqHV (ref Kapoor 2013)
AK4630R1 (NS3r)	TACCCTGTCATAAGGGCRTC	nt 4655-4636 of EqHV (ref Kapoor 2013)
EqHep_C-E1f [1]	CTTGTrCGrTTTGTkGAGGA	nt 886-905 of EqHV
EqHep_C-E1r[1]	GCYTCRCCAATWCCNAGAGTAGA	nt 1244-1222 of EqHV
EqHep_E1E2f[1]	caccATGGGCATGGGyTTCTCTAT	nt 937-953 of EqHV with TOPO cloning tag
EqHep_E1E2r[1]	TTAAGCYTCRGCYTGCA	nt 2565-2549 of EqHV
Qanti-5UF1 (5' UTRf)	GAGGGAGCTGRAATTCGTGAA	nt 196-216 of EqHV (ref Burbelo 2012)
Qanti-5UR1 (5' UTRr)	GCAAGCATCCTATCAGACCGT	nt 351-331 of EqHV (ref Burbelo 2012)
EqHep_E1E2_Or[1]	CTAAARGCNGCNGTAACAGCACTAG	nt 2786-2762 of EqHV
EqHep_E1E2_OF [1]	CTTGTrCGrTTTGTkGAGGA	nt 892-1102 of EqHV
Theiler's disease-associated virus (TDAV)		
EqHep_TDAVf[1]	GCTCAGCGGATTCTTGAGTG	nt 2707-2726 of TDAV
EqHep_TDAVr[1]	TTGTCCTTCCCCATCATGCT	nt 2906-2887 of TDAV
Equine pegivirus (EPgV)		
EqHep_PegiD&Ef[1]	GAGAAGATGATyCTmGGnGAC	nt 9235-9255 of EPgV
EqHep_PegiD&Er[1]	TATCTTNCGACARGCNGCA	Nt 9627-9608 of EPgV
Equine parvovirus with hepatitis (EqPV-H)		
EqHep_PARVf[1]	ATGCAGATGCTTTCCGACC	nt 3218-3236 of EqPV-H
EqHep_PARVr[1]	GCCCCAGAAACATATGGAAA	nt 3386-3367 of EqPV-H

Cloning		
T7 (sense)	5’ TAATACGACTCACTATAGGG 3’	`863 - 882 (upstream of insert) - Invitrogen
bGH (antisense)	5’ TAGAAGGCACAGTCGAGG 3’	1111 - 1128 (downstream of insert) - Invitrogen

2.6 Agarose gel electrophoresis

5 µL of the amplified PCR products were loaded on a 2 % agarose gel (Fisher Scientific, Belgium, FW) containing 0.5 µg/mL ethidium bromide, and electrophoresis was conducted in Tris-Acetate-EDTA (TAE) (60 mM Tris-acetate 50 mM EDTA, pH 7.8) buffer. After electrophoresis at 90 volts for 36 minutes, DNA bands were visualized by UV transillumination.

Products with desired target size were diluted ten-fold (1:10) and sent for confirmatory Sanger DNA sequencing (Source BioScience Life Sciences, Nottingham, United Kingdom) with the same sets of primers used at 3.2 pmol/µL. Finch TV software version 1.4 (Perkin Elmer package, Genesifter Lab edition, USA) was used to check manually and base-call the obtained sequences uploaded to the NCBI database with accession numbers noted. Sequences were then confirmed based on the highest identity score in BLAST (Basic Alignment Search Tool) with Megablast programs using scoring parameters.

2.7 Purification of PCR products

Before subsequent sequencing or cloning PCR products of verified size, PCR products were purified using NucleoSpin® Gel and PCR Clean-up Kit (Takara) according to manufacturers' protocol.

One volume of PCR product was mixed with two volumes of Buffer NT1. The mixture was transferred to the NucleoSpin® Gel and PCR Clean-up silica membrane column to bind DNA and centrifuged for 30 seconds at 11,000 x g with flow-through discarded. 700 µL buffer NT3 was added to the NucleoSpin® Gel and PCR Clean-up column to wash the silica membrane by centrifugation for 30 seconds at 11,000 x g and flow-through discarded. The silica membrane was dried by centrifuging for 1 min at 11,000 x g to remove buffer NT3 altogether, and flow-through was discarded. The NucleoSpin® Gel and PCR Clean-up column was transferred into a new 1.5 mL microcentrifuge tube, and 15-30 µL elution solution NE was added and centrifuged for 1 minute at 11,000 x g after incubating at room temperature (18-25 °C) for 1 minute. DNA was quantified by spectrophotometry and stored at -20°C. Measurements were performed with a NanoDrop spectrophotometer (Thermo Scientific) which allows accurate quantification of DNA concentration in 0.5-2 µL sample. The peak of light absorption is at 260 nm for DNA and 280 nm for protein (225). NanoDrop software calculates DNA concentration and absorption value and

informs its purity based on 260/280 ratios (226–228). The equation to determine the absorption value is given below:

A= ε (260) x c x p.

Where A = absorbance

P = Path length of Nanodrop, usually it is 1cm

ε (260) = extinction coefficient of the DNA sample at 260 nm.

2.8 GNA capture ELISA

All horse serum samples were tested for antibodies directed to EqHV E1/E2 glycoproteins. High-bind 96 well plates (NUNC Maxisorp) were coated overnight with GNA (*Galanthus nivalis* lectin) (Sigma) at a final concentration of 5 µg/mL at 4 °C. Plates were washed with 300 µL of 0.05% Tween 20/PBS (phosphate buffered saline) (v/v) and coated wells were blocked with 200 µL of 5 % milk in 0.05 % Tween-20/PBS) for 2 hours at room temperature. After flicking off the blocking solution and washing it once with PBS-Tween, pre-diluted E1E2 preparations (lysate: E1E2 from strain PAA (a cloned E1E2 from a PCR product generated by Eike Steinmann and colleagues, in the pcDNA3.1 expression plasmid)/H28.4, and a mock-transfected negative control (see section 2.18)) 1:5 in PBS –Tween was added and incubated for 2 hours at room temperature. The wells were washed three times with 200 µL of 0.05 % Tween 20/PBS (v/v), and then the test samples which have been diluted 1:50

in 100 µL of 0.05 % Tween 20/PBS (w/v) were then added to each well and incubated at room temperature for one hour. After three rounds of washing, a 1:1000 dilution of anti-horse immunoglobulin-G AP conjugate (Sigma) in PBS-Tween was added to the well and incubated for one hour at room temperature. After three rounds of washing, plates were developed with pNPP (p-Nitro phenyl phosphate) substrate (SIGMAFAST; Sigma-Aldrich) to each well per the manufacturer‘s protocol. Plates were allowed to develop for 15-20 minutes and were read at 405 nm. Background reactivity to mock-transfected cell lysate was subtracted, and the immunoreactivity of samples to control wells was compared, and three times the experimental control standard deviation plus the mean was set at the cut-off.

2.9 Phylogenetic analysis

Phylogenetic analyses were generated by the maximum likelihood (ML) method in MEGA 7 software (229). All published equine hepacivirus reference sequences from GenBank were downloaded and aligned with the isolated positive horse sequences (Core-E1, NS3, and E1E2). These sequences were then aligned using the program MUSCLE as implemented in the MEGA 7 software. To ascertain the robustness of the generated trees, confidence values were determined for the internal branches by 1000 bootstraps replicates using amino acids p-distances based on aligned amino acids sequences (FASTA format (Fast Alignment search tool)) from full-length or

partial proteins (230–232). Furthermore, maximum parsimony was used to build trees were carried out to confirm the ML tree.

2.9.1 Definition of a transmission cluster

The transmission cluster was determined by using nodes that were statistically supported in the phylogeny using a quick non-parametric version of an approximate likelihood ratio test (aLRT) referred to as Shimodaira-Hasegawa (SH)-aLRT (233). Clusters were defined as aLRT-SH > 0.85 (based on detailed studies on Human Immunodeficiency Virus clusters, Joakim Esbjornsson, unpublished observations).

2.10 Bioinformatic analysis

Dinucleotide odds ratios (DORs) were calculated in ''R'' using a published function (234). Statistical analysis was carried out in GraphPad Prism 8.0. GC content, Pairwise Distance Estimation and Maximum Likelihood phylogenetic trees were calculated in MEGA 7 (229). SignalP-5.0 was used to calculate signal peptidase cleavage probabilities (235). Positive selection analysis was carried out in DataMonkey using the Fixed Effects Model of Evolution (236). HCV E2-constrained *in silico* modelling of HVA E2 was performed in I-TASSER using the crystal structure

of the HCV E2 core ectodomain (6MEI) (237). Electropherograms were visualised using Codon Code Aligner (www.codoncode.com).

2.11 Infusion cloning of PCR products into pcDNA3.1

Linearized pCDNA3.1 vector digest was generated using EcoR1 restriction enzyme. The vector in a total amount of 3ng was digested with 5 µL of EcoR1, 5 µL 10X buffer and made up to 50 µL with water. The setup was incubated at 37 °C overnight, after which the enzyme was heat-inactivated at 65 °C for 20 minutes then ran through the NucleoSpin clean-up kit to remove the enzyme. The linearized vector was confirmed by running it on a 2 % agarose gel and observed band sizes changes.

An E1E2 PCR product was purified, as described in section 2.7. Cloning reactions were assembled at a vector: insert molar ratio of 1:3-1:4, with a total DNA concentration of 50-100 ng in a total volume of 10 µL. A cloning reaction consisted of one volume of linearized and digested EcoR1, purified PCR product, 5X Infusion mix, and two-volume of deionized water. The reaction was incubated for 15 minutes at 50°C in a thermocycler, then placed on ice for 3 – 5 minutes.

Cloned plasmids were transformed into *E.coli* cells, as described in section 2.11. Transformed cells were seeded on agar plates with ampicillin, and after overnight incubation, colonies were screened for the presence of the right size insert (described in 2.12). Screening products were analysed via gel electrophoresis, and

colonies containing the right size insert were grown overnight in Luria Broth to amplify the desired plasmid. Column purified plasmids were sequenced (as mentioned further in section 2.14).

2.12 Transformation of plasmids into Escherichia coli

The transformation was performed on 50 µL aliquots of Stellar competent cells by adding 1-5 µL of plasmid depending on its concentration. The mix was incubated on ice for 3 – 5 minutes immediately after the incubation in a thermal cycler. Afterwards, heat shock was performed at 42°C for 30-45 seconds, followed by a brief incubation on ice for 3 -5 minutes. A 250 µL aliquot of LB was added, and the culture was incubated at 37°C with shaking at 300rpm for one hour without antibiotics. To select bacteria that contain the plasmid, culture was seeded on LB agar plates with ampicillin (100 µg/mL). After overnight incubation at 37°C, colonies from the plate were picked for screening.

2.13 Screening of bacterial colonies

To ascertain which clone had the correct insert, single colonies were screened using PCR. The PCR master mix in 15 µL volumes containing 10X buffer, 0.6 µL T7 and bGH (5pmol) primers (Table 2.2), 0.6 µL of 5 pmol/µL dNTPs, 0.075 µL hot start Taq

polymerase (Qiagen) and 11.625 µL water. Thermoblock cycling parameters were initial denaturation at 95 °C for 15 minutes followed by 30 cycles of 95°C for 20 seconds, 55 °C for 20 seconds and 72 °C for 2 minutes. A final extension at 72 °C for 10 minutes, followed by a final hold of 8 °C was then performed.

PCR screening products were analysed on 2 % agarose gel as described in section 2.61. Colonies containing clones with correct size insert were further picked for overnight culture in a 5mL of LB with ampicillin to obtain 30-50 µL or a 150 mL LB with ampicillin to obtain 1000 µL of plasmid preparations.

2.14 Plasmid purification from 1-5 mL overnight culture

2.14.1 Miniprep

The silica membrane-based method was used to purify the plasmids with the GenEluteTM Plasmid Miniprep kit's aid. An overnight culture of *E.coli* was centrifuged at 12,000 x g for 1 minute. Bacterial pellets were completely re-suspended in 200 µL of chilled resuspension solution (containing RNase A solution at 0.1mg/mL). The re-suspended cells were lysed by adding 200 µL of lysis solution, mixed gently and allowed to stand for 3 minutes. Cell debris was then precipitated by adding 350 µL of 'Neutralization/Binding solution' immediately followed by centrifugation at maximum speed for 10 minutes.

The GenElute™ Miniprep Binding Column was then prepared by adding 500 µL of column preparation solution and centrifuged at 12,000 x g for 30 seconds. This step is essential as it maximizes DNA binding to the membrane, resulting in more consistent yields. Then 350 µL volume of the cleared lysate after neutralization was transferred to the prepared column and centrifuged at 12,000 x g for 30 seconds. The column was then washed with 750 µL of the diluted wash solution and centrifuged at 12,000 x g for 30 seconds and then finally at maximum speed for two minutes. This wash step removed any residual salts and other contaminants introduced during the column load. The column was then transferred to a fresh collection tube, and 100 µL of elution solution was added and centrifuged at 12,000 x g for 1 minute. The DNA is now present in the eluate, and the concentration measured with the aid of a Nanodrop machine (PerkinElmer package, Genesifter Lab Edition, USA). The concentration can be serially diluted, if needed, to a required concentration. The measured eluted DNA is either used immediately or stored at -20°C.

2.15 Plasmid purification from 50-150 mL overnight culture

2.15.1 Midiprep

In order to perform mammalian cell culture experiments, large volumes of EqHV E1E2 containing plasmids had to be used. Plasmids were purified using GenElute™

HP Plasmid Midiprep kit as recommended by the manufacturer. A 50 mL volume of an overnight culture was harvested and centrifuged at 5,000 x g for 10 minutes. Pellets were resuspended entirely in 4 mL of resuspension/RNase A solution. The resuspended cells were lysed by adding 4 mL of lysis solution, gently mixed and allowed to sit for 4 minutes. The lysed cells were neutralized by adding 4 mL of chilled neutralization solution and mixed gently for 5 seconds.

A 3 mL volume of binding solution was added to the mix and immediately poured into the filter syringe barrel. The cell lysate was left in the syringe in an upright position for 5 minutes to ensure adequate separation of lysate from cell debris. The GenElute HP Midiprep Binding Column was prepared by it on a vacuum and adding 4 mL of Column preparation solution. The Column preparation solution was allowed to pass through the column under vacuum pressure. Individual samples were filtered into their respective columns, and vacuum pressure applied until all the lysate passed through. Two washes were carried out using 4 mL each of Wash solution 1 and 2. The latter contained 99.9 % ethanol. Following the wash steps, the vacuum was left for 10 – 20 minutes to dry depending on the number of columns being processed to ensure complete ethanol removal. Plasmid DNA was eluted in 1 mL of elution solution and stored at -20 °C. The concentration can be serially diluted, if needed, to a required concentration.

2.16 Sequencing and contiging of clones

Sequence analysis of each clone was carried out to ascertain if the cloning process was successful. Sanger sequencing (with conditions described in section 2.6) was performed using T7 and bGH primers (see Table 2.2) to reveal 5' end and 3' end insert sequences and the associated sequence of plasmid flanking these regions. Contigs assembled with DNAStar (Lasergene) and uploaded to GenBank (accession numbers: partial C-E1 genes, MN637684–MN637694; contigs of C (partial)-E1-E2 genes, MN615275-MN615289).

2.17 Cell culture

The HuH7 cells, a well-differentiated hepatocyte-derived carcinoma cell line, and HEK (Human Embryonic Kidney) T 293 cells were maintained in DMEM (Dulbecco's modified Eagle medium) (ThermoFisher Scientific), supplemented with 1 % NEAA (Non-Essential Amino Acids) (ThermoFisher Scientific) and 10 % FBS (Fetal Bovine Serum) (ThermoFisher Scientific) without antibiotics and incubated in a humidified atmosphere at 5 % $C0_2$ and 37 °C. Cells were passaged and seeded regularly to maintain cells continually at log phase and optimum confluence at 1.5-2.0 million cells per mL every 2-3 days following an established protocol (238).

2.18 Transfection

A first-generation system was used to generate the pseudoparticles, where the lentiviral backbone pNL 4.3 Luc R^-E^- (239,240) encodes the luciferase reporter gene. HEK 293T cells were seeded at 1.2 million cells in 100mm diameter Primaria coated cell culture dishes (Corning) and incubated overnight at 37 °C and 5 % CO_2. Three different reaction A, B, C, was made. Reaction A contained a mixture of 24 µL of a stable cationic polymer called polyethyleneimine (PEI) and 276 µL of Opti-MeM; Reaction B was made up of a mix of 2 µg of PNL4.3 E^-r^- luc made up to 300 µL with Opti-MeM; Reaction C was made up of 2 µg of test plasmid encoding the E1E2 genes of EqHV. Reaction A and B were mixed first, then followed by C, after which the resulting mixture was then mixed and incubated at room temperature for one hour. The final resultant Plasmid-PEI mixture was incubated with 293T cells containing fresh 7 mL Opti-MeM medium for 6 hours at 37 ^oC and 5 % CO2. After 6 hours, the Opti-MeM is removed and replaced with 10mL of DMEM and the cells incubated at 37 °C and 5 % CO_2 for 72 hours.

2.19 Infection and Luciferase Assay

HuH7 cells were seeded 15000 cells/well in a sterile flat-bottom 96-well white plate and grown in 100 µL DMEM and left overnight at 37 °C and 5 % CO_2. Cells were then incubated with 100 µL of the pseudoparticles in sextuple for 4 hours at 37 °C and 5

% CO_2. After 4 hours, each well was topped up with 200 µL of DMEM for 72 hours. At the end of 72 hours, the luciferase assay system (Promega) was used to carry out the luciferase assay. 50 µL of cell lysis buffer (made using the manufacturer's protocol, Promega) was added to each well to lyse the cell, after which it was incubated on a rocker at room temperature for 15 minutes and vortexed for 15 seconds. FLUOStar Omega filter-based multi-mode chemiluminescence microplate reader (BMG LABTECH) was used to inject 50 µL of the luciferase substrate into the wells and measure the chemiluminescence from the cells at a machine gain value of 3600.

3 Prevalence studies and infection profile of EqHV in horse cohort

3.1 Introduction

EqHV is presently the closest genetic relative of HCV with similar infectivity and chronicity profile. Presently, horses are assumed to be the natural host for EqHV (64–66,74), and detected in all continents in the world except Antarctica (65,69,138,74,81,82,85–88,127). Of all known horse breeds, Thoroughbreds have been found to have the highest prevalence, although the reason for it is yet to be determined (84,127,147). The phylogenetic tree and spread of EqHV data showed that these viruses cluster with other viruses worldwide and not restricted to a particular location, with the reason yet to be determined. Therefore, suggesting no distinct correlation between the geographical area and the virus's strain (82). Determining if these infections are acute or persistent is important to understanding this virus's transmission and pathogenesis.

3.2 Results

In this study, we investigated whether EqHV prevalence, viral copies, reactivity and phylogeny in our Thoroughbred racehorse cohort correlates with what has been reported in other data worldwide. Polymerase chain reaction (PCR) assays were designed for several regions (Core-E1 and E1E2) (see Figure 3.1) of the hepacivirus genome and were compared to a standard assay targeting the non-structural (NS)

three and 5’ UTR genes (65,121). Furthermore, this section aims to identify if persistent EqHV infections apply to our cohort as well as investigating its seroreactivity. To achieve this, we were able to retrospectively identify several sera from some horses at different time points. These serum samples were then screened for EqHV RNA, and phylogenetic analysis was performed.

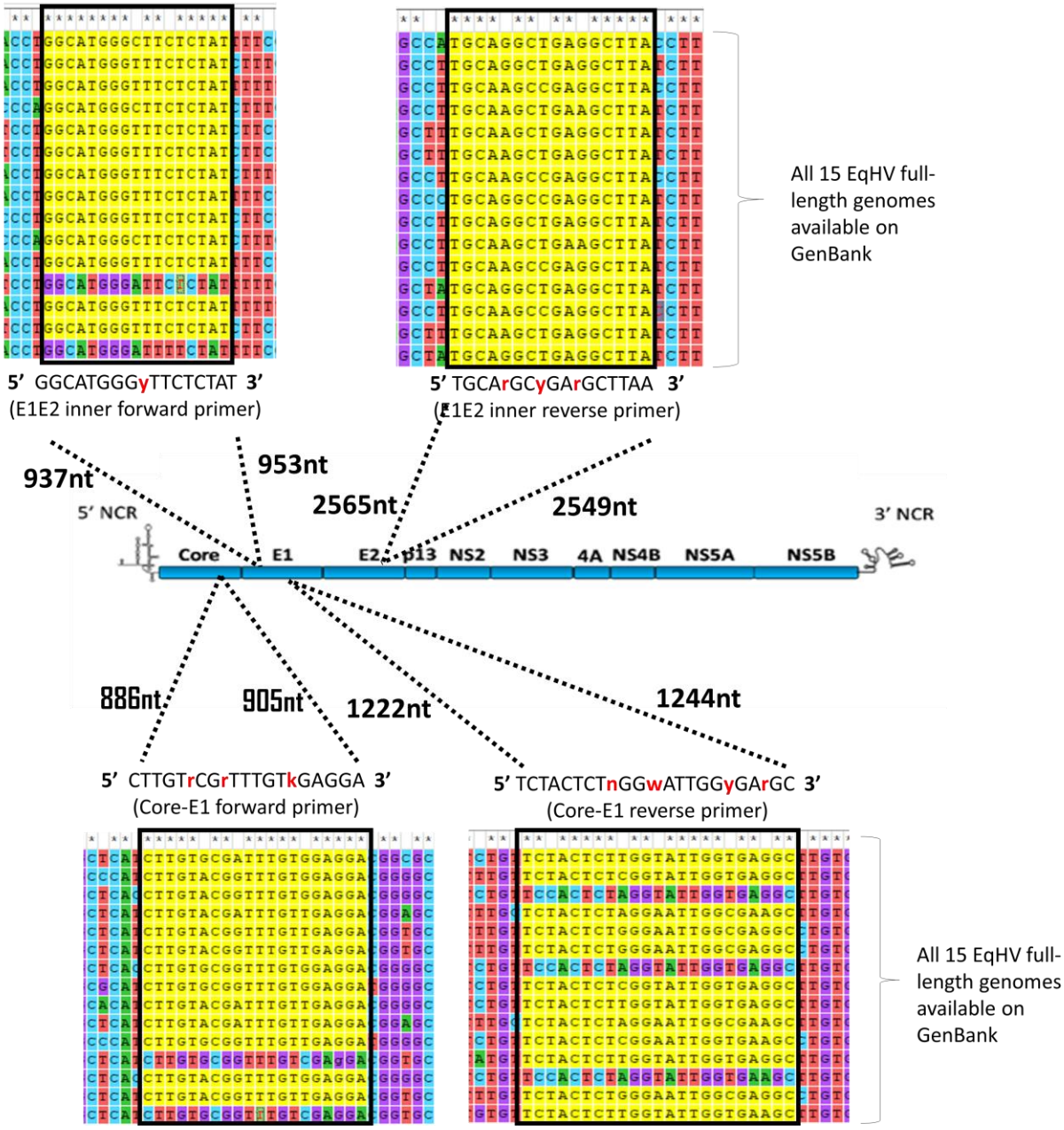

Figure 3.1: Primer design (E1E2 (a) and Core-E1 (b)) in the conserved aligned region of all available EqHV full length sequences on GenBank. Yellow: Conserved region; red: degenerate bases

3.2.1 Amplification of different regions of EqHV genome

To determine the prevalence of EqHV in Thoroughbred sera samples. Sixty-six serum samples taken from discrete horses were sourced from Newmarket Equine Hospital, UK. Subsequently, viral RNA was extracted using a QIAmp RNA extraction kit (see section 2.2).

New PCR assays were designed targeting the most conserved regions of the EqHV genome with minimum permissible degeneracy. All available full-length EqHV reference genome sequences were downloaded from GenBank, MUSCLE aligned and carefully studied to discover new conserved sites. Primers targeting the most conserved regions of the Core-E1 genome region were designed manually aided by the Primer3 primer design tool. Priming sites to amplify the entire E1E2 genes were chosen to amplify all Core-E1 positive samples' expression constructs.

Gradient assays involving using a matrix of several PCR amplification conditions were carried out to determine the optimum PCR amplification conditions using a donor serum positive control (kindly provided by Dr Patrick McClure and confirmed using Sanger sequencing). This gradient assay was essential to validate both the standard assay protocols (NS3 and 5'UTR) and determine the optimized PCR conditions for the newly designed assays (Core-E1 and E1E2) as well as other assays (EpgV and TDAV). Each PCR assay was carried out at different annealing temperatures as well as other cycling parameters, and the most optimal thermal

annealing temperature for all assays (5'UTR, Core-E1, NS3, and E1E2) were found to between 52°C to 58°C with an elongation step of 72°C for 20 seconds (Figure 3.2).

Lane	Anneal temperature (°C)
1	52.3
2	54.4
3	56.2
4	58.5
5	60.7

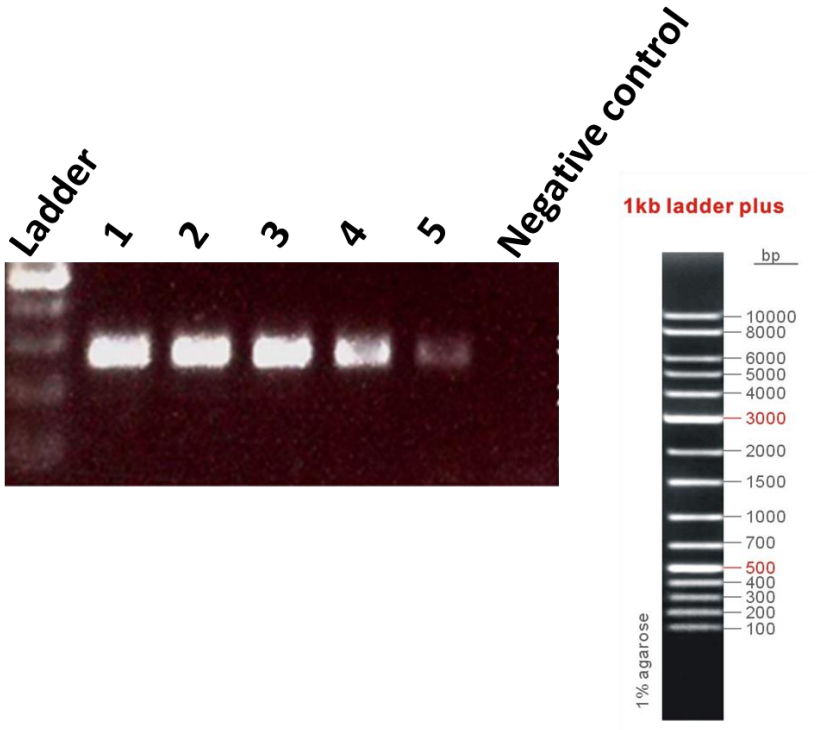

Figure 3.2: Gradient for a NS3 Polymerase reaction assay (PCR). Agarose gel photograph showing a NS3 PCR optimization assay using a positive control (Donor serum) sample with different annealing temperatures ranging from 54.9^{0}C up to 69.5C. Positive control: Donor serum; Negative control: Water

The amplification of several EqHV genomes regions, including the standard PCR assay (NS3 and 5'UTR) and newly designed assays (Core-E1 and E1E2), were performed using randomly primed viral cDNAs generated from horse sera. All samples were amplified with optimized PCR conditions, and the resultant products sequenced (Figure 3.3). Of all the newly designed and published assays, the assay amplifying the Core-E1 region (Figure 1.1, 3.1 and 3.3) was found to be the most sensitive, identifying 22 positives in the 66 samples (33.3 %) (Table 5). The previously described 5' UTR and NS3 primer sets identified 18/66 (27.2 %) and 13/66 (19.6 %) positives, respectively. All samples detected by the NS3-specific primer set were amplified by the primers detecting Core-E1. Three of the samples determined to be negative in the Core-E1 assay (horses 27, 30 and 56) were positive in the assay detecting the 5'UTR. Overall, the prevalence of infection in this population was found to be 37.9 %.

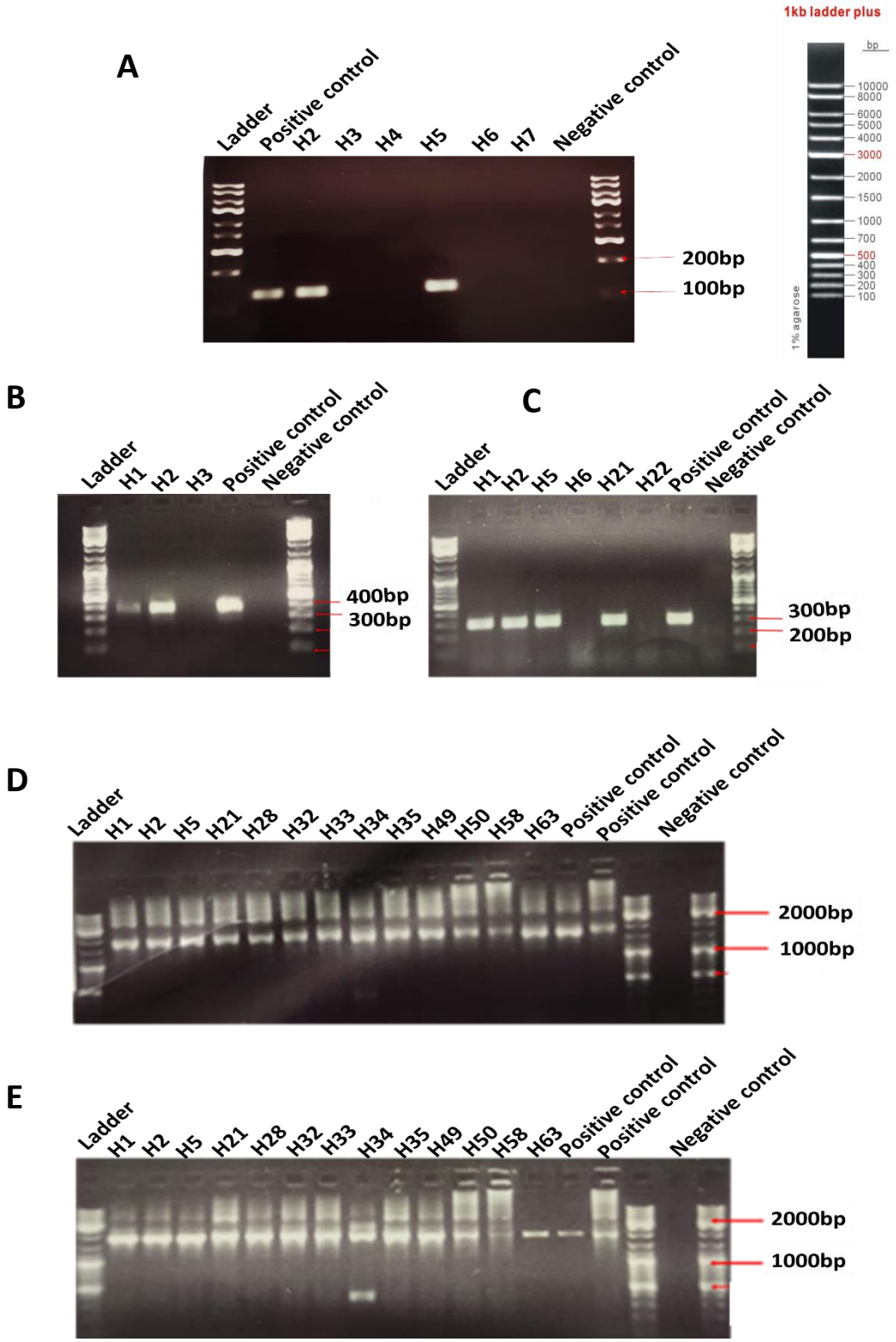

Figure 3.3: PCR screening assays for the presence of EqHV in Thoroughbred racehorses using representative samples. Each of the screening assays had both a positive and negative control to validate the integrity of the assay. All 66 horse Thoroughbred sera were screened for EqHV RNA using the following PCR screening assays; but selected representative samples were used for illustration purposes: **A**. 5' UTR screen, **B**. Core-E1; **C**. NS3; **D.** E1E2 outer screen **E**. E1E2 screen. Positive control = Donor serum; Negative control = Water; H= Horse

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Taken together, this showed that Core-E1 is the most sensitive assay of the three, with a total prevalence of 37.9 %. The Core-E1 sensitivity was twice as sensitive as the standard screening assays, indicating that this virus's prevalence could be underreported.

3.2.2 Resolved contaminations issues

During the preliminary optimization process, contamination was noticed during the initial course of the experiment. This contamination was noticed when some samples that were known to be negative from the first screen started showing positive results in repeat screening assays (Figure 3.4). On further analysis with Sanger sequencing aid, these new positives were identical to each other or a particular sample. So the mechanical and chemical barrier methods were applied to trace the cause of the contamination and resolve it (241) (Figure 3.5).

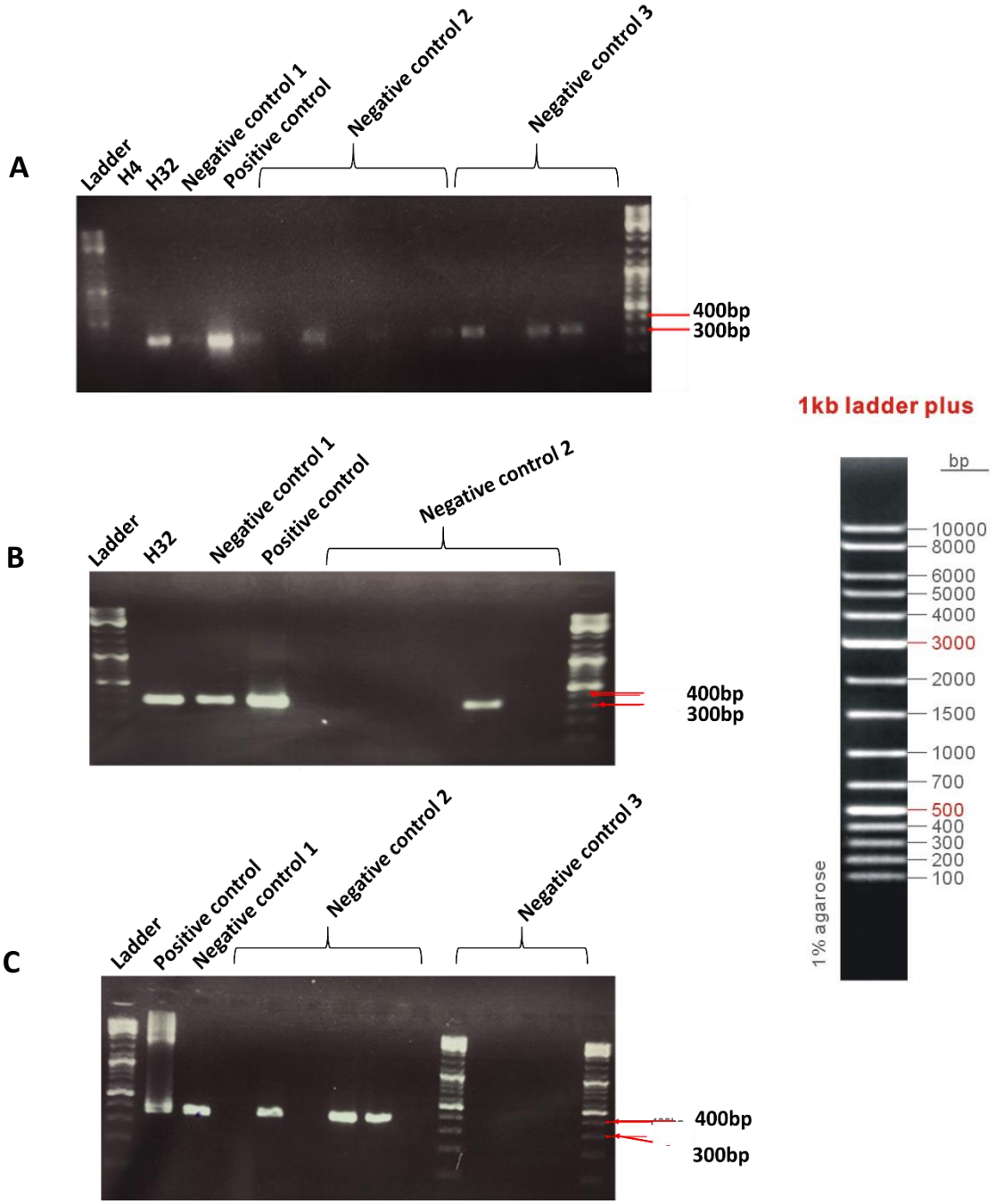

Figure 3.4: Identification of PCR contamination issues using Core-E1 assays. A. Contamination noted with the Core-E1 polymerase reaction assay. B. Repeat of Core-E1 PCR assay with new aliquot of primers and dNTPs C. Repeat of Core-E1 with new primer sets Positive control: Donor serum; Negative control 1: Goat serum cDNA; Negative control 2: Water only; Negative control 3: Master mix alone.

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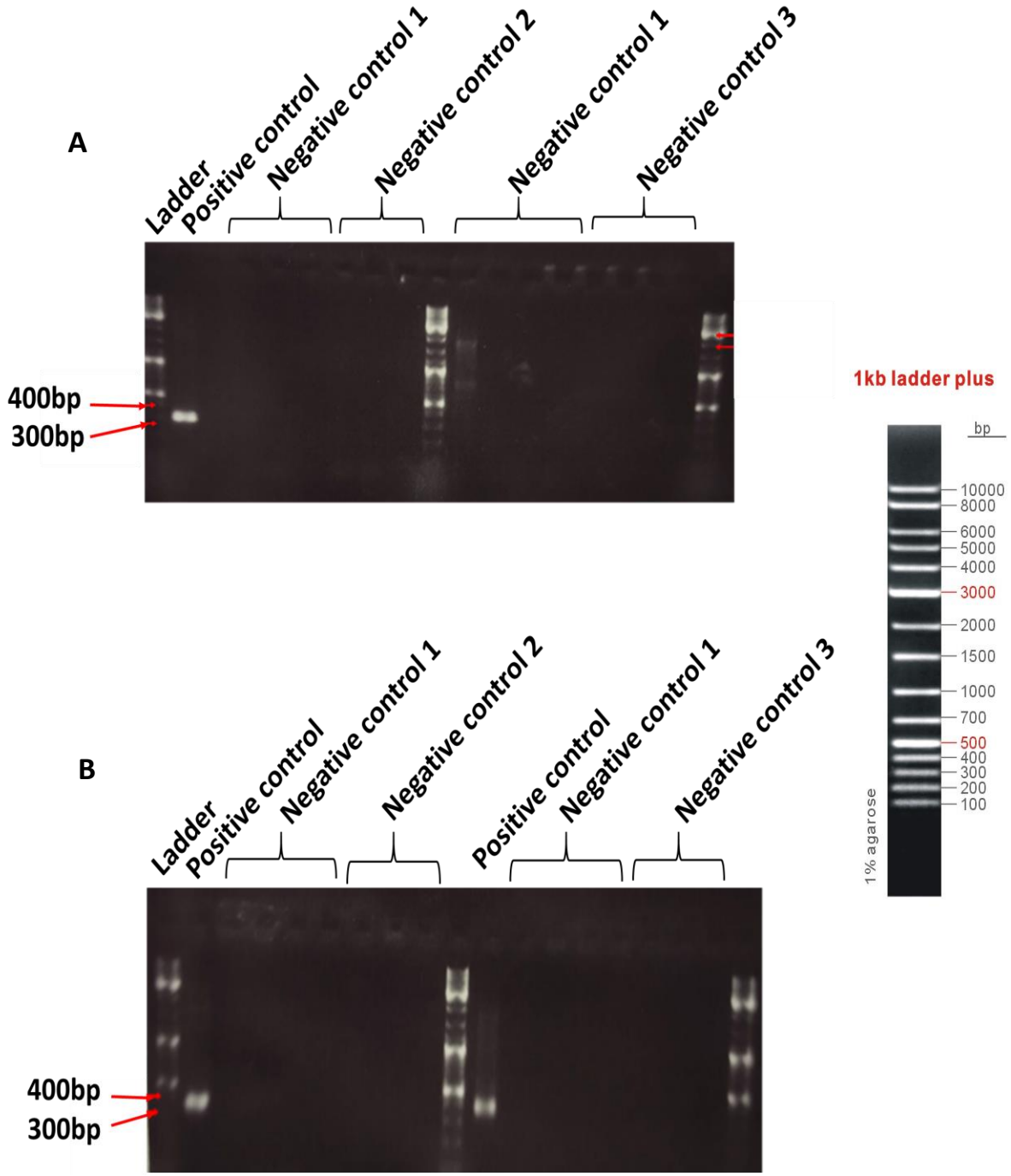

Figure 3.5: Resolving Contamination PCR issues using Core-E1 assays. A and B show independent repeats of resolved contamination using new Core-E1 PCR primer stock, new aliquot of water, use of filter tips throughout, new PCR buffer aliquot and after decontaminating all laboratory apparatus with DNAse. Positive control: Donor serum Negative control 1: Goat serum cDNA; Negative control 2: Water only; Negative control 3: Master mix alone.

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3.2.3 Epidemiology relationship/ characteristics

The prevalence of infection was determined for the groups managed by the 18 different trainers represented in the cohort. Nine of the eighteen trainers (50 %) had at least one EqHV-positive horse (Figure 3.6A). It should be noted that the majority of the horses in this study cohort were sampled from three training yards, therefore raising a question of intra - and inter - herd transmission.

Following amplification with primers amplifying Core-E1, PCRs were performed with the previously described PCR amplifying a fragment of NS3. The reason why 5'UTR was more sensitive with three horse samples (27, 30 and 56) with all below the limit of detection but negative with Core-E1 is yet to be determined.

Although a higher proportion of male horses were EqHV-positive than female horses, the difference was not statistically significant (Figure 3.6B), likely due to the small sample size. At the time of sampling, the horse's age was neither associated with the prevalence of infection (Figure 3.6C and D) nor the viral load in an animal (Figure 3.6E).

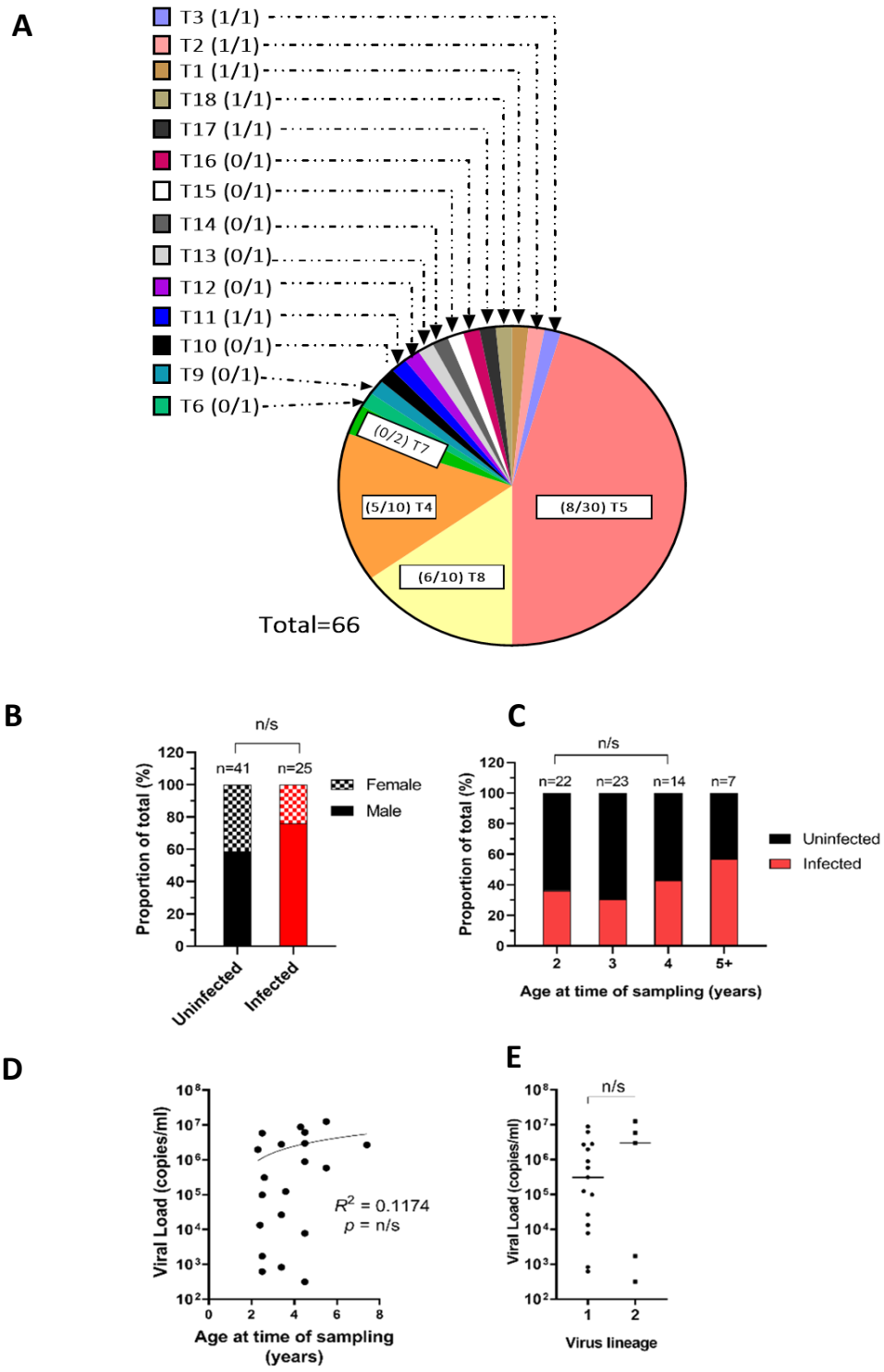

Figure 3.6. EqHV positivity is not associated with training group, sex, or age. A. Proportion of horses managed by individual Trainers (T). EqHV-positive horses and total number of horses are shown in brackets. Proportion of EqHV-positive horses by **B** sex or by **C** age at time of sampling. Total number of animals in group noted above each column. Statistical analysis of B was performed using Fisher's exact test. **D** Viral load does not correlate with age of horse at time of sampling. Correlation was assessed by linear regression (R^2 and p values are shown). **E** Viral load of samples are shown by EqHV subtype and compared by Mann-Whitney test. n/s, not significant.

3.2.4 Quantification of EqHV in serum samples

To determine the quantity of virus circulating in each of the horses in the study cohort, the viral loads of all the positive samples were measured using the assay amplifying the Core-E1 genome region, as this was the most sensitive assay. To ascertain the limit of detection of the assay, a series of 10-fold dilutions (10^7 – 10^1 copies/µl) of 1 X 10^{10} plasmid copies/µL of positive control plasmid clone (section 2.11 – 2.15) were performed. The limit of the assay detection was ten copies/transcripts with the minimum Ct value at 33.70. From the generated standard curve, the copies of cDNA were calculated, and extrapolating back to the dilution from a serum sample to the RNA phase and cDNA, we then calculated the viral loads ranged from below the lower limit of detection (357.14 copies/mL) to approximately 1 x 10^7 copies/mL (see section 2.5 and Table 3.1). Every qPCR reaction product was confirmed using agarose gel electrophoresis, showing the desired size band (Figure 3.7).

All the positive samples were screened using the Core-E1 assay, and four samples (3, 20, 30, and 53) (Table 3.1) were found to be below the detection limit of the assay (< 3 x 10^2 copies/mL). Three samples (27, 30 and 56) were not included because they were positive with the 5'UTR assay alone. The range of the viral load ranged from 1.26 x 10^7 – 6.19 x 10^2 copies/mL.

Together, these results show an appreciable high level of circulating EqHV among Thoroughbred racehorses, supporting other published findings (Table 1.2). Another striking feature here was horse samples with an increased number of viral copies/ML were positive to all the assays, while those with smaller quantities mainly were detected with some assays but mostly Core-E1.

Table 3.1. Sixty-six horses were tested by 2-step RT-PCR using four PCR primer pairs for EqHV RNA. Viral load was assessed by qPCR using Core-E1 primers. BLD, below limit of detection of assay (3.57 x10^2 copies/mL); n/a, not applicable.

Horse	EqHV RNA Screening Assays				Viral load (copies/mL)
	5' NCR	Core-E1	E1-E2	NS3	
32	+	+	+	+	1.26 x10^7
58	+	+	+	+	8.79 x10^6
49	+	+	+	+	6.16 x10^6
5	+	+	+	+	5.86 x10^6
50	+	+	+	+	5.84 x10^6
35	+	+	+	+	2.98 x10^6
63		+	+	+	2.80 x10^6
34	+	+	+	+	2.69 x10^6
33	+	+	+	+	1.96 x10^6
1	+	+	+	+	8.84 x10^5
2	+	+	+	+	3.09 x10^5
22	+	+	+		1.23 x10^5
28	+	+	+	+	9.81 x10^4
6		+			2.64 x10^4
57		+			1.33 x10^4
21	+	+	+	+	7.75 x10^3
31	+	+			1.71 x10^3
51		+			8.26 x10^2
55		+			6.19 x10^2
53		+			BLD
20	+	+			BLD
30	+		+		n/a
3		+			BLD
27	+				n/a
56	+				n/a
Total: 25	18	22	15	13	

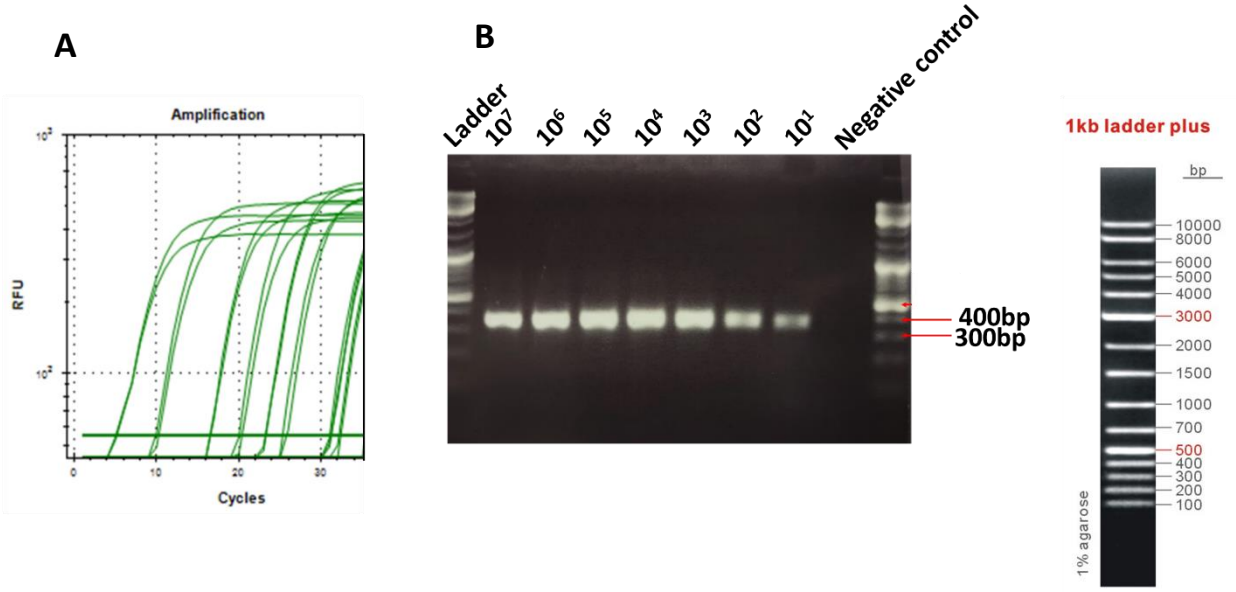

Figure 3.7: Real-time polymerase chain reaction. A. Core-E1 amplification plot of a 10- fold standard dilution replicates carried out before testing the samples, in order to determine the minimum detection level of the assay. **B.** Agarose gel electrophoresis image of the standard carried out in 'A'.

3.2.5 Prevalence of other hepatotropic viruses

To investigate whether the high rate of EqHV was linked with transmission of any other equine hepatitis viruses, we performed PCR-based screening for EqPV-H, EPgV and TDAV on our samples (section 2.5). Only two samples were positive for EqPV-H, and one sample each for EPgV and TDAV. H02 was co-infected with EqHV and EpgV, and H28 was co-infected with TDAV (Table 3.2). There was no link between EqHV prevalence and co-infection with another hepatotropic virus.

Table 3.2: Sera were screened for co-infection with three other equine hepatotorpic RNA viruses. EqPV-H, Equine parvovirus with hepatitis; EPgV, Equine pegivirus; TDAV, Theiler's disease associated virus; EqHV, equine hepacivirus.

Horse	Virus RNA Screening Assays			Co-infected with EqHV
	EPgV	EqPV-H	TDAV	
2		+		+
28			+	+
36	+			
48		+		
Total: 4	**1**	**2**	**1**	**2**

3.2.6 Acute, chronic and reinfection

The detection of a high prevalence of EqHV in our Thoroughbred cohort made it essential to investigate the possibility of persistent infections. Similar to HCV infection, chronic infection is defined as any persistent infection lasting more than six months. From the study cohort, 25 horses were positive for EqHV using the Core-E1 PCR assay, eight samples (H05, H31, H33, H34, H35, H50, H53, and H55) had follow-up samples greater than six months after the first detection of infection (Table 3.3). Ethical considerations were strictly adhered to during sample collection, and this study was considered an extended form of diagnosis for these horses. Using two-step quantitative RT-PCR, of the eight horses, three (H05, H33 and H50) were found to be persistently infected, with the rest resolving the infection. Although of

the resolved infections, it was discovered that one of the horses (H31) was subsequently re-infected with a virus with a very different nucleotide sequence (Figure 3.8A). The three persistently infected horses (same virus isolated from different time-points) were representative of three different sub-lineages of EqHV (Figure 3.8B).

Horse H05 showed persistent infection with high viral loads greater than 1×10^4 copies/mL over one year and peaked at 2×10^7 copies/mL (Table 3.1). When the viral Core-E1 sequence was analysed phylogenetically, there was no significant nucleotide substitution observed in the amplicon analysed by Sanger sequencing (Figure 3.8B). A progressive and constant decline in viral load was observed with H33 and H50 (albeit with only one follow-up sample available for H50). The EqHV strains infecting these horses did not change significantly over the sampling period. Therefore, providing evidence of the myriad interactions of the virus with the immune system of the host.

For H31, the infection at time-point 1 (sample H31 (1)) with a low viral load was spontaneously resolved, re-emerging 13 months later with a high viral load infection representing a virus from a different genetic subtype (H31 (4)), more closely related to the infection in H05 (Figure 3.8B). This data provides evidence of new infections naturally occurring in adult horses and the lack of protective immunity generated by prior infection in this case.

1917 **Table 3.3:** Consecutive sera samples from eight horses used in this study.

Horse	Number of consecutive samples after 6 months	Virus RNA Screening Assays		
		Core-E1	NS3	E1E2
H05	8	+	+	+
H31	6	+		+
H33	12	+	+	+
H50	2	+	+	+
H34	2	+	+	+
H35	3	+	+	+
H53	1	+	+	+
H55	1	+	+	+
Total: 8	**35**	**8**	**7**	**8**

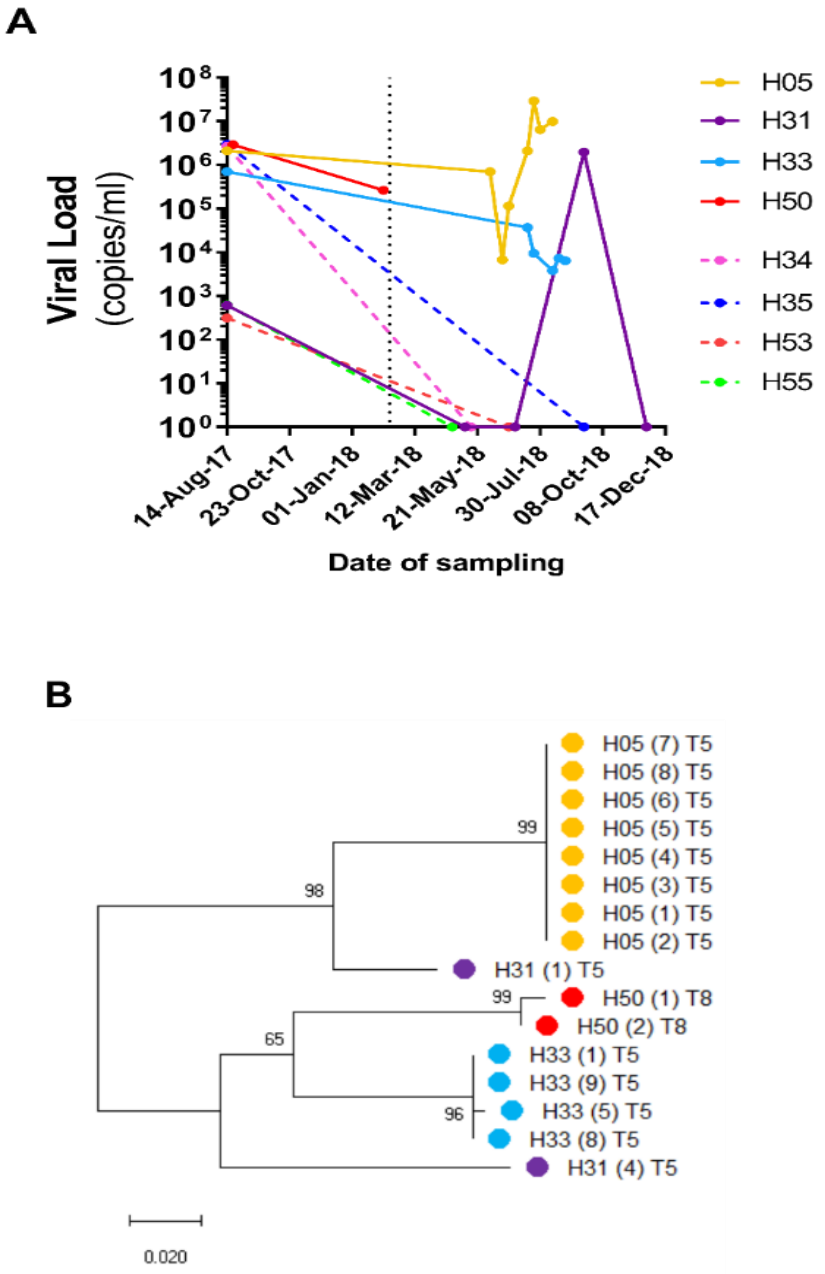

Figure 3.8. Evidence of chronic and acute infection in adult Thoroughbred horses. A Eight EqHV-positive horses had follow-up samples taken > 6 months after (vertical dotted line) the initial sampling date (14th - 21st August 2017). Viral loads were determined by qPCR, following RNA extraction cDNA synthesis, using primers for amplification of Core-E1. The horse number (H) is noted. Data points from the same horse are linked for illustrative purposes only. **B** Phylogenetic analysis of Core-E1 region of EqHV sequences amplified from repeat samples. The evolutionary history was produced from alignment of 277 nucleotides in Mega 7 and inferred by using the Maximum Likelihood method and Tamura-Nei substitution model. The tree with the highest log likelihood is shown with branches drawn to scale and bootstrap values noted. Horse identifiers (Hxx), timepoint (in brackets) and Trainer number (Tx) are shown.

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3.2.7 EqHV serum reactivity to H28 E1E2

To investigate the prevalence of past exposure and current infection to EqHV, a GNA capture anti-E1E2 binding ELISA was performed using proteins expressed from the cloned H28 E1E2 sequence (see section 2.8). A mock-transfected sample was used as a negative control target protein in this assay. Of the 66 sera, 49 samples were available and screened using this assay. All samples were screened using binding GNA (*Galanthus nivalis* lectin)-captured E1E2, with serum antibodies' reactivity to E1E2. The EqHV assay cut-off was set conservatively as the mean serological reactivity (measured at 405nm) plus three SDs of unreactive samples and was used to categorize samples as anti-E1E2 positive or negative.

All unique sera from our Thoroughbred racehorses were screened for anti-E1E2 IgG antibodies. Samples were considered positive only on the conditions set above. A plot of the serology results for EqHV E1E2 absorbance at A405nm (Figure 3.9) showed a lower seroreactivity in the horse sera. Overall, 23 from the 49 samples tested positive (46.94%) to the ELISA assay for anti E1E2 antibodies, with the rest (53.06%) negative. Furthermore, 6 of the 49 RNA positive Thoroughbred racehorse samples had antibody reactivity, with most having no reactivity. Unlike the RNA positive sample, there was an almost equal distribution of anti E1E2 antibody reactivity among the RNA negative Thoroughbred racehorse samples (Table 3.4).

1939 **Table 3.4:** EqHV anti- E1E2 seroreactivity in Thoroughbred racehorses

		Anti- E1E2 status		Total (Percentage)
		Anti E1E2 positive	**Anti E1E2 negative**	
RNA status	**RNA positive**	6 (Current/chronic infection)	11 (Acute infection)	**17 (34.69)**
	RNA Negative	17 (Past infection)	15 (No infection)	**32 (65.31)**
Total (percentage)		**23 (46.94)**	**26 (53.06)**	**49 (100)**

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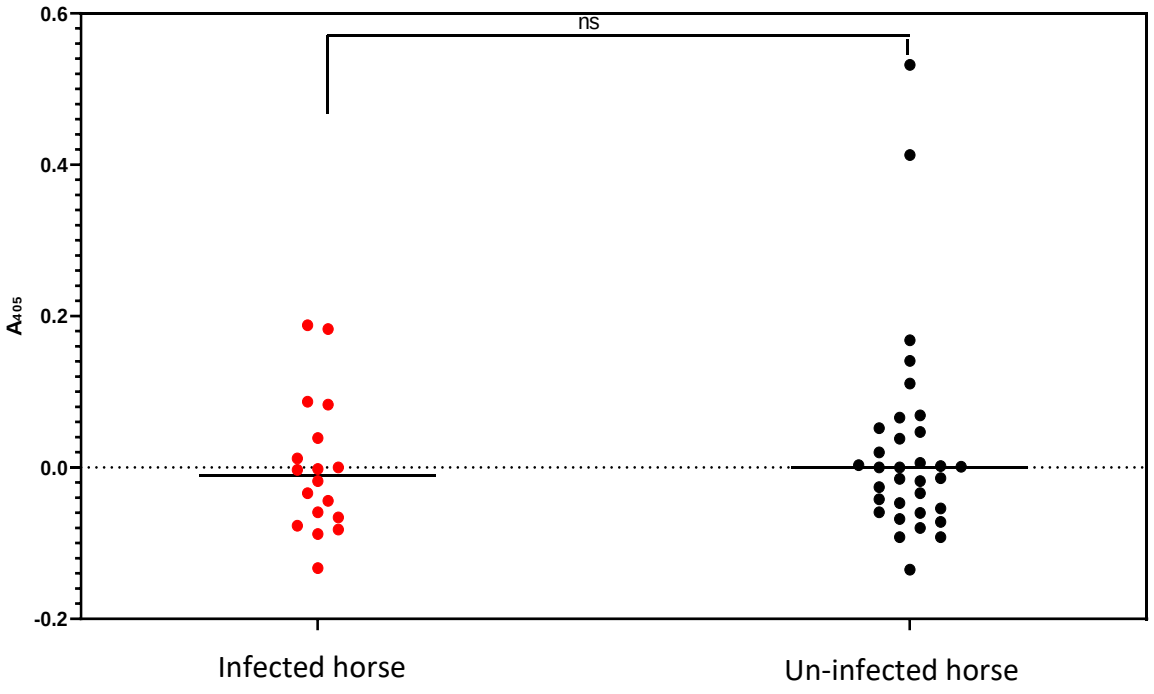

Figure 3.9. EqHV Seroreactivity to viral glycoprotein. Horse sera samples seroreactivity to E1E2 antibodies in both infected and uninfected horses with the negative background score subtracted. The red dots represent infected horses and the black dots uninfected horses. The absorbance value was described as (Δ absorbance) because it is the absorbance achieved with HEK cell preparation expressing H28 E1E2 subtracting the ELISA signal generated when a mock transfected HEK cell lysate was used. Mann-Whitney statistical analysis showed that there was no statistical significant difference between the infected and un-infected horses.

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3.3 Discussion

Hepaciviruses have been isolated from several hosts ranging from mammals to non-mammalian hosts (67,68,121,69,70,72–77). The hepaciviruses isolated from horses were determined to be the closest genetic relative to HCV (66,123), and it presently assumed to be the natural host for the virus (64–66,74). Determining the prevalence rate in horses is crucial and the first step in understanding the importance of this virus and ascertaining if the standard detection assay under-reports this virus's prevalence. Recent research investigating the prevalence of chronic EqHV infection over four-months showed between 20 % - 40 % (83), which is lower than HCV with about 70 % chronicity (73,74). Similarly, EqHV has been documented in a study where it persisted in horses for more than six months using tested serial samples (73,74) and was in more extended periods in other horses (83). This study investigated the prevalence of EqHV infection in a population of Thoroughbred racehorses, the potential for chronic infection, and evidence of prior infection in this population using seroreactivity to the E1 and E2 glycoproteins.

Degenerate primers are PCR primer sequences with some positions having several possible bases and a unique number of sequence combinations called degeneracy (242). Like regular unique primers, degenerate primers are easy and cheap to produce and can amplify several related genetic sequences (243). An essential application of degenerate assays is used in search of a novel or uncharacterized

genetic sequence related to a family of genes (244). A new set of degenerate PCR assays (Table 2.2) were designed by targeting two conserved genomic regions (Core-E1 and E1E2) of EqHV based on the available reference sequences on GenBank (Figure 3.1). During the design of these PCR primers, several considerations were taken to improve the PCR assay: a selection of amino acids with least degeneracy to increase specificity, consideration was given to codon bias for translation, and contain 15 – 20 nucleotides with degeneracy less than 500 (242–245). The newly designed degenerate primers were optimized to find conditions representing the optimum balance between specificity and efficiency.

These new assays' performance was compared with the previously published assays (5'UTR and NS3) (65,121). All samples positive with NS3 assay were positive with Core-E1 assay, and no sample was positive for NS3 alone and negative with Core-E1. The NS3 PCR assay lacked sensitivity and generally only detected infections with higher viral loads compared to other screens (see section 3.2.4 and Table 3.1). This finding suggests that Core-E1 primers could be up to 50 % more sensitive than the published NS3 screening assay. This lower NS3 detection could imply that the prevalence recorded/published (Table 1.2) might be under-reported; hence, the worldwide burden of infection in Thoroughbred horse populations may be currently underestimated by 50 %.

Published data (246,247) suggest that the 5'UTR assay should be more sensitive than Core-E1, but the reverse was observed with seven samples (H6, H57, H51, H55, H53, H63 and H3) (Table 3.1) that failed detection by 5'UTR assay. This irony could be due to the low amount of 5'UTR reference sequences in GenBank compared to other regions of the EqHV genome, and therefore lack of sampling of different sequence variants. This hypothesis can be further seen with two samples (27 and 56) positive with only 5'UTR assay. Thus, the prevalence of active infection (37.9 %) was higher than the averagely reported data, representing the second-highest prevalence of infection reported so far in a horse population. This high prevalence rate was only exceeded by investigations of a mixed horse population in South Africa (88).

In recent years, several surveys were designed to determine the prevalence of EqHV in horses. Understanding this is one of the bases of enhancing HCV prophylactic vaccine research (4,17,205). The most common factors used in studies to evaluate EqHV prevalence in horses include age, gender and breed (77,129,148). All studies generally showed no significant correlation with the prevalence of EqHV in Thoroughbred horses, albeit with slight disparities. This study follows already published data, which indicates no correlation between infection and age or gender in horses (85).

In the clinical management of hepatitis C, accurate knowledge of viral nucleic acid levels has been shown to form a crucial part of patient care, early diagnosis, and

monitoring treatments. Determination of the starting viral load in serum provides a clue to treatment success chances (248,249). Therefore, this concept suggests that precise and standardized diagnostic criteria are essential in interpreting viral infections (249). Quantitative real-time PCR is a widely used molecular assay in quantifying viral nucleic acids, mainly due to its high analytical sensitivity with a detection limit below the limit of quantification (usually < 100 IU/mL) (250–252). This quantification is essential as antiviral treatments' primary objective is to eliminate the blood virus, especially when acute infections have very low viral loads (253,254). Several researchers have a different interpretation of hepacivirus load results. However, this can be grouped into three broad groups: high viral load (4x 10^5 - 8 x 10^5 copies/mL), low viral loads (<4 x 10^5 copies/mL), and undetectable/ below limit of detection (BLD) viral load (depends on the sensitivity of the assay (here <3.57x 10^2 copies/mL) and does not necessarily means no viruses)(255). The result (Table 3.1) showed that about 48 % of all positive samples in the Thoroughbred cohort had a high viral load, with 25 % and 16 % having low and below the detection limit, respectively. Therefore, this data suggests that there is a possibility that up to 50 % of Thoroughbred racehorses could have a high EqHV viral load.

We investigated the potential for chronic infection in those horses from our cohort, where repeat samples were available. Three of the eight horses with samples

available greater than six months after initial sampling had evidence of chronic infection, more substantial than previously observed in adult horses (83) but lower than the rate of establishment of chronic infection in foals (148). Of all the screened sera, the least number (12%) of horses had current/chronic infections infection with the most having past infection. Hence, indicating either a current or prior exposure of the horses to EqHV infection. This result is similar to other findings, showing a lower EqHV chronicity rate in Thoroughbred racehorses or a higher clearance rate (59,66,83,137,198). Also, this study showed that re-infection is possible with EqHV. Re-infection was evident when one horse (H31) cleared an initial infection but succumbed to a second infection with a genetically distinct isolate that was rapidly cleared. This data suggests a lack of cross-protective immunity but a successful and potentially anamnestic immune response. Thus, although numbers are small, these data imply that individual horses vary in their ability to clear EqHV infection. The cause of this variation is unknown currently but might indicate differing exposure to the pathogen or genetic determinants of susceptibility to infection (84). These variations may have implications for the development of any future vaccines. Furthermore, no significant differences were observed among horse samples that indicated past infection, chronic infection or no prior exposure. This observation is remarkably different from HCV, which has a higher rate of chronic infections than EqHV (256–258).

PCR contamination has been one of the significant concerns in molecular analysis and as a diagnostic tool. These contaminations can arise from several sources, especially repeat amplification of the same target sequence and cross-contamination due to large quantities of target organism/plasmids/template (259,260). Researchers have suggested several strategies, including pre-and post-amplification sterilization techniques and measures to prevent carry-over contamination from previously generated amplicons (241,261–265). During this study, contamination was observed in samples that tested negative initially but became positive in subsequent re-runs/experiments. This contamination was also observed with the negative controls coming up positive during PCR amplification. Also, when PCR products were sequenced, they were identical to previously identified samples. This similarity, therefore, implied cross-contamination of PCR reaction with previously amplified amplicons. This conclusion led to a strategic trace of the cause of contamination involving implementing mechanical and chemical barriers (241) and a complete overhaul of reagents and some equipment. Resolving all the contamination issues was key to moving on to other experiments and proving data reliability.

4 Transmission and phylogenetic analysis

4.1 Introduction

It is vital to understand the genetic and phenotypic determinants that aid viral transmission in any natural infection. To understand the epidemiology of EqHV, two essential questions about viral transmission among hosts and how this virus is sustained in any natural population arise. To date, research has focused on phylogenetic analysis of the gene encoding NS3 protein because it is highly conserved (123) and known to encode serine protease and viral helicase, both of which antagonizes host immune responses (266). These analyses suggest that HCV originated from a virus with a common ancestor with viruses infecting dogs or horses. It is more likely that an independent cross-species transmission of hepaciviruses occurred by a yet to be identified source (76,267). Also, EqHV G+C content is more similar to GBV-B but lower than that seen in HCV or pegiviruses and elevated in most other flaviviruses family members, especially pestiviruses (64). CpG (and UpA) dinucleotides are diminished in EqHV. This similar trend is seen in GBV-B and HCV but not in pestiviruses and flaviviruses (64).

This study investigated potential transmission networks between our horse population members and provided evidence of vector-borne transmission of EqHV.

4.2 Results

4.2.1 Phylogenetic analysis

Firstly, phylogenetic analysis of the amplified genetic regions (Core-E1, E1E2 and NS3) and the glycoprotein encoding genes' analysis were carried out. Nucleotide sequences were consistent across each pair of extractions. Therefore, to exclude the possibility of molecular contamination in our assays, samples with identical sequences were re-extracted and re-amplified by PCR separately. Core-E1 products generated using the Core-E1 assays were Sanger sequenced and MUSCLE aligned with all available EqHV reference genome downloaded from GenBank. The aligned sequence bases were individually inspected for misreads and corrected appropriately with the aid of MEGA 7 and Finch TV software (see Section 2.9). Phylogenetic analysis of the Sanger sequenced Core-E1 PCR products determined the circulating consensus sequence for each infection, revealing that the EqHV strains infecting this horse population were broadly representative of the genetic diversity of viral isolates discovered worldwide (Figure 4.1A). This analysis indicated multiple introductions of EqHV into this population.

Additionally, four discrete transmission clusters (TC1-4) of related sequences were evident, indicating a common infection source for specific animals and suggestive of transmission events occurring within this population. These clusters were heuristically defined based on two statistics; the Subtype Diversity Ratio (SDR) and

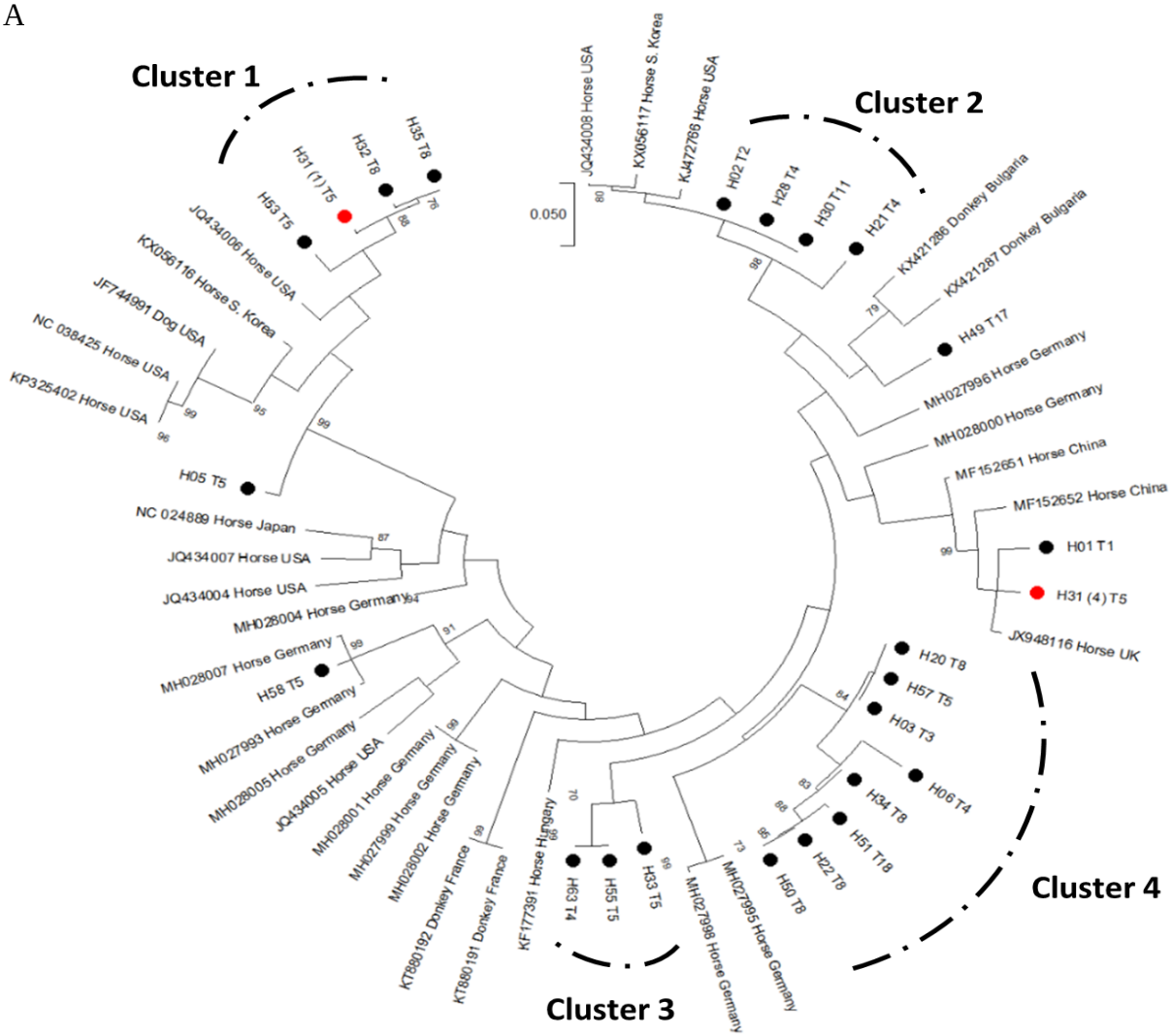
Subtype Diversity Variance (SDV) (233). Similar transmission clusters and spread were seen with the standard NS3 and E1E2 sequence data (Figure 4.2 and 4.3). Furthermore, there were some incongruent branching in the Core-E1, NS3 and E1E2 phylogenetic trees. Of note were H58 and H49, which clustered out of the defined clusters and clustered differently, respectively. Therefore, this data provided a measure of a level of data robustness and the hypothesis stated above.

Pairwise distance analysis of Core-E1 PCR sequences revealed that the clusters differed at the nucleotide level by 9-18 %, depending upon the samples compared, and by up to 5 % within the clusters (Figure 4.1B). Some horses appeared to be infected with identical consensus sequences, albeit with differing minority polymorphisms observable in the Sanger sequencing data (Figure 4.4). These electropherograms were confirmed with repeat sequencing (data not shown). Four distinct clusters of apparent transmission events could be defined in our study population, with five infections occurring outside of a cluster (Figure 4.1A).

Interestingly, these apparent transmission clusters were not restricted to specific training yards but occurred both within and between yards. Trainer 5 (T5) and Trainer 8 (T8), where most horses were sampled, contributed to two transmission clusters (TC1 and TC4), where clear evidence of similar EqHV strains was observed initiating from a common infection source. Horses from Trainer 5 were involved in a transmission cluster with Trainer 14 (TC3). Of the five horses that harboured viruses

outside of a defined transmission cluster, two were from trainers with no other infected horses in a specified cluster, while three horses were from Trainers with multiple infected horses.

A



B

Inter-cluster variation	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Cluster 1				
Cluster 2	16-18%			
Cluster 3	14-17%	9-13%		
Cluster 4	12-17%	10-13%	9-12%	
Intra-cluster variation	0-3%	0-4%	0-5%	0-5%

Figure 4.1. Phylogenetic analysis of Core-E1 region of EqHV sequences amplified from 66 Newmarket Thoroughbred horses. **A** Sequences isolated during this study cluster to both lineages and multiple sub-lineages. The horse (H) and Trainer (T) number for samples from this study are noted. The accession number, species and country of isolation for reference isolates are noted. Tree was assembled using the Maximum Likelihood method and Tamura-Nei model in MEGA 7. The tree with the highest log likelihood is shown. Analysis involved 277 nucleotides across an alignment of 67 unique sequences. The tree is drawn to scale with branch lengths measured in the number of substitutions per site. Boot strap values are shown. Sequences obtained during this study are highlighted (black dots). Two divergent sequences were obtained from different sampling time-points for Horse 31 (red dots). Transmission clusters 1-4 are noted. **B** Pairwise-distance variability (as percentage) between and within transmission clusters. P-distances were calculated based upon the nucleotide sequences.

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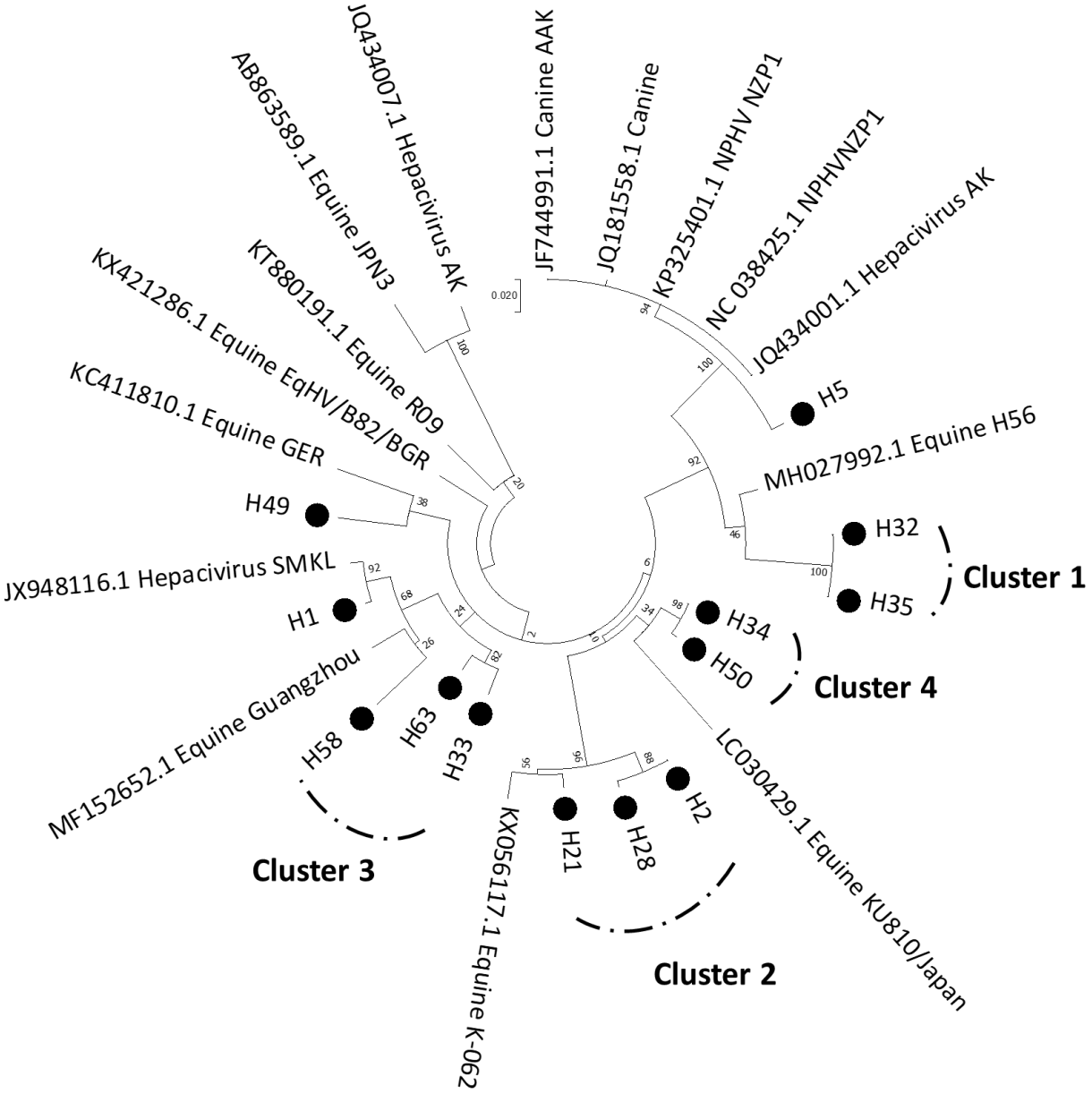

Figure 4.2: Phylogenetic analysis of NS3 region of EqHV sequences amplified from 66 Newmarket Thoroughbred horses. NS3 sequences isolated during this study cluster to both lineages and multiple sub-lineages. The horse (H) samples as well as the reference accession number, species and country samples were noted. Trees was assembled using the Maximum Likelihood method and Tamura-Nei model in MEGA 7. The tree with the highest log likelihood is shown. Analysis involved 270 nucleotides across an alignment of 28 unique sequences. The tree is drawn to scale with branch lengths measured in the number of substitutions per site. Boot strap values are shown. Sequences obtained during this study are highlighted (black dots). Transmission clusters 1 – 4 are noted.

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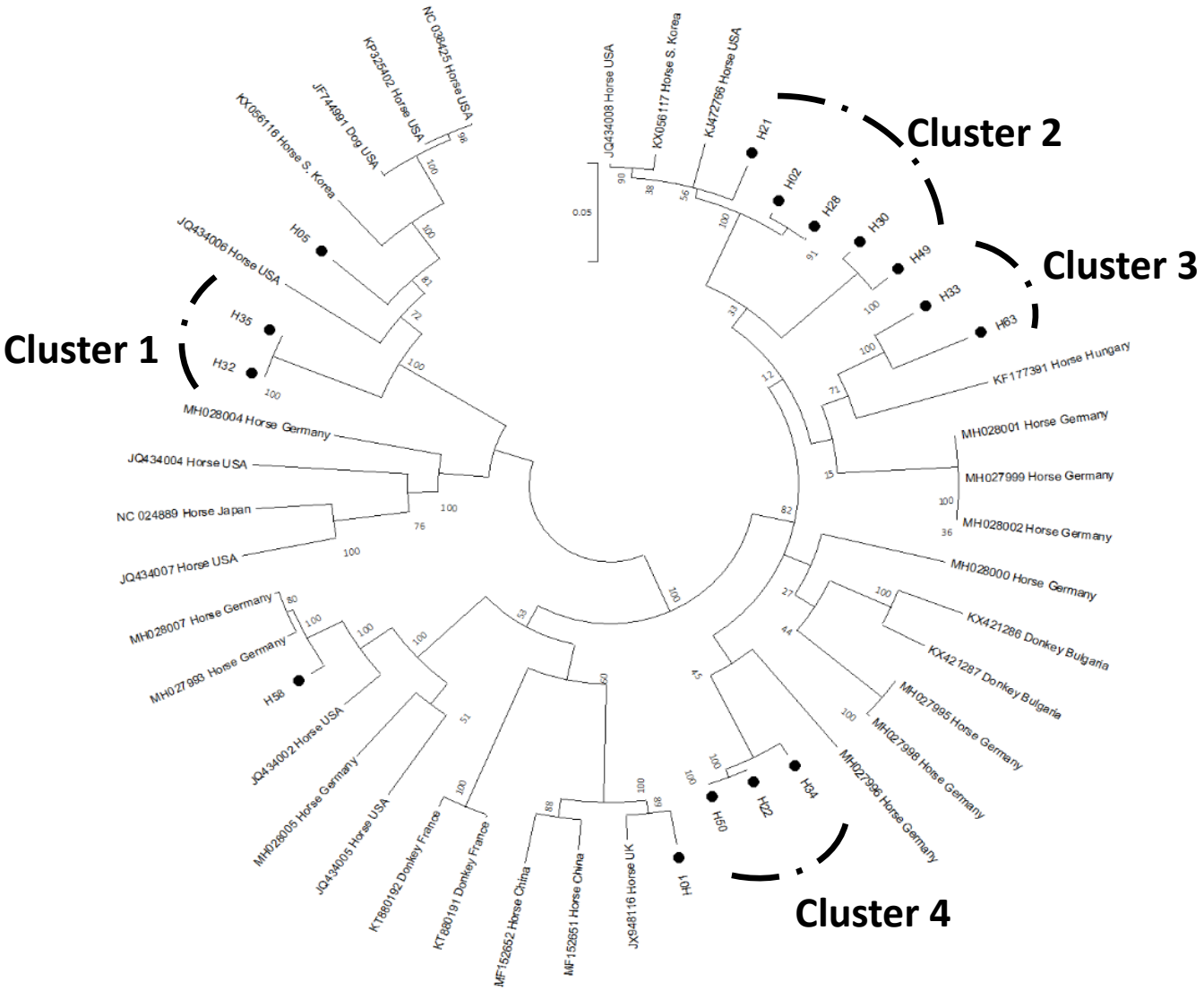

Figure 4.3. Phylogenetic analysis of complete E1-E2 region of EqHV sequences amplified from 66 Newmarket Thoroughbred horses. Sequences isolated during this study cluster to both lineages and multiple sub-lineages. The horse (H) number for samples from this study are noted. The accession number, species and country of isolation for reference isolates are noted. Tree was assembled using the Maximum Likelihood method and Tamura-Nei model in MEGA 7. The tree with the highest log likelihood is shown. Analysis involved 1538 nucleotides across an alignment of 47 unique sequences. The tree is drawn to scale with branch lengths measured in the number of substitutions per site. Sequences obtained during this study are highlighted (black dots). Virus transmission clusters 1-4 described in figure 2 are noted.

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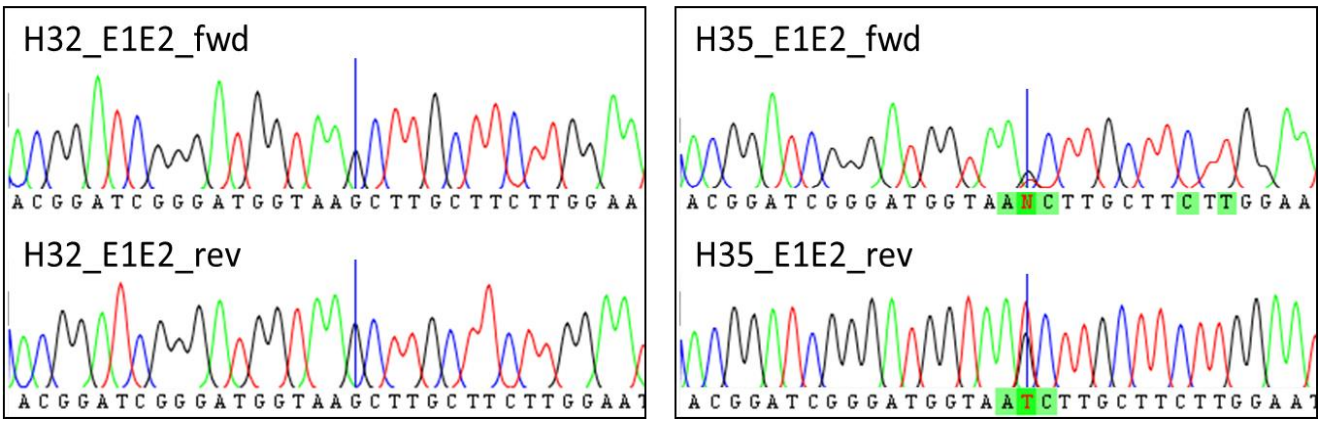

Figure 4.4. Minor variant polymorphisms distinguish otherwise identical virus isolates. Sanger sequence electropherograms for E1E2 regions of EqHV amplified from horses (H) 32 and 35. Blue vertical line indicates position of polymorphism relative to both sequences. Forward and reverse sequencing reaction traces are shown for each sample.

4.2.2 Analysis of variability in envelope glycoprotein-coding regions

The E1 and E2 envelope glycoproteins mediate viral entry and are the target of neutralizing antibodies. They are also under constant selective pressure, resulting in high variability levels, useful for high-resolution phylogenetic analysis and bioinformatic interrogation. To investigate the extent of genetic variability in E1/E2 of viruses circulating in this horse population, phylogenetic analysis was performed on the nucleotide sequences of the 15 PCR products encompassing the E1/E2 genes. Sampling a much broader region of the genome provided a higher phylogenetic resolution. The defined transmission groups TC1-4 were consistent with the previous analysis of the Core-E1 region (Figure 4.1 and 4.3). Interestingly, some of the high viral load samples representing transmission cluster 4 failed to amplify in

this PCR assay, implicating sequence diversity in the priming sites resulting in a lack of detection.

4.2.3 Prediction of polyprotein processing

To determine where the exact termini of E1 and E2 occurred, signal peptidase cleavage sites in the core/E1 and E1/E2 junctions were predicted using SignalP (Figure 4.5). All sequences identified had a conserved 'SVV' motif at the N-terminus of E1 (Figure 4.5A) and a 'VSC' motif at the C terminus (Figure 4.5B). The sequence of the N-terminus of E2 was very variable, with an eight amino acid length polymorphism (Figure 4.6), but the C-terminus of E2 displayed a completely conserved 'AEA' motif.

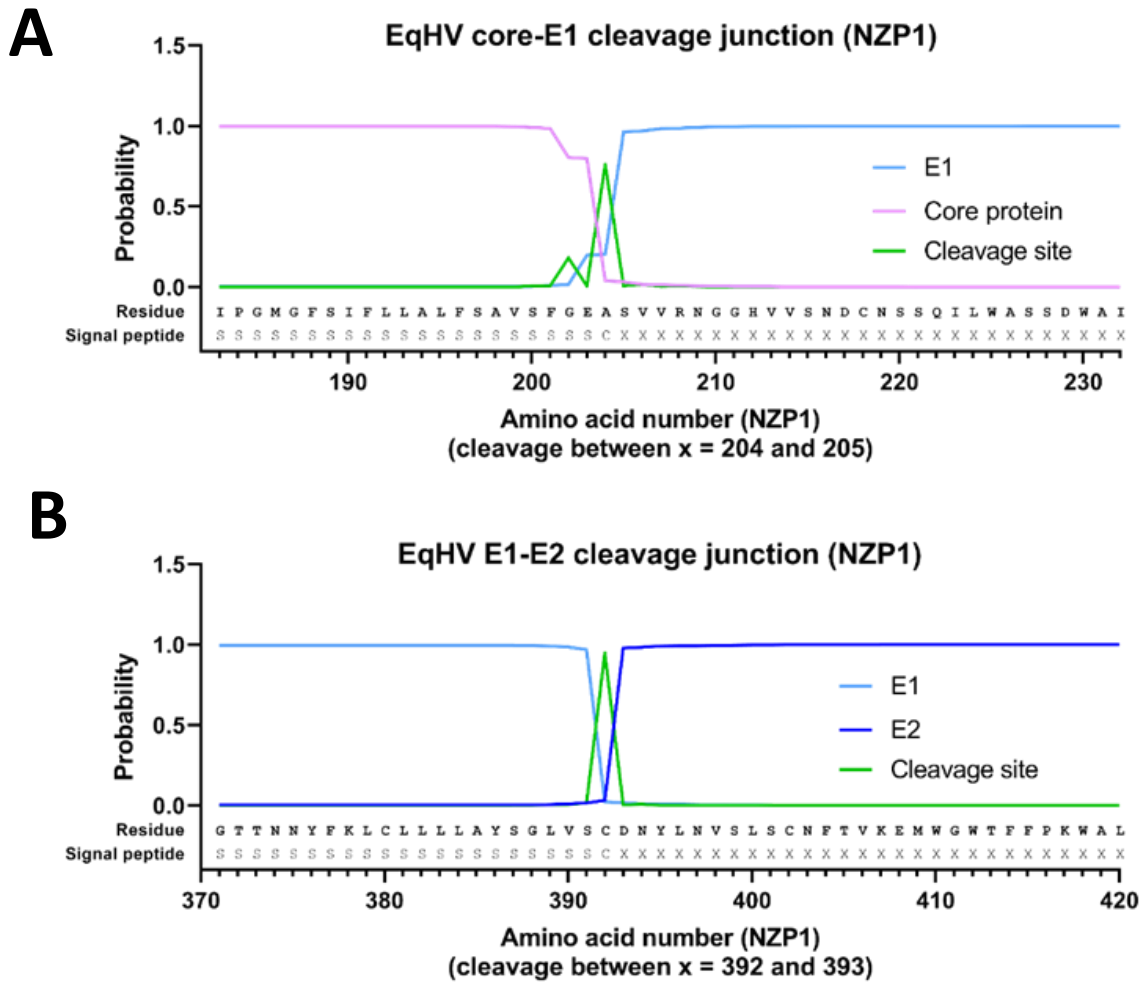

Figure 4.5. EqHV core-E1 and E1-E2 cleavage junctions. Prediction of the core-E1 (**A**) and E1-E2 (**B**) cleavage junctions were performed for EqHV isolate NZP1. The amino acid sequences are noted and the residue at the cleavage site (position 'C' on the Signal peptide text line) is the C-terminal residue of the upstream mature protein. Multiple EqHV sequences were analysed and gave the same predicted C-terminal residues. Predictions were performed using SignalP-5.0.

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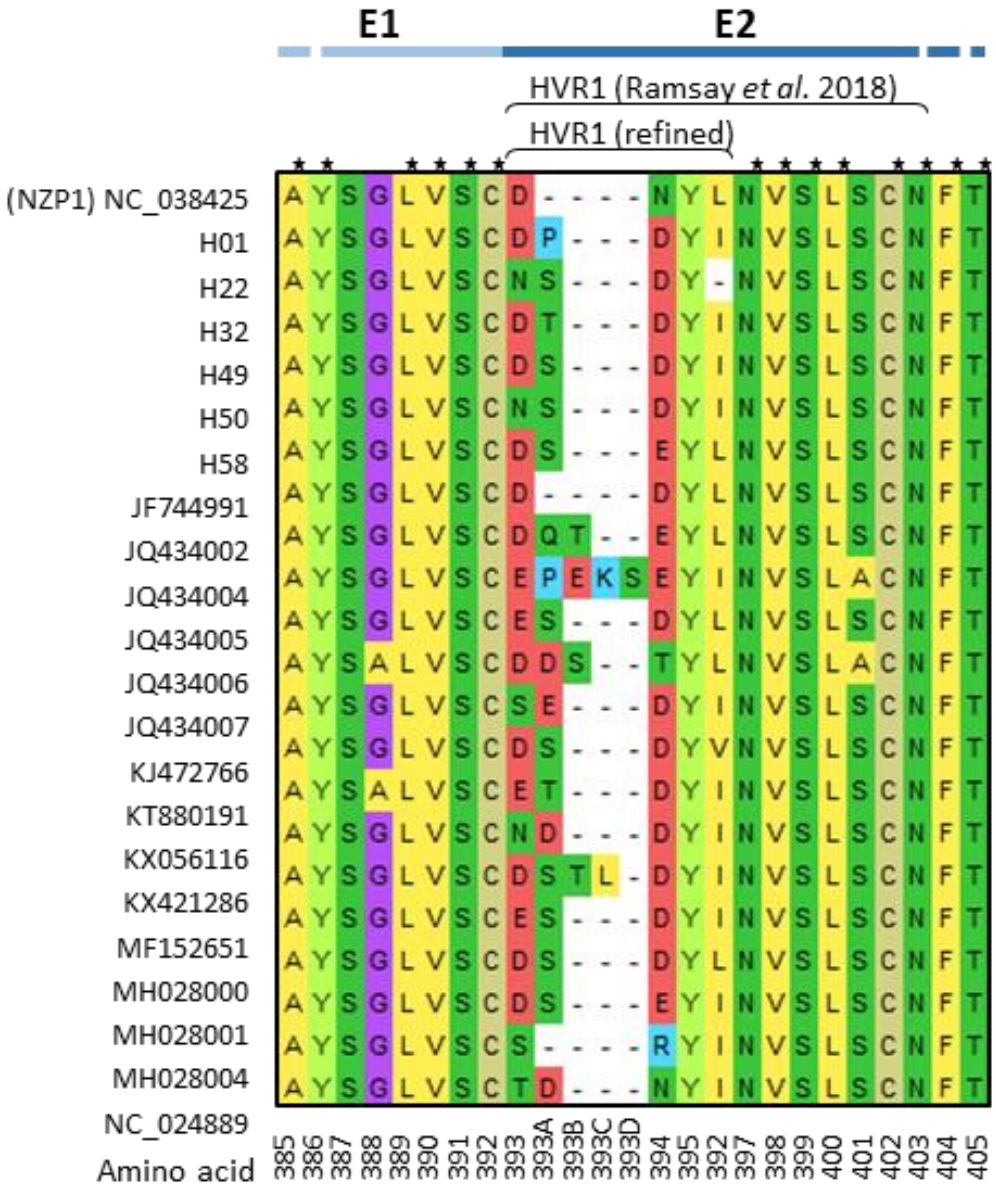

Figure 4.6. Length and sequence variability at the N-terminus of EqHV E2 protein. Examples of unique amino acid sequences spanning the EqHV E1-E2 junction. The conserved cysteine at the C-terminus of E1 is shown. The hypervariable region 1 (HVR1), originally described by Ramsay et al. 2018 and refined in the study, is noted. The EqHV reference strain, NZP1, is noted. Completely conserved residues are marked with an asterisk. Alignment was generated using MEGA 7. Amino acid numbering is based upon the NZP1 coding sequence.

4.2.4 EqHV Darwinian selection in the E1 and E2 genes

The MEME (mixed-effects model of evolution) used a mixed effect-effects maximum likelihood approach testing the assumption if a particular site has been exposed to diversifying or episodic positive selection (268). To investigate this hypothesis, a selected site analysis of the 15 E1/E2 sequences generated from the horse population combined with reference sequences recovered from Genbank revealed that one site in E1, three sites in E2 ectodomain and one site in the stem region of E2 were potentially under Darwinian selection (amino acids 290, 394, 551, 558 and 633 in respect to the first residue of the Core protein of NZP1 KP325401) (Figure 4.7A). Residues used at each of the five sites are shown in Figure 4.7B. To visualise if these selected sites were likely to be accessible on the surface of the glycoproteins, a crystal structure of the HCV E2 core domain was used as a scaffold on which to model the N-terminal E2 sequence of EqHV. Three of the selected sites were located in this core domain, appearing to be surface accessible (Figure 4.7C) and pointing to potential antibody-accessible epitopes for neutralizing antibodies present in this protein.

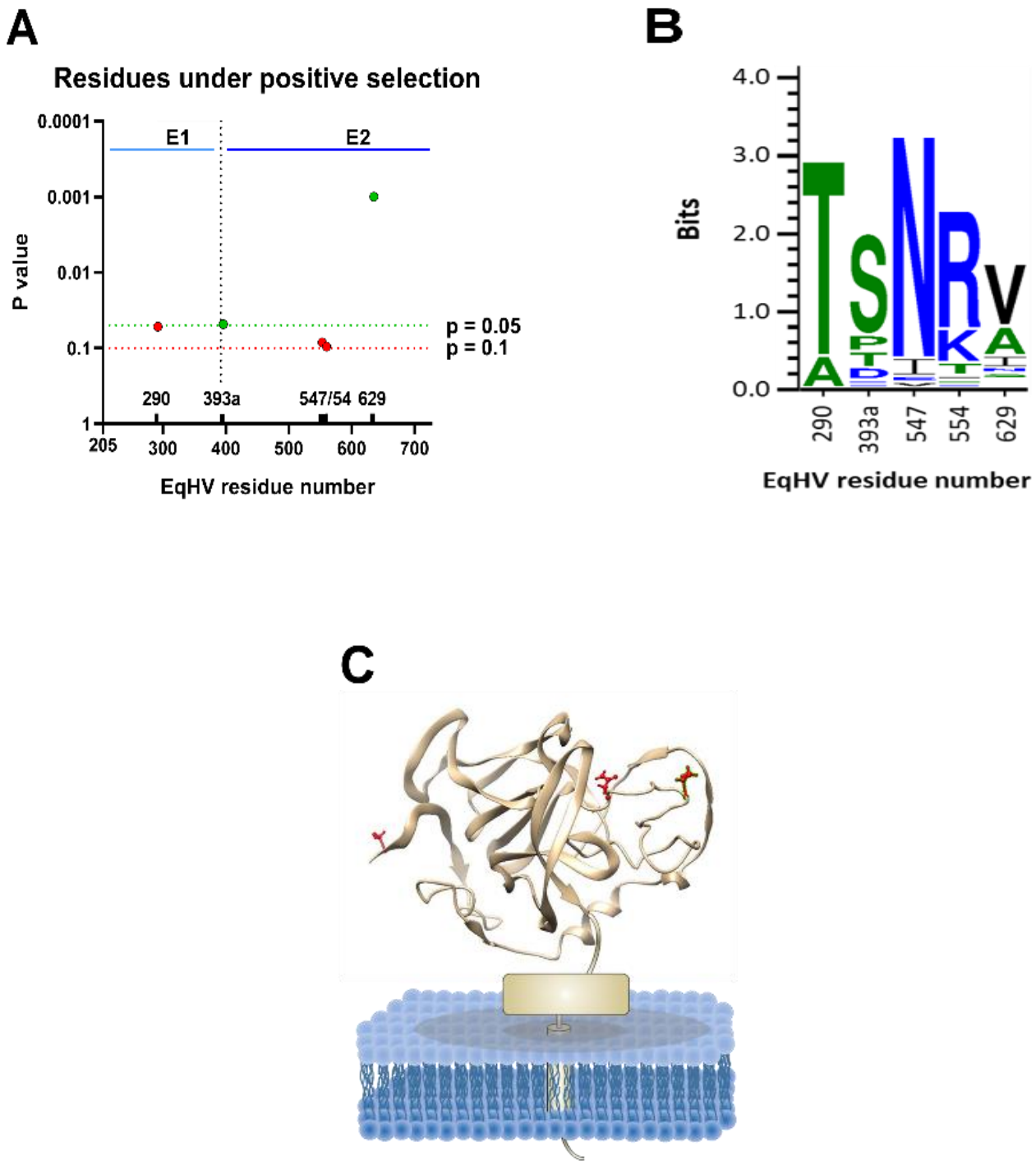

Figure 4.7: Equine Hepacivirus (EqHV) Darwinian selection sites in the E1E2 region A. Five residues were predicted to be under positive selection; three with p values of <0.1 (red dots), two with p values <0.05 (green dots). Amino acid numbering started from the first residue of E1. The E1/E2 junction is marked (vertical dotted line). Positive selection analysis was performed in Datamonkey using the Mixed Effects Model of Evolution (MEME). **B** Amino acid usage at sites with positive selection. Image generated using WebLogo 3.7.3 from an alignment of all EqHV E1E2 sequences (GenBank and this study). **C** Cartoon of EqHV E2 with respect to a membrane, highlighting the position of the sites under Darwinian selection (red). Structure of the N-terminal ectodomain was predicted by constraining the sequence to the HCV E2 x-ray crystal structure in I-Tasser. Residues of E2 predicted to be under positive selection are highlighted (ball and stick projection, red)

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4.2.5 Variability across E1E2 EqHV Darwinian positive selected residue

Three hypervariable regions have previously been posited for the EqHV E2 protein. Having generated a substantial amount of new sequence data for the E1 and E2 genes, we interrogated the variability of the regions of E1 and E2, possessing these positively-selected amino acids. The three amino acids present in the core domain of E2 (192, 349 and 356) were all present in the proposed HVR1 or HVR3 (Figure 4.8A). No positive selection was observed for HVR2. The cysteine residues were conserved entirely in EqHV E1 and E2, with six cysteines in the ectodomain of E1 and 18 cysteines in the ectodomain of E2. Comparing the hypervariable regions to those in the HCV envelope glycoproteins (Figure 4.8B), these regions and sites of selection were in similar locations in the primary amino acid sequence, supporting the use of a structural model of EqHV E2 based on the published HCV E2 structure.

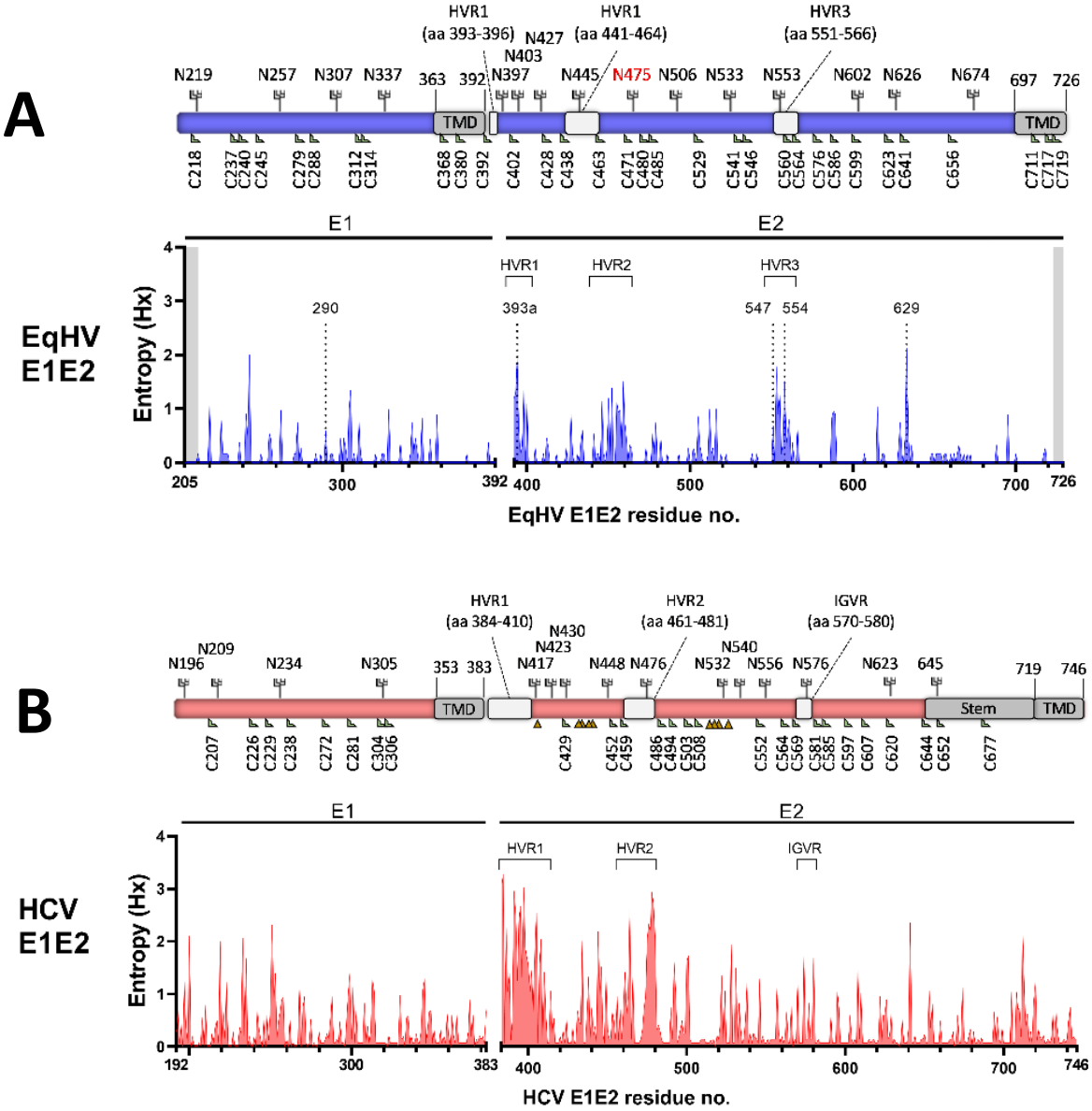

Figure 4.8: EqHV E2 shares conserved and variable sites with HCV E2. The HCV E2 structure omitted the N-terminal 27 aa (corresponding to the HVR1 domain) and the C-terminal 73 aa (corresponding the 'stem' region and the transmembrane domain, TMD). The C-terminal stalk and TMD are shown by membrane-proximal and membrane-spanning structures, respectively. By comparison with the HCV E2 protein, EqHV E2 has a highly truncated, or absent, HVR1. Linear schematic and variability plots Shannon entropy analysis) for the E1E2 proteins of EqHV (**A**) and HCV (**B**) from alignments of 42 and 119 sequences, respectively. Entropy analysis was performed using the Protein Variability Server (PVS). N- and C-termini of the EqHV alignment were trimmed to exclude regions of incomplete sequence coverage (indicated by grey strips on entropy plot). Completely conserved cysteines and N-linked glycosylation sites are noted and numbered with respect to N-terminal residue of each protein. Linear schematics are scaled and annotated to match entropy plots. All amino acid numbering is given with respect to the reference strains NZP1 (NC_038425) for EqHV with additional HVR1 nomenclature (Figure 4.6) or H77 (AF_009606) for HCV.

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4.2.6 Vector-borne transmission of EqHV

The mechanism of EqHV transmission is currently unknown. To determine if any evidence for insect-borne transmission exists, this study used the sequence data generated to interrogate whether EqHV has an intermediate vector. The nucleotide sequence data generated for the E1/E2 genes were further analysed. This region accounts for 16 % of the EqHV genome. Firstly, the dinucleotide odds ratios (DOR) for EqHV E1/E2 genes were compared with the glycoprotein-encoding genes of viruses from the same genus (hepatitis C virus (HCV), bovine hepacivirus (HVN), bat hepacivirus (HVM)), other members of the Flaviviridae (Dengue virus genotype 1 (DENV-1), equine pegivirus (EPgV /Pegivirus E)) and the distinct Togaviridae family (Chikungunya virus (CHIKV), Rubella virus (Rubella)). EqHV conformed to the known pattern of under-representation of CpG and UpA dinucleotides common to many eukaryote hosts' viruses (Figure 4.9). CpG bias pattern was broadly similar to that of HCV and the other hepaciviruses HVM, HVN. Surprisingly, linear regression analysis revealed that the DOR pattern for EqHV glycoproteins most closely correlated with that of DENV-1 (R^2=0.876) followed by the other hepaciviruses.

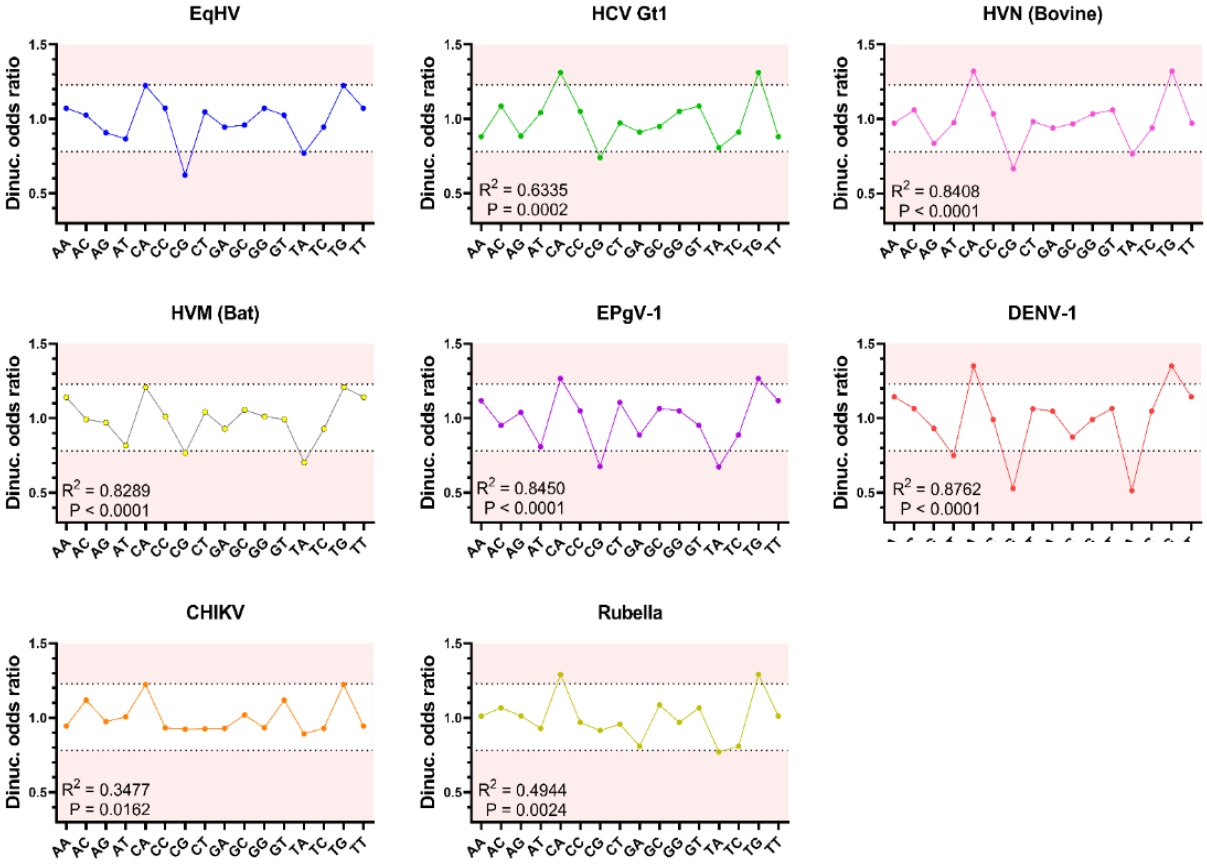

Figure 4.9. Comparison of DOR between glycoprotein encoding genes (E1E2) between EqHV and other related and distant viruses. Dinucleotide odds ratios (DOR) were calculated in R and generated using envelope genes for EqHV and closely related viruses. Points are joined for illustrative purposes on graphs and do represent linked data. R^2 and P values represent linear regression analysis of EqHV DOR against each of the other viruses. Linear regression was performed on data in an X/Y table with EqHV as the X data set. EqHV, hepacivirus A; HCV Gt1, hepatitis C virus genotype 1; HVN, hepacivirus N; HVM, hepacivirus M; EPgV-1, Equine pegivirus 1; DENV-1, dengue virus genotype 1; CHIKV, chikungunya virus; Rubella, rubella virus.

When GC content in these sequences was compared, EqHV was observed to have a relatively low %GC proportion compared to HCV and other non-vectored viruses (Figure 4.10). EqHV %GC content was closely similar to the mosquito-vectored viruses (DENV-1 and CHIKV) and viruses of unknown transmission routes (HVM and HVN).

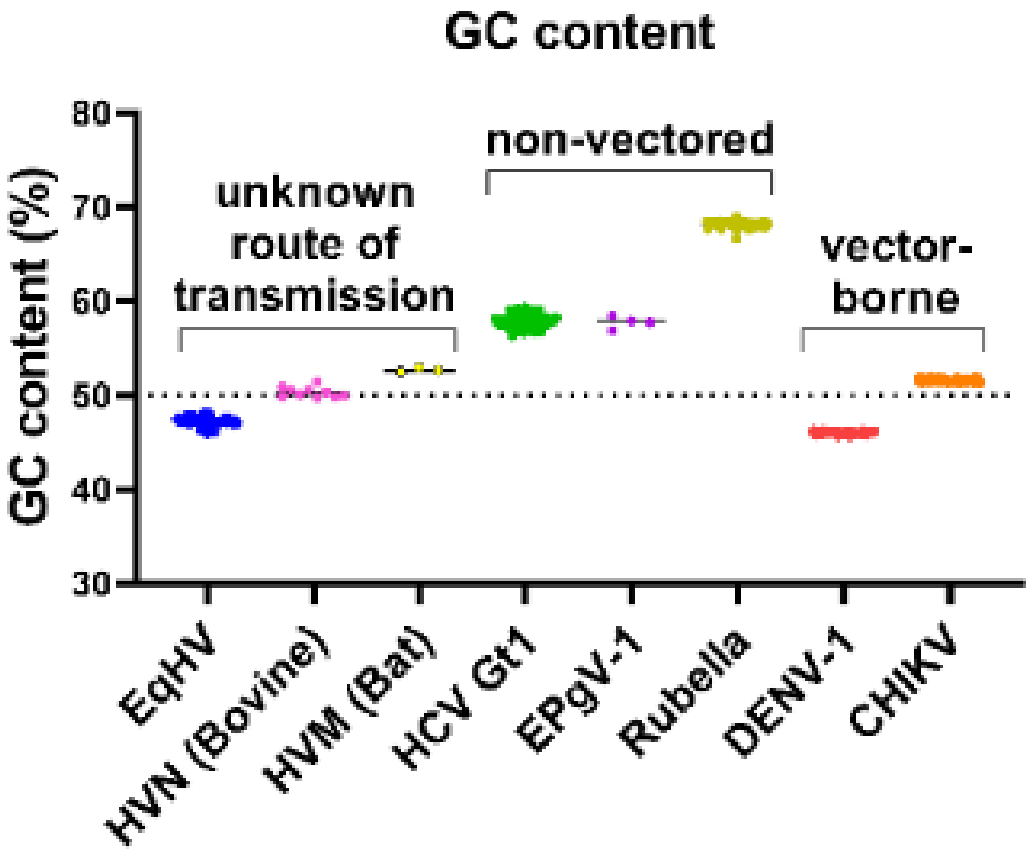

Figure 4.10: Percentage GC content in E1E2 region of viruses with different route of transmission. GC content was calculated for the glycoprotein-coding region (E1E2) for each virus species using Mega 7. 50% GC content (dotted horizontal line) indicates no bias between AT and GC content. Viruses are grouped by known route of transmission. EqHV, hepacivirus A; HCV Gt1, hepatitis C virus genotype 1; HVN, hepacivirus N; HVM, hepacivirus M; EPgV-1, Equine pegivirus 1; DENV-1, dengue virus genotype 1; CHIKV, chikungunya virus; Rubella, rubella virus.

Viruses with a very restricted host range often adapt to possess relative synonymous codon usage (RSCU) patterns similar to their host. Comparison of the RSCU of EqHV and other viruses (HCV, HVN, EPgV-H, GBV-B and Rubella virus) with that of their

primary vertebrate host revealed that while HCV, BVN and EPgV-1 had a strong association of RSCU with their hosts (humans, cows and horses, respectively), EqHV had no association with the RSCU of *Eq. caballus* (Figure 4.11). While Dengue virus had a weak association with H. sapiens, GBV-B had no association with the RSCU of *C. jacchus*. This data provides evidence that EqHV has a genetic architecture closely similar to that of vector-borne viruses than strictly blood-borne transmitted viruses with a restricted host range.

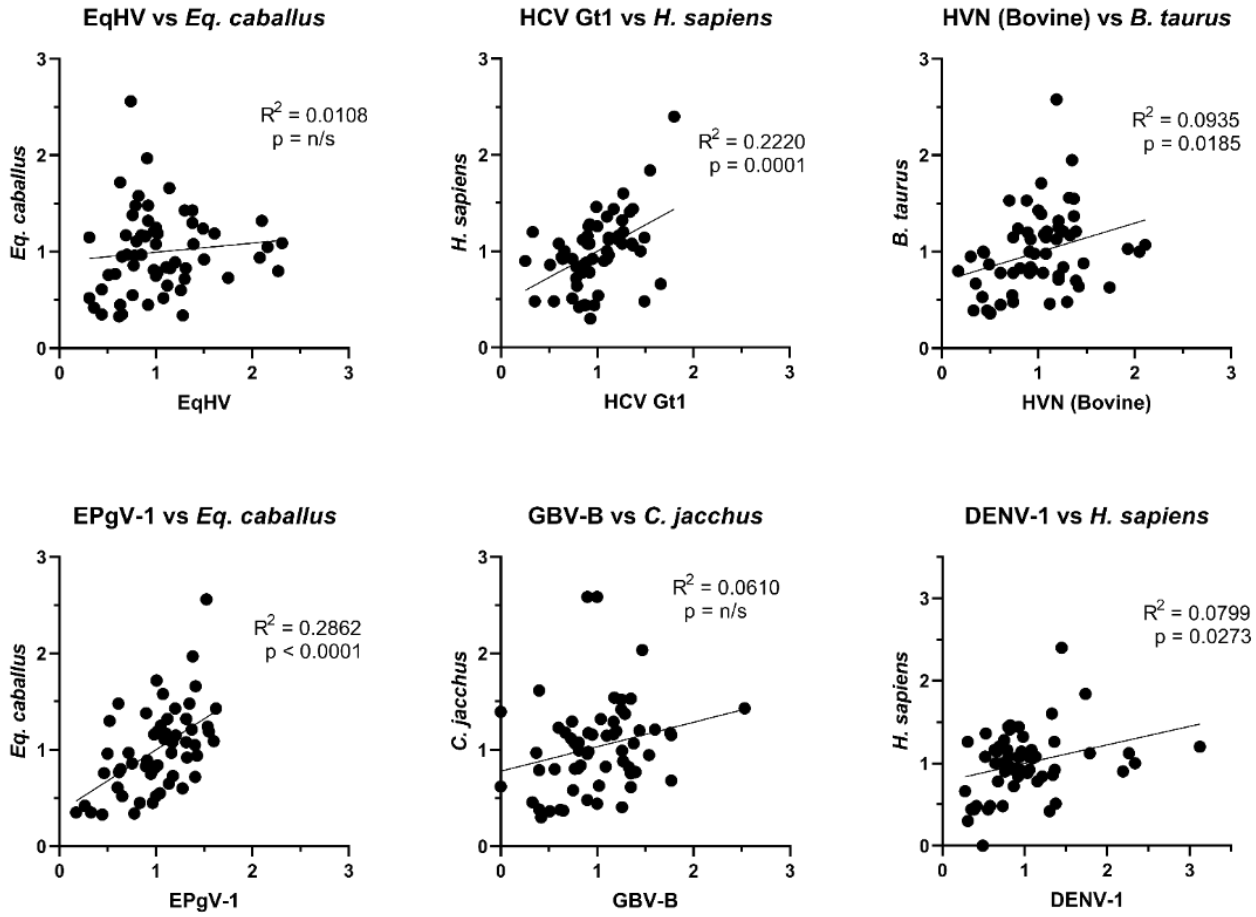

Figure 4.11. Relative synonymous codon usage (RSCU) between flaviviruses and their primary mammalian hosts. RSCUs, calculated in Mega 7, for the surface glycoprotein genes of 6 members of the *Flaviviridae* were compared with those of their respective primary mammalian host. R^2 and *p* values represent linear regression analysis of each comparison. EqHV, equine hepacivirus; HCV Gt1, hepatitis C virus genotype 1; HVN, hepacivirus N; EPgV-1, Equine pegivirus 1; GBV-B, GB virus B (aka hepacivirus B); DENV, dengue virus serotype 1; *Eq. caballus, Equus caballus; H. sapiens, Homo sapiens; B. Taurus, Bos Taurus; C. jacchus, Callithrix jacchus;* n/s, not significant.

4.3 Discussion

Investigations of the prevalence of EqHV have shown a worldwide distribution of the virus in all continents except Antarctica (65,74,138,140,269,81–83,85–88,135). Nonetheless, the natural transmission route of EqHV is yet to be determined irrespective of a singular study on vertical transmission route (129). Similarly, based on high prevalence in Thoroughbred racehorses and specific geographical regions (83,85,88,135), vertical transmission is not likely to be the only route of transmission of EqHV, but there might be a possibility of a horizontal route of transmission. The phylogenetic clustering of EqHV isolated from independent horse serum within respective herds indicated a possibility of a horizontal transmission route (129).

The parenteral transmission route has been established for HCV (270,271); but, the mechanism of transmission of EqHV is still elusive. Furthermore, several studies to establish this evasive transmission route have been carried out with in-bred horses for reproduction (272), broodmares and breeding stallions (135). Similarly, with HCV, iatrogenic and experimental transmission have been demonstrated for EqHV (66,84,113). Furthermore, in general, flaviviruses are known to be transmitted horizontally between vertebrate hosts and hematophagous arthropods (273,274).

The isolated EqHV-sequences in this study were broadly segregated into two subtypes, similar to that previously proposed (85). Within these two subtypes, evident transmission clusters were identified, including viruses infecting hosts with

diverse geographic origins, discrete breeders and training grounds. While vertical transmission has been described (129), the lack of association of age and infection suggests that transmission occurs mainly between adult animals when co-located. As transmission events between animals housed in the same training location appeared to be no more frequent than transmission between horses from different training yards, transmission through direct contact was unlikely. These results are impressive, primarily because a study showed a possibility of horizontal transmission among horses in their pasture herds (129).

The results of this study corroborate previous observations during the first discovery of EqHV in dogs. First was the narrow genetic diversity of EqHV variants isolated from dogs in distinct shelters or separate kennels, which is uncommon for RNA viruses, especially HCV. Secondly, subsequent studies aimed at exploring EqHV epidemiology and genetic diversity in dogs could not isolate any EqHV RNA or antibodies (65,74,110), except for a single seropositive farm dog (73). These results suggest a subclinical infection and a different route or recently introduced into dogs. Furthermore, an iatrogenic transmission or vector-borne transmission is the more probable routes of transmission. Given the much lower prevalence of other equine blood-borne viruses observed in this cohort and the stringent infection control practices used with these animals, selective transmission by an intermediate vector is a plausible transmission mode.

Vertical transmission of EqHV has been reported (129), and although horizontal transmission occurs, the precise mechanisms are unknown. Pathogens can be influenced by the genetic patterns and constraints of the host species (275). These include the host-specific tRNA abundances, the dinucleotide composition (DOR), %GC content and RSCU (276). Pathogens with single-host life cycles may adapt to that host's genetic constraints, while pathogens with a multi-host life cycle, such as arboviruses, may less closely match a specific host or vector's patterns. Instead, vector-borne viruses may require a greater degree of genetic plasticity or 'robustness' to replicate in several hosts (277). Comparison of biases in dinucleotide usage, %GC content, and the relative codon usage of this genomic region together revealed that, in contrast to HCV, EqHV has multiple signatures of vector-borne transmission. While this indirect evidence requires confirmation by direct detection of EqHV of the need for a replicative intermediate vector, classification of arboviruses by RSCU has been demonstrated to predict vectors and primary host species (275). Flies such as horseflies (genus *Tabanidae*) or stable flies (*Stomoxys calcitrans*) have been implicated in the mechanical transmission of equine viruses (278), but this would not result in altered codon usage bias. This data might implicate other vectors, such as ticks (*Ixodes spp*), known to act as vectors for some flaviviruses (279). Furthermore, caution should be applied in bioinformatics data

analysis given its limitations: lack of data, dirty data, and read-length of NGS data (280).

Significant heterogeneity in the N-terminal hypervariable region of the E2 protein was observed. We propose that the primary site of variability is the first eight amino acids of E2; this is shorter than the HVR1 of HCV (281), and that predicted for EqHV by Ramsey and colleagues (282). In contrast to HCV, HVR1 length polymorphisms appear familiar for EqHV. This data is suggestive of an immune-mediated selection of viral variants in different hosts. One of the three sites under Darwinian selection (also known as positive selection) was present in HVR1 (aa393). This data is similar to the amino acid selection observed in the HVR1 in HCV (283).

5 Analyses of EqHV Entry using pseudoparticles

5.1 Introduction

EqHV, as with all viruses, has ‘‘entry’’ as an essential step in the viral life cycle. In contrast to HCV, entry of EqHV into cells has not been well characterized. It could be hypothesized that EqHV, like other hepaciviruses, interacts with the cellular membrane via attachment, internalization, and fusion ensures delivery of viral genome into the host cell cytosol and hence start of its replication (159). Thus, the initial stages of hepacivirus (using the most characterized virus, HCV, as an example) infection is characterized by a multistage and slow process. The initial binding of HCV particles by lipoproteins and glycosaminoglycan receptors is accompanied by a systematic interaction that involves CD81 tetraspanin, among other receptors (158,159). Retroviral pseudotype assays have been used to study several enveloped viruses (e.g. HCV) entry pathways. This assay is a highly tractable model with several extensive applications in the preclinical screening of antiviral antibodies, assessing the phenotype of viruses derived from patient biological samples using HCV pseudoparticles (HCVpp) possessing the HCV E1E2 glycoprotein and especially the HCV entry pathway (284).

5.2 Results

In previous chapters, the data generated suggest that the overall structure of E2 may be the same as for HCV; hence, the receptors may be the same. This phenomenon

has been described for other virus families. For example, coronaviruses use the same receptor across different species in a genus (285). In this chapter, we sought to investigate if the entry of EqHV is dependent on CD81 based on our knowledge on HCV entry receptors, using HuH7 cells with the use of pseudoparticle assays.

5.2.1 Gradient and optimization assay to determine best infection assay parameters

The production of the pseudoparticles and infection of HuH7 cells were optimized before the start of the experiment. This step was primarily due to this process's novelty, with little or no published data to rely on as a basis. Three essential variables (pseudoparticles infection volume, the quantity of pNL4.3 $E^{-}r^{-}$ luc and plasmids) had to be determined using already established HCV pseudoparticle production and infection protocol (173). The experiments were first performed in 12-well plates, partly due to the large volume of cells/reagents needed hence increase accuracy, after which it was scaled to an assay using 96-well plates to enable higher-throughput screening.

The optimum infection volume needed to infect the HuH7 cells was first determined using a range of volume from 50 µL to 1500 µL, and controls (PAA and H77) (see section 2.8) were kindly provided by my supervisor, Dr Alexander Tarr. A general increase in luciferase signal as the infection volume increases (Figure 6.1). 1000 µL

was found to be a tractable volume for the assay. Although while the positive signal was higher in different scenarios, the background signal was also seen to increase. However, but in many of the assays performed, the background signal was significantly lower. As such, this volume of inoculum was used throughout the experiments.

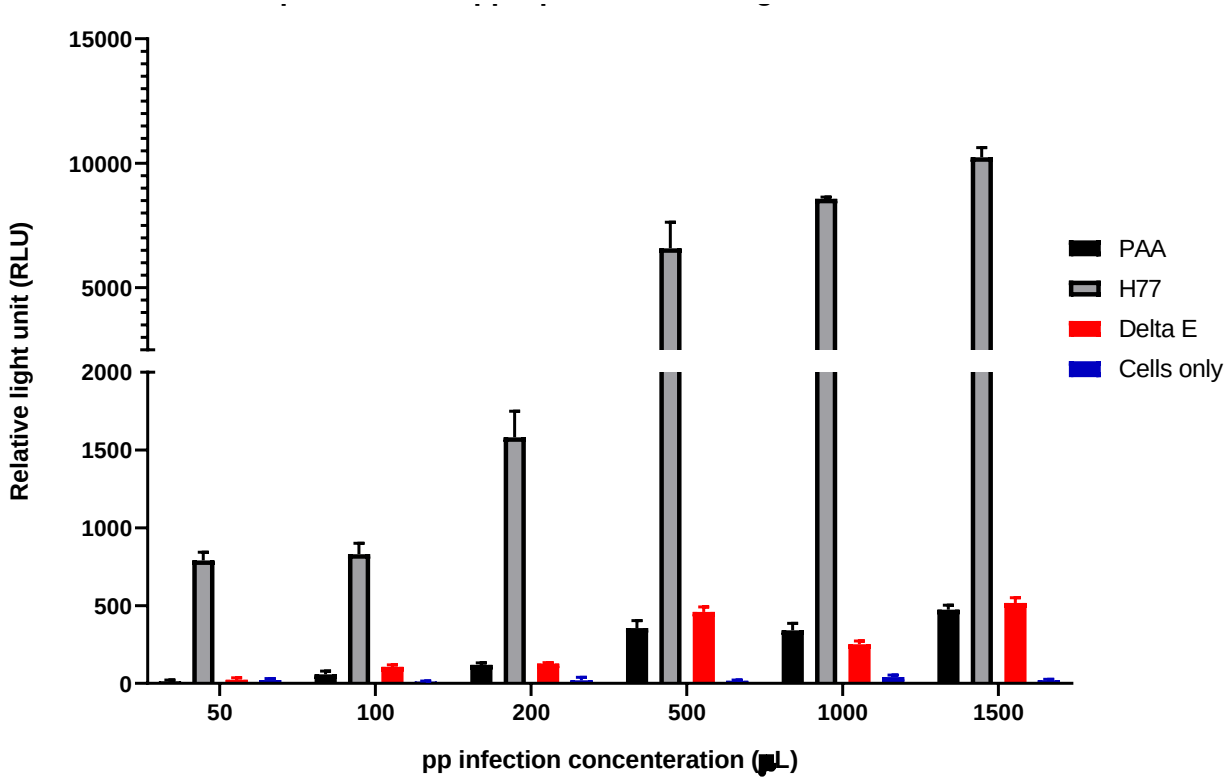

Figure 5.1: Optimum pseudoparticles infection volume using HCV and EqHV controls: A gradient of infection volume experiment of EqHV (PAA) and Hepatitis C (H77) derived pseudoparticles was used to infect on HuH7 cells for 72 hours. The various Relative Light Unit (RLU) were measured to see the optimum infection volume.

5.2.2 Further optimization of pseudoparticles production by varying plasmids and pNL 4.3 lentiviral backbone concentration

Retroviral pseudoparticles are a highly tractable model suitable in investigating enveloped viruses entry pathway, including hepaciviruses (160,286). Studies have shown some pseudoparticles to be sensitive to the concentration of plasmids and pNL4.3 $E^{-}r^{-}$ luc used; hence, making optimum volumes is vital for efficient pseudoparticle production and reproducibility of data (284).

This study then addressed if it is possible to improve the infectivity profile of pseudoparticles infectivity by optimizing the pNL4.3 $E^{-}r^{-}$ luc and plasmid concentration using gradient concentrations of one infectious plasmid (H33), a negative control (H49) and standard concentration of positive controls (HCV strain H77 and vesicular stomatitis virus G protein (VSV)). Interestingly, two different trends were seen with a change in pNL4.3 $E^{-}r^{-}$ luc and plasmid concentrations, with the infectivity of pseudoparticles decreasing as pNL4.3 E-r- luc gradient increases vice versa with changes in plasmid concentration (Figure 6.2). Putting the data together, it could be seen that the 2 µg of plasmid and pNL4.3 $E^{-}r^{-}$ luc is the optimum concentration for an efficiently infective pseudoparticle, similarly to published data (284).

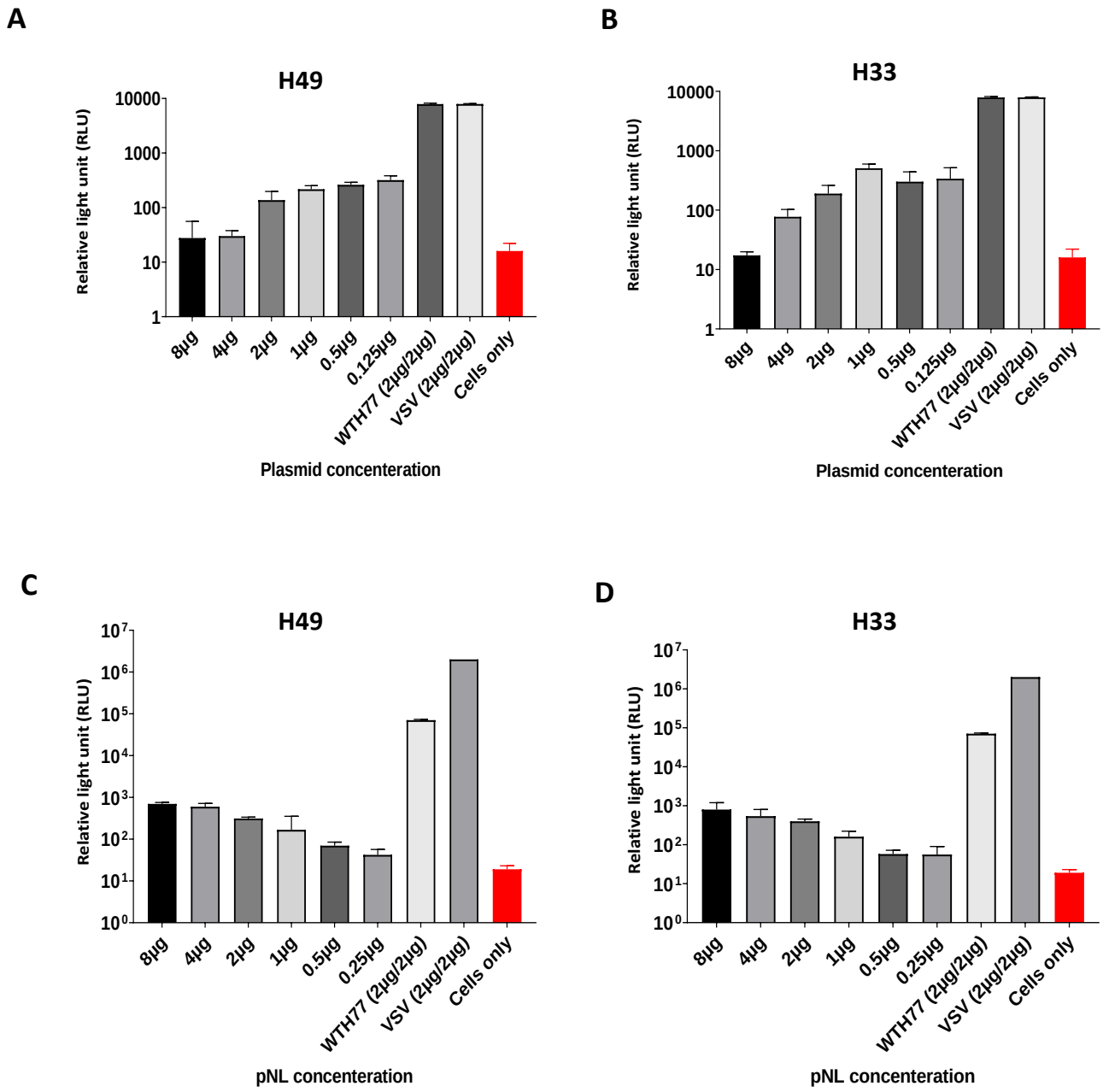

Figure 5.2: Optimization of pseudoparticles production by varying pNL4.3 $E^{-}r^{-}$ luc and plasmid concentration. Gradient concentration of Plasmids (A and B) and pNL ranging from 8 µg to 0.125 µg using a negative control (H49) and infectious pseudoparticles (H33). The positive controls (CD81 knockout H77 (WTH77) and VSV) were carried out using published concentration of 2 µg each of pNL and plasmids.

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5.2.3 HuH7 Infection and luciferase assay experiment

This study further sought to investigate the EqHV entry pathway and if the CD81 receptor is involved in the virus entry into cells during infection. A titration of plasmids was performed and that of the size of transfection. 2 µg of packaging construct and 2 µg of E1E2- expressing plasmid was observed to provide the best discrimination between positive and negative signal. (284). Compared to the background signal (cells only) and positive controls (H77, PAA and VSV); H49 was consistently the lowest in several independent experiments with similar relative light unit (RLU) units and was therefore considered to be true negative control for the downstream experiments, possibly due to the several substitutions in the E1E2 amino acid residue (Figure 6.4). Of the 15 samples, two samples were not infectious (H16 and H49), two samples (H51 and H33) could be considered as exhibiting borderline infectivity, while the rest samples showed varying degree of infectivity, with H28 being the most infectious of all samples (Figure 6.3).

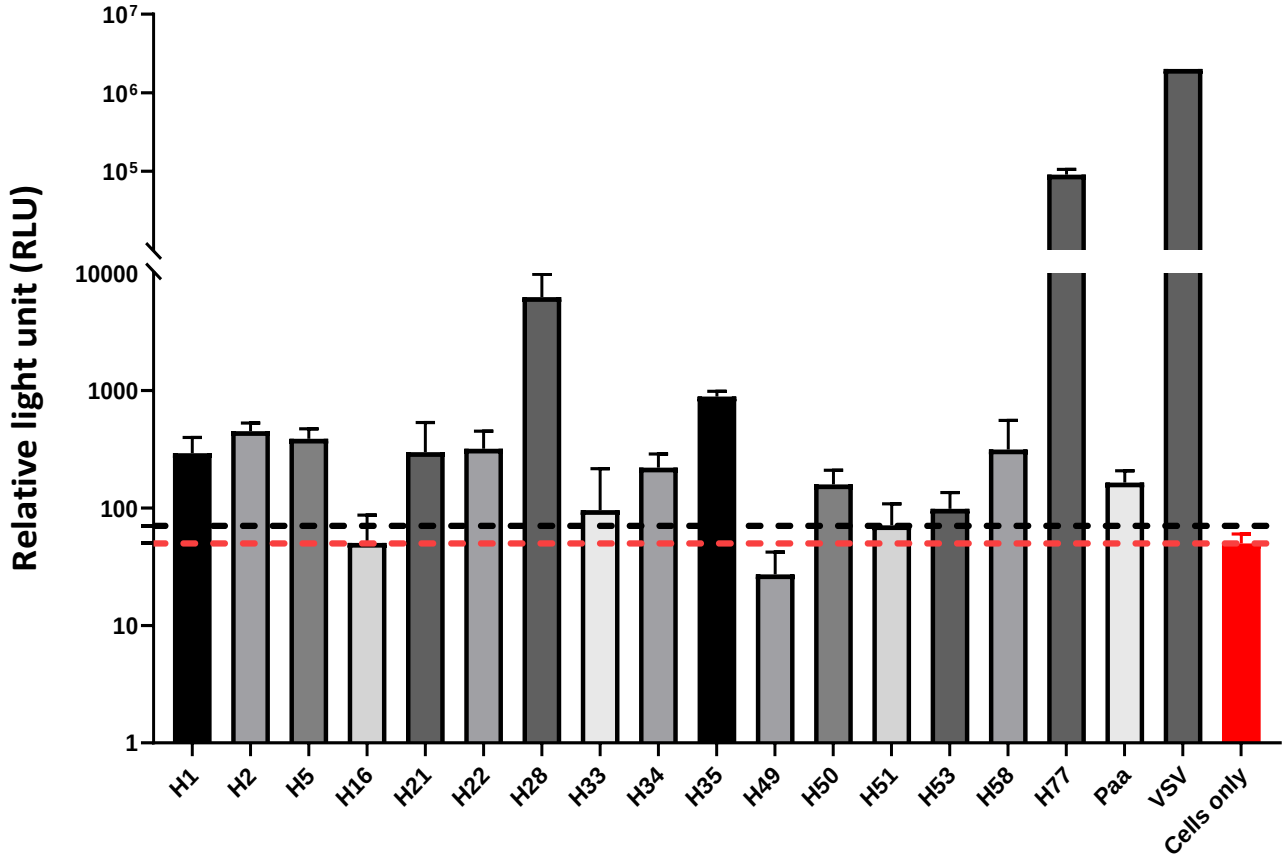

Figure 5.3: Infection of EqHV pseudoparticles from two combined independent experiments in HuH7 cells. HuH7 cells were infected with EqHV pseudoparticles on pNL4.3 Luc R-E- lentiviral backbone and incubated at 37 °C and 5% CO_2 for 72hours. HCV wild type, PAA and VSV were used as a positive control for the experiment while pNL 4.3 lentiviral backbone plasmid was used as a background control. n=15, data are shown as means ± SD. Red line represents background signal. Black line represents mean + 2SD above background signal. SD represents standard deviation. ANOVA: $P < 0.0001$ significant difference among means. $R^2 = 0.999$.

5.2.4 Amino acid comparison between Horse 49 and Horse 28

Repeated independent experiments showed a consistent low signal to background ratio with H49 pseudoparticles, while H28 showed the reverse. This study further investigated the differences in amino acid residues in the E1E2 region and the HVR1 site to determine possible amino acid differences impacting the viruses' entry.

About two times more amino acid differences were observed in the E2 region than the E1 region, with no amino acid substitution in the HVR as demonstrated by this study and another study (282). (Figure 4.6 and Figure 6.4).

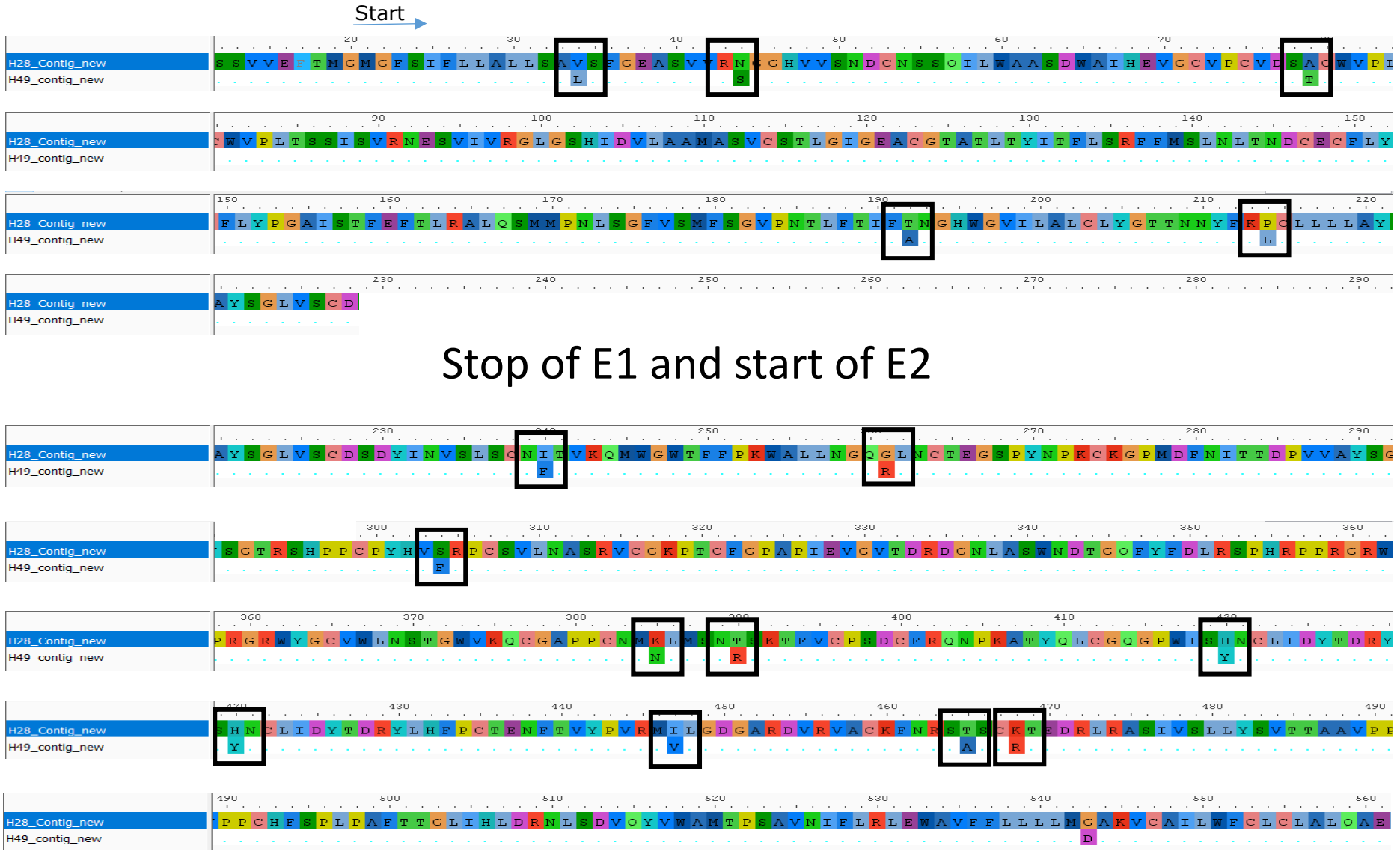

Figure 5.4: Alignment of amino acid sequences of H28 and H49 contigs. These E1E2 amino acid sequences were aligned so as to note the difference in the E1E2 regions as well as the HVR1 site between the two amino acid residues. Identical amino acid residues are marked with dot sign. Differences between both residues are highlighted with a box sign.

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5.2.5 Pseudoparticles stability experiment

Studies show that HCV pseudoparticles' infectivity reduces by two to five-fold after each freeze-thaw, even up to a 50 % decrease compared to a freshly prepared pseudoparticle (287,288). This study further aimed to investigate if EqHV pseudoparticles reduce infectivity as well. The three most infectious pseudoparticles (H28, H35 and H58) and negative control (H49) were freeze-thawed and tested after three weeks. Similar to the trend seen with HCV pseudoparticles, there was one $\log_{10}$ decrease with all pseudoparticles except the negative control that showed little change (Figure 6.5).

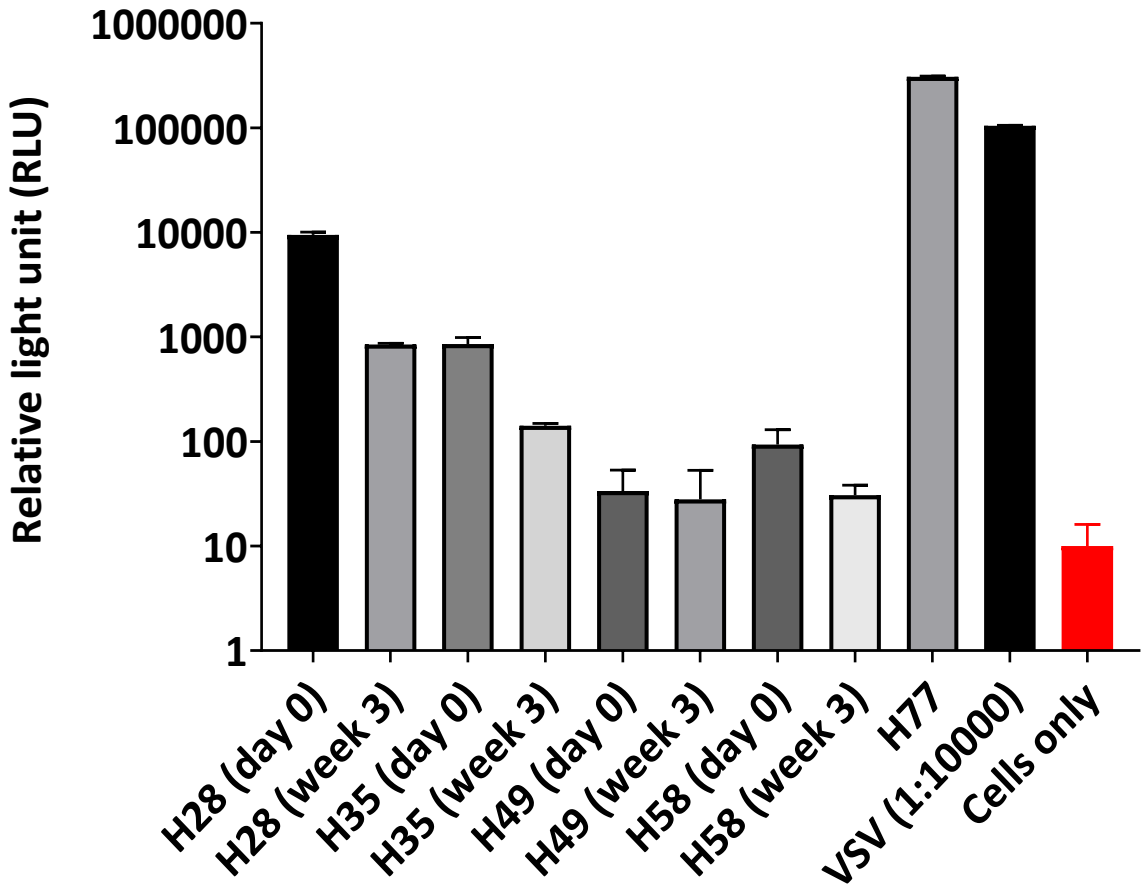

Figure 5.5: Influence of freeze-thaw cycles on EqHV pseudoparticles infectivity over a 3-week storage period. The stability of EqHV pseudotype was evaluated by subjecting aliquots of virus to 2 cycles of freeze-thaw. One biological replicate of each pseudotype virus was used to generate three technical replicates. Error bars represent standard deviation.

5.3 Discussion

HCV inherent sequence diversity is one of the contributing factors to its treatment. HCV envelope glycoprotein (E1E2) *in vivo* has been documented to rapidly adapt to host cellular and humoral responses (289–291). However, for EqHV, little is known about the virus entry molecular mechanisms and immunological responses. In the life cycle of any virus, cellular entry is an essential step and usually serves as a potential drug target. These potential targets, either therapeutic or prophylaxis, aims at the functional and structural conserved region in the virus glycoproteins. Understanding the viral entry mechanisms in terms of its cellular and viral components and the evolution of the envelope glycoprotein in response to host targeting should elucidate the entry process's molecular mechanisms. In the previous chapters, this thesis aimed at describing the prevalence, evolutionary relationship, possible route of transmission and infection profile of EqHV. This prior work generated EqHV E1/E2 expression clones that could be used for phenotyping. Here, we then optimised and evaluated the stability of EqHV pseudoparticles and infection of human cell lines.

Due to the novelty of this technique in EqHV research. The first step involved performing a gradient assay to determine the optimum infection volume to be used in downstream analysis of entry of EqHV variants. This was essential to determine the maximum volume of pseudoparticles needed to perform the downstream experiments. Although the background signal was high, 1000 µL was used in this

study, as it generally provided the best resolution compared to other infection volumes. Furthermore, this study showed an optimized assay with the signal to noise for H28 using H49 as the negative control; neutralization assay can be carried out due to a decent range.

A previous study (284) showed that by varying the pNL4.3 E^-r^- luc packaging construct concentration during transfection, the infectivity observed for pseudotyped viruses varied. We then further optimized the infection volume by carrying out a matrix of pNL4.3 E^-r^- luc and HCV E1E2 expression plasmids to determine the optimum concentration for the EqHV infection experiment. An amount of 2 µg each of pNL4.3 E^-r^- luc packaging construct and EqHV E1E2 expression plasmid was found to produce the best infectivity when assessed by relative luminescence.

Repeated freeze-thawing and long term storage are detrimental to samples, including proteins or DNA/RNA (292). Pseudoparticle production is labour and time-intensive, so large-scale manufacturing batches of virus preparations allows higher throughput analysis—however, cold storage (-20 °C to -80 °C) could impact long-term storage results. We investigated EqHV pseudoparticles' stability over three weeks at -80 °C and observed a consistent one-$\log_{10}$ drop in the pseudoparticles' infectivity. These could be attributed to classical factors associated with freeze-

thawing, especially damage of protein structure and cellular membranes due to ice crystals, the concentration of the salts and proteins, and oxidative stress (293).

Furthermore, this study made steps toward making infectious particles. Interestingly, these particles infected human cells. There are differences in the amino acid sequences of infectious and non-infectious E1E2 constructs, some of which are close to the CD81 binding sites proposed in HCV E1E2. Therefore, some horse isolates may be able to infect human cells. Nevertheless, horse hepatocytes will be needed to perform species-matched pseudovirus assays. While we did retrieve some frozen vials of equine hepatocytes, there was no signal generated in these assays. However, this could be due to the low plating density of these cells or the lack of viability of frozen cell preparations.

6 Final conclusion

Since the HCV discovery, extensive research into finding a prophylactic vaccine has been impeded due to the lack of a tractable animal model. The discovery of EqHV in 2011 provided insight into the possibility of a tractable model suitable for mechanical analysis and evolutionary relatedness of HCV. In this thesis, I attempted to broaden our understanding of EqHV in Thoroughbred racehorses via four distinct settings: prevalence, seroreactivity, transmission, and entry.

This study used a novel PCR assay to demonstrate a high prevalence of EqHV RNA in UK Thoroughbred horses than other breeds of horses in the UK population. Although, the reason for this high prevalence is not known, which might be due to genetic characteristics, lots of travel history due to being an athletic animal or just being a super-spreader. The prevalence of this virus among horses might be underreported due to the two-fold increased sensitivity of our Core-E1 assay compared to the standard NS3 PCR screen used by other laboratories. Therefore, more horse populations have to be screened with this study assay. Also, liver infection in Thoroughbred racehorses could have significant economic importance on the racing industry. Although we are yet to determine the effect on performance, this could demonstrate the importance of screening performance animals. Therefore, further work is urgently required to determine whether EqHV infection impacts racehorse athletic performance by assessing this virus's effect on a control group shown to be positive and another shown to be negative for EqHV. Hence,

providing evidence for these high-value animals. This knowledge is particularly relevant for the horse racing industry because HCV infection often results in fatigue and lethargy (39), and these are likely symptoms common to prolonged EqHV infection. The economic impact of EqHV infection in racehorses is currently unknown, but the introduction of a protective vaccine for EqHV to prevent chronic infections in these horses is likely to have a positive impact on the racing industry.

Racehorses have poor immunity due to inbreeding, and it might be that the immune response in Thoroughbreds is compromised. We identified naturally occurring acute, chronic and re-infection among Thoroughbred racehorses by comparing retrospective EqHV sequences obtained from horse sera at different sampling time points. This data also provided insight into the diversification of the viral population among Thoroughbred racehorses. Comparative sequence analysis provided insights into potential transmission clusters and revealed that EqHV has genetic patterns consistent with being vector-transmitted. Furthermore, multiple signatures of vector-borne transmission were observed on analysis of the %GC content, biases in dinucleotide usage and relative codon usage. We suggested an immune-mediated selection of viral variants due to the common HVR1 length polymorphism. We also proposed that the primary site of variability in the first eight amino acids of E2 is shorter than HVR1 of HCV. This study also showed that antibodies could be produced to EqHV in acute infections.

This study attempted to optimize an EqHV pseudoparticle assay. While only partial progress was made with this study, this assay could prove essential to elucidating the EqHV entry pathway, providing further characterization to show if its entry is CD81 dependent similar to HCV. This entry data has to be further investigated by using horse and liver cells lines. Earlier experiments have shown EqHV to express receptors on the surface of horse cells, including CD81 and SRB1 (Janet Daly, personal communication). Horse CD81 and SRB1 are genetically well conserved between horses and humans, but it will be interesting to investigate the entry pathway of EqHV and if genetic diversity displayed by the virus has consequences for receptor usage entry efficiency. This experiment can be done using two hepatic cell lines (Human and horse), having one cell line with CD81 and SRB1 and another having both SRB1 and CD81 receptor expression knocked out. By assessing infectivity in both cells, the receptor dependency can be determined. We planned to use CD81-negative Huh7 cells provided by a collaborator to investigate entry by the pseudoparticles generated in our initial experiments. Having created pcDNA3.1 based CD81 expression constructs for both the human and equine genes, the next stage would have been to transfect these genes into CD81- cells, assess expression by flow cytometry, and then perform infection experiments, using HCVpp as controls for infection of human-CD81 transfected cells, and testing infection of EqHVpp in

the equine CD81-transfected cells. Due to the Covid-19 crisis and subsequent lockdown, these experiments were only briefly started.

Having demonstrated that infectivity of at least some strains of EqHV can be shown using the pseudotype experimental setup, this assay would lend itself to several different investigations. Assessment of neutralizing antibodies generated during natural infection and in immunized animals could be easily assessed. The efficacy of entry inhibitors (such as recombinant antibodies, soluble lectins, and peptides) could be evaluated using this system. It would also allow assessment of the mechanism of receptor binding and fusion for hepaciviruses. Interestingly, EqHV can demonstrate the possibility of infection in human cells. Given the possibility of cross-species transmission of viruses and the potential impact on human health, the need to investigate viruses that can transmit to humans is urgently needed. Given the close association of humans and horses in many countries, EqHV may pose an immediate threat to human health.

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