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INSIGHTS INTO EQUINE HEPACIVIRUS: ASSAY DEVELOPMENT AND TRANSMISSION PATTERNS

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INSIGHTS INTO EQUINE HEPACIVIRUS: ASSAY DEVELOPMENT AND TRANSMISSION PATTERNS

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This thesis is submitted to the University of Nottingham, School of Veterinary Medicine and Science, for the degree of Doctor of Philosophy

April 2023

Funded by The Horse Trust

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Declaration

I declare the work presented in this thesis was carried out in accordance with the requirements and regulations of The University of Nottingham. This dissertation is original and result of my own work and does not result from collaboration with other institutions except where specifically indicated. I declare that the work included in this thesis has not already been submitted or accepted for any other degree or qualification in any other institute. I confirm this thesis does not exceed the prescribed word limit.



Mirelle Amelia Radley

Date: 26th April 2023

Dedication

Dedicated in memoriam to my little brother, Miles (1998-2021), my partner in crime, who always encouraged me to think differently, take on anything and to remember to dance in the moonlight.

Carpe diem

Acknowledgements

I would like to express my deep gratitude to the following people who have supported me throughout my PhD journey. Firstly, I would like to thank my supervisor Dr Janet Daly who provided such immense continuous support, guidance, and encouragement throughout. I couldn't have asked for a better supervisor. I would also like to thank Dr Alexander Tarr, Dr Terry Akagha, Dr Ge Wu, Dr Toshana Foster, and Dr Anitha Varghese for all their support, and advice in the lab. A huge thank you to Dr Jeremy Kemp Simmonds, Dr Jessica Stokes and all the staff at Bransby Horse Rescue for supporting my field work venture and The Horse Trust whose financial support made this project possible.

I am so grateful for the friends I made along the way, the fellow SB liveries Heather and Ella, always up for a summer evening pub ride to destress. My B31 "ET" friends, Chloe my fellow brain twin and main character inspiration, Scott my distractable desk buddy, Kieran my reluctant agony aunt, and Colman who all regularly made me laugh till I cried and made coming into the office so much fun.

And finally, a big thank you to my parents for their continuous support, encouragement, and enthusiasm from day one. As well as my chosen family, my closest friends; my friends from home, Nicola, for the daily phone calls and continual grounding, Jess, for being there whenever I need it most, and my friends from undergrad, Lizzie, for always providing sanity and rational advice and Ellie, for providing the opposite and driving with me across Europe in a campervan for a thesis writing escape. Thank you all for keeping me going through the hardest times.

Abstract

Equine hepacivirus A (EqHV) is most genetically similar to hepatitis C and causes liver disease in horses. EqHV has been detected in equids globally since 2011, but several aspects about the virus are yet to be established, including transmission and clinical impact.

This study developed a novel assay to detect EqHV antibodies, using EqHV viral proteins expressed by *Agrobacterium tumefaciens* in *Nicotiana benthamiana*. Prevalence of EqHV was assessed in a population of rescue horses from Bransby horse sanctuary over several months of 2018. Results showed a new significant seasonal dependence of EqHV prevalence, with most positive samples obtained in the summer months only. An *in vivo* experiment demonstrated biting flies are a potential transmission vector and may explain this seasonality.

Clinical chemistry data was assessed and compared to data from a Thoroughbred population. Potential implications of haematological parameters were identified for Thoroughbred horses with EqHV. Thoroughbred horses positive for EqHV via PCR had a significantly higher level of haemoglobin, MCH, PCV and MCV and significantly lower MCHC. The haematological parameters for Bransby rescue horses positive for EqHV were not affected, however a have significantly lower levels of CK, highlighting the differences and implications EqHV infections may have on horses in intense work.

Covid-19 Impact

The work undertaken for the submission of this Thesis commenced on the 2nd of October 2018. The School of Veterinary Medicine & Science at the University of Nottingham closed laboratories as a part of the national Covid-19 lockdown from the 13th of March 2020 until the 20th of August 2020, a total of 22 weeks, after which only part time scheduled lab access was available to comply with social distancing laws for the following 12 months. This severely impacted the overall laboratory time available and restricted what research could be conducted to strict time slots when lab access was available. This particularly impacted the development of the ELISA due to the lengthy protocols and access to multiple labs required for the plant expression, protein extraction and purification, which were incompatible in the short time slots available. Field work was also impacted as due to travel restrictions I was unable to travel to the trapping sights to set up traps and collect specimens.

In summary, the work conducted for this thesis has been adversely affected by the Covid-19 pandemic in multiple ways, resulting in work which has been revised and modified as a consequence of the global pandemic.

Abbreviations

Δv	Control pseudovirus (no envelope)
6XHis	Polyhistidine tag
A. tumefaciens	*Agrobacterium tumefaciens*
AHS	African horse sickness
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate aminotransferase
bp	base pairs
BSA	Bovine serum albumin
CCH	Canine chronic hepatitis
cDNA	complementary DNA
CK	Creatine kinase
CPK	Creatine phosphokinase
CPMV	Cowpea mosaic virus
CUB	Codon usage bias
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
E. coli	*Escherichia coli*
E1	Envelope protein 1
E2	Envelope protein 2
EDTA	Ethylenediaminetetraacetic acid
ELD	Equine liver disease
ELISA	enzyme-linked immunosorbent assay
EpgV	Equine pegivirus
EqHV	Equine Hepacivirus A
EqPV-H	Equine parvovirus-hepatitis
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
GLDH	Glutamate dehydrogenase
GLIPS	*Gaussia*-luciferase-immunoprecipitation system
GNA	*Galanthus nivalis* agglutinin
GOT	Glutamic-oxaloacetic transaminase
HCV	Hepatitis C virus

HEK cells	Human endothelial kidney cells
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus 1
h	Hour(s)
Huh	Human hepatocyte
IFNα	interferon alpha
KC	Kupffer cells
kDA	kilodaltons
LB	Lysogeny broth
LDH	Lactate dehydrogenase
LIPS	Luciferase immunoprecipitation system
LSEC	Liver sinusoidal endothelial cells
MCH	Mean cell haemoglobin
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
MHz	Megahertz
min	minute(s)
miR	microRNA
ml	millilitre
mm	millimetre
N. benthamiana	*Nicotiana benthamiana*
NATs	Nucleic acid amplification techniques
NCBI	The National Centre for Biotechnology Information
nd	no data
nl	nanolitre
No	Number
NPHV	Non-primate hepacivirus
ns	not significant
nt	nucleotide
NVS	The University of Nottingham Vet School
OTS	one-to-stop (codon)
O/N	Overnight
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
Pv	Pseudovirus
PVNT	Pseudotype virus neutralisation test
P#	Passage number

QMC	Queen's Medical Centre, Nottingham
qPCR	quantitative polymerase chain reaction
RNA	Ribonucleic acid
rSAP	recombinant shrimp alkaline phosphatase
RSCU	Relative synonymous codon usage
RT	Reverse transcription
SDH	Sorbital dehydrogenase
TAE	Tris-acetate-EDTA Buffer
Tb	Thoroughbred
TBS	Tris-buffered saline
TBST	TBS-Tween
TDAV	Theiler's disease–associated virus
TE	Trypsin EDTA
UK	United Kingdom
UTR(s)	Untranslated region(s)
UV	Ultraviolet
WB	Warmblood
WBC	White blood cell
yr(s)	Year(s)

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Chapter 1: Equine Hepacivirus and Liver Disease

1.1 Overview

Equine Hepacivirus A (EqHV) emerged as a novel virus in 2011, classified within the *Flaviviridae* family, sharing close genetic ties with the human hepatitis C virus (HCV). Primarily detected within equine liver cells, EqHV is believed to induce liver disease akin to HCV. However, significant gaps persist regarding its transmission routes and pathology.

Numerous aspects of EqHV remain elusive, primarily stemming from its recent identification, the inability to cultivate the virus in vitro, and the absence of conclusive evidence delineating its impact on equine health. Given the genetic resemblances between HCV and EqHV, research advancements in one virus are likely to offer valuable insights for understanding the other. The limited knowledge surrounding EqHV underscores the challenges posed by its recent discovery, the complexities involved in culturing the virus outside the host, and the need for comprehensive studies to discern its implications on equine well-being.

1.1.1 Discovery and Nomenclature

EqHV, was initially identified in canine respiratory secretions in America via a study undertaken to characterise viral flora of companion animals. EqHV, previously named canine hepacivirus (CHV), was identified via qPCR in respiratory samples from 9 of 33 dogs, representing 5 different outbreaks of respiratory disease. Nasal swabs of 60 healthy pets were screened and no CHV was found. CHV RNA was found in five liver tissue samples, but no CHV was identified in lung tissue samples (Kapoor et al., 2011). Several studies since its initial discovery have failed to find EqHV in dogs and it has since been concluded that dogs are not the primary host (Burbelo et al., 2012a; Lyons et al., 2012a).

As EqHV was initially isolated from dogs, it was originally named canine hepacivirus (Kapoor et al., 2011). Shortly after this, the virus was discovered in horses and was therefore renamed non-primate hepacivirus (NPHV) (Lyons et al., 2012), EqHV was the first reported non primate homologue for the hepacivirus genus. Since then, little evidence has been found to support dogs as natural reservoirs for the virus. The virus had been predominantly found to infect horses and donkeys (Burbelo et al., 2012a; Walter et al., 2016a).

Following the discovery of other non-primate hepacivirus, the name of the virus underwent subsequent alterations. Initially termed equine hepacivirus, it was later reclassified as Hepacivirus A by the International Committee on Taxonomy of Viruses in 2020 (ICTV 2020). Despite this official alteration, it is frequently still referenced as equine hepacivirus within scientific discourse.

1.1.2 Flaviviridae

Flaviviridae is a family of viruses comprising of 4 genera: *Flavivirus*, *Pegivirus*, *Pestivirus*, and *Hepacivirus*. The *Flaviviridae* are small, spherical viruses 40–60 nm in diameter, with a lipid envelope (Simmonds et al., 2017). The capsid consists of a single protein, with the envelope containing two or three virus-encoded membrane proteins. The genomes are relatively short (9 –13 kb) and always positive sense, single-stranded RNA (Westaway et al., 1985). The majority of viruses in this family are pathogenic and host specific, and many are arthropod borne, including mosquitoes, ticks and unknown insect vectors (Simmonds et al., 2017). Replication complexes for this family of viruses are intricate and use enzymes including serine protease, RNA-dependent RNA polymerase and an RNA helicase; these enzymes are homologous among all members of this genus (Simmonds et al., 2017).

1.1.2.1 Flavivirus genus

The *Flavivirus* genus consists of over 70 viruses and almost all of these are arthropod-transmitted or zoonotic viruses (Thomas et al., 2015). Representative viruses include dengue virus, West Nile virus and yellow fever virus. Some flaviviruses are pathogenic to humans, such as yellow fever virus (Monath, 2001) and Zika virus (Qureshi, 2018). Some flaviviruses infect both humans and the horse such as West Nile virus (Angenvoort et al., 2013; Cardinale et al., 2017) and Murray Valley encephalitis virus (Prow et al., 2013).

1.1.2.2 Pegivirus genus

The *Pegivirus* genus comprises 11 virus species with a large global distribution and a wide range of mammalian hosts including humans, horses and rodents (Simmonds et al., 2017). Transmission via invertebrate vectors has not been observed, rather transmission occurs via sexual, parenteral and maternal routes (Santos et al., 2017; Taklual et al., 2019). Two species of *Pegivirus* are known to infect equids, *Pegivirus E* and *Pegivirus D*. *Pegivirus D* is also known as Theiler's disease associated virus (TDAV) and was originally associated with equine hepatitis (Chandriani et al., 2013) however this is now disputed (see section 1.6.4.1).

1.1.2.3 Pestivirus genus

The *Pestivirus* genus comprises of 11 virus species that infect mainly pigs and ruminants (Vilček and Nettleton, 2006), making *pestiviruses* among the most important flaviviruses economically to livestock agriculture (Tautz et al., 2015). No invertebrate hosts have been found; transmission occurs via infected secretions (e.g. faeces, saliva, urine) (Simmonds et al., 2017) and the viruses cause diseases such as bovine virus diarrhoea and mucosal disease. Susceptible host species are estimated to have persistence infection prevalence of 1–2% (Schweizer and Peterhans, 2014). Currently, there are no reports of *Pestiviruses* being found in horses.

1.1.2.4 Hepacivirus genus

The *hepacivirus* genus, in which EqHV is now classified, previously comprised of only Hepatitis C and GB virus B since their classification into the genus in 1996. However, since 2011, 12 new species of non-primate

hepacivirus (NPHV) have been identified in various host species; including equids, rodents, bats, cattle and catsharks (Drexler et al., 2013; Quan et al., 2013; Baechlein et al., 2015; Shi et al., 2015). Currently, HCV is the only hepacivirus to infect, replicate and cause disease in humans. A study on people in occupations with a high risk of exposure to EqHV found they showed low seropositivity for EqHV antibodies; viral RNA was not detected (Pfaender et al., 2015b).

1.2 Features and genetic structure of EqHV

1.2.1 Host range

In two studies examining canine chronic hepatitis (CCH) and hepacivirus, no correlation was found (Bexfield et al., 2014; van der Laan et al., 2014). Additionally, neither study found any evidence of carrier status or the presence of antibodies specific to hepacivirus in 100 CCH samples from UK dogs or in 267 samples from The Netherlands. It is plausible that the initial detection of an outbreak in the kennels was due to cross-species transmission, potentially occurring during ingestion of infected equine tissues fed as raw meat. This suggests dogs are not the natural hosts. This could be similar to the case of equine influenza found in foxhounds, presumed to be due to ingestion of raw contaminated horse meat (Daly et al., 2008). This may also explain the presence of the virus in unusual tissues, although one study (Pfaender et al., 2015a) did find the presence of EqHV RNA in equine bronchial epithelial cells.

1.2.2 Structure of the genome

EqHV, like all other members of the *Flaviviridae* family, has the same classic structure – a small spherical virus encased in a lipid envelope, internally containing one viral core of single strand positive-sense RNA genome. This genome has an open reading frame encoding a multifunctional polyprotein bordered either end by untranslated regions (UTRs) (See

Figure 1). The polyprotein precursor is processed by the host and viral encoded proteases into 10 protein products. Knowledge of EqHV A genetic structure specifically variability and conservation between genes has been used to create primers for detection of the virus in PCR techniques. Notably, NS3 is the initial gene targeted for the first screening of PCR in Kapoor 2011.

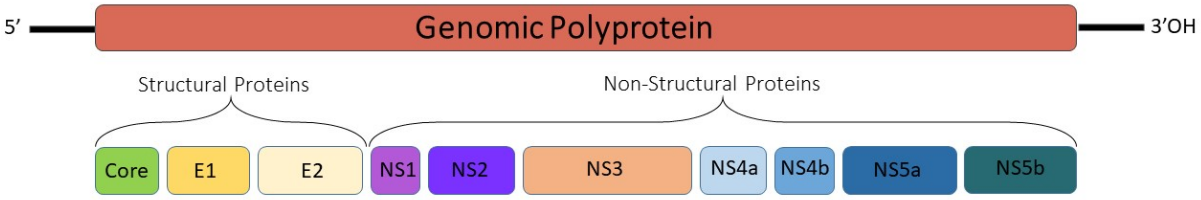


Figure 1 Structure of the EqHV RNA genome, classic Flaviviridae genetic structure depicting genes which encode the structural and non-structural proteins.

1.3 EqHV Replication

The exact method of EqHV replication has yet to be determined. The virus has mainly been detected in the liver cells but also in the spleen, bronchial epithelial cells (lung), cerebellum, and tracheobronchial lymph node (Pfaender et al., 2015a). EqHV possesses a binding site for microRNA (miR)-122, which is a liver-specific micro-RNA and removal of this site prevents translation (Scheel et al., 2015). This indicates that translation happens in the liver cells. EqHV and HCV p7 protein have structural and functional homology, p7 is localised in the endoplasmic reticulum (ER) membranes and is important in production of infectious particles. Similarly to HCV, the 5'UTR region is also essential for translation and replication (Tanaka et al., 2018).

1.4 Hepatitis C virus

The closest genetic homologue to EqHV is HCV, which naturally only infects humans and chimpanzees (Sandmann and Ploss, 2013), and is a major global human health problem and has spread epidemically in western countries in the last 60 years. This is largely due to increased injectable drug usage allowing for more frequent transmission via blood-borne routes. Approximately 3–4 million people are newly infected with HCV every year (Mohamed et al., 2015). Of these cases, 55–85% of individuals who contract HCV are unable to clear the virus and develop chronic hepatitis, defined as infections of greater than 6 months duration (Seeff and Hoofnagle, 2002; Maasoumy and Wedemeyer, 2012). As a result, an estimated 130–175 million people have chronic hepatitis.

1.4.1 HCV Epidemiology

Hepatitis C virus is genetically diverse with 7 genotypes possessing >30% nucleotide (nt) sequence diversity (Mohamed et al., 2015). Genotypes from southeast Asia, central and west sub-Saharan Africa harbour the greatest diversity of subtypes, suggesting long-term endemic circulation of HCV in these geographical areas (Simmonds et al., 2005). It has been suggested that HCV originated from a progenitor virus in animals, however even with the discovery of 12 new *Hepaciviruses* since 2011, there is still no evidence to confirm this. In addition, the dispersal of some HCV genotypes displays similarities with human immunodeficiency virus 1 (HIV-1), which originated from chimpanzees (Gao et al., 1999), implying HCV could also

have originated from a central African reservoir (Pfaender et al., 2014; Martinez et al., 2015).

1.4.2 HCV Transmission

Hepatitis C virus enters the body usually directly into the bloodstream due to contaminated blood products or needles, e.g. shared needles during drug use (Mohamed et al., 2015) or contaminated blood transfusions; especially if the transfusion occurred before 1992 (Maasoumy and Wedemeyer, 2012). Studies have shown cases of vertical transmission are possible and happen in around 5% of pregnancies where the mother is infected (Canadian Paediatric Society, 2008; Yeung et al., 2014).

1.4.3 Detection

As many patients infected with HCV have subclinical disease, most remain undetected for years. Detection usually only occurs either by chance on a routine medical examination or after severe irreversible damage has been done, and signs of chronic liver disease sets in (Mohamed et al., 2015). Currently, there are several diagnostic options including various assays to detect the presence of specific HCV antibodies or nucleic acid amplification techniques (NAT) to detect and quantify viral genomic RNA (Warkad et al., 2018). Using an assay to detect antibodies it is not possible to determine if an individual is currently infected or has been infected previously, as antibodies will remain in the blood after clearance of the virus. In contrast to NAT which can only determine if the individual is currently infected.

1.4.4 Treatment

Historically, HCV was treated with interferon alpha (IFNα) but this treatment was discontinued due to its significant and common side effects, including fever, headache, fatigue, arthralgia, myalgia and in some cases neuropsychiatric and autoimmune problems (Dusheiko, 2004).

Significant improvements in HCV treatment have been developed, namely oral, interferon-free, direct-acting antiviral drugs such as sofosbuvir / velpatasvir (NS5B and NS5A (

Figure 1) inhibitors, respectively) (Vespasiani-Gentilucci et al., 2015); however, these are not necessarily without side-effects. Some drawbacks also remain in the high cost and failure to reduce the risk of re-infection with HCV after treatment. Nevertheless, direct acting anti-viral drugs are more effective and cause far fewer side effects than interferon alpha (Reddy et al., 2017). Re-infection risks could be overcome with the development of a vaccine.

The highest prevalence of HCV is found in developing countries such as Africa and central Asia (Manns et al., 2006) where most of the population is poor, cannot afford expensive drugs and also have a high re-exposure risk as the treatment does not prevent reinfection. Therefore, this population would benefit most from the development of a prophylactic vaccine.

Research on vaccines against HCV has been challenging due to the narrow host tropism of this virus. This has prevented the development of an

effective small animal model that can be used to test the safety and efficacy of novel vaccines. However, with the emergence of several closely related, non-primate hepaciviruses, the possibility of an animal model has arisen. EqHV may be a suitable model for the study of HCV because this virus shows ~50% sequence homology with HCV (Petruzziello et al., 2016). However, using the horse as a model would not be as straightforward as using animals such as rodents. This is due to several factors including the physical size of horses and therefore their space requirements as well as the cost of their upkeep for their management and dietary requirements.

1.5 Equine liver structure and functions

The equine liver is the second largest single organ in the body after the skin (Dyce et al., 2010). In the adult horse, it is located ventrally near the ninth and tenth intercostal space and is completely enclosed within the ribcage. The liver is suspended in the abdominal cavity, attached via six ligaments (Sisson & Septimus, 1974) and consists of multiple lobes as shown in Figure 2.

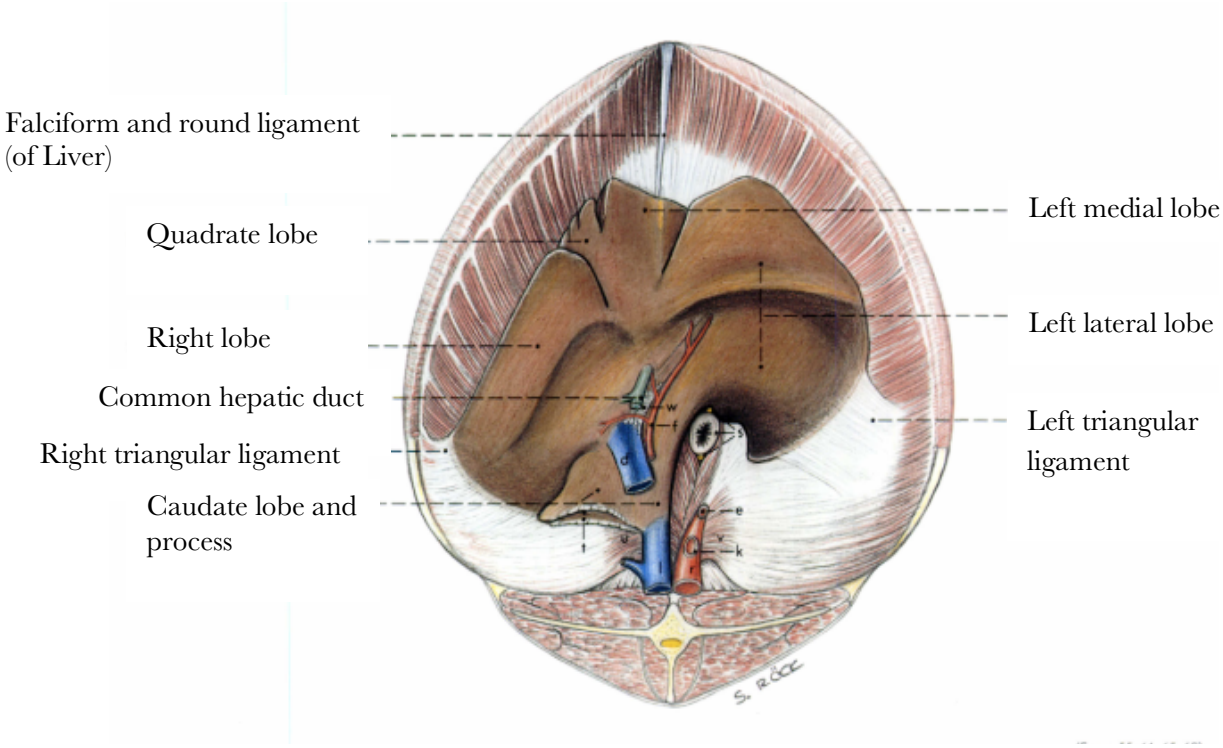


Figure 2 Cross section through the equine abdomen to show the liver, its lobes and ligaments. Adapted from Sisson, Septimus, R, (1974).

The liver comprises of around 70-80% hepatocytes intertwined with bile ducts and blood vessels. Sinusoids, the smallest of these vessels, are lined with liver sinusoidal endothelial cells (LSEC) and Kupffer cells (KC). These form many structural units called hepatic lobules shown in Figure 3. The liver has four fundamental functions: vascular, metabolic, secretory and excretory (Cullen and Stalker, 2016).

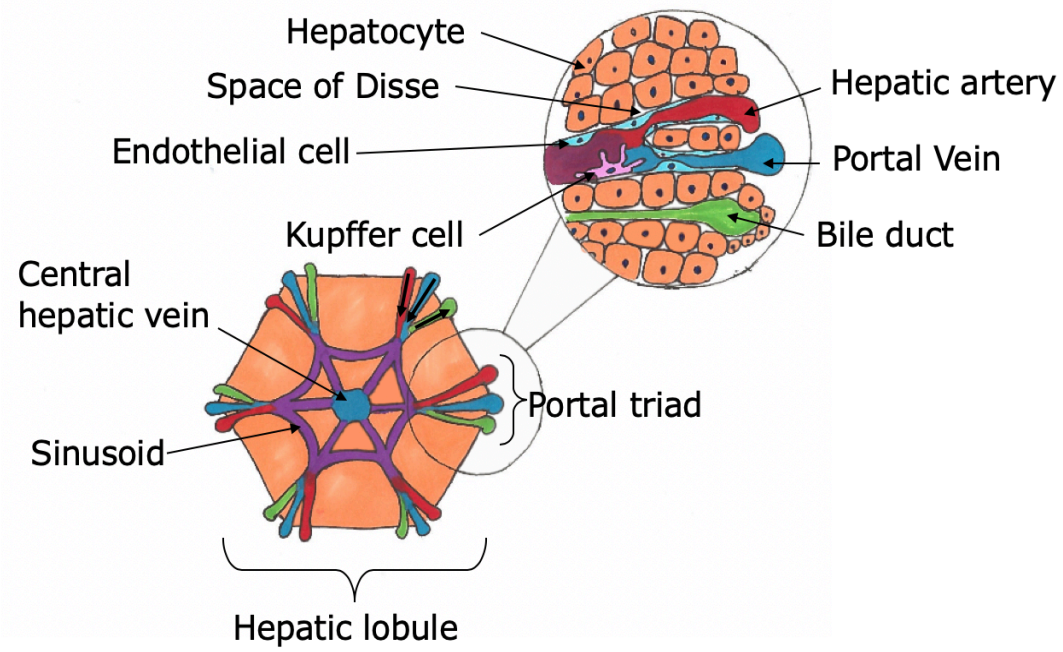


Figure 3 Diagram showing hepatic lobule structure displaying portal triad and tissue organisation, authors original drawing.

1.5.1 Vascular functions of the liver

The liver is the only organ with two blood supplies: the hepatic artery and the hepatic portal vein. Around 75% of blood that enters the liver is venous. The liver has a dynamic vascular system meaning it can both store and release blood, acting as a reservoir for blood during vascular volume changes. The liver also produces between 25 and 50% of lymph in the body (Durham et al., 2003a). The sinusoidal endothelial cells have large pores which allow the free flow of protein and fluid into the space between hepatocytes and the endothelial cells (the "space of Disse") to form lymph (Ohtani et al., 2003; Chung and Iwakiri, 2013). The liver also has its own phagocytic system; Kupffer cells are phagocytic, and form 80–90% of tissue macrophages present in the body. Due to their positioning downstream from the portal vein in the liver sinusoids, Kupffer cells are continuously exposed to gut-derived bacteria, endotoxins and other microbial debris (Poisson et al., 2017), which they effectively remove, preventing infiltration into systemic circulation.

1.5.2 Metabolic functions of the liver

The liver has several key metabolic functions which are essential to homeostasis. The liver synthesises and employs carbohydrates, lipids and proteins and is the main organ involved in regulation of nutrient distribution (Rui, 2014). All animals must maintain a physiological blood glucose concentration within a narrow range. To compensate for the fluctuations in blood glucose levels, extra glucose is stored in the liver and muscle cells as glycogen. Although there is more glycogen in muscle cells, they lack glucose-6-phosphatase which is essential for conversion of

glycogen to blood glucose, meaning muscle glycogen stores can only contribute to the demands of respiration and not replenish blood glucose (Jensen et al., 2011).

Hepatocytes possess multiple pathways to regulate blood glucose concentration. A sharp rise in blood glucose, for example after eating leads to glycogenesis and storage of glycogen in the liver and muscles. In contrast, a decline in blood glucose stimulates glycogenolysis whereby glycogen is converted into glucose and released into the blood. Should hepatic glycogen stores be diminished completely, gluconeogenesis using amino acids or non-hexose carbohydrates occurs (Rui, 2014). Equids are trickle feeders meaning they require and consume a constant supply of forage. Due to the continual supply of forage, there should be fewer extremes of glucose concentrations compared to humans, provided the domesticated horse is managed correctly and has access to this forage (Edouard et al., 2008). Starvation of the horse or intake of large concentrate feeds will cause extremes in blood glucose concentrations.

Hepatocytes also metabolise lipids, excess glucose, carbohydrates, and proteins by converting them into fatty acids, which are broken down at a higher rate than the hepatocytes require. The liver exports ketone bodies such as acetoacetate, into the circulation and provide metabolic fuels for extrahepatic tissues during fasting (Rui, 2014). Hepatocytes use plasma-derived fatty acids, oxidised in the mitochondria as an energy source to perform metabolic activities in the liver (Campbell, 2006). One of the key aspects of protein metabolism in the liver is the removal of ammonia from the body and conversion into urea. This maintains physiological levels of blood ammonia (Gebhardt and Matz-Soja, 2014).

The liver also produces blood coagulation factors as well as most plasma proteins, including albumin, lipoproteins and glycoproteins. The glycoproteins transferrin, hemopexin and haptoglobin are involved in iron transport, contributing to the liver's involvement in iron homeostasis (Heinz and Braspenning, 2015). Iron is stored intracellularly within the liver, spleen, and bone marrow in the form of ferritin.

1.5.3 Secretory and excretory functions of the liver

The liver is the largest gland in the body, with both endocrine and exocrine functions (Bergero and Nery, 2008). One of the most important functions is the secretion of bile, a complex alkaline fluid which is released into the bile duct for transport directly into the duodenum (Boyer, 2013). Most animals including humans store bile reserves in the gall bladder prior to secretion. However, the gall bladder is absent in the horse (Budras, 2009). Bile is responsible for the digestion and absorption of fatty acids and vitamins and the excretion of bilirubin into faeces (Campbell, 2006). The majority is absorbed by the small intestine then transported via the portal vein for re-use within the liver.

Absence of the gall bladder means the horse must be slowly adapted to high-fat diets to allow for the liver to adjust to the requirement of bile. Rapid increase in high fat feeds in the horse's diet will mean poor digestibility and absorption of fats and gastric upset (Marchello et al., 2000).

1.6 Equine Liver Disease

Equine liver disease (ELD) or hepatitis is not uncommon, it can be caused by several factors, many of which are well documented. Age, sex and breed are not risk factors for ELD, however a study in 2003 found ponies were more likely to present with ELD (Smith et al., 2003).

1.6.1 Non-viral causes of equine liver disease

The liver is highly susceptible to specific and unspecific toxins. Although the liver has a high threshold for toxins, long exposure induces degenerative and necrotic changes. Depending on the duration, type, and dosage of the toxins the liver will respond to toxic injury with fibrosis, cirrhosis or regeneration. Toxins affecting the liver generally are derived from plants, fungi, chemicals, drugs and heavy metals (Buergelt and Piero, 2013).

Due to the grazing habits of horses, and the fact many grazing fields are not regularly managed for the removal of unwanted plants or vegetation, it is easy for them to ingest toxic plants or substances (West, 1996). Generally, horses avoid ingesting toxic plants such as ragwort due to unpalatability. However, ingestion can occur where horses are kept in stark, poorly managed grazing pastures, or where forage is contaminated with dried plants (Cortinovis and Caloni, 2015). One of the most common plant-produced toxins in the UK are pyrrolizidine alkaloids, also present in ragwort (Bergero and Nery, 2008). Alkaloids are produced by plants as a defence mechanism against herbivores. Pyrrolizidine alkaloids are

carcinogenic, mutagenic, genotoxic, fetotoxic and teratogenic (Wiedenfeld and Edgar, 2011). The inability of horses to vomit means detoxification is performed solely by the liver, therefore ingestion of toxins results in damage to the liver.

Domesticated horses are fed a wide range of grains and forages. If these are stored in damp conditions mould may grow and mycotoxins are produced. Mycotoxins are a chemical secondary product of mould metabolism often produced when another limiting factor is inhibiting growth. There are various types of mycotoxins such as fumonisins or aflatoxin found in grains (Osweiler, 2001). The quantity of mycotoxin produced does not always correlate with the amount of mould visible. Ingestion of mycotoxins can result in toxicosis (Bergero and Nery, 2008).

Foals' susceptibility to toxicity from low doses of iron compounds increases the likelihood of liver failure and death in the first few days of life (Mullaney and Brown, 1988). Excessive iron intake orally by adult horses over a period of < 8 weeks is unlikely to cause iron toxicosis (Pearson and Andreasen, 2001).

Hepatic lipidosis and hyperammonaemia are metabolic causes of liver disease. Hyperammonaemia is the presence of excess ammonia in the blood often caused by an imbalance of urease-producing bacteria in the intestine. Some papers state this is a genetic abnormality in Morgan foals(Divers and Perkins, 2003; Foreman, 2019; Mair and Simon, 2020), however there is only one publication documenting this and describes a case study of 2 foals who were genetic cousins (McConnico et al., 1997).

Hyperlipaemia syndrome is a metabolic condition, occurring mainly in ponies and donkeys (rarely horses) and causes hyperlipidaemia (an increase in serum triglyceride concentrations). This results in fatty infiltrations into the liver and other vital organs (Bergero and Nery, 2008).

1.6.2 Fasciola hepatica

Fasciola hepatica (liver fluke) infection is less common in horses than other herbivores such as cows. There is currently no rapid and reliable method of diagnosis for liver fluke infection. It is likely risk of infection is increased when horses are kept in the same pasture as other herbivores with endemic liver fluke. Horses in tropical climates have a higher prevalence, and donkeys and mules are more susceptible; mules have 8 times higher risk for *F. hepatica* infections compared to horses (Villa-Mancera and Reynoso-Palomar, 2020). An abattoir study of 200 horses in Ireland found the presence of liver fluke in 19/200 (9.5%) (Quigley et al., 2017). A similar study in the UK examining 183 horses in an abattoir found 4 (2.2%) horses with adult fluke in the liver (Howell et al., 2020). Reports of prevalence of *F. hepatica* in horses vary widely according to geographical region and sensitivity of available diagnostic assays. In north-west Spain, 60% of horses tested positive on an indirect ELISA based on the recombinant *F. hepatica* 2.9 kDa antigen (although faecal egg counts were negative in all animals) (Arias et al., 2012).

1.6.3 Obstructive, inflammatory, and other causes

Cholelithiasis (gallstones) is the most common cause of biliary obstruction in horses (Gerros et al., 1993). Other causes can be due to right dorsal colon displacements, duodenal ulcers, neoplasia, hepatic torsion and portal vein thrombosis (Axon et al., 2004). Additionally, congenital portosystemic shunts in foals may occur but are rare vascular abnormalities.

1.6.4 Viral hepatic disease

Three other viruses are associated with equine hepatitis additionally to EqHV; Theiler's disease–associated virus (TDAV), pegivirus E, also known as equine pegivirus (EpgV), and equine parvovirus-hepatitis virus (EqPV-H).

1.6.4.1 Theiler's disease-associated virus

Theiler's disease, first described in 1918 as a condition of acute hepatic necrosis. Identified after occuring among thousands of horses in a vaccine trial against African horse sickness (AHS) performed by Sir Arnold Theiler. The initial trial involved 1148 horses and 2% developed fatal hepatic necrosis. Since the initial discovery, the condition has been reported in many countries globally in association with administration of a broad variety of equine biological products such as tetanus antitoxin (Guglick et al., 1995), equine plasma (Aleman et al., 2005) and most recently allogeneic stem cells (Tomlinson et al., 2019a). In 2013, TDAV was first identified in a serum sample from a horse suffering from nonfatal serum hepatitis

(Chandriani et al., 2013). This was assumed the cause of Theiler's disease and was thus named TDAV. The virus is scarcer than originally thought and has not been found in epidemiological surveys in multiple countries but has been found in pooled commercial sera (Postel et al., 2016). It is now disputed as to if TDAV is actually associated with Theiler's disease or not. The virus has been classified in the *Flaviviridae* family and *Pegivirus* genus and is officially named as pegivirus D (Smith et al., 2016).

1.6.4.2 Equine Pegivirus E

Equine pegivirus E (EqPV-E) was discovered in 2013 during a study deep sequencing sera from horses showing signs of hepatitis (Kapoor et al., 2013). EqPV-E belongs to the family *Flaviviridae*, genus Pegivirus and has been officially classified as Pegivirus E (Smith et al., 2016). EqPV-E is a common infection worldwide, according to a meta-analysis study approximately 1 to 32% of horses are viraemic and up to 66.5% are reported seropositive (Tomlinson et al., 2019).

1.6.4.3 Equine parvo-hepatitis virus

Parvoviruses belong to the *Copiparvovirus* genus and are single-stranded DNA viruses enclosed in a protein capsid. The genome is so small it does not encode proteins required for viral replication. It is typically thought parvoviruses require either helper viruses to provide replication machinery or actively dividing cells (Tomlinson et al., 2019b).

Discovered in 2018, EqPV-H is the most recently discovered hepatitis-associated virus (Divers et al., 2018). It has been found to be pathogenic and transmitted through biological products e.g., tetanus antitoxin. However,

the complete range of transmission modes are not yet understood. Transmission through biting flies may be possible as EqPV-H is also present in horses which have not received contaminated biological products (Tomlinson et al., 2019a). A preliminary study of 100 clinically normal adult horses found a DNA prevalence of 13% and antibody prevalence of 15% (Divers et al., 2018).

1.6.4.4 Equine herpesvirus

Currently nine equid herpes viruses (EHV) have been identified belonging to the order *Herpesvirales*, and family *Herpesviridae*, five of which can produce disease in horses (EHV-1, 2, 3, 4 and 5). Two produce disease in donkeys (EHV-6 and 8) and are also known as asinine herpesvirus (AHV, AHV-1 to 3), and EHV-9, also known as gazelle herpes, virus infects Thompson's gazelles (Oladunni et al., 2019). Equine herpes virus 1 and EHV-4 are the most relevant as they are ubiquitous to the global equine population. Equine herpes virus 4, is often referred to as respiratory herpesvirus and is restricted to the respiratory tract epithelium and associated lymph nodes (Ma et al., 2013). Equine herpes virus 1 is often referred to as neurological herpes virus, is the causative virus for 'equine virus abortion' (Jönsson et al., 1989) and has a predilection for vascular endothelium. Lesions are often found in the spleen, lungs, and liver as well as significant liver necrosis (Jönsson et al., 1989; Walter et al., 2013). A study on foals with neonatal equine herpesvirus-1 infection found that despite profound necrotic lesions of the liver, the liver enzymes were not elevated, and were not significantly different from the control group (Perkins et al., 1999).

1.7 Diagnosis of equine liver disease

It is important to establish the difference between liver disease and liver failure. The liver requires a loss of at least 60–80% of organic mass before showing signs of liver failure (Satué et al., 2013). Equine liver disease (ELD) is typically subclinical, any clinical signs are generally subtle and non-specific, therefore ELD is usually detected in the latter stages or once the liver is already failing. Multiple methods of detection for ELD are available, typically more than one are used in conjunction to confirm the diagnosis. The liver has a limited number of ways to respond to various forms of damage, therefore, the clinical and laboratory changes tend to be similar regardless of the underlying cause (Mair and Love, 2012)

1.7.1 Clinical signs

Reported clinical signs of animals with confirmed liver disease can include most commonly weight loss (present in 50% of cases with clinical signs of liver disease), depression/lethargy (68%), anorexia (56%), colic (50%), icterus (42%), photosensitisation (16%), coagulopathy (10%). Less commonly diarrhoea, pruritus, fever, coronitis and encephalopathy may be observed. Some horses also present with behavioural abnormalities or neurological manifestations, most commonly depression (present in 68% of neurological manifestations) as well as yawning, blindness, disorientation, ataxia, compulsive wandering, head pressing, circling, gastric impaction, and bilateral laryngeal paralysis (Satué et al., 2013). Neurological issues

have been linked to increased ammonia, hypoglycaemia, and haemoglobinuria (Mair and Love, 2012). Less commonly observed signs are bleeding abnormalities, oedema, and ascites (Bergero and Nery, 2008). Some causes of liver failure are accompanied by specific clinical signs, for example, cholangiohepatitis induces fever and hepatic lipidosis causes a ventral oedema (Satué et al., 2013).

As a large portion of liver function must be lost before functional liver failure occurs, and therefore development of clinical signs. Presence of clinical signs of hepatic dysfunction suggests a significant degree of liver pathology (DeNotta and Divers, 2020).

1.7.2 Biochemical testing

Biochemical testing is imperative when attempting to diagnose liver disease or liver failure. Liver enzyme levels can be tested via blood sample analysis; this is the most common form of testing and diagnosis. Levels are checked against an expected normal range (West, 1996). Values above the expected range indicate liver damage. Most common liver enzymes/ liver function indicators screened are gamma-glutamyl-transferase (GGT), sorbitol dehydrogenase (SDH), lactate dehydrogenase (LDH), aspartate transferase (AST), glutamate dehydrogenase (GLDH), ornithine carbamoyl transferase (OCT), alkaline phosphatase (AP), bilirubin, bile acids (Bergero and Nery, 2008; Mair and Love, 2012).

Biochemical results can be helpful in narrowing the differential diagnosis for liver analyte changes and give a prognosis. For example, GGT activity can be used to identify subclinical hepatotoxin exposure, such as during

outbreaks of pyrrolizidine alkaloid toxicity. Increased GGT activity is highly specific for liver disease, and it is rare that a horse with moderate to severe liver disease does not have increased GGT activity (DeNotta and Divers, 2020).

It is important to remember liver enzyme activities are not indicators of hepatic function. Biochemical results are best interpreted in combination with other clinical examinations (DeNotta and Divers, 2020).

1.7.3 Diagnostic ultrasound

Transabdominal ultrasonography is generally performed with either a 2.5 MHz or 5 MHz transducer (7.5 MHz or 10 MHz in neonatal foals). The liver is imaged from the right immediately caudal and ventral to the lung. Imaging can be variable due to factors such as normal age changes (right lobe atrophy in old horses), degree of gas distension of the colon, extent of lung field, amount of subcutaneous fat (Mair and Love, 2012)

Visibility of the left liver lobe is problematic and it may not be seen in some horses (le Jeune and Whitcomb, 2014). Discrete lesions such as abscesses/masses, choleliths (gall stones), biliary sludge and dilated bile ducts can be identified as well as chronic fibrosis and enlargement of the liver.

1.7.4 Liver biopsy

A biopsy can be taken to directly assess the health of the liver tissue, the horse is lightly sedated and given local anaesthetic. A small incision is made in the right flank in the 13th intercostal space (West, 1996) and a biopsy needle is used to obtain a small sample of liver tissue. In several studies, a biopsy is considered the 'gold standard' technique for hepatic disease diagnosis (Spycher et al., 2001; Durham et al., 2003b). Biopsies are best performed after an ultrasound has been performed to visualise the liver and identify the extent of fibrosis and inflammation.

The tissue sample extracted is then analysed histologically. Histology can then be used to indicate potential causes of liver disease. For example, there are specific lobular patterns of response to blood borne toxic injuries to the liver. Recognition of these exact patterns may be used as indicators to the nature of toxin involved (Buergelt and Piero, 2013). Although not commercially available, biopsy sections can undergo a variety of further tests including immunohistochemistry and immunohistochemical staining methods which adequately identify and localise viruses (Nayak and Sathar, 1999; Roskams, 2002).

1.7.5 Radiopharmaceutical techniques

Techniques using radiopharmaceuticals are commonly used in humans and occasionally in dogs to examine hepatic function and have been found to be a reliable method (Morandi, 2014). Normal hepatocytes are able to extract radiopharmaceuticals from the blood rapidly, however when hepatic function is compromised, the extraction rate is decreased (Morandi et al.,

2005). However, acquiring the radiopharmaceuticals and adequate imagery machinery is not practical or applicable to many smaller vet practices. For equids radiopharmaceuticals are primarily used for research applications.

1.7.6 PCR

Polymerase chain reaction (PCR) is used to definitively identify if one of the 4 viruses that have been linked to equine liver disease are present. Specific primers for each virus are used. It is possible to do this as a straightforward single round PCR (after conversion of RNA to cDNA for EqHV and EpgV) or as a nested PCR where multiple primers are used to show the presence of viral nucleic acid in one PCR product (Bexfield et al., 2014; Lu et al., 2017).

1.8 Epidemiology

1.8.1 Prevalence

EqHV RNA has been detected in 5 of the 7 continents with RNA prevalence ranging from 2–35% and antibody prevalence ranging from 22.6–83.7% (Badenhorst et al., 2018). The UK equine populations average around 2% viraemia (Lyons et al., 2012). Global prevalence (accurate according to data available as of 2022) can be seen in Table 2 and Figure 4. A small epidemiological survey has also been done on donkeys and mules in some countries (Walter et al., 2016b) (see Table 1).

It's important to note that the data presented here do not represent comprehensive population surveys but rather constitute a subset of the equine population within each county. Most studies did not collect samples at specific or consistent timepoints and often used samples obtained from veterinary practices as such, they may exhibit inherent biases.

Table 1 Antibody prevalence of EqHV in donkeys from different countries. n=sample population, Ab+ = %of the population positive for EqHV antibodies. Adapted from (Walter et al., 2016b)

Country	***Sampling period (yr)***	***n***	***Ab+ (%)***
France	1974/1979	2	1 (50.0)
Germany	2007/2008/2015	56	6 (10.7)
Spain	2011	86	7 (8.1)
Italy	2004-2015	350	140 (40.0)
Bulgaria	2015	201	114 (56.7)
Israel	2014	44	0 (0)
Kenya	2015	34	0 (0)
Mexico	2016	94	10 (10.6)
Costa Rica	2016	15	0 (0)

Table 2: Seroprevalence prevalence of EqHV in different countries, showing sample size and paper published. (nd = no data) Tb = Thoroughbred

Country	***Viral RNA Prevalence (%)***	***Sample size***	***Gene amplified***	***Antibody prevalence (%)***	***Reference***
Australia	11.2	188	NS5B	nd	(Fortier et al., 2021)
Austria	4.15	386	5'UTR & NS3	45.9	(Badenhorst et al., 2019a)
Brazil	13	202	NS5B	nd	(Figueiredo et al., 2015b)
Brazil	8.30	300	NS3	nd	(Gemaque *et al.*, 2014a)
Brazil	13.40	231	NS5B	nd	(Figueiredo *et al.*, 2018)
China	3.60	163	NS3, NS5B 5'UTR	nd	(Lu et al., 2016)
France	6.20	512	NS3	nd	(Pronost et al., 2017)
France (Tb)	8.30	521	NS3	nd	(Pronost et al., 2017)
Germany	2.40	1158	5'UTR	nd	(Schlottau et al., 2019)
Germany	2.50	433	5'UTR	31.4	(Pfaender, *et al.*, 2015)
Germany (Tb)	18.30	733	NS3	61.8	(Reichert et al., 2017)
Italy	4.70	1932	5'UTR	nd	(Elia et al., 2017)
Japan	35.60	31	5'UTR	22.6	(Tanaka *et al.*, 2014)
Japan	nd	335	nd	23.4	(Hayashi *et al.*, 2018)
Japan	13.68	453	5′UTR	33.55	(Matsuu *et al.*, 2015)
Mongolia	40	299	5′ UTR	nd	(Tomoko *et al.*, 2020)
Morocco	10.5	172	5'UTR & NS3	65.7	(Abbadi *et al., 2021)*
South Africa (Tb foals)	7.93	454	5' UTR	83.7	(Badenhorst *et al.*, 2018)
South Korea	18.90	74	NS3	nd	(Kim *et al.*, 2017)
UK	2	142	5'UTR & NS3	nd	(Lyons *et al.*, 2012)
UK	<1%	328	5'UTR & NS3	43	(Lyons et al., 2014)
USA (New York)	7.78	103	NS3	34.95	(Burbelo *et al.*, 2012)

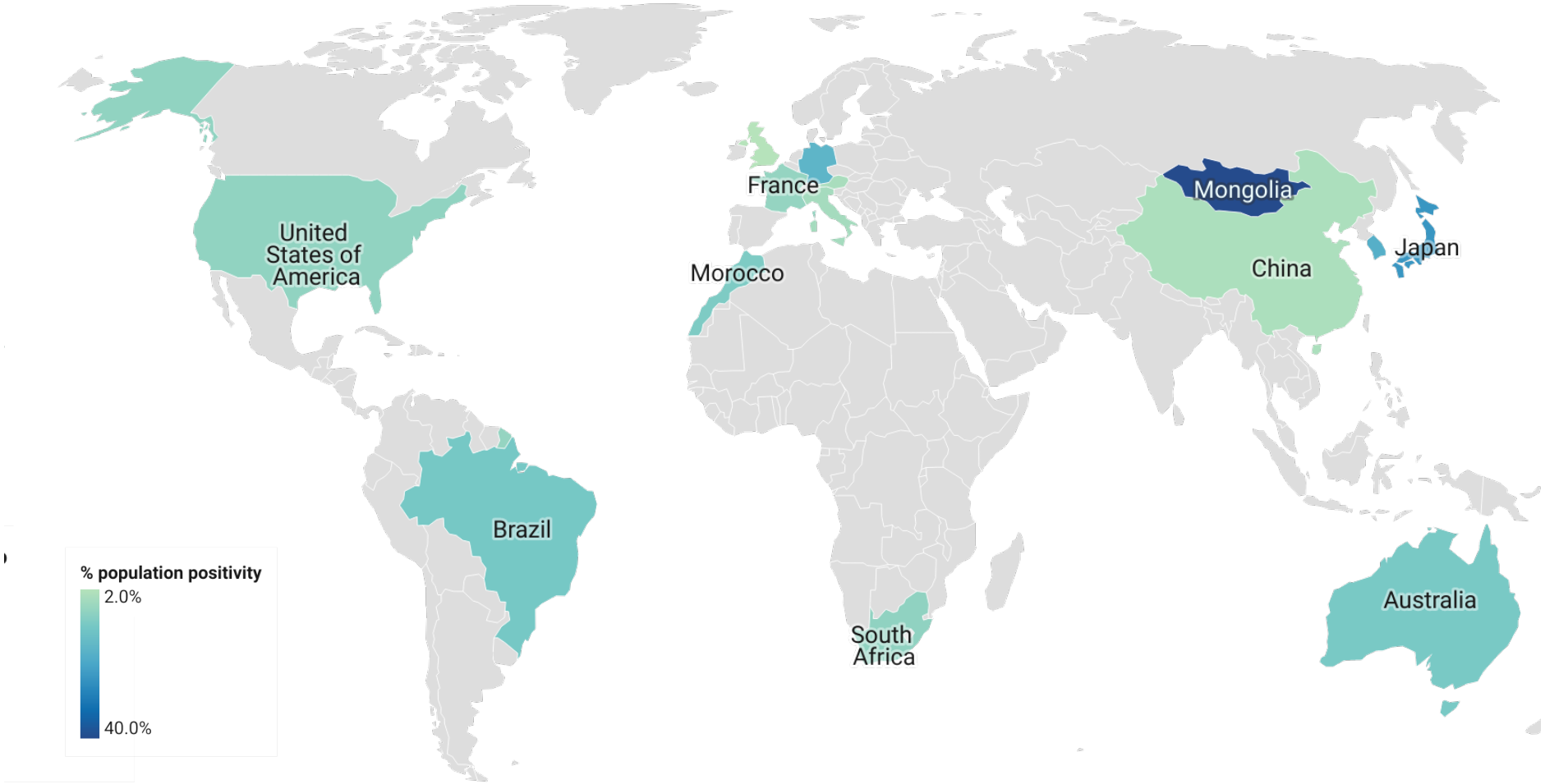


Figure 4 Epidemiological map displaying average viral RNA prevalence of EqHV according to studies (available in 2022) per country. Colour scale represents percent of population found to be positive for EqHV RNA through PCR testing.

1.9 Risk factors

A limited number of risk factors for EqHV infection of horses have been assessed. The Thoroughbred breed appears to have a higher prevalence of viral RNA and seropositive status compared with non-Thoroughbred populations (Pronost et al., 2017a) as seen in Table 2. Some studies examined both viral, and antibody seroprevalence in Thoroughbred populations, all of which had significantly higher levels than general equine populations. In Germany, the average horse population seropositivity was 2.5% whereas in the Thoroughbred it was 18.3%. The cause of this is yet to be identified as to if genetics or difference in management may increase prevalence (Pfaender et al., 2015a). Racehorses are transported more frequently than privately owned horses and therefore may have more opportunity to be exposed to other EqHV infected horses. A study from Brazil also supports this hypothesis as there was significantly higher EqHV seroprevalence in competition horses (19.1%) compared with farm and cart horses (6.2%) (Postel et al., 2016).

Some studied found prevalence of EqHV is more likely in younger horses. Reichert et al., (2017) found 3–6-year-old horses twice as likely to be infected compared to 16–19-year-old horses.

Antibody and viral RNA levels were examined in 20 pregnant mares and their foals until 6 months post-partum (Gather et al., 2016b). In total, 80% of the 20 mares were EqHV seropositive, and 20% were viraemic at the time of sampling. A follow-up study including 7 of these foals showed new infections within 6 months post-partum (Gather et al., 2016a). All foals originated from mares seropositive for EqHV NS3 antibodies at the time of

foaling, by six months of age, all foals displayed a decrease in antibody titres. Although this study has some interesting findings the sample size is very small and further studies with larger samples sizes are needed.

Some studies have highlighted sex as a risk factor, however these have been small studies with conflicting findings. One study found 30% of stallions sampled were viraemic for EqHV compared to 18% of mares sampled (Reichert et al., 2017). Conversely, a separate study conducted in Morocco demonstrated a higher likelihood of infection among mares compared to stallions, with infection rates of 77.14% versus 46.27%, respectively, $p < 0.0001$ (Abbadi et al., 2021).

The ongoing challenge of limited sample sizes in published studies significantly impedes the ability to draw firm conclusions, emphasising the critical necessity for larger-scale investigations. Achieving a more comprehensive understanding necessitates robust and expansive studies. Hence, extensive research encompassing diverse equine populations is imperative to elucidate the intricate role of risk factors in EqHV infection.

1.10 Transmission of EqHV

While research on EqHV transmission routes exists, the understanding remains notably limited. Reported instances suggest the potential involvement of both horizontal and vertical transmission pathways. However, the specific mechanisms and predominant routes facilitating EqHV spread within the equine population remain poorly understood.

1.10.1 Horizontal transmission

Horizontal transmission is the spread of an infectious agent from one individual to another, which can be via direct or indirect contact with sweat, urine, blood, or salivary secretions from an infected horse. Several studies have shown it is possible to experimentally infect healthy horses with EqHV via injection of plasma from horses infected with EqHV (Ramsay et al., 2015; Pfaender et al., 2017).

Another possible transmission route may be via biological products with equine origin, for example intravenous administration of donor horse immunoglobulin to protect new-born foals with low colostrum immunoglobulin or tetanus antitoxin. This would be similar to equine parvovirus; a study in the USA showed an equine parvovirus to be transmitted via equine-origin tetanus antitoxin (Divers et al., 2018a).

Furthermore, the potential for EqHV transmission via vectors, such as biting flies, has been considered, drawing parallels with the transmission mechanisms observed in equine infectious anaemia (Issel and Foil, 2015). Biting flies, including Tabanids (commonly known as horse flies) and

Stomoxys calcitrans (referred to as stable flies), possess large mouthparts capable of retaining remnants of infected blood from previous feedings. This characteristic allows them to potentially mechanically transmit the virus to subsequent hosts (Foil et al., 1983). For a detailed discussion on this aspect, refer to section 3.1.

1.10.2 Vertical transmission

Vertical transmission has been documented in two studies, one of which investigated four viraemic mares during pregnancy (Gather et al., 2016b). Among these mares, one transmitted EqHV to her foal, confirmed by detecting EqHV RNA in the umbilical cord blood. Moreover, the genetic sequences of the virus in both the mare and foal were identical, further supporting the transmission link. However, among the remaining three viraemic mares, no viral RNA was detected in their foals' umbilical cord blood.

The second study examined 394 tissue samples from aborted foetuses/neonatal foal deaths in France, collected between January 2013 and December 2016. Of the 394 samples 3 (0.76%) were found positive for EqHV by RT- quantitative PCR. Two of these positive cases had samples of the allantochorion, both of which also tested positive by RT- quantitative PCR (Pronost et al., 2019).

The observed instance of vertical EqHV transmission in the primary study stands at 1 in 4 cases (Gather et al., 2016b). However, this study's limited dataset comprising only four cases precludes an accurate determination of the vertical transmission rate. In contrast, the second study, involving a

more substantial sample size of 394, was retrospective, examining deceased foals and aborted foetuses intended for autopsy or routine analysis. However, crucial details, such as the number of samples concurrently obtained from the reciprocal mare or tested alongside sera, remain unclear. These omissions hamper the accurate calculation of a percentage rate of vertical transmission, limiting assessment solely to the number of infected foetuses.

Despite these limitations, an average seroprevalence rate of approximately 6.2% observed in a study involving 1033 horses in France (Pronost et al., 2017) juxtaposed with a 0.76% presence in the second study suggests a significantly lower rate of EqHV transmission through vertical means. This discrepancy implies that the majority of EqHV transmission likely occurs via alternative routes, underscoring the need for further investigation into the predominant pathways of EqHV dissemination."

HCV is vertically transmitted from mother to baby 5% of the time (Yeung et al., 2014). The placental structure differs substantially between mares and women (see Figure 5). The human placenta has a haemochorial structure whereby the trophoblast is in direct contact with maternal blood (Yeung et al., 2014). The equine placenta has an epitheliochorial structure, whereby the maternal endometrial epithelium, connective tissue and uterine endothelium are all retained (Robles et al., 2018). Consequently, bloodborne pathogens must pass through twice as many layers in equids compared with humans, potentially making the transfer of infectious virus less likely. This suggests the vertical transmission rate of EqHV could be less than that of HCV.

Figure 5 Differences in placental structures between epitheliochorial (equine) and haemochorial (human).

1.10.3 EqHV in commercial sera

Several studies have shown the presence of EqHV RNA in commercial equine sera available to laboratories (Gemaque et al., 2014). This contamination could have unintended consequences. For example, equine serum is regularly used for cell culture and may thus influence the results of research, including the results of deep sequencing studies to identify novel viruses. For example, horse-derived snake anti-venom sera is administered to humans with snake bites (Guidolin et al., 2016). Additionally, other biological products such as live vaccines may be contaminated with EqHV principally when cell lines used for propagation are grown in media supplemented with horse serum (Lu et al., 2017). Tetanus antitoxin is another possibility and has been shown to be a cause of transmission of EqPV-H (Divers et al., 2018).

1.11 EqHV pathology

HCV causes chronic hepatitis in humans, inflammation, and necrosis of liver cells, but it is still unclear exactly the effects EqHV has on the liver. The liver of a chronically infected horse with a serum titre of $5x10^7$ copies/ml was examined post-mortem. The hepatic lymph node was found to be mildly enlarged, and the liver displayed multiple areas of mild calcification and lipidosis (Pfaender et al., 2015a).

In a more recent study (Tegtmeyer et al., 2019), the liver of another chronically infected horse was examined. The horse presented with elevated gamma-glutamyl transferase, glutamate dehydrogenase, aspartate aminotransferase and bile acids. Additionally, in a transcutaneous abdominal ultrasonographic examination dilated bile ducts and a heteroechogenicity of the hepatic parenchyma was noticeable. The horse was euthanised due to steady deterioration after being chronically infected for over 12 months. The liver was examined post-mortem, highly altered liver tissue and micronodular liver cirrhosis was observed (Figure 6).

Reports of the effect of EqHV infection on liver enzymes are conflicting. One study on naturally infected foals showed mild elevations of liver enzymes in some cases, but no clinical signs of hepatitis (Gather et al., 2016a). A further study found no indication of hepatic impairment in 7/11 EqHV positive horses, however serum GGT concentrations were mildly elevated in 3 horses and highly elevated in 1 horse (Gather et al., 2016a). A study from Brazil found liver enzymes were slightly above their reference range (Figueiredo et al., 2015a) for 11/27 of their EqHV seropositive samples.

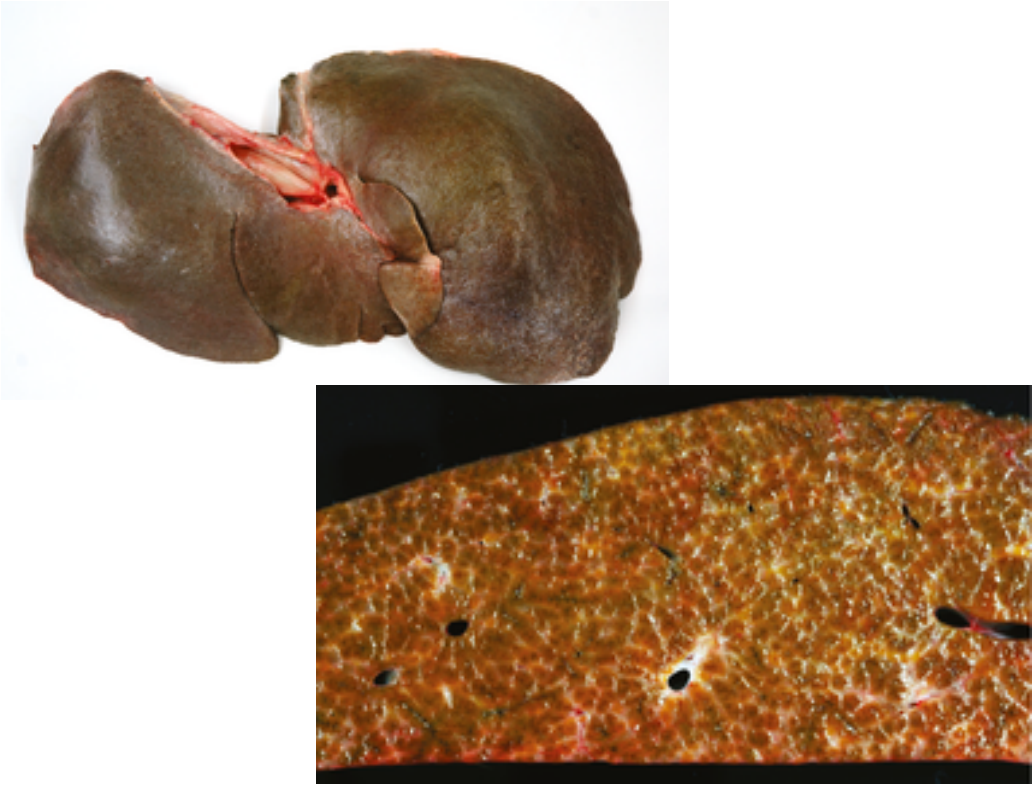


Figure 6 Post-mortem examination of liver chronically infected with EqHV, picture of whole liver and sliced side view. Adapted from (Tegtmeyer et al., 2019).

A case study in Hungary found a horse suffering from chronic wasting was also chronically infected with EqHV. The horse had elevated aspartate aminotransferase, alanine transaminase and lactate dehydrogenase (Elia et al., 2017b), although a liver biopsy was not performed to examine the extent of liver damage.

Table 3: Percent of EqHV infected horses with liver enzyme levels above normal ranges. AST - aspartate aminotransferase, ALT - Alanine transaminase, GLDH - Glutamate dehydrogenase, GGT- Gamma glutamyl transferase.

Horses with significant Liver Enzyme elevation (%)	***Sample size***	***Enzymes tested***	***Country of study***	***Reference***
41	11/27	AST, ALT	Brazil	Figueiredo et al., 2015
0	362, 275, 477	biliary acid, GLDH, GGT	France	Pronost et al., 2017
27	3/11	GGT	Germany	Pfaender et al., 2014
33.3	1/3	GGT	UK	Lyons et al., 2012
0	3	GLDH, Bile acid	UK	Lyons et al., 2012
45	31	AST	Brazil	Figueiredo et al., 2018
19	31	GGT	Brazil	Figueiredo et al., 2018

1.12 Diagnosis of EqHV

As a close homologue of HCV, it is not unexpected that EqHV behaves similarly to HCV in its pathology. Many patients infected with HCV are subclinical or clinical signs are vague and non-specific meaning cases go undiagnosed for years. Diagnosis of HCV usually only occurs either by accident on a routine medical examination or once severe damage has been done and signs of chronic liver disease sets in. This also appears to be the case for EqHV, as seen in a case study in Hungary with a horse suffering from chronic wasting (Elia et al., 2017b). The majority of horses testing seropositive for EqHV show no obvious clinical signs of infection, although as discussed in 1.7.1 clinical signs of equine liver disease, in general, are easily missed. EqHV antibodies are usually only found due to population sampling for research.

Limited knowledge of EqHV and its effects means that most equine veterinary practices are unequipped to test for its presence. Currently the only way to confirm an active infection is via PCR; there are several protocols for this, some more informative than others.

To confirm active infection with EqHV, viral RNA can be detected indirectly in plasma or sera using a standard reverse transcription (RT)-PCR with a single set of primers. Diagnosis of a chronic EqHV infection requires several sequential blood samples over several months. Quantitative PCR, typically a nested version (Pronost et al., 2017a) using multiple sets of specifically designed primers can also be used to not only detect but also quantify the amount of RNA in the blood. Antibodies specific for individual EqHV proteins can be detected using two assays; the

luciferase immunoprecipitation system (LIPS) assay (Burbelo et al., 2012) or *Gaussia* luciferase immunoprecipitation system (GLIPS) (Matsuu et al., 2015). GLIPS uses *Gaussia* luciferase, a more stable protein, increasing the accuracy of the assay. Both assays detect the presence of anti-NS3 antibodies in the blood which confirms the animal has been exposed to the virus.

Diagnosis of EqHV by liver biopsy and histopathology is also an option; this is especially useful for identifying which tissues are infected (Tegtmeyer et al., 2019). This procedure is however much more invasive, involves sedation and is more costly.

1.13 Summary

In summary, many aspects surrounding the emergent Equine Hepacivirus (EqHV) necessitates a more comprehensive exploration. Current diagnostic methodologies, notably the LIPS assay for antibody detection, demand refinement before their translation into mainstream veterinary laboratories. Critical gaps persist in understanding EqHV's transmission routes and the comprehensive pathologies associated with both chronic and acute infections.

This thesis draws from established research in published literature, aiming to delve into various facets of Equine Hepacivirus (EqHV) epidemiology, biomarkers, transmission dynamics, and diagnostic methodologies. The principal goal is to expand our understanding of EqHV prevalence, potential transmission routes, and the refinement of diagnostic tools.

Chapter 2: Evaluation of the clinical impact of EqHV

2.1 Introduction

The focal subjects of this investigation are 'welfare horses' from a rescue centre. Notably, a significant proportion of these equines have been rescued from neglectful environments, frequently arriving at the centre in a state of severe emaciation and compromised health. This context is crucial to consider when interpreting and contrasting the outcomes of this study with those obtained from populations like racing Thoroughbred horses.

Racehorses typically receive meticulous care to maintain peak health, aiming to optimise athletic performance. Consequently, the probability of co-morbidities relating to emaciation and malnutrition is notably lower in this population compared to welfare horses rescued from neglectful conditions. This inherent disparity in the health status between these distinct equine populations underscores the importance of contextual considerations when evaluating and comparing study results.

The rescue population studied in this project were horses under the care of Bransby Horse Sanctuary ('Bransby'). Most horses have been removed from their situation and placed in the care of Bransby due to extreme welfare concerns, a small minority have been given up willingly by the

owner. As Bransby is a charity, all veterinary fees must be funded by public donations; this may have an impact on diagnostic and treatment options due to amount of funds available and number of horses requiring veterinary treatment.

2.1.1 Laboratory diagnosis of liver disease from equine blood

As discussed in section 1.7, diagnosis of liver disease can be challenging as often clinical signs are subtle or nonspecific. Serum biochemistry and the use of liver biomarkers including enzyme levels and other biochemical measurements are generally the first diagnostic tool utilized when liver issues are suspected. A standard 'panel' of parameters is often assessed when equids present as unwell but with no immediately obvious diagnosis. This includes liver biomarkers, which can be used as an indication of liver disease, some markers indicate liver function and others are markers of liver injury (Mcgill et al., 2016).

2.1.1.1 Liver function tests

Test results that provide an indication of liver function become abnormal only after 70% or more of hepatic function is lost. Liver function tests include increased direct (conjugated) and indirect (unconjugated) bilirubin concentrations, ammonia and bile acid concentrations, as well as coagulation tests such as prothrombin and activated partial thromboplastin (DeNotta and Divers, 2020).

2.1.1.1.1 Bilirubin

Bilirubin is a molecule formed from the breakdown of haem, largely from haemoglobin in aged red blood cells and prematurely destroyed erythroid cells in the bone marrow. A small amount is produced during the breakdown of other haem-containing proteins, primarily from the liver and muscles (Kalakonda et al., 2021). Bilirubin is toxic to the body so must be removed and is done so by the liver.

Once bilirubin is released into the plasma it is taken up by albumin, which serves as a transporter to the liver as well as protects the tissues from exposure to toxic bilirubin. This is known as indirect bilirubin or unconjugated bilirubin. The binding affinity for albumin to bilirubin is extremely high and normally there should be no non-albumin bound bilirubin in the plasma (albumin bound bilirubin cannot be filtered by the kidneys). Once at the liver, the process of disassociation of bilirubin from albumin is inefficient (only approximately 20% of bilirubin enters the liver on the first passage through the liver) consequently a measure of unconjugated or indirect bilirubin can be taken from the venous circulation (Kalakonda et al., 2021).

To be excreted, bilirubin must conjugate to glucuronic acid within the hepatocyte to render bilirubin soluble and facilitate excretion into bile. This is known as conjugated bilirubin or direct bilirubin.

Increased bilirubin concentrations are associated with haemolysis, hepatocellular disease and cholestasis. Failure of the liver to take up, conjugate and/or excrete bilirubin may lead to increases in serum unconjugated and/or conjugated bilirubin (Ambrojo et al., 2013).

Assessment of both direct and indirect bilirubin concentrations are useful for diagnosing liver dysfunction. Unconjugated bilirubin is measured by subtracting the direct bilirubin reading from the total bilirubin.

2.1.1.1.2 Serum bile acids

Bile acids are hydroxylated steroids and amphophilic molecules (molecules possessing both hydrophilic and lipophilic properties) synthesised in the liver from cholesterol. They are essential components to the process of solubilization and digestion of dietary lipids (Engelking, 2015). Bile acid levels are generally evaluated from serum. The main limitations of serum bile acid estimation are that liver disease must be quite severe before increased concentrations are detected. Most liver disease cases will be found to have normal serum bile acid concentrations at the time of initial presentation (Dunkel et al., 2015).

Although not routinely examined, a recent study indicated that peritoneal bile acids may be relevant to diagnosis of hepatic disease (Rodríguez-Pozo et al., 2021). Concentrations of total plasma bile acid between 5 and 28 μmol/L have been reported in normal adult horses (Kaneko et al., 1992). Bile acid concentrations above this in the adult horse are a good predictor of liver failure.

2.1.1.2 Liver injury tests

Tests that provide an indication of liver injury include the levels of certain enzymes in the blood. Diagnosis of liver disease from abnormalities in serum enzymes are based on serum enzymes that are

partly or exclusively associated with cells within the liver parenchyma-hepatocytes and or epithelial cells that line the biliary tree and some analytes that assess liver functionality (Satué et al., 2013).

Enzymes occur normally in the body and are distributed differently among organs. Enzymes in the cell cytoplasm include aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and alanine aminotransferase (ALT). Mitochondria-contained enzymes are glutamate dehydrogenase (GLDH) and aspartate aminotransferase (AST). Enzymes found in the cell membrane are alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT) (Hoffmann and Solter, 2008). In general, increased enzyme concentrations in the bloodstream indicate recent organ damage rather than decreased function.

The baseline activity of enzymes in plasma usually reflects the amount or turnover of the tissue that contains this enzyme. However, in cases of chronic liver disease with severe fibrosis and therefore a reduction in the number of functional hepatocytes, plasma levels of liver enzymes may remain within normal limits.

Cytoplasmic enzymes are released in early cell degeneration, while mitochondrial enzymes will be released after advanced cell necrosis (Satué et al., 2022). Enzymes that have a longer half-life and are more stable provide more reliable results when detecting liver disease. Understanding the origin and role of the enzyme elevated helps to determine the type of damage the body has sustained.

Increase of liver enzymes in equids in clinical practice is typically assessed in increments above the upper reference range. Usually, a two-

or three-fold increase is considered mild, four- to five-fold is considered moderate and 10-fold and above is severe (Satué et al., 2022).

2.1.1.2.1 Aspartate aminotransferase (AST)

Aspartate aminotransferase (AST) (or glutamic oxaloacetic transaminase; GOT) catalyses the transamination (removal and transfer of amino acids) of L-aspartate and 2-oxoglutarate to oxaloacetate and glutamate. AST is found mainly in the cytoplasm, but also in mitochondria in hepatocytes, although AST is not liver specific and is also found in the kidneys, cardiac and skeletal muscle as well as erythrocytes (Satué et al., 2013).

Serum levels of AST typically increase as a result of liver disease, reflecting hepatocellular injury. AST activity is increased in reversible and irreversible hepatocyte injury following hepatocellular injury and cholestasis. Hepatic necrosis due to viral hepatitis, acute biliary obstruction, cholangiohepatitis (inflammation of the bile ducts, gall bladder, and surrounding liver tissue), among others, can cause significant increases in this enzyme. The sensitivity of serum AST activity in horses is 72% for hepatic necrosis and 100% for hepatic lipidosis (Satué et al., 2022).

However, as AST is not liver specific, elevated serum levels should only be interpreted as liver disease in conjunction with more specific liver enzymes. Further testing can be performed with more organ specific enzymes. For example, increased AST and creatine kinase (CK) gives a clear indication of muscle damage rather than liver disease (Satué et al., 2013). It is important to note that this enzyme can be increased by

haemolysis, due to its presence in erythrocytes, and by lipaemia (Satué et al., 2022).

2.1.1.2.2 Alanine transaminase (ALT)

Alanine transaminase (ALT) also known as alanine aminotransferase, (formerly called serum glutamate-pyruvate transaminase or serum glutamic-pyruvic transaminase (SGPT)) is a liver transaminase. ALT is predominantly found in the cytoplasm of hepatocytes. It can be detected at low concentrations. Any process that leads to loss of hepatocyte membrane integrity or necrosis results in the release of ALT in high concentrations in the serum (Moriles and Azer, 2022).

2.1.1.2.3 Alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) is found in the membrane of cells, ALP catalyses the hydrolysis of organic phosphate esters present in the extracellular space. ALP is present in many different tissues but catalyses the same reaction. In the liver, alkaline phosphatase is cytosolic and present in the canalicular membrane of the hepatocyte (the apical membranes of hepatocytes, which form the bile canaliculi that collect bile secreted by hepatocytes). The majority of alkaline phosphatase in serum (more than 80%) is released from liver and bone, and in small amounts from the intestine (Lowe et al., 2021).

2.1.1.2.4 Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) is an enzyme utilised in the anaerobic metabolic pathway. LDH catalyses the reversible conversion of lactate to pyruvate with the reduction of NAD+ to NADH and vice versa (Farhana

and Lappin, 2022). LDH is a cytoplasmic enzyme and is present in almost all tissues, but at high concentrations in muscle, liver, and kidney. LDH exhibits five isomeric forms, assembled in tetramers of either of the two types of subunits, namely muscle and heart. LDH isoenzyme 5 (LDH-5) is of importance to liver disease diagnostics, as LDH-5 has significant expression in the liver, although is also present in kidneys, muscle, myocardium, and red blood cells (Satué et al., 2022). Elivated LDH may support the diagnosis of hepatopathy, hepatocellular disease but is not liver specific.

2.1.1.2.5 Sorbital dehydrogenase (SDH)

Sorbital dehydrogenase (SDH) (also known as iditol dehydrogenase or IDH), catalyses the reaction of sorbitol + NAD to fructose + NADH. SDH is a liver-specific enzyme in horses. It is considered a good indicator of active hepatocellular disease. However, SDH has poor stability compared to other enzymes, remaining stable for only 5 hours at room temperature or 4°C and 48 hrs at -20°C (Fouché et al., 2021, Satué et al., 2013). This poor stability makes SDH difficult to measure in clinical settings.

2.1.1.2.6 Glutamate dehydrogenase (GLDH)

Glutamate dehydrogenase (GLDH) is an enzyme found in the mitochondria of hepatocytes and is concentrated to the central areas of the lobule. GLDH catalyses the removal of hydrogen from L-glutamate to form the corresponding ketimine acid that then undergoes spontaneous hydrolysis to 2-osoglutarate (Hoffmann and Solter, 2008). The liver has the highest concentration of GLDH activity. Some GLDH

is found in the kidney and small intestine however the GLDH levels in non-hepatic tissues are much less than in the liver (Ambrojo et al., 2013). Therefore, in horses elevated GLDH is considered highly specific for liver disease.

GLDH is more stable than other enzymes and has a half-life of 12-14 hours, making it useful for detecting acute hepatocellular disease (DeNotta and Divers, 2020). However, GLDH levels may be high in young foals without the presence of liver disease. The sensitivity of GLDH activity for detection of hepatic necrosis, hepatic lipidosis, and hepatic cirrhosis in horses was 78%, 86%, and 44%, respectively (Ambrojo et al., 2013), although horses with severe chronic fibrosis (cirrhosis) may occasionally have GLDH activities within normal reference ranges (Satué et al., 2022).

2.1.1.2.7 Gamma glutamyl transferase (GGT)

Gamma glutamyl transferase (GGT), a membrane bound enzyme, resides on the extracellular membranee. Although GGT is located on the luminal surface of proximal tubular cells of the kidneys and the luminal surface of cells lining the acini and pancreatic ducts, increased serum GGT activity is not generally associated with primary injury to the kidney or pancreas (Hoffmann and Solter, 2008).

In the liver GGT is specifically found in the canalicular surfaces of the hepatocytes (the apical membranes of hepatocytes, which form the bile canaliculi that collect bile secreted by hepatocytes) (ONG and ESPAT, 2007) and in the biliary epithelial cells, therefore it indicates biliary disease rather than hepatocellular injury. The location of GGT in the liver

is noteworthy as the liver likely contributes to most, if not all the serum GGT activity (Satué et al., 2013).

GGT is regarded as an excellent screening test for equine hepatic disease because it indicates biliary disease rather than hepatocellular injury. It is rare that a horse with moderate to severe liver disease does not have increased GGT activity.

2.1.2 Interpreting liver diagnostic data

Research into clinical chemistry however has shown liver marker data is not always black and white, and neither is the interpretation of it. For example:

In a two-year, on-farm investigation of forage-associated liver injury, weanling foals, yearlings, and mature horses all showed an increase of up to 1000 IU/L in GGT concentrations and up to 1200 IU/L in GLDH. However, no animal showed signs of liver failure, and total bilirubin and bile acid concentrations remained within the range of reference values in almost all horses. These studies demonstrate that disease prognosis is not determined by the size of changes in serum hepatocellular enzyme levels does not determine the prognosis of the disease. Therefore, in horses, liver disease is best determined by integrating abnormalities such as function tests, aetiology, degree of fibrosis on liver biopsy, or brain dysfunction caused by the inability of the liver to eliminate toxins (Durham et al., 2003a)

A different study examined the clinical chemistry of 82 horses with suspected liver disease and confirmed with biopsies. The single positive test results of greatest diagnostic value were presence of hepatic encephalopathy (HE), increased GGT, hyperglobulinaemia, hypoalbuminaemia, increased AP, increased total bile acids and increased total bilirubin. Increased AST and increased GLDH were also of good diagnostic value, but only when used in combination with the above tests. However, no single, combination or sequential test was able to discriminate fully between horses with and without biopsy-confirmed liver disease and reliance on the use of non-invasive tests for the prediction of the presence

or absence of significant liver disease may lead to frequent diagnostic errors. Although certain positive results did reliably predict the presence of liver disease, negative test results were invariably poor predictors of the absence of liver disease (Durham et al., 2003a).

Results of clinical chemistry tests vary largely when the causation of liver disease differs. Some liver enzymes are increased depending on the cause or mechanism of liver disease and damage. Therefore, it is important that multiple factors and test results be examined before coming to a clear diagnosis.

2.1.3 Other biochemical parameters

Other parameters examined from blood sample testing, such as fibrinogen can reveal the presence of inflammatory markers.

2.1.3.1 Fibrinogen

Fibrinogen is a soluble glycoprotein complex synthesized in the liver and forms the major structural component of a clot (Gonzalez et al., 2022). Horses with liver failure can have significant decreases in fibrinogen concentrations, However, fibrinogen is an acute-phase protein, and it is a widely used marker for diagnosis of acute inflammatory processes. Therefore, it is important to consider if there is concomitant liver failure when interpreting fibrinogen concentrations in blood (Ambrojo et al., 2013).

2.1.3.2 Ferritin

Ferritin is a major iron storage protein, storing intracellular iron. Ferritin is present in most tissues as a cytosolic protein and is essential for iron homeostasis. Ferritin is often used as a serum marker of total body iron stores. The liver controls iron haemostasis by producing proteins that maintain systemic iron balance, acting as a storage site for excess iron and playing a critical role for the mobilisation of iron from hepatocytes to the circulation to meet metabolic requirements (Anderson and Shah, 2013). Liver damage can lead to dysregulation of the liver's ability to maintain balance of these three parameters leads to iron-related disorders affecting levels of ferritin in the blood.

2.1.3.3 Urea

Urea is synthesised from ammonia in the liver, filtered by the glomerulus and then reabsorbed passively in part by the renal tubules. Low serum urea concentrations have been recognized in association with liver failure and have been suggested to indicate reduced hepatic synthesis of urea from ammonia. Although the majority of equine hepatopathy cases have normal serum urea concentrations, decreased serum urea is associated with more severe hepatopathies and has prognostic relevance (Satué et al., 2013).

2.1.3.4 Serum Albumin and Globulin

Albumin, a protein synthesised by the liver, transports molecules through the blood and constitutes over half of the protein found in serum(Busher, 1990). Albumin has as long serum half-life, therefore marked hypoalbuminemia is uncommon in equine liver disease. Low albumin concentrations occur in 6% of acute cases and 18% of chronic cases (DeNotta and Divers, 2020). Serum albumin concentrations below 20 g/L are very rarely encountered even in severe hepatopathies (Dunkel et al., 2015).

Globulins are increased in 48% of horses with liver failure. The albumin-to-globulin ratio is more likely to be low in horses with chronic versus acute liver disease and failure (Satué et al., 2013).

2.1.4 Haematology

Routine haematology is generally screened at the same time as liver enzymes. This can be useful for identifying key factors such as anaemia. A set of standard parameters are screened for in a typical haematology panel (Table 4). Results of haematology are not liver specific and must be interpreted with other test results.

Table 4 Standard parameters screened for in a typical equine haematology panel and their abbreviations.

Abbreviation	Clinical parameter	Test
RBC	Red blood cell	Number of red blood cells
PCV	Packed cell volume	% of RBC in blood
Hb	Haemoglobin	Concentration of haemoglobin in the blood
MCV	Mean corpuscular volume	Average size of red blood cells
MCHC	Mean corpuscular haemoglobin concentration	Average amount of haemoglobin per unit volume of red blood cells
MCH	Mean corpuscular haemoglobin	Average quantity of haemoglobin present in a single red blood cell
Plts	Platelets	Count of platelets per µl of blood
WBC	White blood cells	Count of white blood cells per µl of blood
Neutrophils	Neutrophils	Count of neutrophils in the blood
Lymphocytes	Lymphocytes	Count of lymphocytes in the blood
Monocytes	Monocytes	Count of monocytes in the blood
Eosinophils	Eosinophils	Count of eosinophils in the blood

2.2 Chapter Aims

In this chapter, the primary objective was to evaluate the prevalence of Equine Hepacivirus (EqHV) within a cohort of horses residing in a rescue sanctuary. EqHV prevalence was determined by screening for the presence of viral RNA. Subsequently, a comparative analysis of clinical chemistry profiles between EqHV-positive and EqHV-negative individuals aimed to discern potential biomarkers indicative of the infection's influence on health.

Additionally, a secondary dataset obtained from a population of Thoroughbred horses was incorporated into the study. This secondary group, characterised by a more uniform and well-maintained physical condition, served as a comparative cohort. The rationale behind this comparison was to potentially highlight more nuanced variations in biomarkers or health parameters, given the heightened homogeneity and optimum health status of the Thoroughbred population.

2.3 Methods

2.3.1 Sample Populations

Samples from horses residing at Bransby Horses Rescue were obtained periodically via Rainbow Veterinary hospital. These samples were surplus after routine resting during intake or adoption checks, or diagnostic testing if symptoms of ill health were detected by staff members. Thoroughbred horse samples were obtained periodically via Newmarket Equine Hospital as surplus post routine testing. All equine blood samples were collected by venepuncture of the jugular vein by the attending veterinary surgeon. Samples consisted of either serum derived from whole blood or EDTA anti-coagulated plasma. Whole blood samples were couriered fresh on ice, on arrival centrifuged for 5 mins at 1510 RCF at room temperature to separate the plasma. Serum or plasma samples were stored at -20°C prior to shipping and shipped on ice, on arrival samples were aliquoted into 1.5 ml Eppendorf tubes and stored at -80°C.

2.3.2 Viral detection

2.3.2.1 RNA extraction

Viral RNA was extracted from 140 µl of equine plasma or sera (stored at -80°C) using a Qiagen RNA kit (Qiagen Ltd, Crawley, UK) according to the manufacturer's instructions. RNA extracts were immediately converted into cDNA or stored at -80°C.

2.3.2.2 Reverse transcription/cDNA synthesis

Conversion of viral RNA to cDNA was done using TakaRa cDNA EcoDry Premix. The protocol according to manufacturer's instructions were followed with the exception that RNA was not diluted prior to conversion to cDNA due to the low concentration of viral RNA present in the extractions.

2.3.2.3 Primer Design

Primers were designed and optimised by Dr Alexander Tarr (School of Life Sciences, University of Nottingham). The predicted amplicon size generated from analysing several full genomes available on NCBI was approximately 359 base pairs (bp).

Table 5 Equine hepacivirus PCR primers used in this project. Primers were designed by Dr Alexander Tarr. [1]Nucleotide numbering relative to reference sequences: EqHV, NC038425.

PRIMER NAME	PRIMER SEQUENCE (5′- 3′)	BINDING REGION
EQHEP_C-E1F	CTTGTrCGrTTTGTkGAGGA	886–905
EQHEP_C-E1R	GCYTCRCCAATWCCNAGAGTAGA	1244–1222

2.3.2.4 EqHV qPCR method refinement

Primers were designed by Dr Alex Tarr for use in PCR. Despite various numbers of attempts to establish a real time PCR method using the E1/E2, including changing all reagent types, reagent concentrations, cycling conditions and ramp times a workable method could not be established.

The initial protocol provided for qPCR using the E1/E2 primers, produced poor results giving extreme levels of primer dimer. A revised protocol was developed giving much lower levels of primer dimer (although some level was still present) allowing for easier identification of positive tests results. Due to the presence of primer dimer, positive results were determined with a combination of examining melt curves and identifying correct weight DNA bands using agarose gel electrophoresis against a DNA ladder and a known positive sample as well as sanger sequencing. A 2% agarose gel was used to help separate the PCR product from the primer dimer. A lower primer concentration of 0.2 µl was attempted to try and minimise primer dimer formation further but was found to be too low and did not yield detectable results for the positive controls.

2.3.2.5 Quantitative polymerase chain reaction (qPCR) for equine hepacivirus

The initial method of qPCR was developed by Dr Alexander Tarr and Dr Terry Akagha at QMC (Akagha, 2021) with the following master-mix per reaction:

10 µl SYBR Green (SensiFAST™ SYBR No-ROX Kit)

0.8 µl Forward Primer (EqHep_C-E1f) (10 µM)

0.8 µl Reverse Primer (EqHep_C-E1r) (10 µM)

6.4 µl Nuclease free water

2 µl cDNA

The recommended thermocycle parameters were as follows:

95°C for 2 minutes

95°C for 20 seconds
55°C for 20 seconds
72°C for 20 seconds
} x55 cycles

72°C for 1 minute

This was later revised to the following master-mix protocol per reaction:

10 µl SYBR Green (SensiFAST™ SYBR No-ROX Kit)

0.4 µl Forward Primer (EqHep_C-E1f) (10 µM)

0.4 µl Reverse Primer (EqHep_C-E1r) (10 µM)

7.2 µl Nuclease free water

2 µl cDNA

The revised thermocycle parameters were as follows:

95°C for 2 minutes

95°C for 20 seconds
55°C for 20 seconds
72°C for 20 seconds
} x32 cycles

72°C for 1 minute

An EqHV E1E2 PCR product ligated into a PGEMT-easy vector plasmid was used as the positive control. A standard curve was created using a set of 10-fold serial dilutions ranging between 2 $x10^{10}$ and 2 $x10^{2}$ copies per µl. Nuclease-free water was used as the negative control.

2.3.2.6 Quantitative polymerase chain reaction (qPCR) for equine parvovirus-hepatitis (EqPV-H)

The primers design and method used to test for EqPV-H was established at Cornell University and provided via direct communication from Joy Tomlinson, DVM DACVIM.

Primer	**Sequence**
EqPV-H F16	CAGAGGTAGTGCACATCACG
EqPV-H R16	TCATCAGTGCTCGTCTCTTG

Mastermix:

10 µl SYBR Blue/Green

0.6 µl EqPV-H F16 Forward Primer from Cornell (10 µM)

0.6 µl EqPV-H F16 Reverse Primer from Cornell (10 µM)

6.8 µl nuclease-free water

2 µl DNA

Cycling conditions:

95°C for 20 seconds

95°C for 3 seconds
60°C for 30 seconds
} x40 cycles

95°C for 15 seconds

60°C for 1 minute

95°C for 15 seconds

60°C for 15 seconds

2.3.2.7 Agarose Gel Electrophoresis

Products from qPCR were separated via agarose gel electrophoresis to identify the amplicon. A 2% agarose gel was cast using 3 g of agarose powder in 150 ml of 1xTAE (Tris-acetate-EDTA) buffer and 3 µl of Nancy Red (Nancy-520) (Sigma-Aldrich, Missouri, USA). PCR products were added to each well at a volume of 5 µl with 2 µl of purple gel loading dye 6X (New England Biolabs Ltd, Hitchin, UK) alongside a low mass ruler ladder (New England Biolabs Ltd, Hitchin, UK), at 95 V for 45 mins in 1x TAE buffer. Following electrophoresis, the gel was viewed on a UV transilluminator (ImageQuant 300).

2.3.2.8 EqHV qPCR product sequencing

Several samples with positive qPCR results were sent for Sanger sequencing through Source BioScience. Samples were prepared as per Source BioScience directions prior to shipping, and samples were sent with accompanying EqHV PCR primers displayed in Table 5.

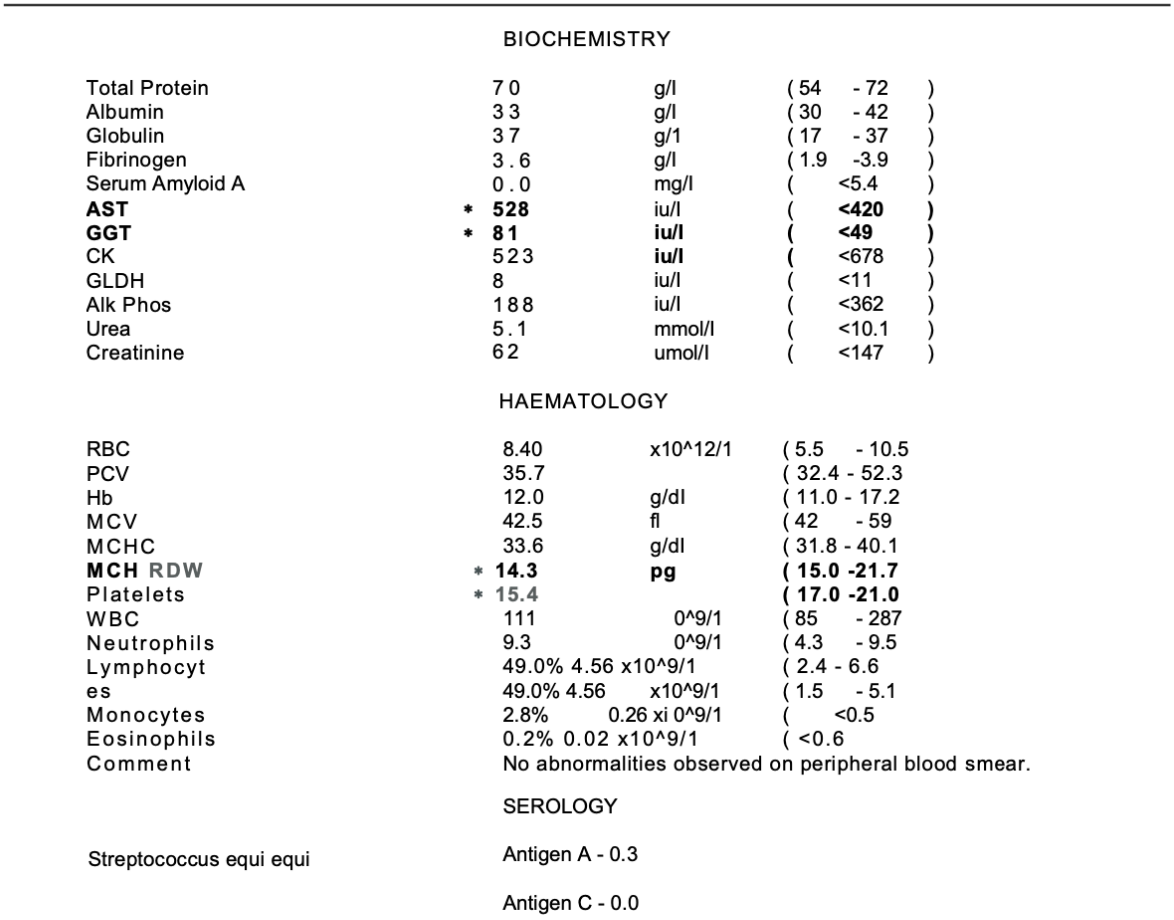
2.3.3 Clinical chemistry methods

2.3.3.1 Diagnostic test results

Clinical notes or test results pertaining to the blood samples from Bransby horses were acquired. These included basic details about each horse, age, sex breed a standard haematological screen, as described in section 2.1.4, an example of test result data can be seen in Figure 7. A further set of clinical chemistry results was obtained from Newmarket Equine Hospital. These related to samples from 66 racing Thoroughbred horses that were screened for EqHV RNA as part of another project (Akagha, 2021).

2.3.3.2 Additional biochemical analysis

Ferritin levels of the blood samples pertaining to the 66 Thoroughbred horses were assessed using a horse ferritin ELISA (MyBioSource, USA) with the assistance of Bethany Edwards. The biochemical analysis of other parameters of the Thoroughbred blood samples was performed by Dr Nigel Kendall and Catherine Williams of Nottingham University's Nutrition Advisory Service using a RX Imola™ (Randox, USA). Prior to analysis, the samples were removed from -80°C storage, thawed and then refrigerated at 4°C for 24 hours. A series of test kits (Randox, USA) were used to measure the levels of ALT, AST, GLDH, GGT, creatine kinase, total bilirubin, direct bilirubin, and bile acids in each blood sample.



		BIOCHEMISTRY		
Total Protein		70	g/l	(54 - 72)
Albumin		33	g/l	(30 - 42)
Globulin		37	g/1	(17 - 37)
Fibrinogen		3.6	g/l	(1.9 -3.9)
Serum Amyloid A		0.0	mg/l	(<5.4)
AST	*	**528**	iu/l	(**<420**)
GGT	*	**81**	**iu/l**	(**<49**)
CK		523	**iu/l**	(<678)
GLDH		8	iu/l	(<11)
Alk Phos		188	iu/l	(<362)
Urea		5.1	mmol/l	(<10.1)
Creatinine		62	umol/l	(<147)
		HAEMATOLOGY		
RBC		8.40	x10^12/1	(5.5 - 10.5
PCV		35.7		(32.4 - 52.3
Hb		12.0	g/dl	(11.0 - 17.2
MCV		42.5	fl	(42 - 59
MCHC		33.6	g/dl	(31.8 - 40.1
MCH RDW	*	**14.3**	**pg**	**(15.0 -21.7**
Platelets	*	15.4		**(17.0 -21.0**
WBC		111	0^9/1	(85 - 287
Neutrophils		9.3	0^9/1	(4.3 - 9.5
Lymphocyt		49.0% 4.56 x10^9/1		(2.4 - 6.6
es		49.0% 4.56	x10^9/1	(1.5 - 5.1
Monocytes		2.8% 0.26 xi 0^9/1		(<0.5
Eosinophils		0.2% 0.02 x10^9/1		(<0.6
Comment		No abnormalities observed on peripheral blood smear.		
		SEROLOGY		
Streptococcus equi equi		Antigen A - 0.3		
		Antigen C - 0.0		

Figure 7 Example of test result data provided with each blood sample from Bransby used in analysis.

2.3.3.3 Statistical analysis

Using R studio, data were assessed for normality using the Shapiro-Wilk test. Each clinical chemistry variable was analysed, and EqHV positive and negative populations were compared using either the Wilcoxon rank test or unpaired t-test depending on the outcome of the Shapiro-Wilk test, to determine any significant difference between means. Significance was assumed with a P value of < 0.05.

2.4 Results

2.4.1 Bransby Rescue Horses

2.4.1.1 Seroprevalence

EqHV prevalence in the Bransby population, determined by qPCR was 21.05% with 28 of 133 samples testing positive for EqHV (Figure 8). All samples were also screened by PCR for equine parvovirus-hepatitis virus (EqPV-H), 3 horses tested positive for EqPV-H; horse 38, 101 and 128. Horse 38 also tested positive for EqHV. Sequencing data from Source Bioscience showed some differences in base pairs between samples confirming individuality of sequences.

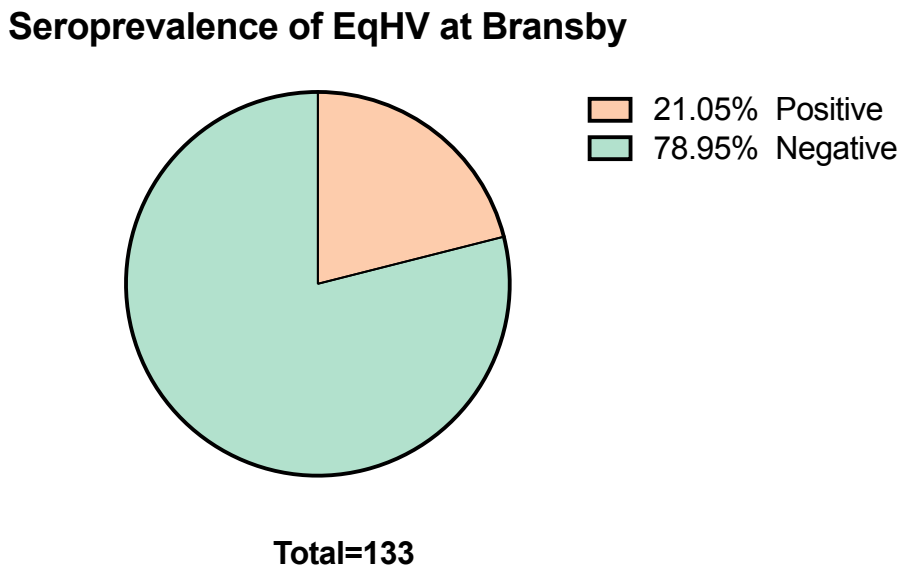


Figure 8 Seroprevalence of equine hepacivirus detected by qPCR targeting the E1/E2 region in 133 blood samples from a population of rescue horses at Bransby Horses Rescue and Welfare in 2018.

Characteristics of the horses sampled including age, sex and breed were analysed using odds ratio and Fisher's exact test and Chi-squared tests. The analysis did not identify any statistically significant differences among the variables examined in relation to presence of EqHV RNA.

Table 6 EqHV prevalence distribution by sex in a population of rescue horses from Bransby horse rescue in 2018. Pearson's Chi-squared test with Yates' continuity correction determined no significance. p-value = 0.2677, X-squared = 1.2283, df = 1

SEX	NEGATIVE	POSITIVE	SUM	POSITIVE (%)
F	49	17	66	26
M	56	11	67	16
SUM	105	28	133	21

Table 7 EqHV prevalence distribution by breed. Pearson's Chi-squared test X-squared = 19.315, df = 19, p-value = 0.4368

BREED	NEGATIVE	POSITIVE	SUM	POSITIVE (%)
COB	53	18	71	25
COB X	2	0	2	0
DONKEY	0	1	1	100
EXMOOR	1	1	2	50
FRESIAN	0	1	1	100
HIGHLAND	1	0	1	0
IRISH DRAUGHT X	2	0	2	0
NATIVE PONY	1	0	1	0
NEW FOREST	1	0	1	0
QUARTER HORSE	1	0	1	0
SHETLAND	19	5	24	21
SHIRE	2	0	2	0
THOROUGHBRED	3	0	3	0
TRAKEHNER	1	1	2	50
TROTTER	1	0	1	0
WARMBLOOD	1	1	2	50
WELSH	9	0	9	0
WELSH B	1	0	1	0
WELSH C	1	0	1	0
WELSH X	5	0	5	0

Frequency of EqHV prevalence is not constant throughout the eight months analysed in 2018, with 53% of total positive samples identified in June (see Figure 9).

No positive samples were identified in August, October, November, and December. In June 94% of all samples tested were positive for EqHV (Figure 10).

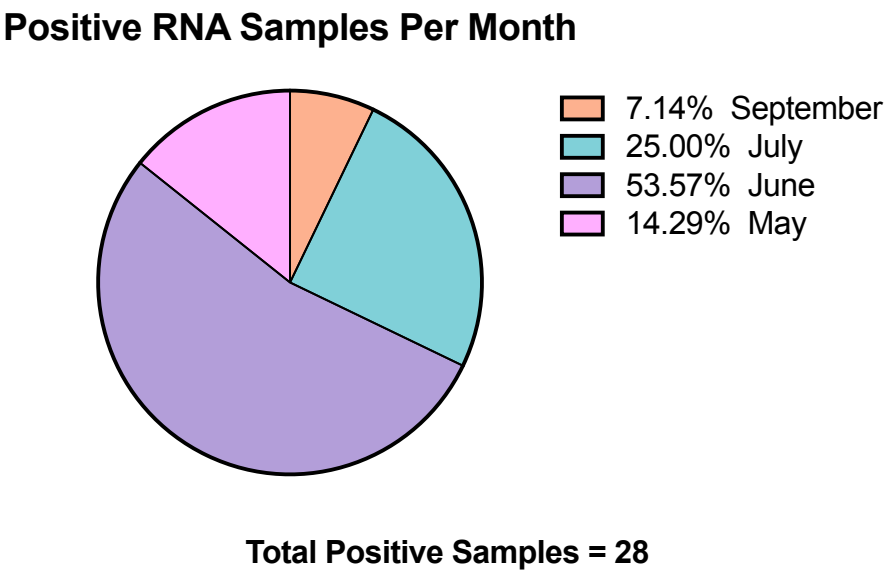


Figure 9 Distribution of blood samples positive for EqHV from Bransby horse sanctuary by month in 2018

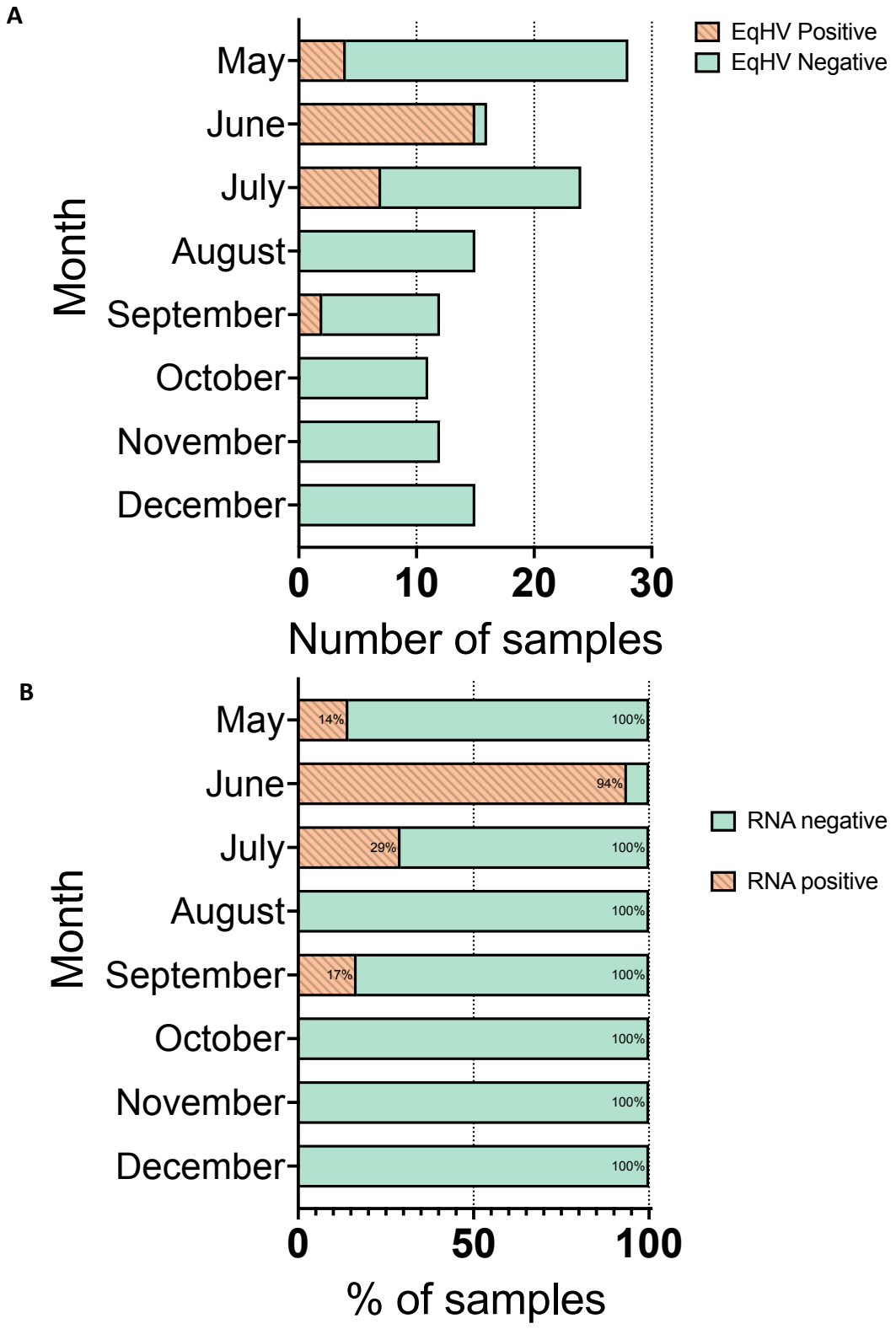


Figure 10 Prevalence of EqHV in the Bransby welfare population by month in the year 2018. A - bar chart showing number of samples tested for EqHV and the frequency of positive and negative samples. B – Percentage distribution of samples for each month positive for EqHV.

2.4.1.2 Bransby population univariate analysis

Blood parameters and clinical chemistry data were analysed in R. Samples from foals (defined as individuals under the age of 1) were removed prior to analysis due to the variation in the expected normal range between foals and adult horses. Data were assessed for distribution normality using the Shapiro-Wilk normality test to determine the appropriate statistical test. Mean values of blood parameters for groups positive or negative for EqHV were compared using either the unpaired t-test or the Wilcoxon test depending on the outcome of the Shapiro-Wilk normality test. Full data for each parameter can be seen as individual box and whisker plots in the appendix (Figure 54 - Figure 76). Normal reference ranges used are the ranges provided with the clinical lab results.

Results of a comparison of haematological parameters between horses either negative or positive for EqHV RNA found a statistically significant difference in the mean lymphocyte count ($p=0.0098$), with positive horses having a higher mean lymphocyte count compared to negative horses. The other parameters did not show a statistically significant difference between the two groups (Table 8).

Comparison of clinical chemistry parameters, shown in Table 9, found the population positive for EqHV RNA, had significantly lower CK compared to the negative population. The other parameters assessed did not show a statistically significant difference between the two groups.

Table 8 Results comparing haematological parameters of horses from Bransby horse rescue, either positive or negative for EqHV RNA by qPCR, using either T-test or Wilcoxon test.

Parameter	***P-value***	***Mean positive***	***Mean negative***	***Mean difference***
Eosinophils	0.8482	0.32	0.33	-0.02
Haemoglobin	0.7064	12.44	12.26	0.19
Lymphocytes*	**0.0098**	**4.18**	**3.28**	**0.90**
MCH	0.4821	16.05	16.28	-0.23
MCHC	0.4005	35.19	39.07	-3.88
MCV	0.0541	45.61	47.27	-1.66
Monocytes	0.2304	0.32	0.37	-0.05
Neutrophils	0.5466	6.39	5.98	0.41
PCV	0.8772	35.36	35.57	-0.21
Platelets	0.6289	142.50	147.98	-5.48
RBC	0.5043	7.76	7.56	0.19
RDW	0.8219	14.91	14.85	0.06
WBC	0.2230	11.19	9.96	1.22

Table 9 Results comparing clinical chemistry parameters of horses from Bransby horse rescue, either positive or negative for EqHV RNA by qPCR, using either T-test or Wilcoxon test.

Parameter	***P-value***	***Mean positive***	***Mean negative***	***Mean difference***
Albumin	0.2683	34.23	32.50	1.73
Alk Phos	0.4760	315.29	292.71	22.57
AST	0.6459	454.36	468.34	-13.98
Creatine kinase *	**0.0260**	**506.00**	**885.29**	**-379.29**
Creatinine	0.5142	61.62	64.29	-2.68
Fibrinogen	0.3777	4.05	3.84	0.21
GGT	0.6410	89.64	69.35	20.29
GLDH	0.6231	9.50	15.12	-5.62
Globulin	0.8294	45.07	44.32	0.75
Ser Amyloid A	0.0531	4.98	38.89	-33.91
Total Protein	0.7035	78.93	77.88	1.05
Urea	0.2272	10.95	7.07	3.88

2.4.2 Thoroughbred Population

2.4.2.1 Seroprevalence

Surplus blood samples were obtained as part of routine health assessments from a cohort of 66 clinically healthy racehorses in active training. These horses were housed across various training stables dispersed throughout England. Regular surveillance of haematological parameters was a standard practice during their racing and training regimens. All samples were obtained in 2017, 66 of which in August and six in September. Seroprevalence was determined by qPCR by Dr Terry Akagha (Akagha, 2021), 25 of 66 samples tested positive for EqHV, sex or age had no significant impact on prevalence of EqHV (Akagha, 2021).

Table 10 EqHV prevalence distribution by age in a population of 66 Thoroughbred racehorses in training. Pearson's Chi-squared test determined no significance. p-value = 0.4331, X-squared = 5.9125, df = 6.

AGE	NEGATIVE	POSITIVE	SUM	% POSITIVE
2	14	8	22	36.4
3	16	7	23	30.4
4	8	6	14	42.9
5	1	3	4	75.0
6	1	0	1	0.0
7	0	1	1	100.0
8	1	0	1	0.0
SUM	41	25	66	37.9

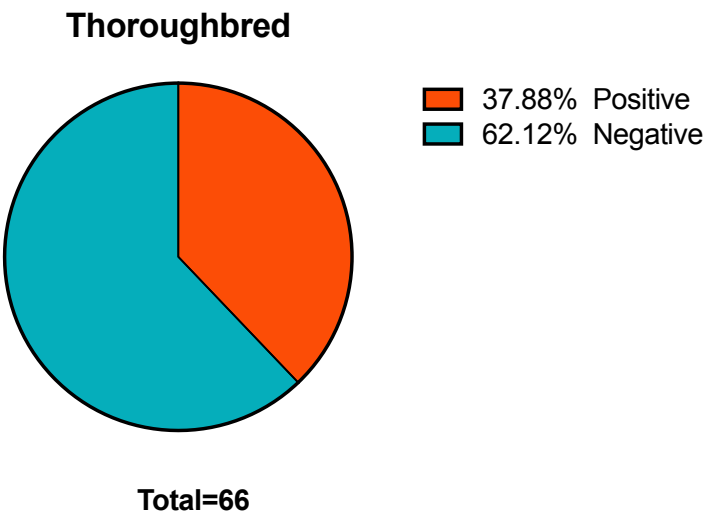


Figure 11 Seroprevalence of equine hepacivirus detected by qPCR targeting the E1/E2 region from a population of 66 Thoroughbred racehorses.

2.4.2.2 Thoroughbred univariate analysis

Blood parameters from Thoroughbred samples were analysed in R. Data were assessed for distribution normality using the Shapiro-Wilk normality test to determine the appropriate statistical test. Nonparametric data were log transformed and reanalysed for distribution normality. Logged data which resulted in parametric distribution was assessed alongside non-parametric original values. Mean values of blood parameters for groups positive or negative for EqHV were compared using either the unpaired t-test or the Wilcoxon test depending on the outcome of the Shapiro-Wilk normality test. Each comparison was plotted in a box and whisker plot (Figure 79 - Figure 100).

Table 11 presents the results of the haematology parameter analysis. Among the parameters analysed, Haemoglobin, MCH, MCHC, MCV, and PCV showed statistically significant differences ($P < 0.05$) between positive

and negative horses. All levels were higher in the EqHV RNA positive group except MCHC which was lower in the positive group. The remaining parameters did not show statistically significant differences between the two groups.

Table 12 presents the results of clinical chemistry parameter analysis. Levels of GLDH differed significantly between populations positive or negative for EqHV RNA, ($p = 0.03$), GLDH was higher in the positive group with a mean difference of 1.26. Other parameters were not found to be statistically significant.

Table 11 Results comparing haematology parameters of 66 Thoroughbred racehorses in training, either positive or negative for EqHV RNA by qPCR, using either T-test or Wilcoxon test.

Parameter	***P-value***	***Mean positive***	***Mean negative***	***Mean difference***
Eosinophils	0.3	0.15	0.12	0.03
***Haemoglobin** **	0.015	14.25	13.31	0.94
Lymphocytes	0.30	3.39	3.65	-0.26
MCH*	0.002	15.64	13.39	2.25
MCHC*	$3.3x10^{-6}$	35.46	37.01	-1.55
MCV*	0.00034	40.13	36.21	3.92
Monocytes	0.17	0.39	0.30	0.08
Neutrophils	0.39	5.35	5.04	0.31
PCV*	0.00023	39.92	35.96	3.96
RBC	0.83	10.03	9.97	0.06
WBC	0.73	9.28	9.11	0.17

Table 12 Results comparing clinical chemistry parameters of 66 Thoroughbred racehorses in training, either positive or negative for EqHV RNA by qPCR, using either T-test or Wilcoxon test.

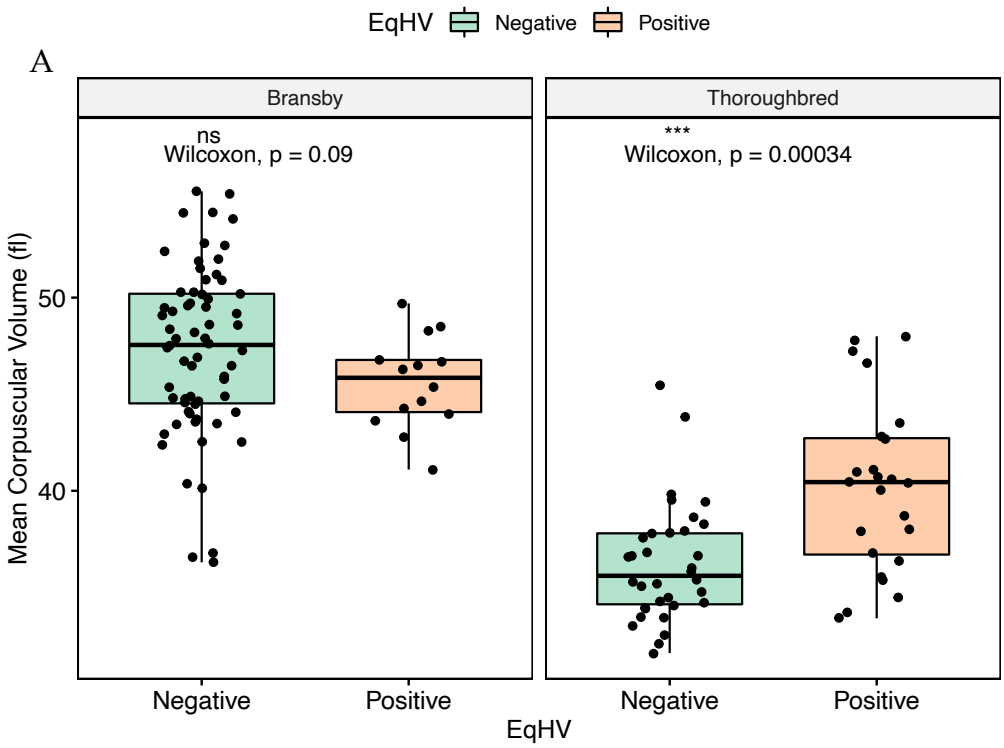
Parameter	***P value***	***Mean Positive***	***Mean Negative***	***Mean Difference***
ALT	0.61	10.02	11.61	-1.60
AST	0.86	244.12	239.78	4.34
Bile acids	0.4	4.96	4.52	0.44
Bilirubin – Total	0.28	23.82	21.61	2.21
Bilirubin - Direct	0.064	4.72	5.15	-0.44
Creatine kinase	0.57	157.80	193.39	-35.59
Ferritin	0.74	104.61	91.05	13.56
Fibrinogen	0.36	3.19	3.68	-0.48
GGT	0.442	25.16	27.91	-2.75
GLDH*	0.032	3.97	2.72	1.26
Serum Amyloid A	0.513	26.52	49.20	-22.68

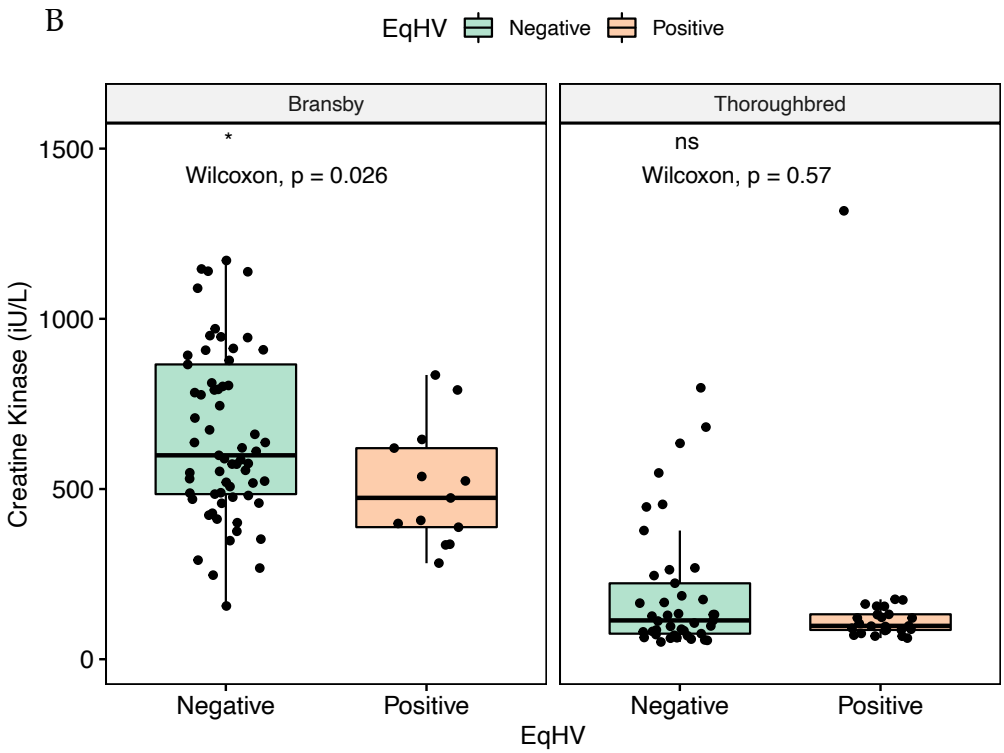
2.4.3 Rescue horses vs Thoroughbreds in training

In the Bransby rescue population, the only haematological parameter that showed a significant difference between the positive and negative groups was lymphocyte count ($p = 0.0098$) which was increased in the positive population. In contrast, in the Thoroughbred population, the haematological parameters that showed significant differences between the positive and negative groups were haemoglobin, MCH, MCHC, MCV, and PCV. Lymphocyte count was not significant in the Thoroughbred population.

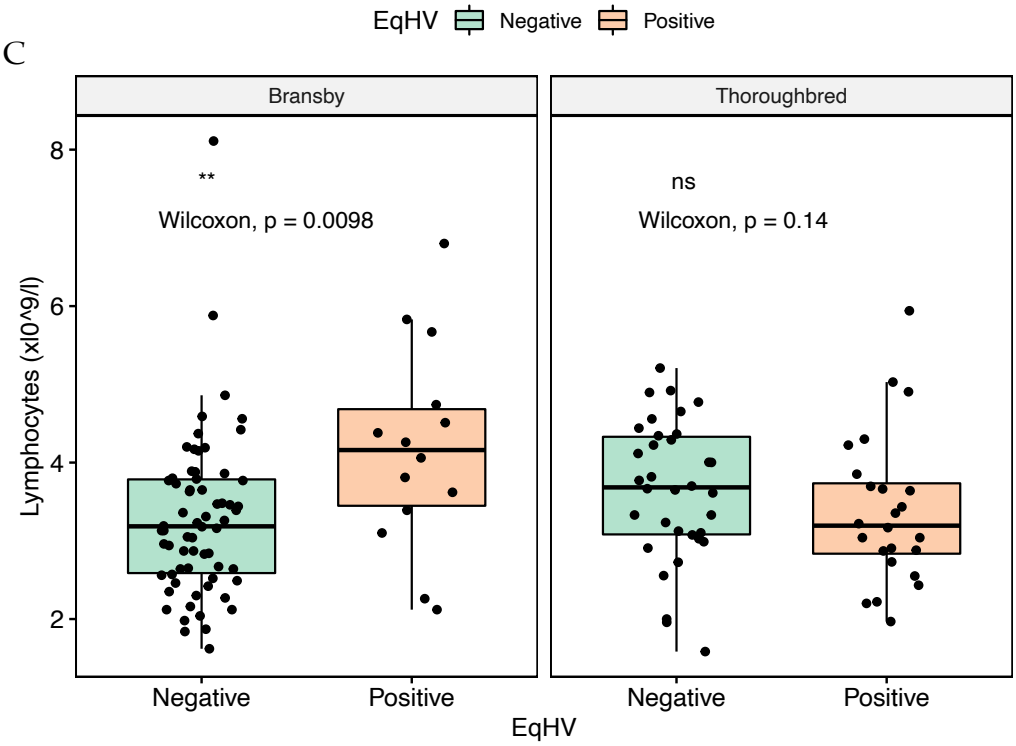
For the clinical chemistry parameters, CK level was significantly lower in the Bransby positive population. In the Thoroughbred population, GLDH levels were significantly higher in the positive population.

Parameters significant for one population were plotted as box and whisker plots alongside data from the other population using the same scale for comparison in Figure 12.

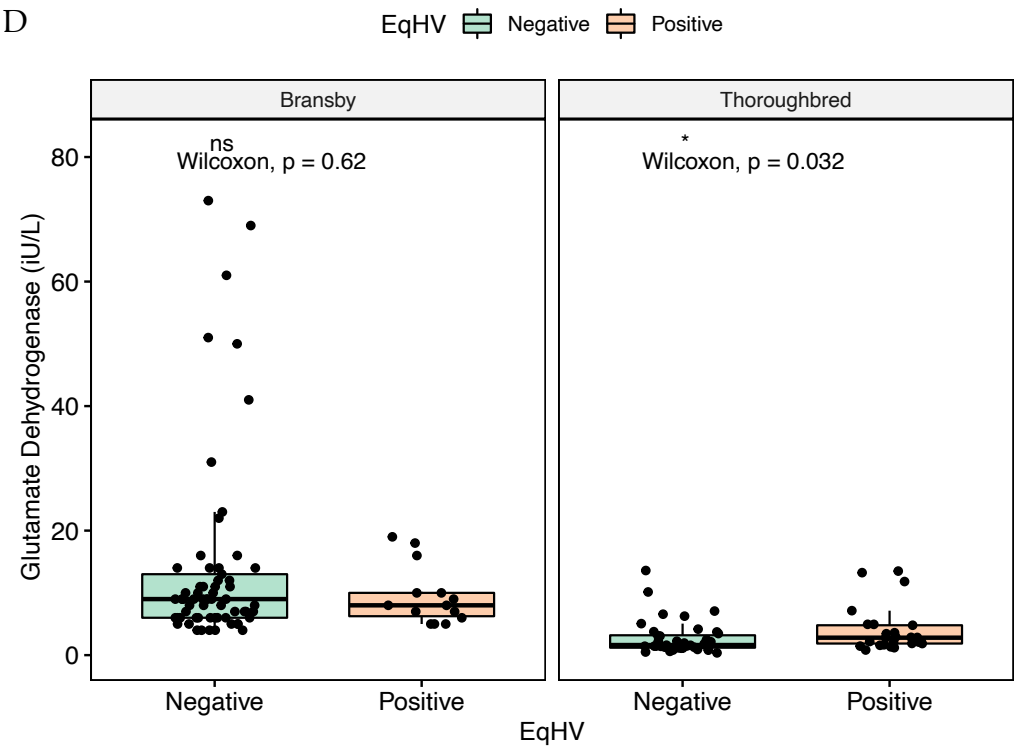

A
EqHV Negative Positive
Bransby
Thoroughbred
ns
Wilcoxon, p = 0.09
***
Wilcoxon, p = 0.00034
Mean Corpuscular Volume (fl)
50
40
Negative
Positive
Negative
Positive
EqHV


B
EqHV Negative Positive
Bransby
Thoroughbred
*
Wilcoxon, p = 0.026
ns
Wilcoxon, p = 0.57
Creatine Kinase (iU/L)
1500
1000
500
0
Negative
Positive
Negative
Positive
EqHV

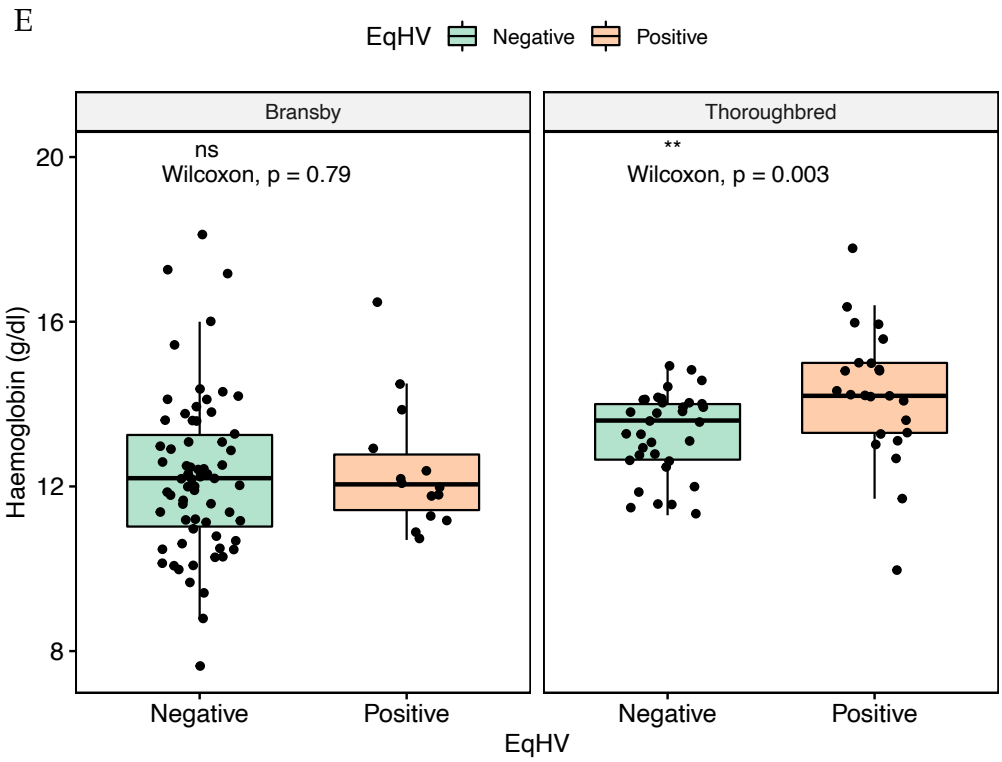
C


EqHV Negative Positive
Bransby
Thoroughbred
**
Wilcoxon, p = 0.0098
ns
Wilcoxon, p = 0.14
Lymphocytes (xl0^9/l)
8
6
4
2
Negative
Positive
Negative
Positive
EqHV

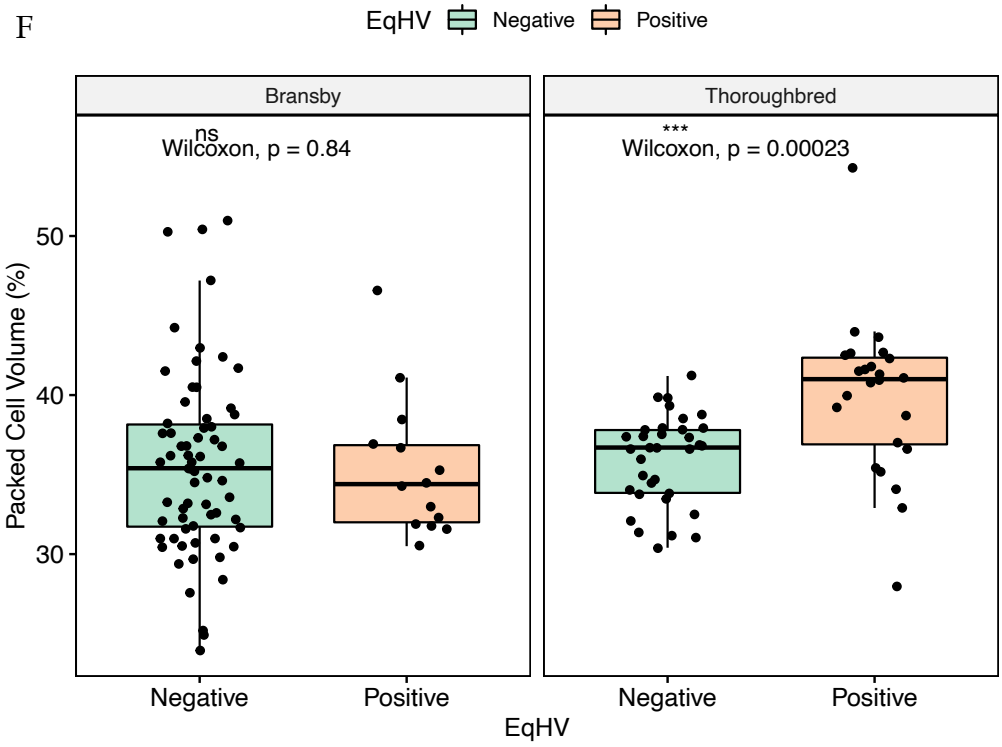
D


EqHV Negative Positive
Bransby
Thoroughbred
ns
Wilcoxon, p = 0.62
*
Wilcoxon, p = 0.032
Glutamate Dehydrogenase (iU/L)
80
60
40
20
0
Negative
Positive
Negative
Positive
EqHV

E


EqHV Negative Positive
Bransby
Thoroughbred
ns
Wilcoxon, p = 0.79
**
Wilcoxon, p = 0.003
Haemoglobin (g/dl)
20
16
12
8
Negative
Positive
Negative
Positive
EqHV

F


EqHV Negative Positive
Bransby
Thoroughbred
ns
Wilcoxon, p = 0.84
***
Wilcoxon, p = 0.00023
Packed Cell Volume (%)
50
40
30
Negative
Positive
Negative
Positive
EqHV

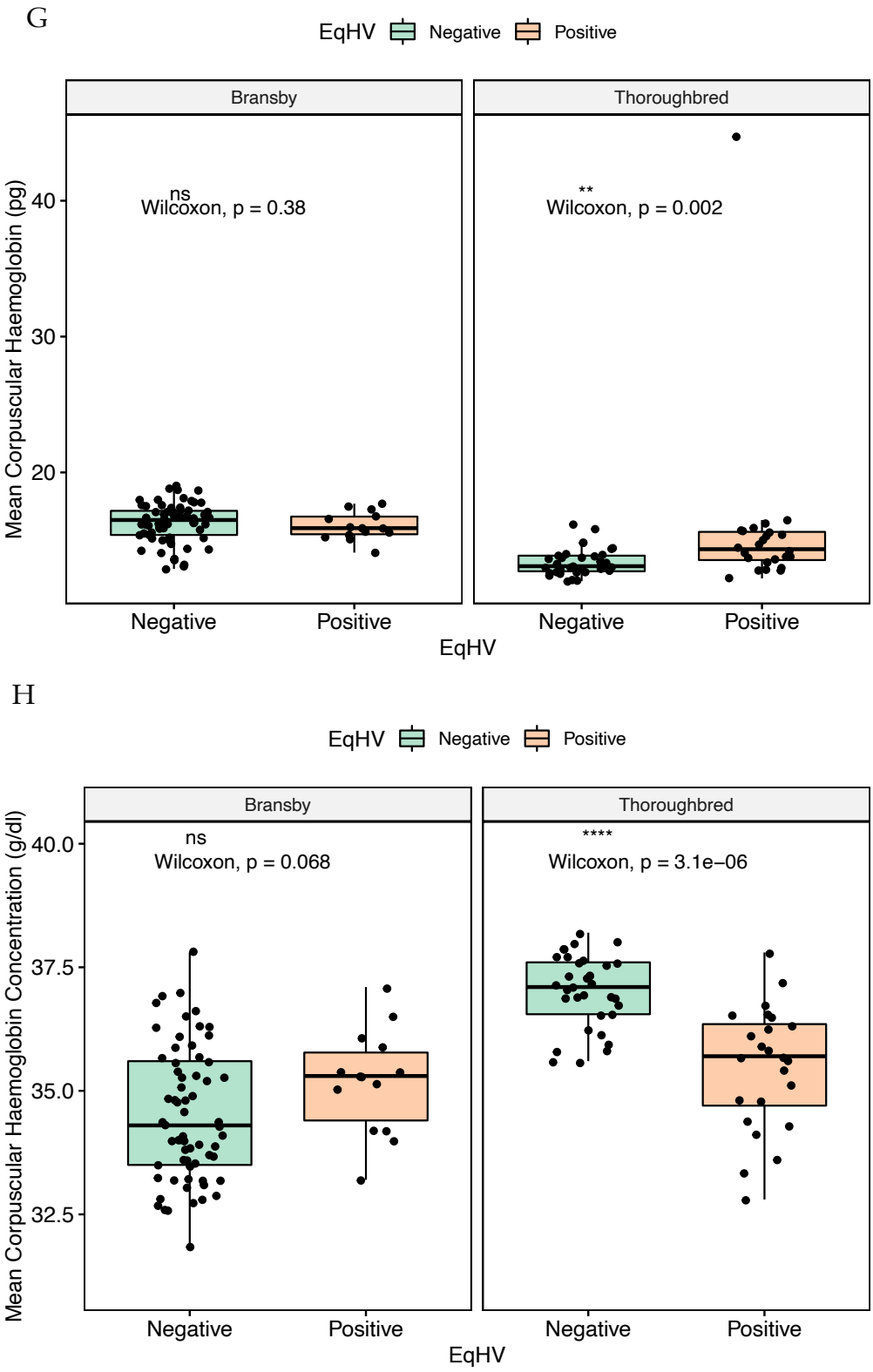


*Figure 12 Box and whisker plots displaying individual values and mean average levels of **A**: MCV, **B:** creatine kinase, **C:** lymphocytes, **D:** GLDH, **E:** haemoglobin, **F:** PCV, **G:** MCH, **H:** MCHC for to populations of horses, Bransby rescue horses and 66 racing Thoroughbreds.*

2.5 Discussion

2.5.1 Prevalence of EqHV

The findings within this thesis chapter contribute to an exploration of Equine Hepacivirus (EqHV) prevalence, enhancing our understanding within the United Kingdom's equine populations. The assessment of seroprevalence in both the Bransby and Thoroughbred cohorts unearthed higher rates compared to prior studies, highlighting the need for a deeper investigation into the underlying factors contributing to these disparities. Methodological advancements, particularly the adoption of more sensitive primers, have notably improved the precision of EqHV detection. Employing a year-long sampling approach has unveiled a potential seasonal pattern in EqHV prevalence, introducing a novel dimension to our understanding of EqHV epidemiology.

The overall seroprevalence of EqHV was 21% in the Bransby population and 37.88% in the Thoroughbred population (Akagha, 2021). These prevalence rates exceeded those reported in two other studies on the UK equine population, which documented a prevalence of 2.1% (3/142) and less than 1% (3/328) (Lyons et al., 2012, 2014) .

Many EqHV prevalence studies, including the two UK-based investigations, typically assess period prevalence rather than point prevalence. This distinction involves evaluating blood samples collected over an extended duration as opposed to examining prevalence at a specific time in a population.

Currently the highest prevalence of EqHV reported in Europe is 18.3% from a population of 733 Thoroughbreds tested in Germany (Reichert et al., 2017).

The difference in reported prevalence may be due to the methodology used in this study. The primers were designed and optimised by Dr Alex Tarr (School of Life Sciences, University of Nottingham), utilising the larger number of EqHV sequences from various regions now available on GenBank, as well as sequenced data from the EqHV strains identified in the Thoroughbred population. A region in the E1 and E2 genes were found to be very well conserved after phylogenetic analysis. When compared to other primers commonly used in published papers; NS3, 3'UTR, NS5 and 5'UTR, the E1/E2 primers were found to be most accurate and give the least number of false negatives compared to other published methods (Akagha, 2021).

As this study collected blood samples with the intention of screening for EqHV RNA samples were always kept at -80°C. Other studies also often utilised convenience samples which may not have been kept stored at -80°C which may have affected the viability of the EqHV RNA.

Prevalence of EqHV in the Bransby population varied greatly by month. With the highest prevalence rate identified in June, 94% of June blood samples were positive for EqHV. Proportionally 54% of the total positive blood samples (n = 28) identified between May – Dec in 2018 were sampled in June. Zero cases of EqHV were identified in October, November and December. To date, no other study has reported assessing prevalence by month. This research identifies a potential seasonal link with case

prevalence of EqHV with a significantly higher number of cases of EqHV in May June and July. Seasonality in the Thoroughbred population could not be assessed as all samples were all obtained from August and September only.

To ensure the accuracy of the findings, multiple EqHV-positive samples identified through qPCR underwent Sanger Sequencing. This rigorous process was particularly critical during the testing phase focused on June, with its remarkably high 94% prevalence. Sequencing analyses revealed minor base pair variations (consisting of one or two bp differences across the sequence in the wobble base region) between each sample and when compared to the positive control. This thorough confirmation validated the accuracy of the results, ensuring the credibility of the identified positive cases. Furthermore, it's essential to highlight that the horses contributing the sequenced positive blood samples were segregated in separate paddocks and had no contact with each other during the sampling process.

The apparent seasonal trend observed in EqHV prevalence, notably the surge in cases during May, June, and July, may indicate a potential association with the activity of biting flies. Biting flies, including horseflies (Tabanids) and stable flies (Stomoxys calcitrans), are known to be active during the summer months and often present in areas with equine populations. These insects are notorious for their biting behaviour and are capable of feeding on multiple hosts, thereby potentially facilitating the transmission of pathogens. Previous studies have indicated the involvement of biting flies in the transmission cycle of various equine diseases. This observation in EqHV prevalence provides preliminary evidence supporting the hypothesis that seasonal changes in biting fly

activity might align with increased EqHV transmission rates. Further exploration and detailed investigation into the role of these insects in EqHV transmission pathways are explored in greater depth in Chapter 3.

Factors including age, breed and sex were found to have no significant impact on the presence of EqHV in either the Bransby or Thoroughbred population.

2.5.2 Clinical chemistry analysis

2.5.2.1 Population selection

The population of racing Thoroughbreds was selected for biomarker assessment and analysis to compare against the Bransby rescue horses. The Thoroughbred population was to provide a more homogenous and comparable population. Additionally, to being the same breed, they are also similar in age and size. Their environments, lifestyle and nutrition are very controlled, and are presumed a 'healthy' population. This reduces the possibility of confounding factors that are more likely to be present in a population such as the horses at Bransby Horses Rescue and Welfare, which may have health concerns. Additionally, routine blood tests are a standard practice for monitoring the health and performance of racehorses. These tests serve as a tool for assessing the haematological parameters and physiological status of the animals during their training and racing regimens. In contrast the horses at Bransby are sampled on the intake to the centre, usually after removal or surrender from their original owners due to neglect, prior to rehoming, or when there is a health concern.

2.5.2.2 Bransby horses clinical chemistry

Analysis of data obtained from the Bransby population revealed horses positive for EqHV had significantly higher lymphocytes compared with the negative population.

These data suggest that EqHV infection may be associated with changes in the lymphocyte count in horses, higher lymphocyte count in horses positive for EqHV is likely due to an immune response and indicates the horse's immune system is mounting a response to the EqHV infection. A significant difference was also found in CK levels (P-value 0.026). The positive horses had significantly lower levels of CK compared to the negative group. CK is an enzyme found in high concentrations in muscle cells, Elevated levels can indicate muscle damage or disease, as the enzyme is released into the bloodstream when muscle cells are damaged. However, in horses, CK levels can easily fluctuate with exercise or rapid movement, levels return to baseline levels over the course of several hours to days.

No significant differences in other liver enzymes were found between the populations. Although liver enzymes generally indicate the level of liver disease in an individual, in cases of chronic liver disease with severe fibrosis and therefore a reduction in the number of functional hepatocytes, plasma activities of liver enzymes may be within normal limits (Satué et al., 2022).

All but two horses within the whole Bransby study sample were deemed below the 'normal range' of red cell distribution. This raises the reliability of the 'normal range' reference values currently used to assess results of clinical chemistry. 'Normal levels' of clinical chemistry data are determined by the vet practice; these are guided by a reference range. The "normal

ranges" are often set using Thoroughbred populations as the baseline (Lumsden et al., 1980). However, horse breeds vary vastly in height and build and athletic tendencies which all play a factor in determining standard 'normal levels' of clinical chemistry. These reference ranges were also established prior to the identification of various subclinical equine hepatitis viruses, potentially affecting assumed 'healthy horses' sampled to determine a 'normal range'.

The population of horses at Bransby Horses Rescue and Welfare also vary vastly in age and height and generally tended to be native type breeds (smaller, stocky, thick coated, hardy breeds with lower metabolisms, well adapted to suit living in harsh exposed conditions). These factors may affect baseline levels of clinical chemistry for example the 'normal' level of haemoglobin for a small cob is lower than that of a Thoroughbred. This creates difficulty when attempting to determine if a value is actually abnormal or just abnormal based on the values determined off a Thoroughbred population.

Non-specific results from the clinical chemistry are not unsurprising; as EqHV is similar to HCV they share the same initial subclinical nature.

2.5.2.3 Thoroughbred clinical chemistry

Analysis shows a significant increase in haemoglobin, packed cell volume (PCV), mean cell volume (MCV) and mean cell haemoglobin (MCH) in horses positive for EqHV compared to those negative for EqHV. Mean cell haemoglobin concentration (MCHC) was significantly lower in horses positive for EqHV.

The combination of elevated MCH and decreased MCHC levels is usually indicative of a regenerative red cell response or regenerative anaemia. This is when there is an increased production of new red blood cells to replace those that have been lost, resulting in an increase in the number of immature red blood cells (reticulocytes) in circulation. As reticulocytes are larger and contain more haemoglobin than mature red blood cells, their presence can cause an increase in the MCH. However, the MCHC may decrease as the reticulocytes have a lower concentration of haemoglobin compared to mature red blood cells. This is because reticulocytes contain residual RNA and other cellular components that contribute to their larger size, which displaces the haemoglobin and reduces its concentration per unit volume (Walton and Lawson, 2020).

However, no significant decrease in red blood cell levels was found to indicate anaemia as the cause. Interestingly there was also no significant difference in any white blood cell counts between the infected and non-infected Thoroughbred populations.

It is established that intense exercise causes a reduction in immune function, and young horses are particularly susceptible to exercise-induced immune suppression (Cappelli et al., 2020; Miglio et al., 2021). Erythropoiesis (generation of red blood cells) can happen as a response to hypoxia, intense training that causes temporary hypoxia will induce erythropoiesis through hypoxia-inducible factors (HIF) (Mairbäurl, 2013)

These results are comparable to the impact of HCV infection on haematological parameters, studies show HCV increases HIF-1α (Zhu et al., 2014) therefore increasing erythropoiesis, and a study assessing

predictor for early identification of HCV infection found HCV positive patients also had significantly higher MCH, haemoglobin, and PCV compared to a non-infected population (Tsai et al., 2015).

GLDH was significantly higher in the positive population (p value = 0.032), indicating EqHV causes hepatocellular damage. This finding correlates with findings of other studies (Badenhorst et al., 2019b; Tegtmeyer et al., 2019)

2.5.2.4 Population comparison

In the Bransby horse rescue population, the only haematological parameter that showed a significant difference between the positive and negative groups was lymphocyte count (p = 0.0098), with a mean difference of 0.9. The average lymphocyte count in the positive group was 4.9 x 10^9/L, while the negative group had an average count of 3.28 x 10^9/L. In contrast, no significant difference was found in the lymphocyte counts between the positive and negative groups in the Thoroughbred population. The average lymphocyte count in the positive group was 3.3 x 10^9/L, while the negative group had an average count of 3.6 x 10^9/L. This suggests that only the rescue population of horses were mounting an immune response to the presence of EqHV.

Data also showed that the positive Thoroughbred population had significantly higher mean values of haemoglobin, MCH, PCV, and MCV but a significantly lower MCHC value. In contrast, the rescue horse population did not show any significant differences in these haematological parameters. This may be that within a population undergoing intense training, EqHV infection impacts the susceptibility of training-induced

erythropoiesis. This effect would not be seen in a population not undergoing intense exercise.

As this is the first study to highlight potential links between EqHV and haematological parameters, further research is needed to confirm this association and to better understand the underlying mechanisms, likely they are similar to HCV(Saifan et al., 2013; Zhu et al., 2014; Tsai et al., 2015). If a link between EqHV infection and the aberrant haematology results can be confirmed to cause a significant impact on the horses' ability to perform, this may have implications for the racehorse industry as well as other sports horse industries that rely heavily on the health and fitness of horses.

In both populations, the mean CK levels were lower in the EqHV RNA-positive group compared to the negative group. In the Bransby population, the mean CK level was 506 U/L in the positive group and 885.29 U/L in the negative group, with a significant mean difference of -379.29 U/L ($p = 0.026$). In the Thoroughbred population, although not statistically significant, the mean CK level was 157.8 U/L in the positive group and 193.39 U/L in the negative group, with a mean difference of -35.59 U/L ($p = 0.57$). Lower levels of CK could be due to lethargy and decreased activity caused by the infection. Further studies are needed to confirm any relationship and to determine the potential clinical implications of these findings.

In the Thoroughbred population, a significant increase in GLDH levels in the positive group was shown which was not reflected in the Bransby population. However, the mean average GLDH levels for the Bransby horses was much higher than the Thoroughbreds, and the EqHV RNA

negative population had several values over the 'normal' threshold. Due to the nature of the Bransby horses there may be some horses with existing liver disease.

Overall, these results suggest that EqHV infection affects both clinical chemistry and haematological parameters differently in two populations, as evidenced by the different parameters that showed significant differences between the positive and negative groups. This could be due to a combination of the vast differences between the two populations as well unknown aspects of EqHV infections Further studies are needed to explore the underlying mechanisms of these differences and their implications for the health and performance of horses, as well as thoroughly investigating the potential seasonal trends of EqHV identified in this thesis.

2.5.3 Limitations

All blood samples were donated to the project after routine diagnostic testing by a qualified veterinary surgeon as surplus to requirements. This project is ethically favourable as no invasive techniques were performed on horses for the sole purpose of researching in this project. This did, however, mean obtaining large numbers of samples from the welfare population was challenging as, unlike racehorses, welfare horses are less routinely tested. Samples are only taken on their arrival to the centre or if any disease is suspected. This puts some limitations on obtaining a representative prevalence in the welfare population as well as potentially biasing sample selection in favour of animals with poor health.

Chapter 3: Biting flies as a potential route of transmission of equine Hepacivirus A

3.1 Introduction

At present, the method of transmission of equine Hepacivirus A (EqHV) is unknown. Given the substantial genetic resemblance—approximately 50% homology—between EqHV and the hepatitis C virus (HCV), which is known to be transmitted via blood-to-blood contact, it is plausible that EqHV might share a similar transmission route involving infected blood. In humans, HCV primarily spreads through needle-sharing and transfusion of unscreened blood products (Mohamed et al., 2015).

However, the modes of HCV transmission in humans are unlikely to be prevalent in horses. Blood transfusions are rare among horses (Mudge and Williams, 2017), and needle-sharing does not occur in veterinary practice. A more likely occurrence of blood-to-blood contact in equids would be from biting flies.

Other equine viruses within the *Flavivirus* genus, are known to be transmitted by biting insects. West Nile virus (WNV) and Japanese encephalitis virus (JEV) are transmitted by ornithophilic mosquito species, mainly *Culex* spp and tick-borne encephalitis virus (TBEV) and louping-ill

virus (LIV) are both transmitted by the same ixodid tick vector, *Ixodes ricinus* (Cavalleri et al., 2022).

Data from the findings in chapter 2 indicated a seasonal pattern to the EqHV prevalence within the Bransby population tested, this pattern could coincide with biting fly activity in the UK. This chapter explores biting flies as a potential mode of transmission.

3.1.1 Biting flies in the UK

There are five families of biting flies found in the UK: *Culicidae* (mosquitoes), *Ceratopogonidae* (biting midges), *Muscidae* (Stable flies and horn flies), *Simuliidae* (black flies) and *Tabanidae* (including horse flies, deer flies and clegs) (Berenger and Parola, 2017).

3.1.1.1 *Culicidae* - Mosquitos

Mosquitos are a known vector of viral diseases and there are several known mosquito-borne viruses that affect equids world-wide including: West Nile virus, Japanese encephalitis virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus, Eastern equine encephalitis virus, Murray Valley encephalitis virus, Ross River virus (Chapman et al., 2018), Barmah Forest virus and Sindbis virus (Ong et al., 2021).

Only female mosquitoes require blood meals (to be able to produce eggs); they extract blood by piercing the skin using their fascicle housed in their proboscis mouth part. The fascicle consists of a bundle of six coherently functioning stylets, thin hollow needle like mouthparts (Gurera et al., 2018).

Blood is sucked up through the labrum stylet like a straw. Mosquito bites are typically painless, the fascicle is very small in diameter (~ 80 μm) and saliva containing both numbing and clotting agents is deposited into the skin. Mosquitos are biological vectors (Lounibos, 2003) whereby the agent of disease is able to develop or propagate within the vector for onwards transmission (Onyango et al., 2020). Transmission of diseases, including equine encephalitis, typically occurs during a bite due to the insertion of infected mosquito saliva into the host during a bite (Stauft et al., 2022). Mosquitos have previously been studied as possible vectors for EqHV. In a large study conducted in Austria, in which 5000 mosquitos were analysed by RT-qPCR, no presence of EqHV was detected (Badenhorst et al., 2019b).

3.1.1.2 Culicoides - Midges

Culicoides, commonly known as midges or gnats, are among the most abundant hematophagous insects worldwide, and are vectors of multiple arboviruses of veterinary importance (Sick et al., 2019). *Culicoides* often feed in swarms with an attack rate as high as 110 per minute. *Culicoides* are 'pool' feeders rather than vessel-feeders like mosquitos, they possess specialised mouthparts to pierce through epidermis causing significant mechanical dermal and vascular damage (Lehiy et al., 2018), blood is then ingested off the surface of the skin. Like mosquitoes, midges also use their saliva during feeding which contains antihemorrhagic factors and immunomodulatory proteins. *Culicoides* spp. have been shown to transmit several animal-associated pathogens in their saliva during feeding. Most notably for the equid population, African horse sickness virus (AHS) (Zientara et al., 2015; MartÍnez-de la Puente et al., 2017).

3.1.1.3 *Muscidae* – house fly / stable fly

The *Muscidae* include approximately 5,000 species in 200 genera. Not all species are biting flies and only a few of these genera are of medical or veterinary concern. Important non biting species include the house fly and false stable fly. Important biting species include the stable fly and horn fly (Moon, 2018). Flies belonging to the genus *Stomoxys* (18 different species) are obligate blood sucking insects; males of some species also feed on blood. *Stomoxys* are known mechanical transmitters of several pathogens that affect multiple species including the horse such as equine infectious anaemia virus and West Nile virus (Baldacchino et al., 2013).

3.1.1.4 Tabanidae

In the UK, species within the *Tabanidae* are known by 3 common names. Horsefly is used generally and can be inclusive of all five genera within the family or specifically species within the *Atylotus, Tabanus* and *Hybomitra.* Deer fly is used for species within the genus *Chrysops*, cleg for *Haematopota* spp.. Horse flies are typically relatively large (10–30 mm) whereas deer flies and clegs are smaller (6–13 mm). To differentiate, from here Tabanids will be used to signify all UK species.

Adult tabanids have large stocky bodies and large coloured eyes often with bold iridescent patterns, owing to the presence of corneal interference filters (Baldacchino et al., 2014).

Only the female requires a blood meal as it is needed as a source of protein to produce eggs (Mugasa et al., 2018). A single blood meal is used as a source of energy for egg production (100–1000 eggs per meal) (Foil and

Hogsette, 1994). In temperate climates tabanid activity is seasonal, with higher activity occurring in the summer months.

Female mouthparts include paired blade-like mandibles and paired maxillae (Figure 13), which are used in a scissor-like fashion to cut the skin, piercing subcutaneous capillaries, inflicting a painful bite and creating a micro-haematoma. Saliva is introduced into the wound via the salivary duct and blood is drawn up into the feeding canal. This feeding method is known as pool feeding or telmophagy (Baldacchino et al., 2014). As males do not produce eggs they do not need to feed on blood and therefore lack mandibles, instead they feed on nectar and honeydew, which are also important additional food sources for female Tabanids (Mullens, 2018).



Figure 13 Photo of Tabanus autumnalis species depicting mouthparts.

3.1.2 Tabanidae as vectors of disease

Species of the *Tabanidae* are important vectors of both human and animal disease, however, they are poorly studied. Tabanids are known vectors of animal disease agents, including viruses, bacteria, and parasites. As shown in Table 13, Tabanids have been described as mechanical vectors of over 35 livestock pathogens, including viruses, bacteria, and parasites (Barros and Foil, 2007).

Tabanidae can transmit pathogens via both biological and mechanical transmission. Mechanical transmission occurs when the disease agent is transmitted without amplification within the fly. Tabanids make for excellent mechanical vectors. They are highly mobile and frequently engage in interrupted feeding, whereby they begin a meal on one host, become interrupted (often by the host noticing the painful bite) and immediately move to a fresh host (Baldacchino et al., 2014). For horse flies, the success of initial feeding attempts is often poor. It has been estimated that only 10% of horse flies successfully feed to repletion during the initial feeding attempt on cattle, though this varies among species of horse fly (Mullens, 2018). The Tabanid pool feeding method ensures their mouthparts and legs become covered in the hosts blood making cross contamination of blood between hosts very likely.

The inclination of *Tabanids* to return to the same host animal or relocate to another depends on the fly species and the distance between animals. The larger tabanids are more likely to transfer. It has been estimated that almost 90% of certain horse flies that feed or attempt to feed on horses will return to the original horse if other hosts are more than 35–50 m away. The

moderate-sized tabanid *T. fuscicostatus* has an average of 10 nl of blood residue on its mouthparts after feeding (Mullens, 2018).

Tabanidae are already a known mechanical vector for various diseases including several affecting equids, such as equine infectious anaemia. Equine infectious anaemia is a notifiable disease with an extremely poor prognosis, very few horses recover. Studies have shown that at high viral titres (10^6/ml), single tabanid bites can transmit the pathogen. Potential for transmission is high when multiplied by a large number of persistent flies feeding at a given time (Mullens, 2018). Other significant diseases transmissible by tabanids can be seen in Table 13.

The study of natural mechanical transmission of animal pathogenic agents is complex due to numerous pivotable variables. Factors like pathogen titre within the vertebrate and vector, the proximity between infected and susceptible hosts, the virus's stability when exposed to environmental elements on the fly's mouthparts, and the variety and density of potential vectors must be considered in field studies of mechanically transmitted diseases (Issel et al., 1988). Demonstrating natural mechanical transmission necessitates the alignment of numerous variables, many of which are beyond human control, such as their association with annual weather patterns and fly populations.

Table 13 Significant tabanid-associated diseases adapted from (Mullens, 2018)

Disease agent/ Disease	Vectors	Geographic occurrence	Transmission	Affected host
Viruses				
Equine infectious anaemia	*Tabanus, Hybomitra, Chrysops* spp.	Worldwide	Mechanical	Equids
Bovine leukaemia	*Tabanus* spp.	Worldwide	Mechanical	Domestic cattle and water buffalo
Classical swine fever	*Tabanus* spp.	Worldwide; eradicated from North America, Australia, New Zealand, South Africa	Mechanical	Pigs
Bacteria/Rickettsia				
***Anaplasma marginale* / anaplasmosis**	*Tabanus* spp.	Worldwide (Tropics, Subtropics)	Mechanical	Ruminants and ticks
***Francisella tularensis* / tularemia**	*Chrysops* spp.	North America, Russia, Japan	Mechanical	Humans and small mammals (rabbits, hares etc)
***Bacillus anthracis* / Anthrax**	*Tabanus, Haematopota, Chrysops* spp.	Worldwide	Mechanical	Humans, wild or domestic livestock, such as sheep, cattle, equids and goats
Protozoa				
***Besnoitia besnoiti* / besnoitiosis**	*Tabanus, Atylotus* spp.	South America, Southern Europe, Africa, Asia,	Mechanical	Livestock, deer, small carnivores, rodents, reptiles
***Trypanosoma evansi* / Surra**	*Tabanus, Haematopota, Chrysops* spp.	South America, North Africa, Asia, India	Mechanical	Domestic animals, dromedaries, cattle, equids and dogs
***Trypanosoma vivax* / Trypanosomosis**	*Tabanus* spp.	South America, Africa	Mechanical	Domestic and wild mammals
Filarial nematodes				
***Loa loa* / Loa loa filariasis**	*Chrysops* spp., esp. *C. dimidiatus, C. silaceus*	Central Africa	Biological	Humans
Elaeophora schneideri	*Hybomitra, Tabanus* spp.	North America, southern Europe	Biological	Deer, elk and sheep

3.1.3 Trapping biting flies

Several different styles of biting fly traps have been developed; this project focuses on 'Nzi' traps. Nzi (Swahili for fly) traps were designed originally in Kenya to trap tsetse flies. Traps were required to aid in controlling the spread of "sleeping sickness" also known as African trypanosomiasis, caused by the parasite *Trypanosoma brucei*, in sub-Saharan Africa. This trap was shown to be up to eight times more effective at trapping Stomoxys than their previous method (Mihok, 2002).

The traps are specially designed to attract large biting flies that select and locate their prey by movement and colour contrast rather than smell (Vaduva, 2020). The trap relies on species-specific attack behaviours and preference for feeding sites on mammals. Horse flies predominantly attack the under-belly area or legs. The upper bright blue is attractive to the fly and the lower black material mimics the shadow created by the underbelly of a large mammal (Figure 14). The fly enters from below, becomes trapped in the netting and flies up to escape becoming trapped in the capture bottle attached on top.



Figure 14 Front view set up of NZI trap before capture bottle is attached

Flies can be collected alive or dispatched and stored in solution. The ideal technique is dependent on the downstream intended use of the samples. Live collection allows for the dispatch of flies to be delayed until immediately prior to storage in -80°C or DNA/RNA preserving solutions thus preventing nucleic acid degradation, as would be present with traps that immediately kill the flies. Flies that trap within solution are a good choice for population counts as they prevent the possibility of escape.

3.1.4 Relative synonymous codon usage (RSCU)

The comprehensive analysis of viral transmission pathways often involves a multi-pronged approach, blending field observations with genetic investigations. As earlier explored through fly trapping methodologies, the identification of potential vectors forms a fundamental aspect in understanding the ecological interactions of viruses. These preliminary observations serve as a starting point in for subsequent genetic analyses, such as investigating codon usage bias.

Codon usage bias delineates the preferential utilisation of specific codons encoding identical amino acids within the genetic code of an organism. Within viruses, this bias serves as a proxy to ascertain adaptation to the host's cellular machinery. Through meticulous analysis of codon usage patterns, insights into a virus's host specificity emerge. Should a virus display a codon usage pattern closely mirroring that of a particular host, it implies a stronger affiliation or adaptation to that specific host. Such analyses also unveil clues regarding a virus's potential to engage multiple hosts within its life cycle. Furthermore, an in-depth examination of codon usage bias sheds light on the virus's propensity to exploit diverse species as biological vectors or intermediate hosts during transmission and life cycle progression. This interplay between field-based identifications of vectors and genetic examinations offers a crucial framework to decipher viral adaptations and transmission dynamics across various hosts within the ecosystem.

Codons are sets of 3 DNA base pairs used to code for one of 24 amino acids. Each amino acid can be coded for by more than sequence of DNA or codon. Which codon is selected within the genes is somewhat down to chance, however, organisms have 'bias' for selecting some over others. This is known as synonymous codon usage bias (CUB) and has been investigated in many organisms, and generally exists in prokaryotes and eukaryotes.

Some codon bias is for functional reasons, such as codons containing TpA dinucleotides due to the destabilizing effect of TpA on RNA (Duret and Galtier, 2000). Additionally, codons that are one mutation away from becoming a stop codon (a one-to-stop codon, OTS). Both TpA-containing codons and OTS codons are avoided in vertebrates and invertebrates.

Largely, the variability within codons, is in the third base position, often referred to as the 'wobble base' as proposed by Francis Crick (Doherty and Guo, 2016).

CUB also allows organisms to recognize codons as foreign if they encounter DNA or RNA that does not coincide with the codon bias preferences of their particular species.

As viruses utilize host machinery for propagation, close interactions with host cells are a necessity. Therefore, co-evolution occurs, and viruses mimic codon usage patterns similar to their target host to aid in preventing detection by the host's immune system.

Relative synonymous codon usage (RSCU) is a method for analysing CUB and was developed by Sharp and Li (Sharp et al., 1986). It can be used to determine the level of bias in the use of codons for a particular amino acid.

RSCU is calculated by dividing the observed number of occurrences of a particular codon by the expected number of codons if there was no bias. The expected value is calculated as the total number of codons that result in the amino acid of the codon being investigated divided by the codon degeneracy (number of codons resulting in that amino acid) as shown in the equation below. Codons with RSCU values above 1.0 are regarded as having positive codon usage bias.

$$RSCU_{ij} = x_{ij} / \frac{1}{n_i} \sum_{j=1}^{n_i} x_{ij}$$

It is evident that EqHV can be transmitted via the blood as transfusion is used in experimental infection studies (1.10.1). However, neither blood transfusion nor use of contaminated needles, as is the cause for HCV in people, is likely to explain the high prevalence of EqHV observed in equids. Although only one study has been performed (Badenhorst et al., 2019b), there was no evidence that mosquitos transmit EqHV. The observation (Chapter 2) that infection of equids at a rescue centre coincided with the height of the horse fly season suggested that horse flies might be a vector for this virus. Viruses with insect mechanical vectors do not utilise the insect cells in the viral replication phase, therefore a CUB similar to insects would not be beneficial or expected.

3.2 Aims

The primary goal of this chapter was to investigate the plausibility of biting flies serving as vectors for the transmission of EqHV. Building upon the data generated in Chapter 2, this research aimed to explore the seasonal pattern of EqHV infections identified at Bransby.

Using a multidisciplined approach comprising of fieldwork, *in vivo* experiments using laboratory based captive biting fly populations, and bioinformatics analyses, this chapter aimed to unravel the potential role of biting flies in EqHV transmission. Furthermore, the integration of bioinformatics tools aims to discern any genetic associations or patterns between EqHV prevalence and biting fly activity.

3.3 Methods

3.3.1 Codon Analysis

A list of viral DNA sequences was obtained (Lobo et al., 2009), those available were collated. Accession codes were compiled into plain text format, https://www.ncbi.nlm.nih.gov/sites/batchentrez website was used to search and identify all codes needed. Only the CDS regions were download in FASTA format and imported into MEGA. Statistical codon analysis was performed on groups of viruses according to type.

3.3.2 *In vitro* mechanical transmission

The *in vitro* mechanical transmission method outlined in Figure 15 utilised a plasmid containing EqHV Core-E1 PCR product in PGEMT-easy vector (as made by Terry Akagha (Akagha, 2021). This was added to whole horse blood at a concentration of $1x10^6$ copies/ml. Cotton wool balls were soaked in the blood before being provided to *Stomoxys calcitrans* to feed on for 20 minutes prior to dispatch by placing at -20°C for 30 minutes. Flies were transferred into universal tubes using sterile forceps, containing 250 µl of wash solution. Five different wash solutions were tested: 70% ethanol, water, RNA later, phosphate buffered saline (PBS), PBS with RNA later added after the fly was washed.

Three different methods were trialled for the washing technique. H- The mouthparts and head only were submerged, B- the body only was submerged, WB- the whole body was submerged and the tube vortexed briefly.

The above section of work was kindly completed by Dr Jess Stokes at the Pirbright Institute. The wash solutions were shipped to University of Nottingham School of Veterinary Medicine and Science by courier on dry ice. DNA extraction was performed on the wash solutions using DNAeasy blood and tissue kit (QIAGEN) according to manufacturer's instructions. DNA elutions were assessed via qPCR as described in section 2.3.2.5.

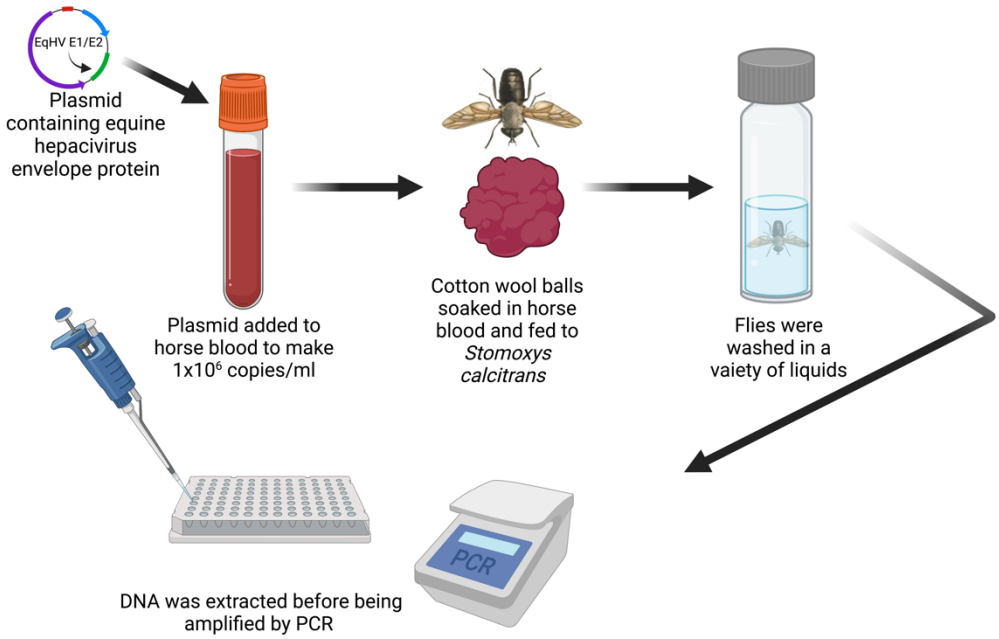


Figure 15 Schematic depicting the method utilised for theoretical in-vitro mechanical transmission of equine hepacivirus A by Stomoxys calcitrans

3.3.3 Fly trapping for identification

NZI traps were set up in several locations across both the Bransby Horses Rescue and Welfare main site comprised of >600 acres, (Figure 16) and the secondary Barlings site comprised of >800 acres (Figure 17) in Lincoln, UK. Traps were pegged in each corner with electric fencing posts and supported with a wooden post in the centre (Figure 18 & Figure 19). For the capture bottles, 2 litre plastic bottles were cut circumferentially around 4–5 inches from the top. The top section was rotated 180 and inserted into the bottle cavity to form a funnel and held in place with strong gaffer tape (Figure 20). The capture bottle was placed over a small shallow plastic funnel sewn into fabric of the trap and taped in place. Traps were set up in between biting fly resting areas (primarily thick hedgerows) and horses.

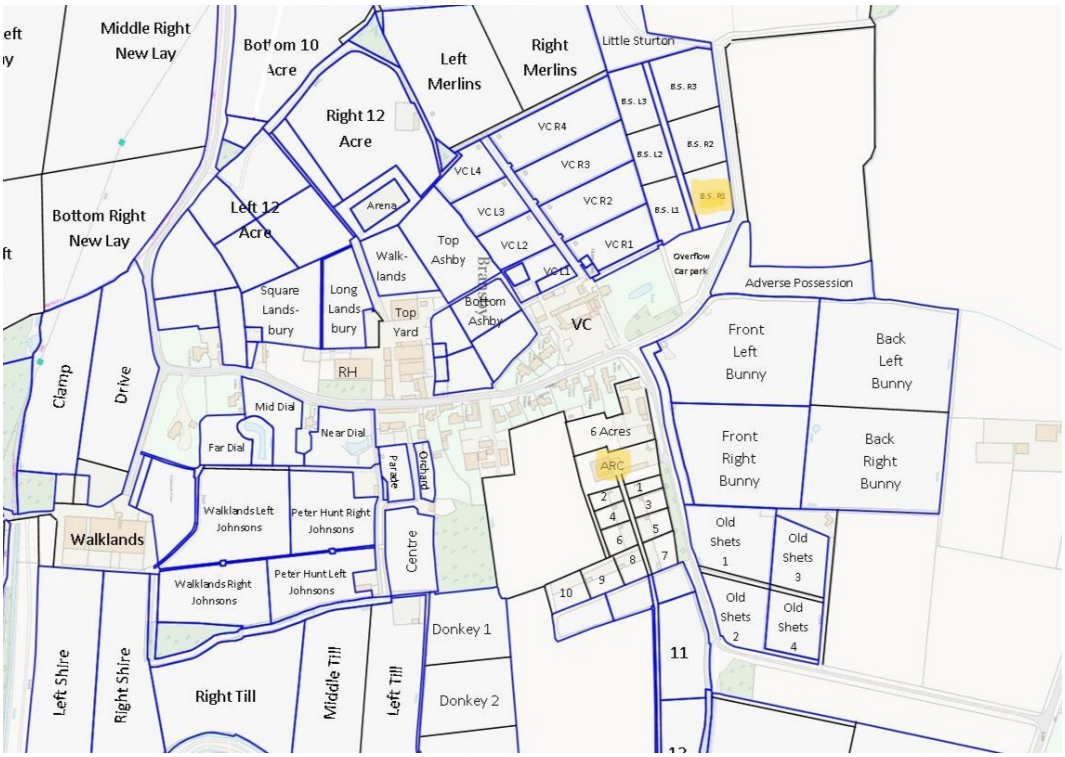


Figure 16 Map of Bransby main site, new animal intake occurs at the ARC, highlighted in yellow.



Figure 17 Map of Bransby horses secondary Barlings site.



Figure 18 Front view set up of NZI trap before capture bottle is attached.



Figure 19 Side view of NZI trap in situ set up with capture bottle live for trapping.



Figure 20 Fly capture bottle made using a plastic bottle cut in half and inverted, ready for attachment to the NZI trap.

3.3.4 Live fly trapping on animals

A small clear plastic funnel was used to trap live flies whilst directly feeding on horses at the Bransby Horses Rescue and Welfare sites. When a biting fly was observed landing on a horse, the plastic funnel was slowly lowered, the largest side facing the horse, from directly above to trap the fly. Round ended tweezers were inserted through the smaller opening in the funnel to grasp the fly. The fly was then transferred into a universal tube containing 200 µl of washing solution, comprised of 70% ethanol and RNAlater. The tube was inverted vigorously several times for 1 minute. The immobilized

fly was then removed with tweezers and placed into a second universal tube containing 5 ml of 70% ethanol.

RNA was extracted from the tubes containing the fly wash using the method in 2.3.2.1, cDNA was created as per 2.3.2.2 and RT-qPCR was performed as per 2.3.2.5.

3.4 Results

3.4.1 Codon Analysis

Viruses were grouped by mode of transmission and their RSCU values plotted on a superimposed scatter dot plot (Figure 21). Codons with an RSCU over 1 have a positive bias. The codons GGA and UCA had a strong positive bias in all 3 insect vector groups of viruses. EqHV did not have a positive bias for either of these codons.

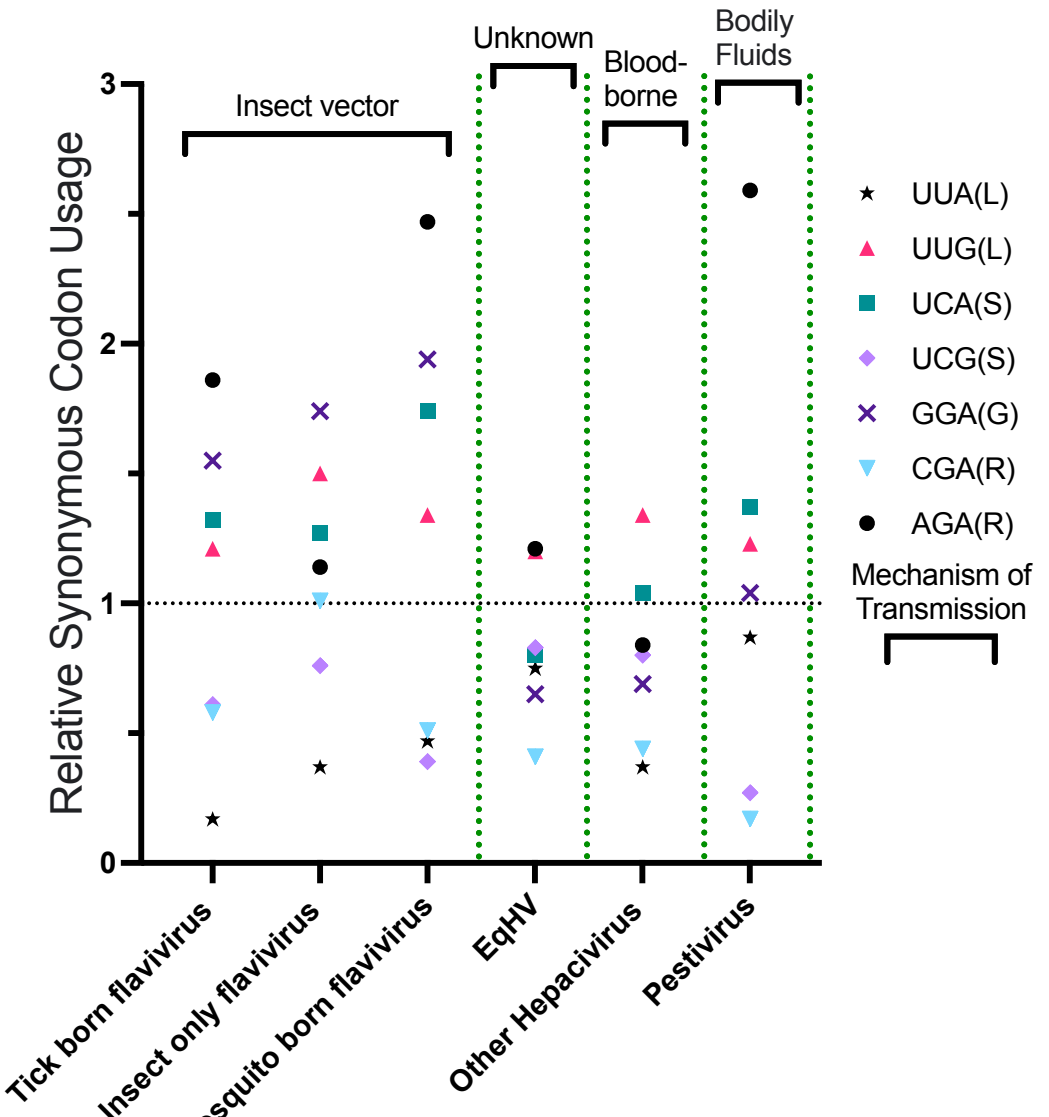


Figure 21 Plot displaying the relative synonymous codon usage for groups of viruses with different mechanisms of transmission.

3.4.2 *In vitro* fly transmission

RT-qPCR successfully detected the presence of EqHV plasmid in several of the experiments (Figure 22). Positive bands on the agarose gel could be seen for whole body wash in 70% ethanol, body and whole-body wash with RNA later, whole body with water and head, body and whole body with PBS and RNAlater.

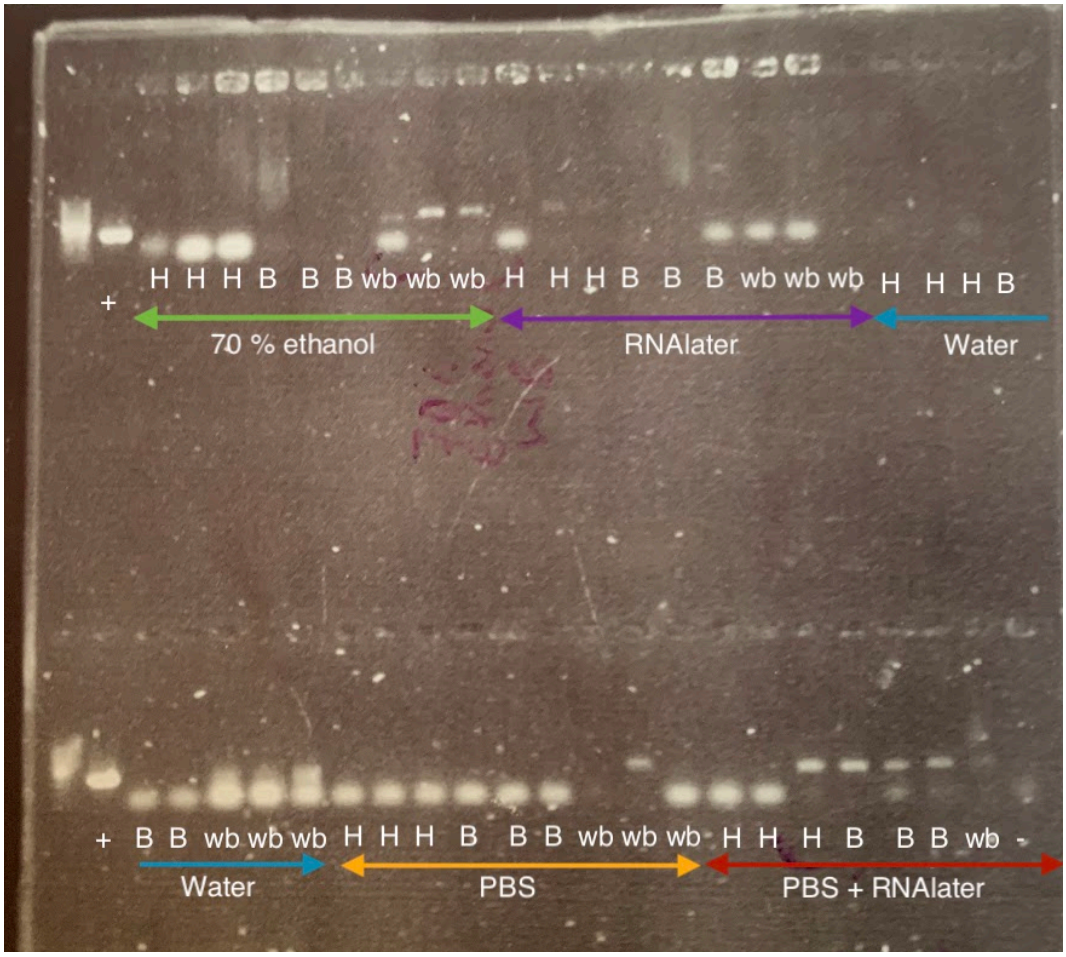


Figure 22 Agarose gel containing RT-qPCR products of fly wash. + = Positive control plasmid, B = Body of fly was washed, H = Head of fly was washed, wb = Whole body of fly including head was washed, - = Negative control (nuclease free water)

3.4.3 *In vivo* fly trapping

Four flies were successfully collected off live horses, one horsefly, one cleg and two deer flies. All flies screened negative for EqHV via qPCR. Flies were formally identified by entomologist Dr Jessica Stokes using the keys of Stubbs and Drake (Stubbs and Drake, 2014)to be as follows:

Tabanus autumnalis also known as the large marsh horsefly (Figure 23), identifying features: no hairs on the eyes, no ocellar tubercle (Figure 24). Wing length ~16 mm, whole body length ~18 mm (Figure 25). Dark stripe down centre of sternites (the ventral part of each body segment).

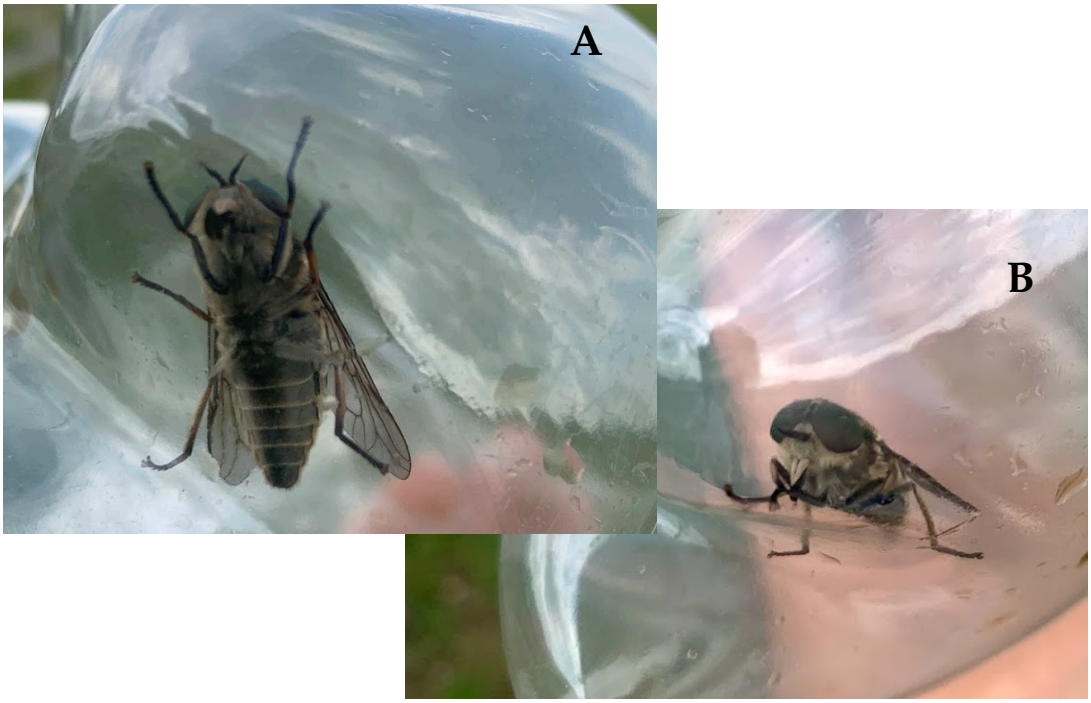


Figure 23 Ventral (A) and anterior (B) view of a Tabanus autumnalis specimen trapped in the field.



Figure 24 Antennae on Tabanus autumnalis.

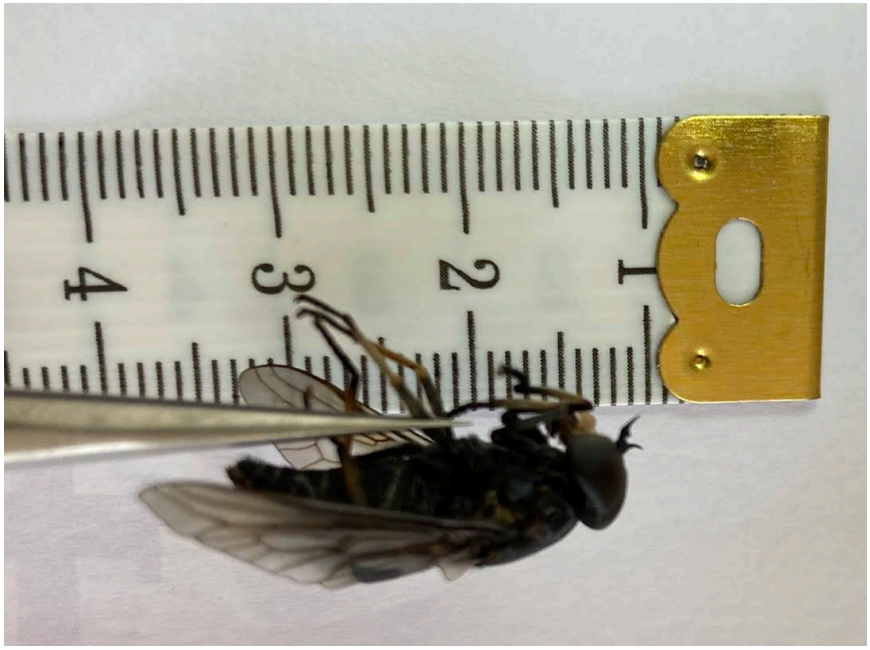


Figure 25 Tabanus autumnalis with scale in cm

The deerflies caught were pertaining to two different species. Both have yellow/orange and black patterns on their abdomens (Figure 26). One is of the species *Chrysops relictus* (twin-lobed deerfly) as it has orange hind tibia and half an upturned heart shape on the second tergite (the dorsal side of the body segments) (Figure 30). The other is of the *Chrysops caecutiens* (splayed deerfly) as it has black hind tibia and an upside-down Y on the second tergite (Figure 29).



Figure 26 Two different species of deer fly caught from live trapping off horses at Bransby. Left: Chrysops caecutiens Right: Chrysops relictus



Figure 27 Colourful eye striations on Chrysops



Figure 28 Colourful eye striations on Chrysops and large mouth parts



Figure 29 Chrysops caecutiens (splayed deerfly) species indicative black hind tibia



Figure 30 Chrysops relictus (twin-lobed deerfly) species indicative orange hind tibia

Unfortunately, adverse weather conditions characterized by low temperatures, high winds, and frequent rain prevailed during the trapping period. Regrettably, no viable insects were successfully captured using the NZI traps throughout the study duration.

3.5 Discussion

3.5.1 Codon usage bias

Codon usage analysis revealed the codon usage of EqHV aligns closer with viruses that do not use insects as biological vectors. GGA and UCA have a strong positive codon usage bias in insect born flaviviruses which is not found in other hepaciviruses and pestiviruses, EqHV also does not have a positive bias for GGA and UCA (Figure 21). This is further supporting evidence suggesting EqHV is not biologically vector born.

3.5.2 Fly trapping

NZI traps failed to collect many specimens. Several issues contributed to this. Weather conditions during the summer of 2021 were cooler, wetter and windier than usual, all conducive of low levels of horse fly activity. Locations were carefully selected to be in the most likely flight path of tabanids. Traps were placed along most likely travelled paths between resting areas and near hosts.

These hunting areas selected were horse paddocks, this proved problematic as once the horses became accustomed to the traps they often wanted to investigate or play with the traps. This resulted in the traps frequently being knocked down and loss of the collection.

'Mid bite' trapping method in 3.3.4 was implemented alongside static traps to provide the freshest blood meal specimens. Flies caught in traps would be captive but live for a period of time between daily trap checks, this may

allow flies to clean their mouthparts of infected blood or allow EqHV RNA to degrade due to temperature or UV exposure.

Specimens that were collected were identified to be specimens of tabanidae, chrysops and Stomoxys and were all common to the area. Specimens collected were tested via qPCR for EqHV but yielded no positive results. However, this was a small pilot study with very few specimens captured.

3.5.3 In vitro fly transmission:

During the trapping period, several challenges emerged that influenced the outcomes. Unforeseen weather conditions, including heavy rainfall and unfavourable temperatures, impacted fly activity and reduced trapping success. Additionally, the trapping sites were accessible to horses, resulting in disturbances and trap displacement, potentially affecting the captured fly population. To mitigate this, future trapping efforts would require fencing around the trap areas to prevent interference by animals. Moreover, logistical constraints induced by COVID-19 impeded regular travel and field checks, limiting the ability to consistently monitor traps and gather an adequate number of specimens.

The *in vitro* EqHV transmission experiment demonstrated it is plausible EqHV could be transmitted mechanically via *Stomoxys calcitrans*. Four of the five methods trialled detected positive EqHV plasmid. The plasmid was diluted to a concentration of copies per ml similar to that of virus particles found in blood samples from naturally infected equids.

Only *Stomoxys calcitrans* were tested for mechanical transmission potential, although this is not the only biting fly species found in the UK, this is the only species available to test there are no other colonies of species available in a controlled lab setting. Tabanid mating occurs in flight, especially in the morning, but has never been induced in the laboratory. This is a key barrier to colonisation, lack of colonisation is a barrier to infection studies (Mullens, 2018). This limitation extends further to the inadequate representation of Tabanid species in captivity and the scarcity of recent literature, impeding comprehensive research into this area of disease transmission.

The use of NZI traps to assess for EqHV may bias the population caught. Species-specific attack behaviours contribute to the disproportionate collections of certain tabanid species in traps. For example, deer flies usually feed high on the body, especially on the head or shoulders, and are poorly represented in canopy or box traps that require entry from below. Horse flies feed in various areas on the host depending on the species. Legs are favoured feeding sites for many horse fly species that attack livestock.

Additionally, the reliability of testing EqHV in biting flies captured by NZI traps might be compromised due to the nature of EqHV as an RNA virus. Given its susceptibility to rapid degradation outside a host, there's a likelihood that viral particles could degrade on the mouthparts of flies. Furthermore, the flies might have had opportunities to clean residual blood off their body and mouthparts, potentially affecting the detectability of EqHV.

Given the limitations encountered, there is a need for future studies employing larger-scale trapping methods and intensified in-lab

investigations akin to those detailed in section 3.3.2. Notably, precautions such as securing trapping sites against animal interference and increased monitoring could enhance trapping efficacy. Furthermore, establishing captive colonies of diverse biting fly species for comprehensive studies would be beneficial. Lastly, considering the current constraints, further research should endeavour to diversify vector species for EqHV and investigate potential infection among equid hosts.

Chapter 4: Development of a novel ELISA for Equine hepacivirus

4.1 Introduction

4.1.1 Antibody monitoring

Determining the level of EqHV antibodies in populations of horses will add to the development of epidemiological studies and is key to the overall understanding of the virus. Antibody data are also needed for potential vaccine development.

From a veterinary perspective, establishing antibody levels in an individual is also important. There have been several cases of chronic infection with EqHV (Gather et al., 2016a; Tegtmeyer et al., 2019). Determining the antibody levels and viral load is not only vital in these individual cases, but will provide insight into the overall frequency of these chronic infections.

As stated in Chapter 1 section 1.12, currently the most common EqHV antibody detection system is a luciferase immunoprecipitation system (LIPS) assay. This is a complicated and expensive non-standard assay which cannot be replicated in a standard veterinary diagnostic laboratory. Therefore, it cannot be used diagnostically, although is used predominantly for research purposes into EqHV.

A pseudovirus neutralisation test (PVNT) has been developed by colleagues at UoN (Akagha, 2021). Although effective it is also a highly

specialised and complicated assay to implement for purely diagnostic purposes. Evidently, a more straightforward assay to measure antibodies is needed.

4.1.2 Aims

The primary aim of this chapter was to develop an ELISA method optimised for the accurate quantification of EqHV-specific antibodies utilising plant-expressed proteins. The initial phase involved evaluating and critically assessing the limitations inherent in prevalent techniques used for detecting EqHV antibodies. Through this critical analysis, the chapter aimed to identify and scrutinise the shortcomings of existing methodologies, thereby providing a foundation for ensuing advancements. The subsequent focus was to address these identified limitations by introducing a novel ELISA protocol tailored to enhance the precision, sensitivity, and specificity in detecting EqHV antibodies.

4.1.3 Overview of plant expression systems

Transgenic plants were first used to express antibodies in 1989 (Hiatt et al., 1989), until a Hypertrans® system was later developed for the utilisation of plants for transient expression of proteins, by Professor George Lomonossoff and Dr Frank Sainsbury at the John Innes Centre (Sainsbury et al., 2007). Transient expression using a plasmid and agrobacterium offers an easier and faster way to manipulate DNA and obtain proteins than with transgenic plants. It is also a cost-effective and higher yielding solution for protein production (Komarova et al., 2014). This method offers a functional advantage as many proteins that are toxic to mammalian or bacterial cells, can be successfully expressed in plants (Gengenbach et al., 2019). Additionally, plant expression is a safer method for production of pharmaceutical proteins as there is a lower risk of contamination and plant pathogens do not infect humans or mammals (Lico et al., 2008). Furthermore, plants are a eukaryotic system, unlike *Escherichia coli* (*E. coli*), meaning they are able to perform some of the complex processing required by some proteins for full functionality.

4.1.3.1 Expression vector pEAQ

The pEAQ vectors are a series of small plasmids, tailored for transient expression. The pEAQ-HT expression vector was developed utilising the cowpea mosaic virus (CPMV) hyper-translational expression system. This provides a high yield of recombinant protein without the need for viral replication *via* high translational efficiency (Peyret and Lomonossoff, 2013).

The vector also has the advantage of the option to fuse a C- or N- terminal histidine (6XHis) tag to the target sequence (Sainsbury et al., 2009) allowing for easy purification.

4.1.3.2 Uses of plant expressed proteins

Plant-made recombinant proteins generally fall into one of three categories (Xu et al., 2018): biopolymers, industrial proteins/enzymes and therapeutic proteins.

Biopolymers are often recombinant protein-based biopolymers such as elastin-like polypeptides (ELPs), spider silk proteins, collagens, and plant gums. Collagen is traditionally extracted from cadavers or animal sources. However, recombinant human collagen type I is now being produced in tobacco plants (Shilo et al., 2013).

Industrial proteins/enzymes are most commonly hydrolases - enzymes which catalyse the cleavage of a covalent bond using water. Corn seed is often used as a platform for industrial protein/enzyme production due to having the highest potential recombinant protein yield per hectare (Christou et al., 2008).

Finally, transgenic plants can produce fully functional mammalian proteins which may be used for therapeutic purposes. These include blood proteins, vaccine antigens, monoclonal antibodies (mAbs), cytokines, therapeutic enzymes, growth factors, and growth hormones (Davies, 2010; Xu et al., 2011). Plants can produce lower-cost, safer therapeutic proteins than mammalian cells and sometimes may be the only production system available, e.g., for production of secretory antibodies (Paul and Ma, 2011).

4.2 Methods

4.2.1 Blood Sample Collection

All equine blood samples were collected by venepuncture of the jugular vein as part of routine diagnostic testing by the attending veterinary surgeon as described in Chapter 2 section 2.3.1. Additional serum samples were available including:

(i) Horse H, identified as a chronic EqHV case with multiple positive results for EqHV RNA PCR tests over a period of 12 months with clinical signs including lethargy and various abnormal blood parameters, horse H was used as a positive control.

(ii) Pony O, used in other equine experimental studies.

(iii) Thoroughbred horses that were previously screened by GNA ELISA (Akagha, 2021)

(iv) commercially available equine serum.

(v) Human sera positive for Hepatitis C Virus, donated by Dr Alexander Tarr from QMC archives.

4.2.2 Cell Culture

Huh 7 cells (p4 when removed from liquid nitrogen) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated fetal calf sera, 1% Penicillin-Streptomycin and 1% L-glutamine. Cells were initially cultured in T25 flasks for 3 days, until 70% confluent, before being resuspended with 0.25% Trypsin/EDTA (TE) and split 1:3 into T75 tissue culture flasks with fresh DMEM. These were grown for a further 48 h, until 70% confluent, before being resuspended with TE and split 1:3 into T175 tissue culture flasks with fresh DMEM. After 48 h, when the cells were between 75 and 85% confluent, some cells were used in neutralisation assays and any spare were centrifuged and diluted with freezing media, before storage in liquid nitrogen.

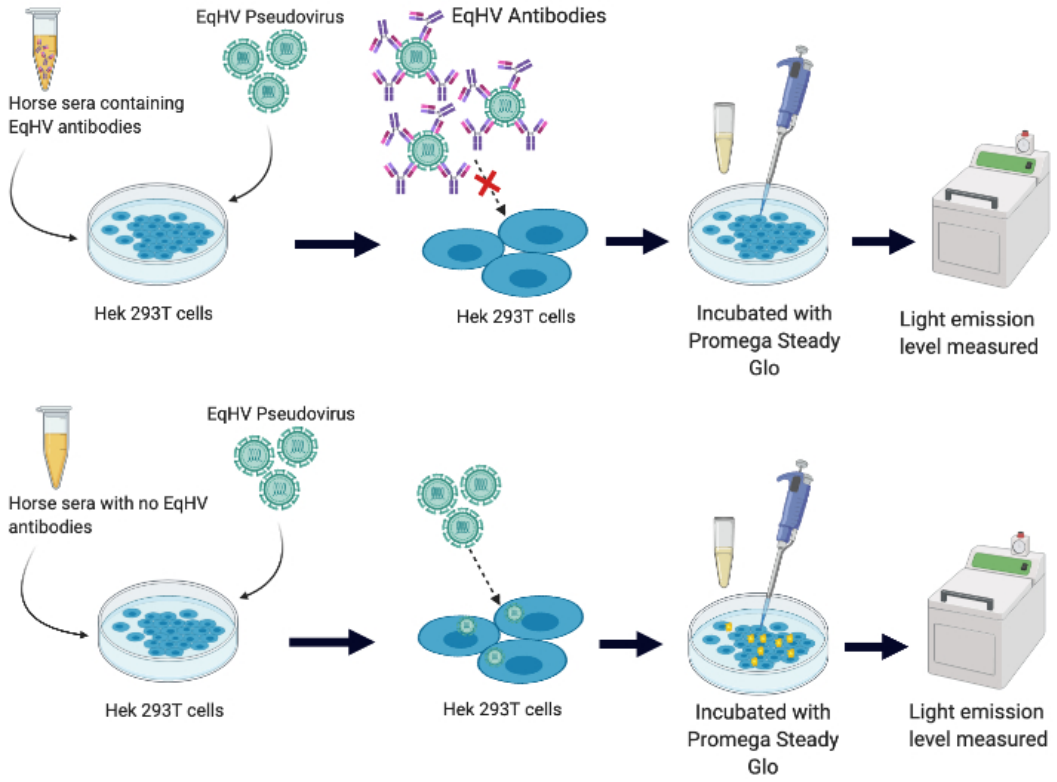
4.2.3 Pseudovirus Neutralisation Test

Pseudovirus particles with the EqHV envelope proteins (E1/E2) on a non-infectious HIV core, and a luciferase gene flanked by HIV long terminal repeat regions were generated by Dr Alexander Tarr (School of Life Sciences, University of Nottingham). White flat bottom 96 well plates were seeded with Huh 7 cells at a concentration of $1.5x10^4$ per well and incubated at 37°C, 5% CO_2 overnight. Sera was diluted 1:50 then a ratio of 9:1 pseudovirus particle: sera was made up. A second volume of the same ratio was made up using a pseudovirus (Δ envelope virus) with no protein on its surface as a negative control. Pseudovirus/sera mixture was incubated for 1 hr at 37°C.

Existing media was removed from the plate and 100 µl of pseudovirus/sera mixture was added to each well in duplicate. The plate was incubated for 4-6 h at 37°C, 5% CO_2. Media was replaced with 100 µl per well of DMEM and incubated at 37°C, 5% CO_2 for 72 h. Media was removed and 50 µl of Promega Steady-Glo® reagent was added and incubated for 5 min (

Figure 31). The plate was then read on a Geneflow Orion L microplate luminometer.

Figure 31 Method and functionality of equine hepacivirus pseudovirus neutralisation assay with equine sera, both positive and negative for equine hepacivirus antibodies.



4.2.4 *Galanthus nivalis* agglutinin (GNA) Assay

Equine sera were screened for the presence of antibodies using a lectin capture ELISA (

Figure 32). Human endothelial kidney (HEK) cells were transfected with a plasmid containing the EqHV E1/E2 genes under control of the CMV immediate-early promoter. As a control, cells were also transfected with a plasmid that does not encode the E1/E2 genes. Seventy-two hours after transfection, cells were lysed with a 50 mM tris-HCl buffer (pH 7.6) containing 1 mM EDTA, 1% Igepal-650 and 150 mM NaCl. The lysate was collected and centrifuged to remove membranes. Lyophilised lectin from *Galanthus nivalis* (snowdrop) (Sigma-Aldrich L8275) was resuspended in PBS at 10 mg/ml then 2.5 µl was added to 5ml of carbonate/bicarbonate buffer (Sigma-Aldrich C3041) to make a coating buffer.

Wells of a Nunc MaxiSorp plate were coated with 50 µl of coating buffer per well and left to incubate overnight at 4°C. Coating buffer was discarded, and the plate tapped dry on paper towel before adding 200 µl of blocking buffer, containing 1% bovine serum albumin (BSA) and 3% non-fat milk made up in PBS-0.1% Tween and incubated for 1 hour at room temperature. Blocking buffer was discarded and wells were washed once with 200 µl of PBS-0.1% Tween. Both EqHV E1/E2-containing cell lysate and a negative control cell lysate diluted 1:10 in blocking buffer were added into an equal number of wells and incubated for 1 hour at room temperature. Wells were washed twice with PBS-0.1% Tween. Horse sera was heat inactivated at

56°C for 30 minutes and diluted 1:100 in blocking buffer, then 50 µl of each sample was added to three wells containing E1/E2 and three wells containing the negative control and incubated for one hour at room temperature.

Wells were washed three times with TBS-0.1% Tween and vigorously tapped on a paper towel until dry. Anti-horse IgG-Alkaline Phosphatase (Sigma A6063) was diluted 1:1000 in TBS-0.1% Tween before being added at a volume of 50 µl to each well and incubated for 45 minutes at room temperature. Following a final three washes with TBS-Tween, 100 µl of p-nitrophynyl phosphatase (pNPP) substrate (Sigma) was added and incubated at room temperature for 10 minutes. After incubation, the plate was read using a BMG Labtech Omega spectrophotometer, reading at a wavelength of 405 nm.

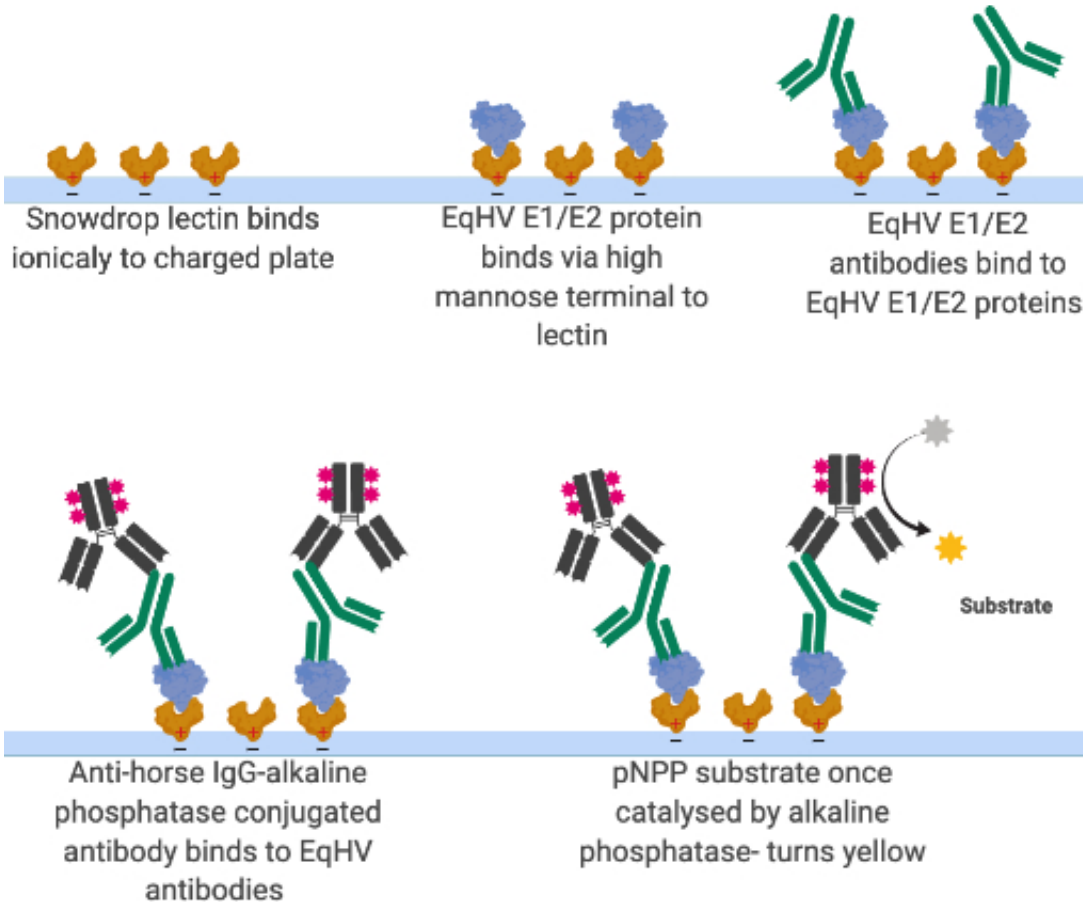


Figure 32 The process of the Galanthus nivalis lectin capture assay with equine sera containing equine hepacivirus antibodies.

4.2.5 HCV peptide ELISA

Two non-biotinylated peptides, HCV peptide 370c or rabies peptide 1074 (used as a control) were diluted to 1 µg/ml in carbonate/bicarbonate buffer, 100 µl of this peptide coating buffer was added to wells of a Nunc Maxisorp plate (Sigma; cat no. M9410) and incubated at room temperature for 1 hour. Coating buffer was then discarded, and excess peptide buffer was removed by tapping dry on a paper towel. Wells were then blocked with 250 µl of blocking buffer containing TBS, Tween 0.05% and BSA 3% and incubated at room temperature for 2 hours. The plate was washed four times with 300 µl of TBS-Tween (TBS-T) and tapped dry. Serum samples were heat inactivated at 56°C for 30 minutes (human HCV positive sera was donated by Dr Alexander Tarr for use as a positive control) were diluted 1/100 in TBS-T and incubated at room temperature for 2 hours. The sera were then discarded, and the plate was washed four times with 300 µl of TBS-T and tapped dry. Anti-mouse IgG conjugate (Alkaline phosphatase bound) was diluted 1/1000 in TBS-T, 50 µl was added per well and incubated at room temperature for 1 hour.

Wells were then washed four times with 300 µl of TBS-T before 100 µl of p-nitrophynyl phosphatase (pNPP) substrate (Sigma) was added and incubated at room temperature for 15 minutes. After incubation, the plate was read using a BMG Labtech Omega spectrophotometer, reading at a wavelength of 405 nm.

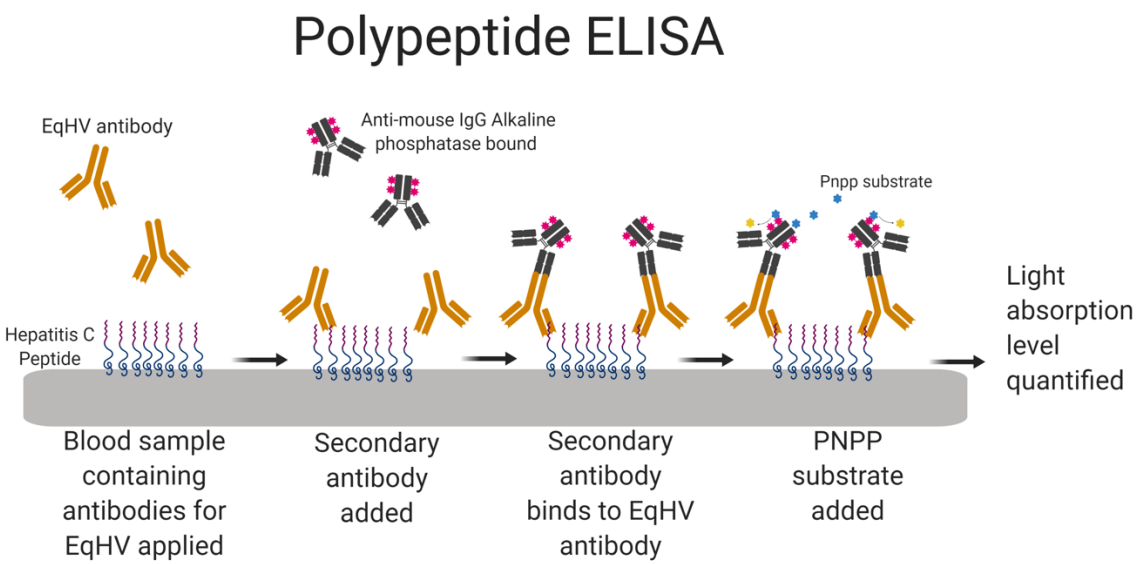


Figure 33 Process of polypeptide ELISA using hepatitis C peptides to detect equine hepacivirus antibodies.

4.2.6 EqHV RNA gene selection and modifications

4.2.6.1 E1/E2 gene

The E1/E2 gene of the EqHV virus was selected for expression and use in the ELISA. As the E1 and E2 genes code for envelope proteins which are expressed and presented on the surface of the virus. These proteins are most likely to have antibodies raised against them by the immune systems of in infected individuals. The selected section was aligned against all existing strains in the GenBank database and found to be almost 100% conserved.

4.2.6.2 NS3 Gene

A section of the NS3 gene that was used for development of the LIPS assay as described in Burbelo et al. (2012) was also used as the basis for an ELISA. Alignments were done on this specific section to create a consensus. The initial alignment consisted of the 8 sequences acquired only from Burbelo et al. (2012) - accession numbers JQ434001 to JQ434008. A secondary alignment containing 22 sequences was completed and deemed to give a better consensus with fewer 'n' base pair results. Alignment was performed by the DNASTAR MegAlign Pro software. The sequence was translated using "Expasy translate" software (Figure 34).

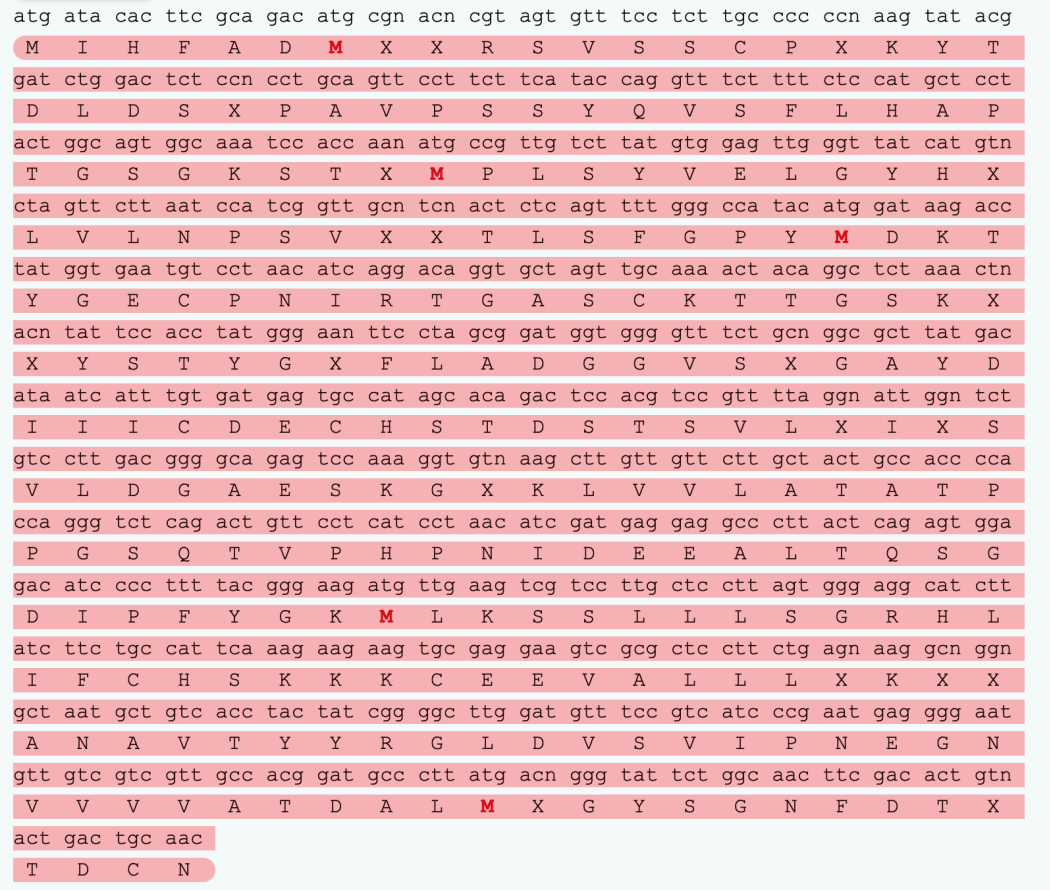


Figure 34: NS3 consensus segment protein alignment.

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
A	MH027992.1		85.19	85.19	85.06	83.80	83.92	85.06	83.54	83.04	85.19	85.19	85.19	83.92	92.15	85.06	83.54	84.43	84.43	84.43	85.95	86.46	93.04
B	MH027996.1	85.19		91.39	91.77	90.38	90.13	89.75	90.13	84.94	91.39	91.65	91.65	89.37	85.32	90.63	89.87	89.11	89.11	89.11	88.10	89.24	85.44
C	MH027998.1	85.19	91.39		90.76	88.35	88.35	89.87	88.10	85.06	100.00	90.63	90.63	87.34	85.32	89.62	89.75	89.62	89.62	89.62	87.47	86.96	85.32
D	MH027999.1	85.06	91.77	90.76		90.51	90.00	90.63	89.75	84.56	90.76	99.87	99.87	89.62	85.19	89.62	89.87	89.37	89.37	89.37	86.96	87.59	85.95
E	MH028007.1	83.80	90.38	88.35	90.51		98.73	88.48	94.30	85.70	88.35	90.38	90.38	88.35	85.70	88.23	88.35	89.37	89.37	89.37	89.37	90.00	86.08
F	MH027993.1	83.92	90.13	88.35	90.00	98.73		88.48	93.29	85.06	88.35	89.87	89.87	87.85	85.82	87.97	87.85	89.24	89.24	89.24	89.49	90.13	85.95
G	MH028000.1	85.06	89.75	89.87	90.63	88.48	88.48		87.97	84.81	89.87	90.51	90.51	87.97	85.57	91.77	91.39	88.61	88.61	88.61	86.84	86.71	84.18
H	MH028005.1	83.54	90.13	88.10	89.75	94.30	93.29	87.97		85.32	88.10	89.62	89.62	88.35	85.70	88.73	88.10	88.23	88.23	88.23	87.59	87.59	84.81
I	MH028004.1	83.04	84.94	85.06	84.56	85.70	85.06	84.81	85.32		85.06	84.43	84.43	83.92	84.94	85.06	85.19	84.05	84.05	84.05	82.78	83.67	82.91
J	MH027995.1	85.19	91.39	100.00	90.76	88.35	88.35	89.87	88.10	85.06		90.63	90.63	87.34	85.32	89.62	89.75	89.62	89.62	89.62	87.47	86.96	85.32
K	MH028001.1	85.19	91.65	90.63	99.87	90.38	89.87	90.51	89.62	84.43	90.63		100.00	89.49	85.06	89.49	89.75	89.24	89.24	89.24	87.09	87.72	85.82
L	MH028002.1	85.19	91.65	90.63	99.87	90.38	89.87	90.51	89.62	84.43	90.63	100.00		89.49	85.06	89.49	89.75	89.24	89.24	89.24	87.09	87.72	85.82
M	KJ472766.1	83.92	89.37	87.34	89.62	88.35	87.85	87.97	88.35	83.92	87.34	89.49	89.49		83.67	88.73	88.48	87.09	87.09	87.09	86.20	86.08	84.43
N	MN734124.1	92.15	85.32	85.32	85.19	85.70	85.82	85.57	85.70	84.94	85.32	85.06	85.06	83.67		84.81	84.18	85.06	85.06	85.06	86.08	86.58	90.51
O	KX421286.1	85.06	90.63	89.62	89.62	88.23	87.97	91.77	88.73	85.06	89.62	89.49	89.49	88.73	84.81		96.46	88.35	88.35	88.35	86.46	86.08	83.54
P	KX421287.1	83.54	89.87	89.75	89.87	88.35	87.85	91.39	88.10	85.19	89.75	89.75	89.75	88.48	84.18	96.46		87.72	87.72	87.72	85.57	85.19	83.54
Q	KT880191.1	84.43	89.11	89.62	89.37	89.37	89.24	88.61	88.23	84.05	89.62	89.24	89.24	87.09	85.06	88.35	87.72		100.00	100.00	88.61	88.86	85.32
R	KT880192.1	84.43	89.11	89.62	89.37	89.37	89.24	88.61	88.23	84.05	89.62	89.24	89.24	87.09	85.06	88.35	87.72	100.00		100.00	88.61	88.86	85.32
S	KT880193.1	84.43	89.11	89.62	89.37	89.37	89.24	88.61	88.23	84.05	89.62	89.24	89.24	87.09	85.06	88.35	87.72	100.00	100.00		88.61	88.86	85.32
T	MF152651.1	85.95	88.10	87.47	86.96	89.37	89.49	86.84	87.59	82.78	87.47	87.09	87.09	86.20	86.08	86.46	85.57	88.61	88.61	88.61		94.30	86.46
U	MF152652.1	86.46	89.24	86.96	87.59	90.00	90.13	86.71	87.59	83.67	86.96	87.72	87.72	86.08	86.58	86.08	85.19	88.86	88.86	88.86	94.30		86.46
V	JF744991.1	93.04	85.44	85.32	85.95	86.08	85.95	84.18	84.81	82.91	85.32	85.82	85.82	84.43	90.51	83.54	83.54	85.32	85.32	85.32	86.46	86.46	

Figure 35 uncorrected pairwise distance of alignment of NS3 segments from 22 sequences of equine hepacivirus

4.2.6.3 Core gene

Similar to the NS3 protein, a section of the core gene that is amplified using the primers used for development for the assay as described in Lyons et al. (2014) was chosen as a potential protein for the ELISA. Alignments were generated with this specific section to produce a consensus. An alignment containing 22 sequences was completed. Alignment was performed by the DNASTAR MegAlign Pro software. The sequence was translated using "Expasy translate" software. All bases designated 'n' from the consensus (Figure 36) were aligned to match the bp from the JF744991.1 sequence.

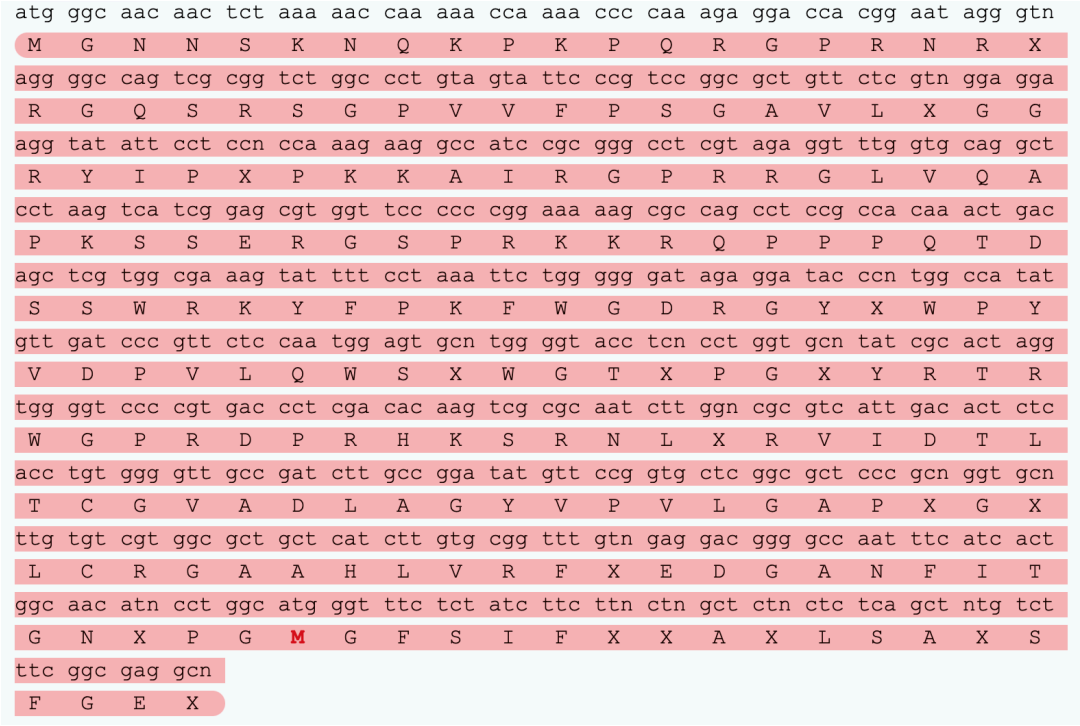


Figure 36: Equine hepacivirus Core gene consensus segment protein alignment.

4.2.6.4 Insert modifications

To allow for extraction of the protein via metal chromatography, 6XHis tags were tagged onto the end of the E1/E2, Core and NS3 sequences. A Kozak consensus sequence; defined as the optimal sequence for protein translation initiation by eukaryotic ribosomes (Kozak, 1986), was added to the beginning of each gene. Two restriction enzymes *AgeI* and *XhoI* were added to the beginning and end of the sequence respectively to allow for removal from the plasmid (Figure 37). Once finalised, the sequence was codon optimised for expression in *N. benthamiana* and sent to be synthesised by GenScript.

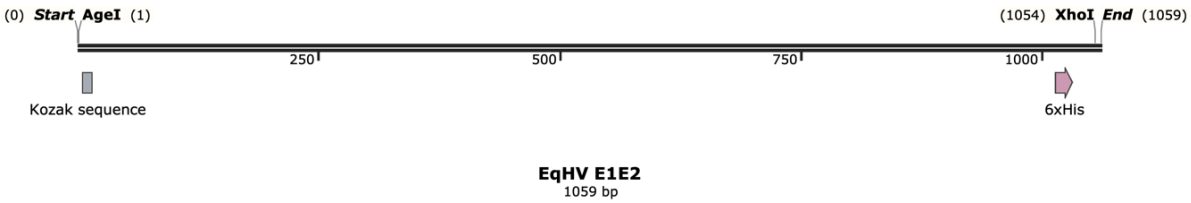


Figure 37 Finalised EqHV E1/E2 sequence for expression in plants

4.2.7 Subcloning

4.2.7.1 Transformation of chemically competent DH5-Alpha cells

Chemically competent NEB DH5-Alpha cells stored at -80°C were thawed on ice. The cells were held on ice for 20 minutes after 1.5 μl of plasmid DNA was added per 50 μl of cells. The cells were heat shocked in a 42°C water bath for 30-45 seconds, then held on ice for a further 20 minutes. Each tube had 500 μl of LB broth added before being incubated for 45 minutes at 37°C. The tubes were spun at 6500 x *g* for 2 minutes to pellet out the cells, supernatant was removed, and the cells were re-suspended in 200 μl of LB broth. Agar plates containing 100 μg/ml of ampicillin were streaked with 100 μl from each tube, left for 5 minutes to settle then incubated at 37°C overnight. One colony was selected using a pipette tip and incubated in 1.5 ml of LB broth with 1:500 ampicillin in the orbital shaking incubator at 37°C, 220 rpm overnight.

4.2.7.2 Culturing pJET1.2-EqHV-E1/E2 plasmid

The EQHV E1/E2 insert was synthesised in pJET1.2 plasmid and the NS3 and Core in the pMA-RQ plasmid by GenScript, both plasmids contain ampicillin resistance genes. A working stock of plasmid was transformed and cultured in DH5-α competent *E. coli* as described in section 4.2.7.1, using ampicillin to select only for bacteria containing the plasmid. The plasmid was extracted using the Jena Bioscience plasmid prep kit according to manufacturer's instructions.

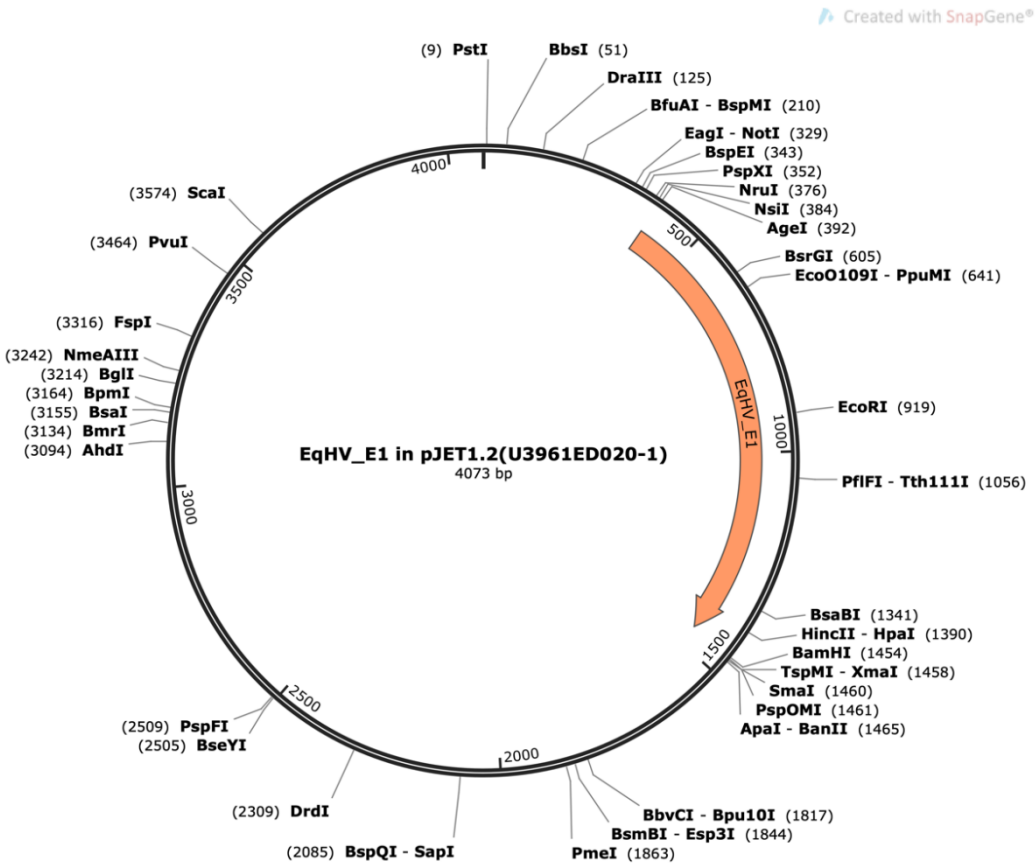


Figure 38 Plasmid map depicting EqHV E1/E2 insert in the PJet plasmid synthesised by GenScript

4.2.7.3 Sequential digests of pJET1.2-EqHV-E1/E2 plasmid

Sequential restriction enzyme digests were carried out to first linearise the plasmid before separating the EqHV insert from the pJET1.2 plasmid. Sequential digests were required to assist in correct identification of the desired DNA sequence, due to the presence of 2 restriction sites for *XhoI* in the pJET1.2 plasmid. Initial digest was prepared with the protocol in Table 14.

Table 14 Reagents and quantities used for restriction digest of pJET-EqHV E1/E2

Reagent	Quantity
Plasmid DNA	1 µg
10x Cut smart buffer	5 µl
***AgeI*-HF**	1 µl
H_2O	Made up to 50 µl

The master mix was incubated at 37°C for 2 h, 5 µl of this was examined via gel electrophoresis with 5 µl of undigested plasmid to ensure total linearisation (see Figure 39). Once linearisation was confirmed via gel electrophoresis, 2 µl of *XhoI* was added to the existing master mix and incubated for a further 2 hours at 37°C.



Figure 39 Image showing the plasmid pJET-EqHV E1/E2 before and after being digested with restriction enzyme AgeI

The full volume of digest was subjected to gel electrophoresis as per section 0, except a voltage of 90 V was used for 1 h, to separate the DNA fragments by size. The band of correct size corresponding to the desired insert was selected under UV and excised using a scalpel. The DNA was extracted using Qiagen QIAquick Gel Extraction Kit according to the manufacturer's instructions.

4.2.7.4 Restriction enzyme digest of pEAQ-HT plasmid

The plasmid pEAQ-HT was digested with the protocol in Table 15 and incubated for 2 h at 37°C. Linearisation was confirmed via gel electrophoresis, the linearised plasmid was excised from the gel with a scalpel and extracted using Qiagen QIAquick Gel Extraction Kit according to the manufacturer's instructions.

Table 15 Reagents and quantities used for digestion of pEAQ-HT plasmid

Reagent	Volume
pEAQ-HT plasmid (1 ng/µl)	5 µl
10x Cut smart buffer	5 µl
***AgeI*-HF**	1 µl
XhoI	2 µl
rSAP (shrimp alkaline phosphatase)	2 µl
H_2O	35 µl

4.2.7.5 Ligation of EqHV-E1/E2 and pEAQ-HT

The EqHV E1/E2 insert and linearised pEAQ-HT vector were ligated to form the plasmid shown in Figure 39 as per the protocol in Table 16, the master mix was incubated overnight at 16°C. A control ligation was performed in duplicate simultaneously with the same reagents and conditions minus the insert, to monitor for re-ligation levels.

Table 16 Reagents and quantities used for ligation of EqHV E1/E2 and pEAQ-HT

Reagent	Volume
Linearised pEAQ-HT vector from gel extraction	1 µl
EqHV E1/E2 insert from gel extraction	6 µl
Ligase buffer	2 µl
T4 DNA Ligase	1 µl
H_20	10 µl

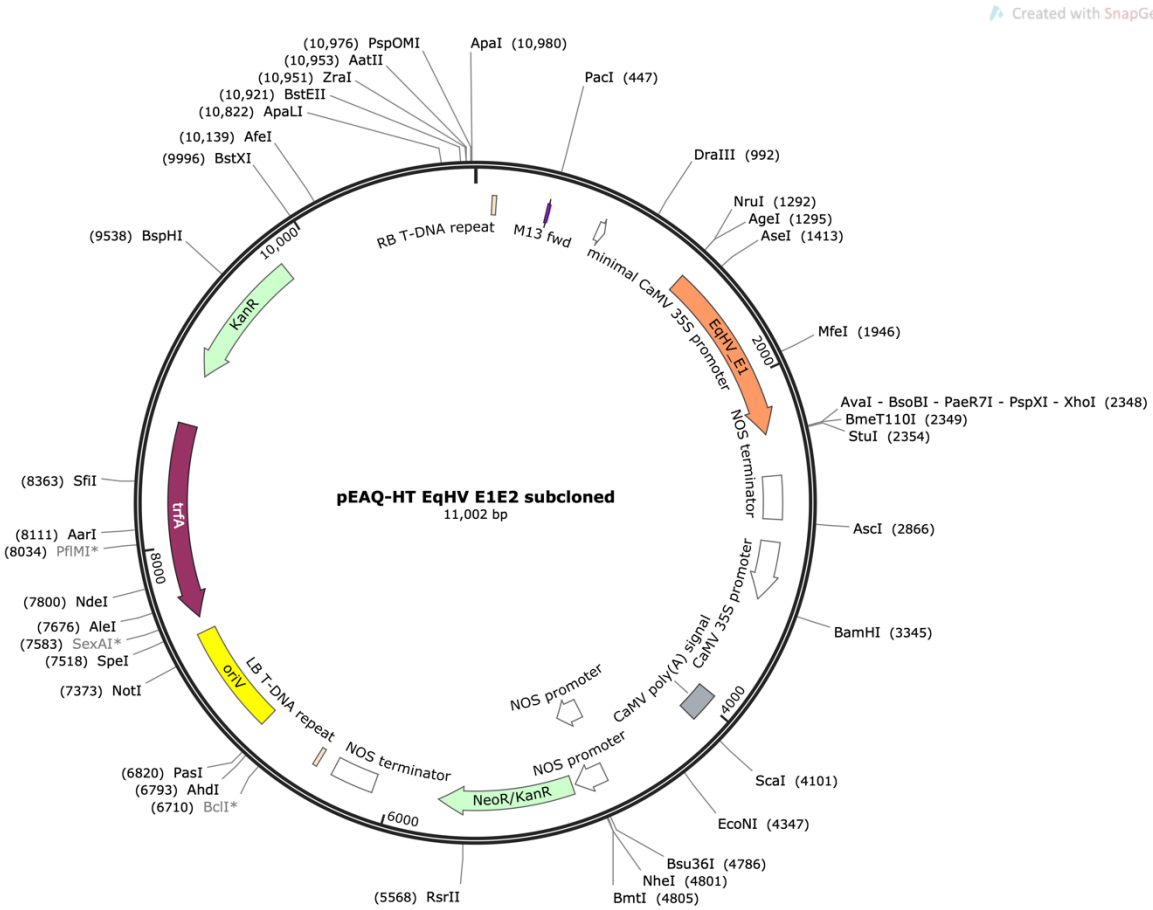


Figure 40 Plasmid map depicting EqHV-E1/E2 insert in pEAQ-HT

4.2.7.6 Culturing pEAQ-HT-E1/E2 EqHV

Ligation products were transformed into chemically competent DH5-Alpha cells as in section: 4.2.7.1, using kanamycin to select for the plasmid. No growth was observed on either of the control plates. Overnight cultures were extracted using Qiagen QIAprep miniprep kit according to the manufacturer's instructions.

4.2.8 Electroporation of *A. tumefaciens*

An aliquot of 1 µl of ligated pEAQ-E1/E2 (~150 ng/µl) was added to 40 µl of thawed ElectroMAX™ *A. tumefaciens* cells (LBA4404) and mixed into a 2 mm cuvette. Transformation was performed at 2500 V, 25 µF, 200 Ω. Bacteria was added to 400 µl of SOC Outgrowth medium (NEB#B9020S) prewarmed to 28°C and incubated in a shaking incubator for 1 h at 28°C and 200 rpm. Bacteria were plated as 60 µl and 380 µl on LB plates with 50 µl/ml kanamycin and 50 µl/ml rifampicin, prewarmed to 28°C, plates were then incubated for 72 h at 28°C.

4.2.9 Agroinfiltration

Single colonies of agrobacterium containing the plasmid were selected and cultured overnight in 5 ml of LB with 50 µl/ml kanamycin and 50 µl/ml rifampicin. The most turbid tubes were selected and spun in a centrifuge for 10 min at 4000xg at room temperature. Supernatant was discarded and remaining bacteria was combined and dissolved in MMA infiltration buffer (prepared as per Table 17) with a ratio of 5 ml MMA per tube of agrobacterium. MMA and agrobacterium were incubated for 2 h at room temperature.

Agrobacterium was forced into the stoma on the underside of the leaves of juvenile (pre flowering) *N. benthamiana*, using a 1 ml blunt end syringe, 3–4 leaves on each plant were infiltrated. Infiltrated leaves were marked with a permanent marker to allow for easy identification. The plants were left to

grow in a secure growing cupboard for 7–9 days before the infiltrated leaves were harvested.

Table 17 Reagents required to make 100 ml of MMA infiltration buffer.

Reagents	**Volume**
MES Hydrate 1M pH 5.6 *(Sterilised by filtration through a 0.45 µm filter)*	1 ml
$MgCl_2$ 1M *(Sterilised by autoclaving)*	50 ml
Acetosyringone 0.1 M (in DMSO)	100 µl
H_2O	97.9 ml

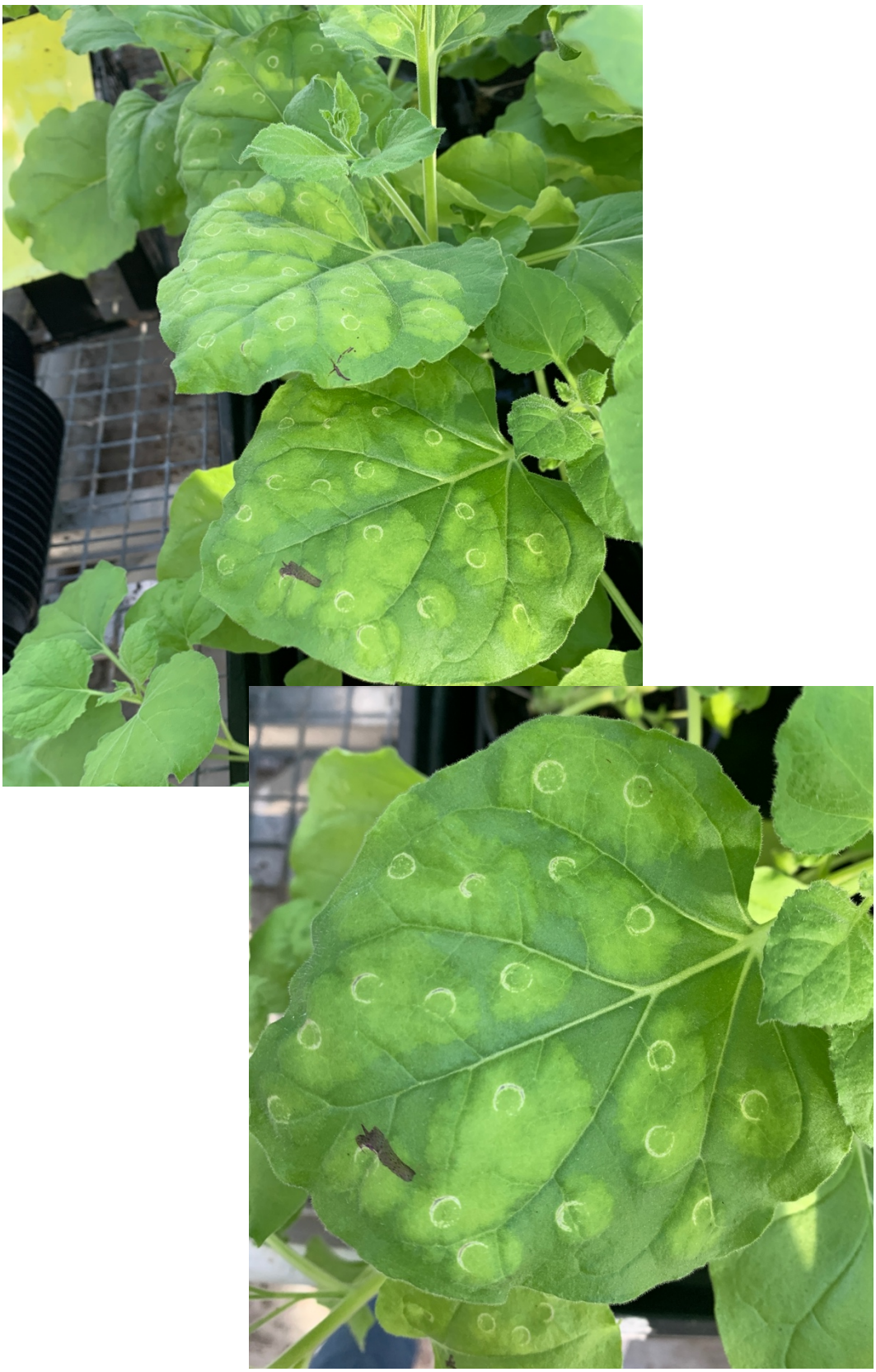


Figure 41 N.benthamiana leaves 8 days post infiltration with agrobacterium transformed with pEAQ plasmid containing EqHV insert. Black mark indicates infiltrated leaf.

4.2.10 Extracting protein from *Nicotiana benthamiana* leaves

Leaves were harvested between 7 and 9 days post infiltration, non-infiltrated leaves were also collected and used as a control during extraction. Leaves were either processed fresh or stored at -20ºC until required. The leaves were weighed, then placed in a mortar in a fume hood. Liquid nitrogen was added as needed and the leaves were ground with a pestle until a fine powder consistency was achieved. The powdered leaves were added to a small blender with 3X their weight (g/ml) in extraction buffer and homogenised for 1–2 minutes. The liquid was transferred to 250 ml centrifuge bottle and centrifuged at 13000 g for 20 minutes at 4ºC. The supernatant was aspirated and filtered through mira® cloth and a funnel over a 500ml beaker. The filtrate was further filtered using a sterile syringe and 20 µm syringe filters. This filtrate was assessed via western blot to confirm presence of desired protein.

4.2.11 Protein Purification

All purification steps were performed in a cold room at 4°C, 2–3 ml of HisPur™ Ni-NTA Resin was prepared according to manufacturer's instructions in 50 ml falcon tubes. Once prepared, 20 ml of filtered leaf homogenate was added to each falcon tube containing resin and incubated on a roller at 4°C to bind overnight.

All filtered leaf homogenate and resin was then transferred into individual His GraviTrap™ columns and preparing according to manufacturer's

instructions. Thermo Scientific™ HisPur™ Ni-NTA Spin Purification Kit was used according to the manufacturers 'native binding' protocol. Protein was eluted with 10 ml of native elution buffer, collected in 1 ml fractions and assessed for quality and concentration.

4.2.12 SDS – Page Gel

Raw plant extracts or extracted protein eluants were added to 4x Laemmli sample buffer containing beta-mercaptoethanol and incubated in a heat block for 10 minutes at 100°C. Precast 12% Bio-Rad Mini-PROTEAN® gels were prepared into cassettes placed in 0.1% SDS Tris/Glycine buffer, 10 µl of each protein sample was added. Five µl of high range rainbow molecular weight marker ladder (Cytiva - RPN756E) was added to one well. Gels were then run for 15 minutes at 50 V then 120 V for one hour.

4.2.13 Coomassie blue gel staining

After the protocol in section 4.2.12 gels were carefully removed from the cassette and placed in a gel staining box. Gels were washed 3 times with water then 20 ml of Invitrogen SimplyBlue™ SafeStain was added and incubated with the gel on a rocker at room temperature for 2–3 hours. The stain was discarded, and 100 ml of water was added to the gel staining box and incubated overnight on a rocker at room temperature. The stained gel was then imaged.

4.2.14 Western blot

After the protocol in section 4.2.12 gels were carefully removed from the cassette and placed onto the Bio-Rad turbo blotting transfer pack blotting paper membrane and into the transfer cassette. The stack was then added on top and rolled to remove any air bubbles before being inserted into the Biorad Trans-blot turbo blot. The pre-set protocol of mixed proteins (7 min) was selected.

The blotting paper membrane paper was removed from the stack using tweezers and placed in a 50 ml falcon tube. ensuring the side containing the protein was facing to the centre of the tube. Blocking buffer containing – was added 2.5% milk powder (0% fat) and TBS-Tween was prepared and 10 ml was added to the falcon tube and incubated for 30 minutes at room temperature on a roller.

Blocking buffer was discarded and 5 ml of fresh blocking buffer and 5 µl of Thermo Fisher His Tag X6 HRP antibody (AB_2536989) was added to the falcon tube and incubated at 4°C overnight on a roller.

Blocking buffer and antibody was discarded and 10 ml of wash buffer (10% TBS and 1% Tween 20, in H_2O) was added and incubated at room temperature for 10 minutes on the roller. This wash step was repeated 2 more times. The membrane was removed and laid flat on plastic wrap protein side up. ECL solution A was mixed with ECL solution B at a ratio of 1:1, 2 ml of the solution was added to the membrane and incubated for 1 minute at room temperature. The membrane was then imaged using ChemiDoc Imaging Systems - Bio-Radx.

4.2.15 Plant Expressed Protein ELISA

Protein fractions identified to have the highest concentration of desired protein were selected. The concentration was quantified using a NanoDrop spectrophotometer through 280 nm absorbance measures. Protein was diluted to 20 µg/ml in carbonate/bicarbonate buffer and added to wells in Nunc MaxiSorb plates at a volume of 50 µl per well. Plates were then covered and incubated overnight at 4°C. Protein coating was discarded, and the plate tapped dry onto a paper towel. Blocking buffer containing 1% bovine sera albumin, 3% milk powder (0% fat) in PBS-Tween (0.1%) was added at a volume of 200 µl per well. The plate was covered and incubated at 4°C for 48 hours. Blocking buffer was discarded and wells were washed twice with 200 µl of PBS-Tween, tapping the plate dry on a paper towel in between washes.

Equine sera were heat inactivated at 56°C for 30 minutes then diluted 1:100 in blocking buffer and added to the washed wells at a volume of 50 µl per well. The plate was incubated for 1 hour at room temperature. The wells were emptied and washed with TBS-Tween six times, tapping the plate dry on a paper towel in between washes. Anti-horse alkaline phosphatase conjugated antibody was diluted 1:1000 in TBS-T and 50 µl added to each well and incubated for 45 minutes. The wells were emptied and washed with TBS-Tween three times, tapping the plate dry on a paper towel in between washes. P-nitrophenyl phosphatase substrate (Thermo Scientific™) was added at a volume of 100 µl per well and the plate was

incubated for 30 minutes in the dark at room temperature. The plate was read on a spectrophotometer at 405 nm wavelength (Figure 53).

The cut off value for a positive result was estimated by the taking the value of positive control sera against negative control, un-infiltrated plant leaves, and adding the standard deviation x3 as described in Lardeux et al., (2016).

4.3 Results

4.3.1 Pseudovirus neutralisation assay

It can be seen from data displayed in Figure 42, the light emission from the control wells containing EqHV pseudovirus (Pv) only, are lower than the Pv with sera added. These both confirm the assay did not function correctly. The control well with Pv only should give the highest value as there are no antibodies present to neutralise the virus. EqHV antibodies should neutralise the Pv preventing the production of luciferase but should not affect the Δv. Moreover, the expected light emission values were in the thousands rather than below 100 further confirming the assay did not function correctly.

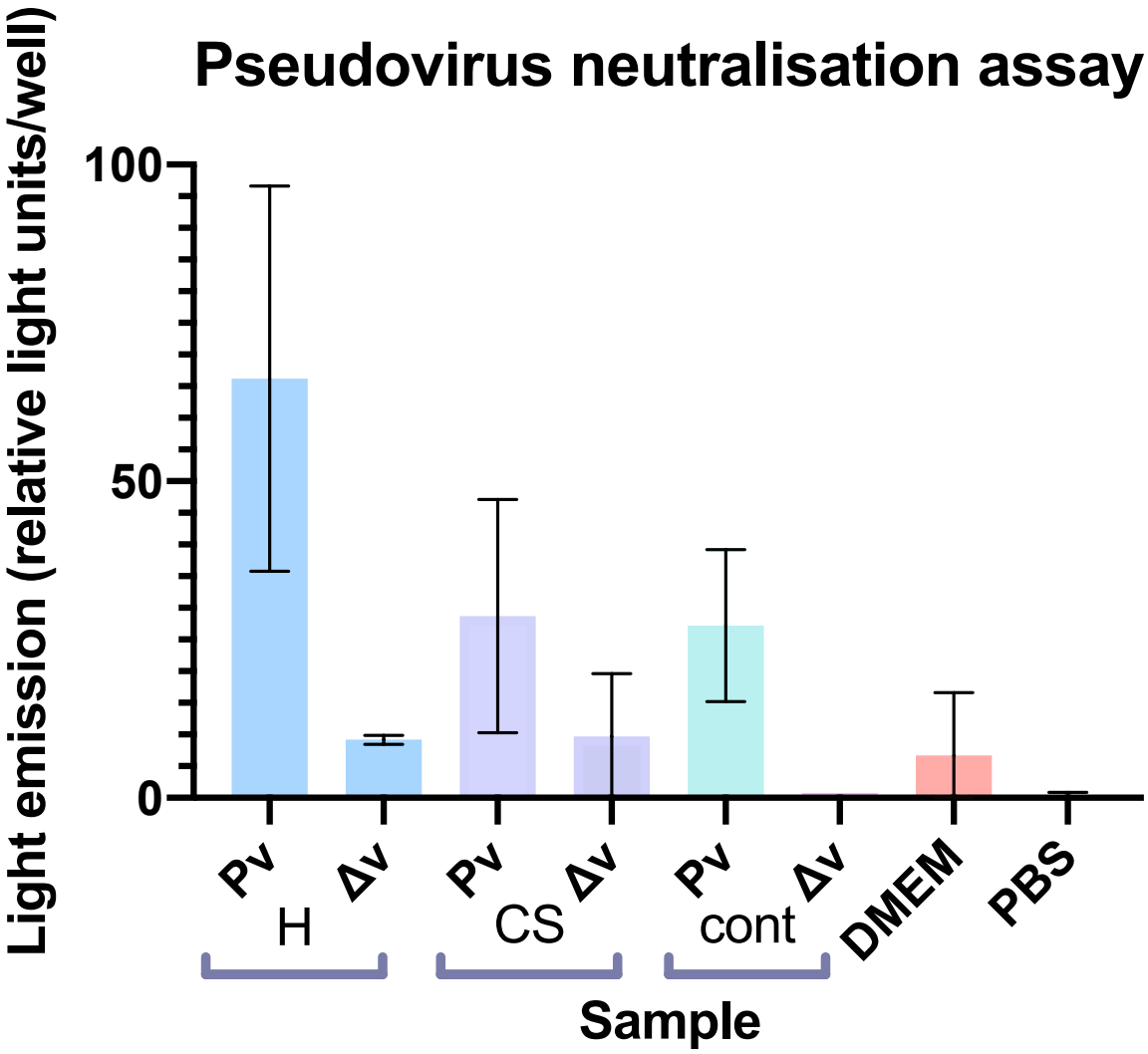


Figure 42 Results of pseudovirus neutralisation test. Mean average light emission levels (with standard error of the mean bars) from Promega Steady-Glo® reagent added to Huh 7 cells infected with EqHV pseudovirus (Pv) and control pseudovirus (Δv) both with known positive samples, Horse H sera (H) and commercial sera (CS) added and without (cont) as well as uninfected Huh 7 cells with Dulbecco's Modified Eagle Medium (DMEM) and phosphate-buffered saline (PBS) as additional controls. All values are minus the average value of background noise from the wells with no Promega Steady-Glo® reagent added.

4.3.2 GNA Assay

For an initial run of the GNA assay executed at QMC shown in Figure 43, a cut-off value of 0 was established by subtracting the background noise from a negative control, values over 0 were considered 'positive'. Error bars for many of the 'positive' samples are large and overlap with the 'negative' sample results. Results gave a big variance between replicates of samples and the known positive control, shown in Table 18.

Table 18 Table showing raw data results and methodology used to derive final value.

Sample ID	E1/E2 positive well 1	E1/E2 positive well 2	Negative cont well 1	Negative cont well 2	+ Mean	- Mean	final value - background noise
988	0.665	0.199	0.422	0.206	0.432	0.314	0.118
1369	0.679	0.212	0.138	0.237	0.4455	0.1875	0.258
569	0.424	0.13	0.125	0.155	0.277	0.14	0.137
544	0.457	0.425	0.176	1.121	0.441	0.6485	-0.2075
1071	0.285	0.345	0.624	0.484	0.315	0.554	-0.239
1124	0.524	0.262	0.152	0.184	0.393	0.168	0.225
1130	0.694	0.586	0.204	0.195	0.64	0.1995	0.4405
H2018 (Pos control)	1.383	0.418	0.211	0.292	0.9005	0.2515	0.649

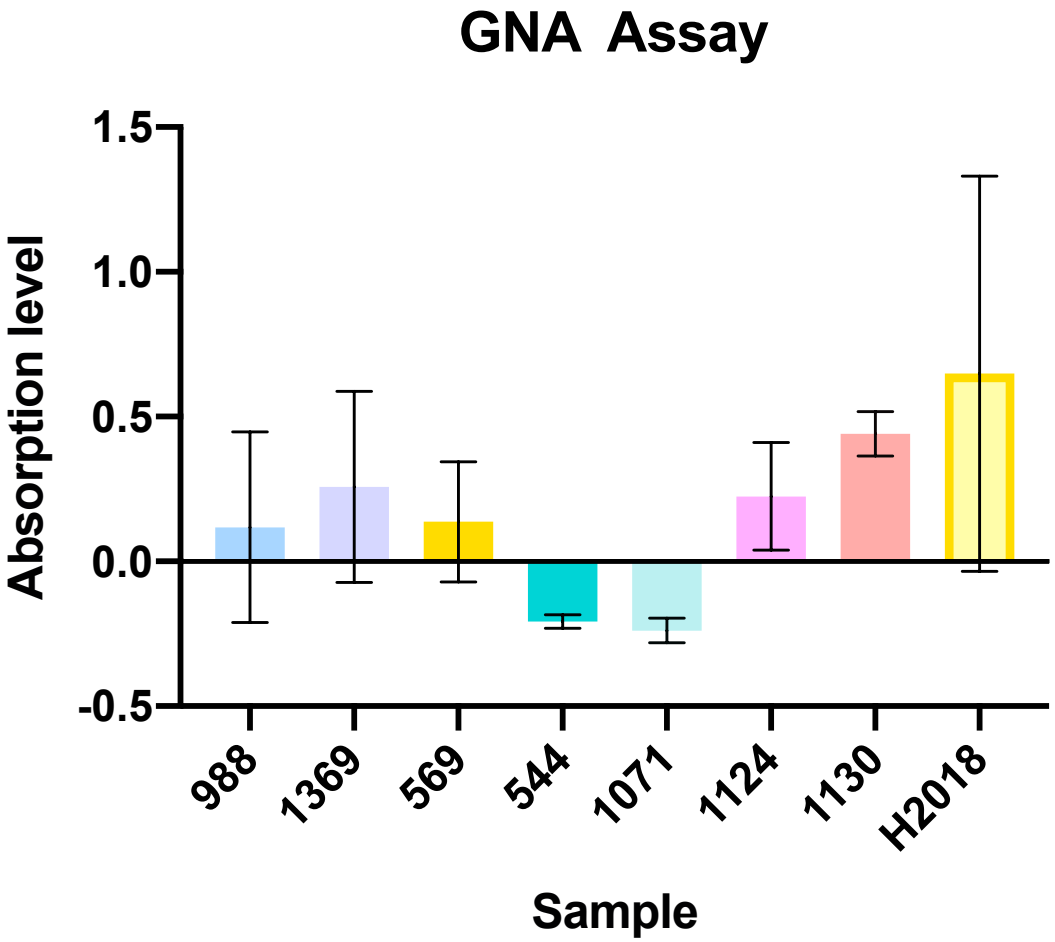


Figure 43 Absorption levels of each sera sample from GNA ELISA assay after data analysis, including standard deviation bars. 0 is the cut off value, values above 0 are positive for EqHV antibodies.

Several repeats were attempted to establish a reproducible GNA assay at SVMS. A methodical troubleshooting process was implemented to identify the cause of error, including increasing the blocking time (Figure 44, Figure 45 and Figure 46). Figure 45 shows a high OD for the navy bar representing wells with no lysate. This suggested either, sera were reacting with the GNA only or the GNA was binding protein other than the cell lysate to the pate which was reacting to the equine sera.

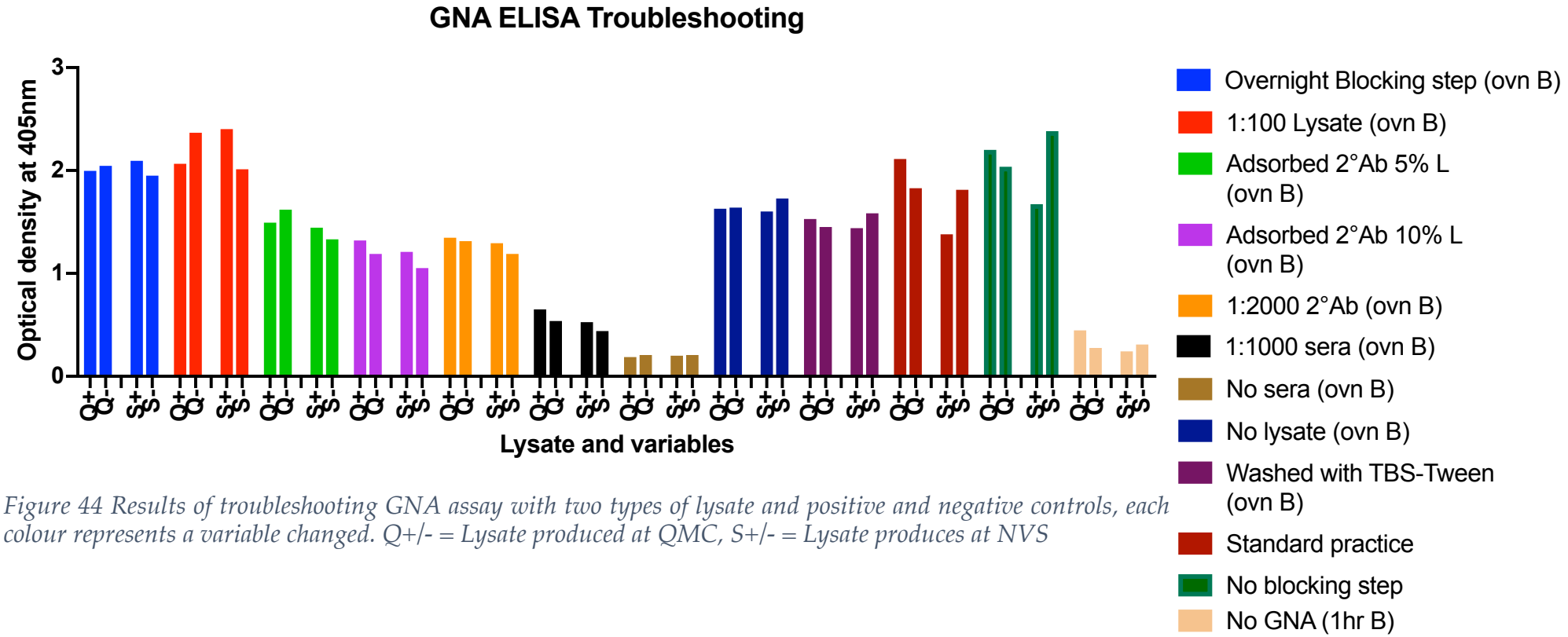


Figure 44 Results of troubleshooting GNA assay with two types of lysate and positive and negative controls, each colour represents a variable changed. Q+/- = Lysate produced at QMC, S+/- = Lysate produces at NVS

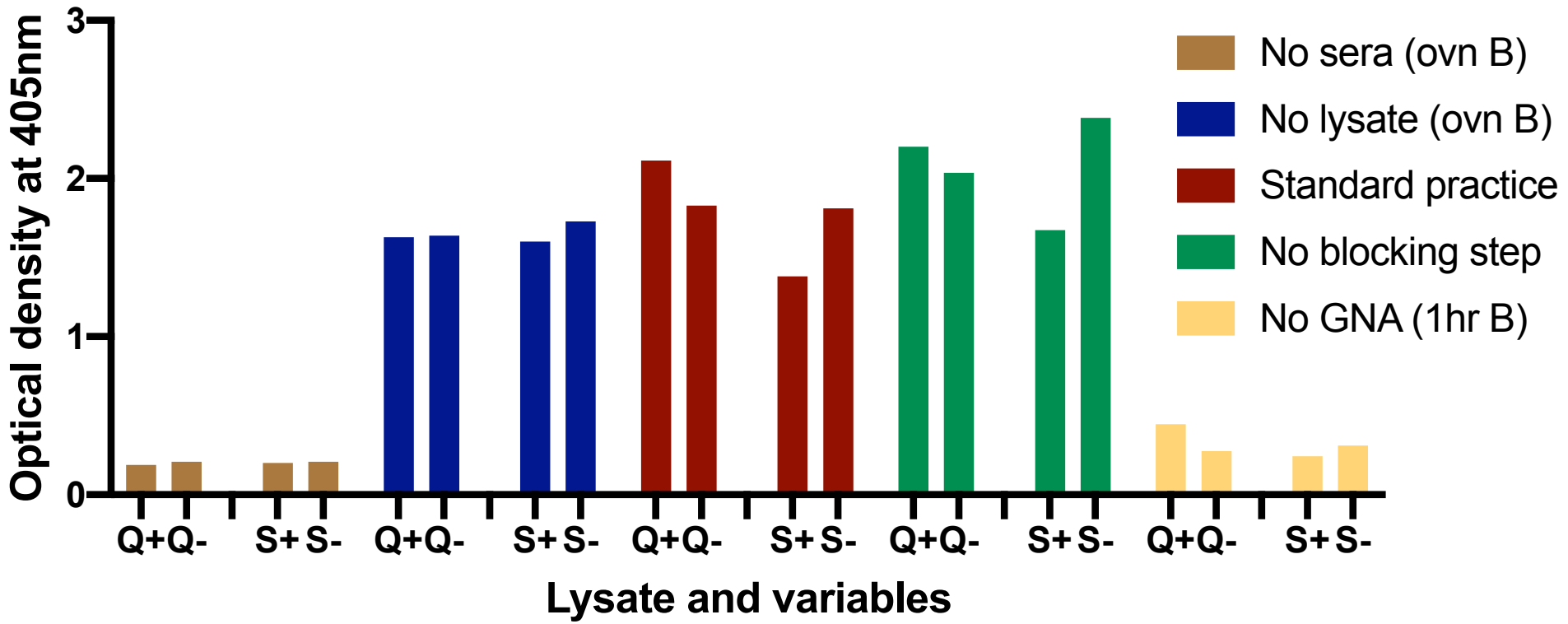


Figure 45 Results of key reagent variables from the GNA assay troubleshooting process.

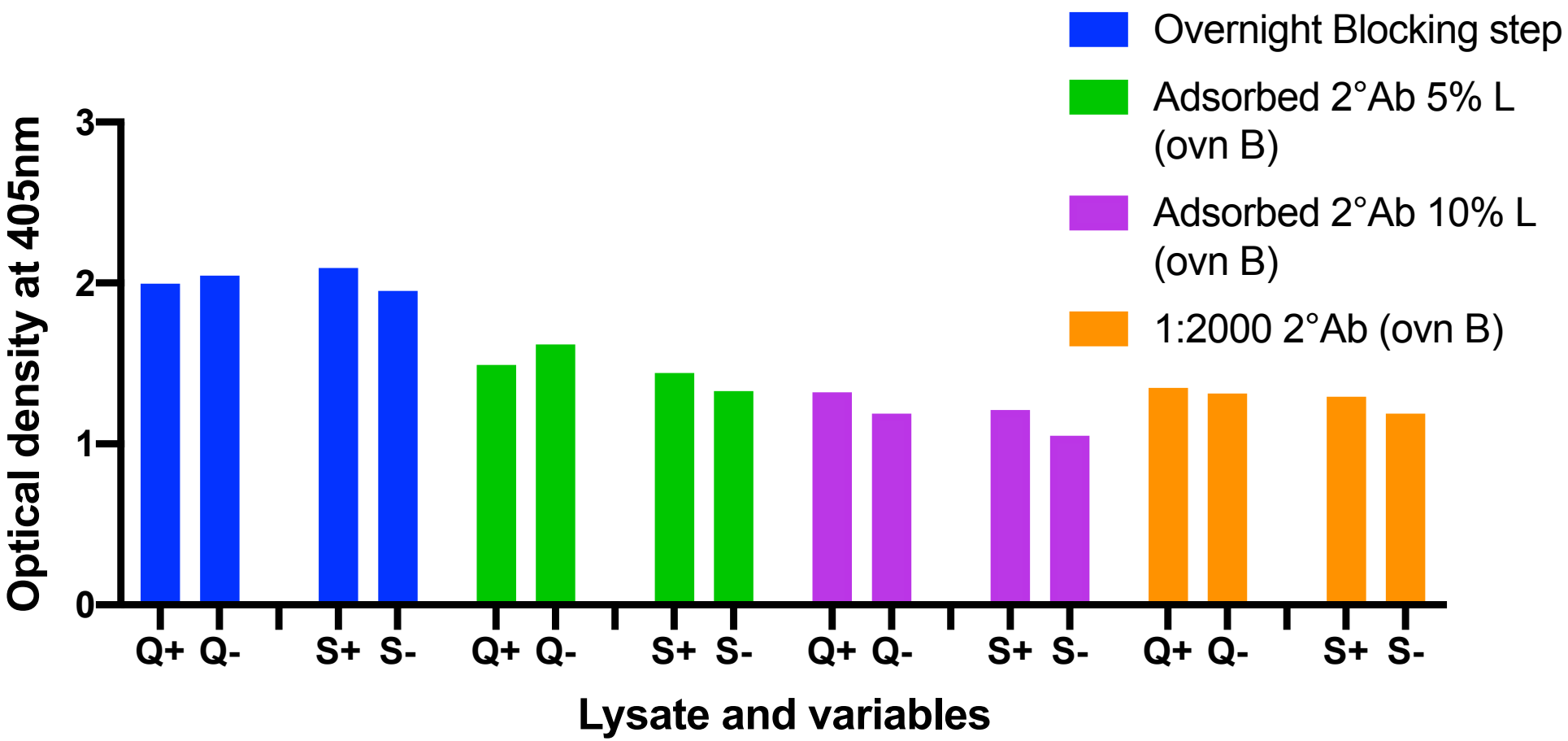


Figure 46 Results of key antibody variables from the GNA assay troubleshooting process.

4.3.3 HCV peptide ELISA

HCV peptide ELISA functioned correctly for the positive HCV sera control, a high OD was measured for the HCV peptide and negligible OD for negative control rabies peptide. Horse sera gave nonspecific results, the positive control sera horse H did not give a high signal for the HCV peptide and many other horse sera samples gave a false positive reaction to the rabies peptide indicating nonspecific binding.

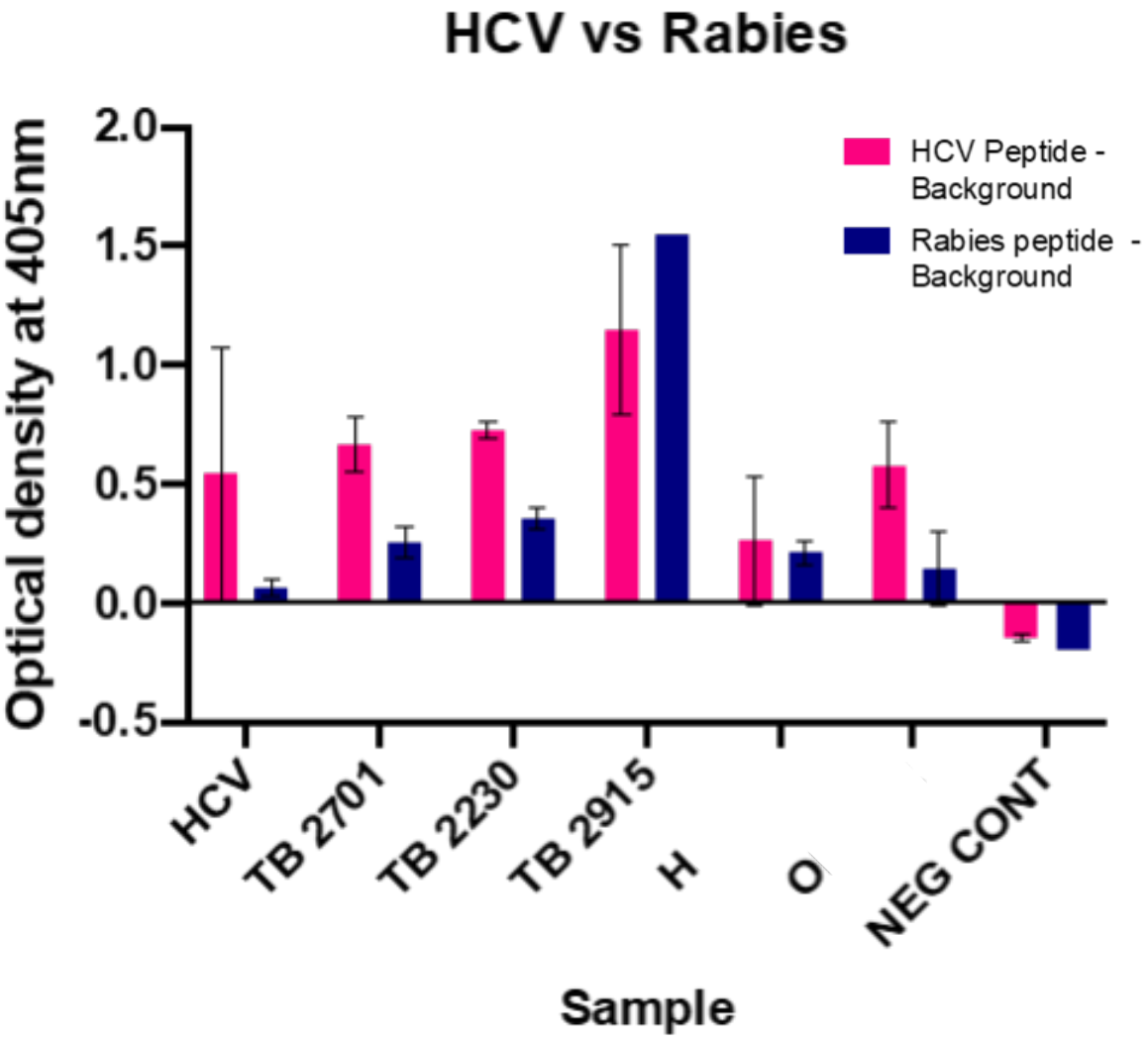


Figure 47 Results of the Hepatitis C peptide assay using human and horse sera, Horse H is a positive control for EqHV. TB = Thoroughbred

4.3.4 ELISA using plant expressed EqHV protein

4.3.4.1 Confirming functionality of expression vector

To ensure the protein expression vector and method was functional, a control vector was used. A pEAQ-HT plasmid with a gene for green fluorescent protein (GFP) in place of the desired EqHV protein. The pEAQ-HT with GFP was transformed into *A. tumefaciens* cells as per section 4.2.8, then infiltrated into *N. benthamiana* as per section 4.2.9. After 7-9 days of growth, plant leaves were harvested, upon examination under UV light the following images in Figure 48 were taken where areas of GFP can clearly be seen in the leaf.

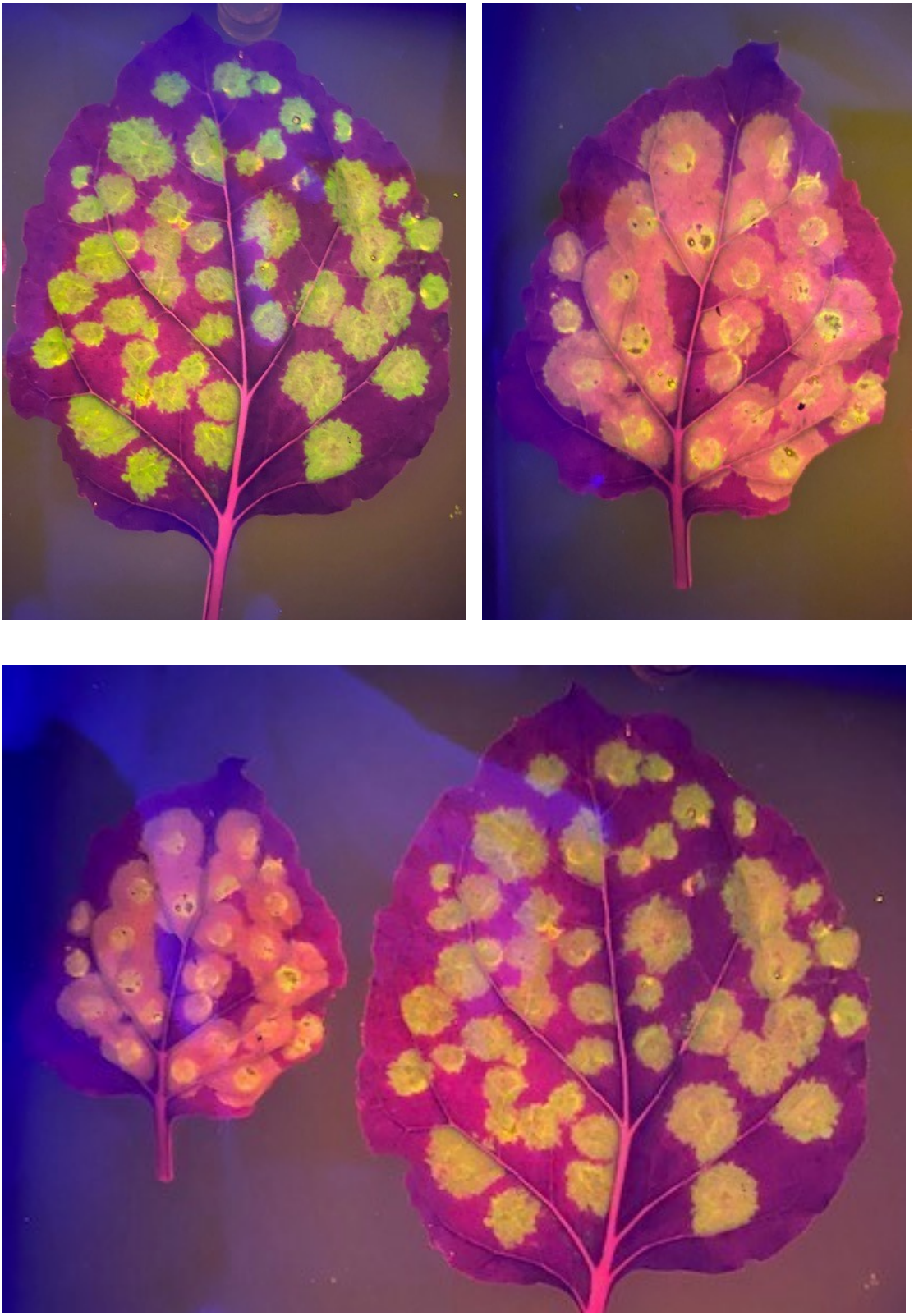


Figure 48 Images taken of Nicotiana benthamiana leaves under UV light showing the green fluorescent protein expression in the leaf.

4.3.4.2 Western blot

Western blot using anti-His antibody was used to ensure the desired His tagged protein had been eluted after protein extraction. Figure 49 shows band containing desired His tagged protein.



Figure 49 Gel image from a western blot depicting extracted and purified expressed proteins. An anti-His (HRP conjugated) antibody was used to detect his-tagged proteins. Circled band shows EqHV E1/E2 protein.

Coomassie blue gel stains

Figure 50 shows different elution fractions in each lane, lanes containing fractions 2+ and 3+ have the largest band of EqHV Core protein ~50 kDa. The fractions with the highest protein concentration were selected, quantified and combined for use in the ELISA.

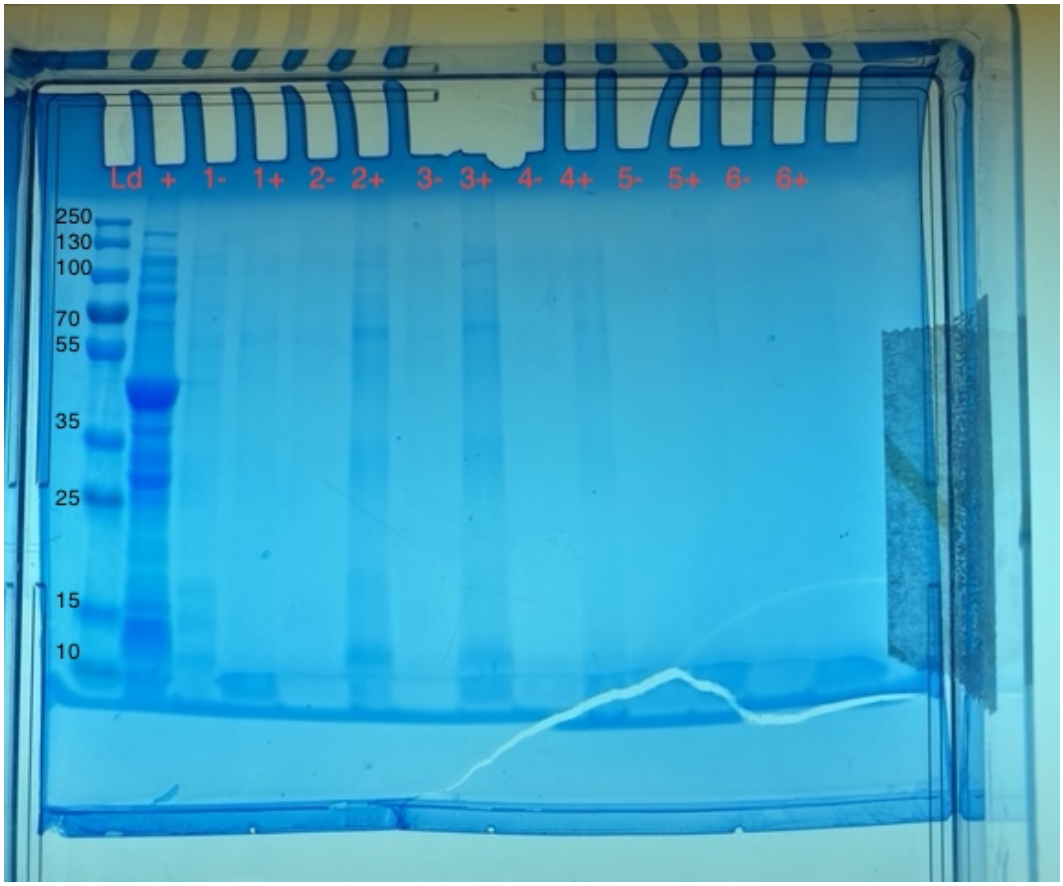


Figure 50 Agarose gel containing protein fractions, stained with Coomassie blue staining Gels were stained with Coomassie blue to assess protein content of elution fractions 1-6 of leaves containing EqHV protein (lanes +1–6) and non-infiltrated leaves (-1–6) as well as pre-purified leaf supernatant (in + lane next to ladder) to compare. Fractions with the largest band of EqHV Core protein were selected for use in the assay. Ladder measuring in kDa.

4.3.4.2.1 Comparison of 3 Equine hepacivirus proteins performance in an ELISA

Three different EqHV proteins (NS3, E1/E2 and core) were expressed in plants and tested with multiple blood samples. This included an HCV positive human blood kindly donated by Dr Alexander Tarr from QMC. Proteins were tested against known positive or negative samples, (see Table 19) and compared to leaf protein not transfected with EqHV plasmids, (see Figure 51).

Core protein was selected for the ELISA for two reasons. Results of comparative ELISAs showed the core protein gave a slightly higher OD value for positive samples and quantification of the yield of protein extractions were consistently higher for the purification cycles for the core protein.

4.3.4.3 Trialling equine hepacivirus proteins in an ELISA

Table 19 Optical density at 405nm from ELISAs using different plant expressed proteins from EqHV

		1	2	3	4	5	6	7	8	9	10	11	12
Non-infiltrated plants	A	*Blocking buffer* 0.194354	*Blocking buffer* 0.192704	*Blocking buffer* 0.183608	*Oliver* 0.3525	*Oliver* 0.409378	*Oliver* 0.402808	*Harry* 0.163682	*Harry* 0.160121	*Harry* 0.16538	*Commercial sera* 0.146314	*Commercial sera* 0.14169	*Commercial sera* 0.037927
Non-infiltrated plants	B	*HCV* 0.235873	*HCV* 0.183633	*HCV* 0.148966	*Tb 1* 0.29765	*Tb 1* 0.332368	*Tb 1* 0.348428	*Tb 2* 0.375062	*Tb 2* 0.347014	*Tb 2* 0.325181	*Tb 3* 0.500486	*Tb 3* 0.406943	*Tb 3* 0.486975
E1/E2	C	*Blocking buffer* 0.16168	*Blocking buffer* 0.159697	*Blocking buffer* 0.148373	*Oliver* 0.735935	*Oliver* 0.922591	*Oliver* 1.14092	*Harry* 0.153096	*Harry* 0.148831	*Harry* 0.148373	*Commercial sera* 0.146906	*Commercial sera* 0.154145	*Commercial sera* 0.18863
E1/E3	D	*HCV* 0.167799	*HCV* 0.154033	*HCV* 0.152359	*Tb 1* 0.494904	*Tb 1* 0.527268	*Tb 1* 0.548183	*Tb 2* 0.455903	*Tb 2* 0.52514	*Tb 2* 0.503402	*Tb 3* 0.724914	*Tb 3* 0.747469	*Tb 3* 0.612804
NS3	E	*Blocking buffer* 0.197772	*Blocking buffer* 0.191526	*Blocking buffer* 0.184549	*Oliver* 0.79774	*Oliver* 0.798488	*Oliver* 1.44742	*Harry* 0.176906	*Harry* 0.197803	*Harry* 0.203061	*Commercial sera* 0.30967	*Commercial sera* 0.160257	*Commercial sera* 0.15954
NS3	F	*HCV* 0.204635	*HCV* 0.209445	*HCV* 0.203374	*Tb 1* 0.621486	*Tb 1* 0.679899	*Tb 1* 0.679701	*Tb 2* 0.558673	*Tb 2* 0.500512	*Tb 2* 0.52628	*Tb 3* 0.641095	*Tb 3* 0.611622	*Tb 3* 0.611537
Core	G	*Blocking buffer* 0.173802	*Blocking buffer* 0.174904	*Blocking buffer* 0.181391	*Oliver* 1.19255	*Oliver* 1.2847	*Oliver* 1.2843	*Harry* 0.230154	*Harry* 0.391289	*Harry* 0.455686	*Commercial sera* 0.23113	*Commercial sera* 0.198176	*Commercial sera* 0.208487
Core	H	*HCV* 0.164037	*HCV* 0.169382	*HCV* 0.174113	*Tb 1* 0.477056	*Tb 1* 0.495853	*Tb 1* 0.609491	*Tb 2* 0.490189	*Tb 2* 0.489185	*Tb 2* 0.558937	*Tb 3* 0.76838	*Tb 3* 0.763198	*Tb 3* 0.893319

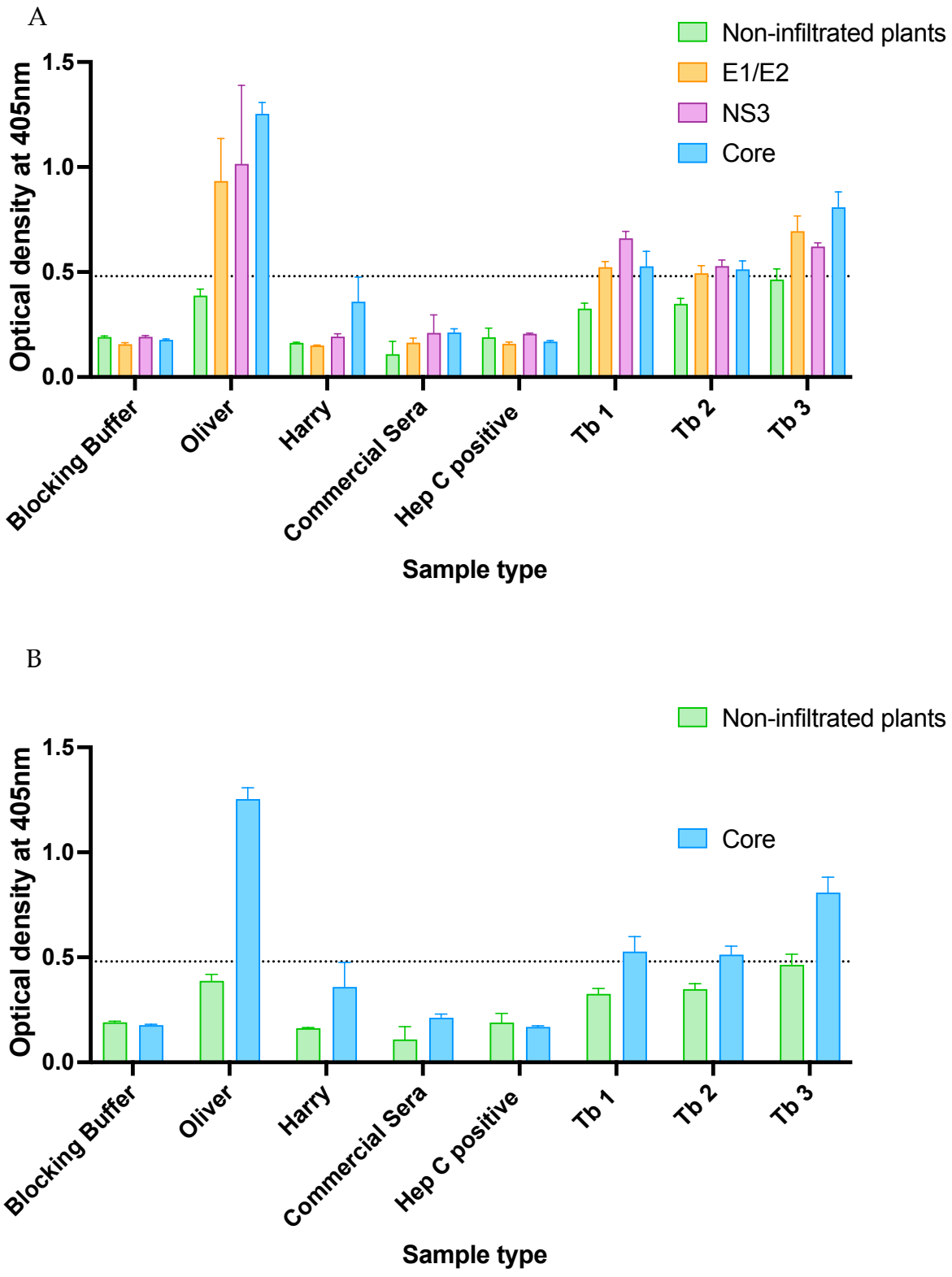


Figure 51 A. OD values of different sera when used in ELISAs with 3 different plant expressed proteins and a control (Tb = Thoroughbred) B. OD values of Core protein chosen to take forward and a control.

4.3.5 Screening Bransby Samples

Once a reliable method had been established, samples from the equine population at Bransby were screened using the new ELISA. Horses with multiple samples were selected to assess the impact of EqHV infection on levels of antibody, shown in Figure 52.

All horses positive for EqHV on PCR showed an increase of EqHV antibodies either at time of positive PCR result or on a sample taken later.

Horse 13 (Figure 52) tested positive for EqHV RNA in September and retested negative in December but retained high levels of EqHV antibody. Horse 6 appeared to have EqHV antibodies in May and was negative for EqHV RNA suggesting previous exposure, however between May and June became infected with EqHV and had an increase in antibody levels, this horse was retested again in November, PCR showed negative for EqHV and antibody levels were decreasing. Horse 12 tested positive in both May and June and had not raised antibodies above the positive threshold of the assay, although this time window is not sufficient to label as a chronic infection.

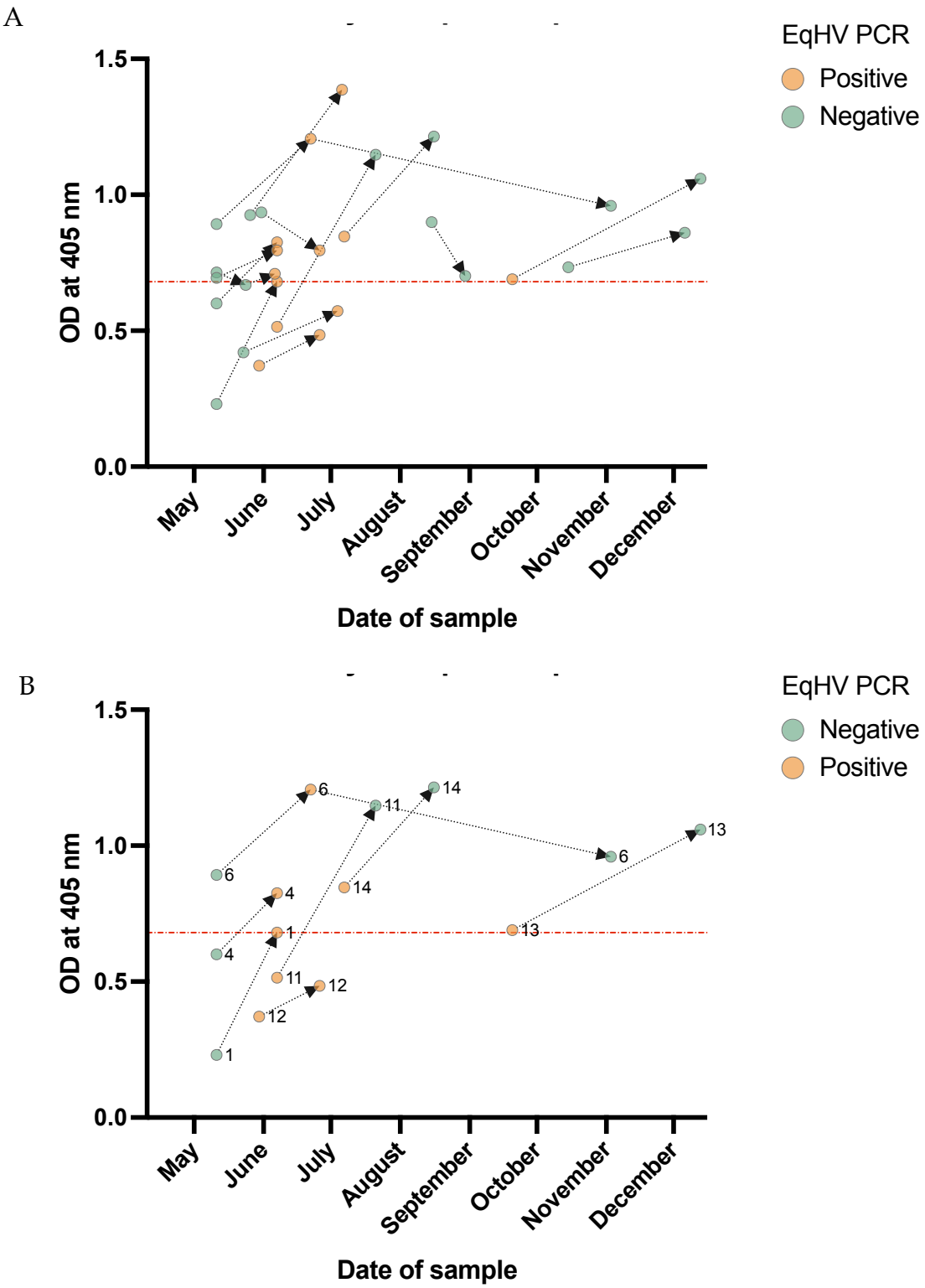


Figure 52 A Results of antibody testing from Bransby equine blood samples, OD values representing antibody level. Red dashed line showing 'cut off' value, values above this are considered positive for EqHV antibody. Samples from the same horse are connected with arrows, orange circles are also positive, and green are negative for EqHV virus on PCR testing. B shows the same data with fewer samples and includes assigned horse numbers.

4.4 Discussion

4.4.1 Existing Assays

Results of already established assays were poor and inconsistent highlighting the need for a novel diagnostic assay. The GNA assay gave inconsistent results with consistent issues with non-specific binding and high background signals. The PVNT assay did not yield meaningful results with low signals and large error bars.

4.4.1.1 GNA Assay

This assay exploits the broad reactivity range of GNA with glycoproteins and the fact that EqHV proteins are retained in the rough endoplasmic reticulum without any further processing in the Golgi by N-glycosyltransferase enzymes. As such these proteins possess the terminal mannose-containing glycans that are able bind to GNA. In the initial assay performed, there were clear differences in absorption signal between the positive and negative samples demonstrating clear detection of EqHV antibodies. However, results of several of the positive samples have large error bars.

Obtaining the lysate containing EqHV protein from HEK cells is a limitation, as the plasmids do not always translate the required protein in the cell and the quantity of lysate produced is variable.

The non-specific binding of the lectin is another limitation of this method, several blocking steps are used to limit the effect of any non-specific binding. It is also possible horses may have lectin sensitivity, and therefore

produce antibodies against lectin which may be present in blood samples and would also bind to the GNA in the assay producing a false positive result, affecting reliability of the results.

The GNA assay is difficult to apply in a commercial veterinary setting as the threshold must be determined each time the assay is performed. Also, to try and account for cross-reactivity, all samples are run against a negative control (serum samples are added to wells with cell lysate that does not contain EqHV protein). This negative control must be contained on the same plate and the operator must manually interpret the standard threshold for each sample, meaning 6 wells are required per sample, reducing the number of samples that can be run at one time and decreasing the ease of the assay. Additionally, GNA is quite a costly reagent.

4.4.1.2 Pseudovirus neutralisation test

Attempts to perform a pseudovirus neutralisation test (PVNT) did not give meaningful results. Although the PVNT gave a small visible difference in signal between the positive and negative control for the known positive sample the error bars were large and overlapped with the negative controls.

This type of assay, when successful, works well however it is particularly difficult to utilise due to the complex nature, and instability of the pseudovirus. There are many potential reasons this assay was unsuccessful. As the Pv was created at QMC, transportation of the Pv could have been a contributing factor. The Pv may not have infected the cells correctly at a high enough concentration. This type of assay would be impractical to implement in commercial veterinary practices due its complex nature adding significant cost and time.

4.4.1.3 Hepatitis C virus peptide Assay

This assay was considered due to the 50% homology EqHV shares with HCV. This peptide assay is already established for assessing the prevalence of HCV antibodies in human sera but has not been attempted for EqHV. Rabies peptide was used as a control as the sampled horses were not expected to have antibodies against rabies. Although signal was detected from the positive horse sera for the HCV peptide there were large error bars and signal was also detected for the negative control rabies peptides, suggesting non-specific binding. This could be due to the methodology of this assay needing more refinement, potentially requiring more blocking or washing steps, or equine sera could be binding non-specifically. The human sera sample positive for hep C worked as intended, giving signal for Hep C (although the error bars were also large) peptide and not for rabies, indicating the assay method was effective and the reagents were functioning as expected.

4.4.2 Plant expressed protein (PEP) ELISA development

Methods and insight learned from trialling and refining the above established assays allowed for a more informed development approach for the finalised protein production and extraction (shown in Figure 53) as well as the methodology required for the finalised PEP assay.

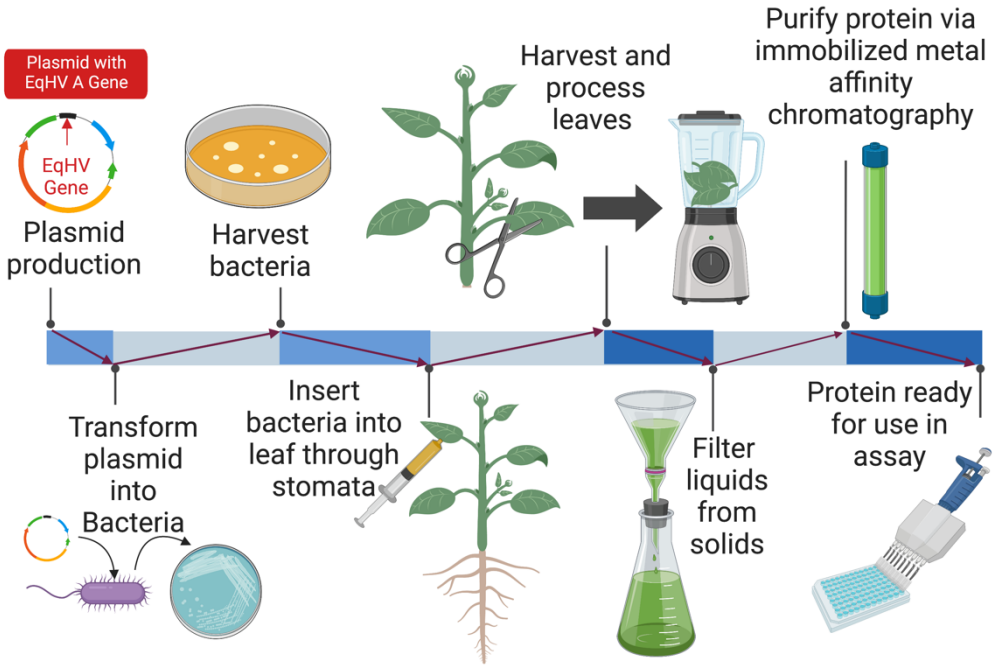


Figure 53 Finalised EqHV protein expression method using N.benthamiana

4.4.2.1 Protein purification

The two main issues encountered during the development of this ELISA were efficient protein purification and non-specific binding (discussed in 4.4.2.3). Initially, a large amount of protein was lost during extraction and purification. Various methods of protein extraction were trialled. Utilising liquid nitrogen to freeze the leaves prior to grinding with a pestle and mortar, was found to produce the most successful yield. Keeping products

at extremely low temperatures and using a protease inhibitor provided the least protein degradation.

The His tag was used to label and identify the desired protein as this is the most convenient tag to attach using the pEAQ vectors. The method for purifying His tagged protein utilises Ni-NTA Agarose; an affinity chromatography matrix for purifying recombinant proteins carrying a His tag. Although Ni-NTA Agarose should be specific at selecting the His-tagged proteins there is still large quantities of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the most abundant plant protein is known to be present in purifications of PEPs (Hanittinan et al., 2020).

Proteins post purification are still contaminated with some other undesired proteins. The possibility of further purifying protein extractions via dialyzing protein samples was explored. RuBisCo contributes to a large part of the contaminant, but as one of the subunits of RuBisCo is ~55 kDa, this is too similar in size to the selected EqHV proteins to be able to be separated by dialysis. Other studies suggest varying the concentration of ammonium sulphate may precipitate out some levels of RuBisCo (Hanittinan et al., 2020). However, the presence of other plant proteins in the samples did not appear to affect the specificity of the assay.

4.4.2.2 Equine hepacivirus protein selection

Three proteins were identified to trial for the plant expressed protein ELISA. NS3, E1/E2 and Core, The NS3 gene is the most frequently used target for PCR detection of EqHV. It is also used as the gene of choice to express in the LIPS assay. E1/E2 is an envelope protein and therefore expressed on the surface of the viral capsid. The E1/E1 protein was trialled

as the location of the protein on the surface is likely to induce high antibody production following infection. Core protein is also used as a target in some PCR assays. Use of a control vector with GFP ensured the protein expression system was functional prior to the protein extraction step.

Human sera positive for HCV was used to assess for specificity to EqHV. HCV antibodies did not bind to any of the three EqHV proteins trialled in this project. Given that there was no detection of EqHV antibodies in the HCV peptide ELISA this is not surprising.

4.4.2.3 ELISA methodology

Non-specific binding was reduced by changing the blocking approach and the wash steps. Wash steps after the sera was added were increased to 6 washes. The blocking buffer used contained both BSA and milk powder. However, the most significant improvement in reducing non-specific binding came from increasing the blocking buffer incubation time to 48 hours after the plate had been coated. This blocking step was effective enough to reduce non-specific binding so much that results for negative sera or negative control blocking buffer were of a similar OD value. For future development it may be possible to dry the plates after the 48-hour blocking step and store them pre-blocked ready for use, similar to a lot of commercially available ELISAS, allowing for results to be obtained in a couple of hours when needed.

4.4.3 Antibody levels in the Bransby population

The new ELISA test developed in this study proved to be a reliable tool for detecting EqHV antibodies, allowing for the assessment of the impact of EqHV infection on antibody levels in horses. The data showed all horses positive for EqHV PCR test also showed an increase in EqHV antibodies either at the time of positive PCR result or on a secondary sample, both demonstrating the accuracy of the assay as well as displaying EqHV infection stimulating the production of antibodies, suggesting acute infections. This is also supported from the clinical chemistry data in chapter 2, which showed an increase in lymphocyte count in the EqHV positive horses.

The results also revealed interesting patterns in the antibody response of individual horses over time. Horse 13, for example, tested positive for EqHV RNA in September and was retested negative in December but retained high levels of EqHV antibody not shown among other horses in this cohort. Horse 6 showed evidence of previous exposure to EqHV, as indicated by the presence of EqHV antibodies in May accompanied with a negative PCR test. However, between May and June, the horse became infected with EqHV and had an increase in antibody levels, then a subsequent decrease in antibody levels after retesting negative for EqHV in November. Horse 12, on the other hand, tested positive for EqHV in both May and June but had not raised sufficient antibodies, however, this is not a sufficient time window to identify a chronic infection.

These findings highlight the importance of monitoring EqHV antibody levels in horses to better understand the dynamics of EqHV infection in equine populations. The ability to detect EqHV infection accurately and quickly through serological testing can aid in the detection of EqHV outbreaks. Further testing would need to be done to ascertain what value or increase in value of ELISA OD represents seroconversion in response to a recent infection as opposed to antibodies being present from a previous infection. This could be assessed by testing blood samples with both anti-IgG and anti-IgM as IgM antibodies indicate a primary infection.

Overall, the development of a more accessible ELISA test for EqHV antibodies could provide significant advancement in the understanding and management of EqHV infections in horses.

Chapter 5: Discussion

The aim of this thesis was to develop a diagnostic assay as and advance the understanding of several aspects of EqHV. This was accomplished through a comparative study involving two distinct equine populations in the UK, primarily focusing on a rescue population but also drawing informative comparisons with a previously studied Thoroughbred population (Akagha, 2021).

Data generated in this thesis established EqHV prevalence in both the Bransby (21%) and Thoroughbred (37.88%) populations, prevalence significantly surpassed previously reported rates of no more than 2% among other UK equine populations (Lyons et al., 2012; Lyons et al., 2014). The higher prevalence observed could potentially be attributed to the utilisation of a more sensitive PCR assay, as demonstrated by Akhaga (2021) in a comparison of various PCR assays. Additionally, differences in sample storage conditions, notably the use of -20°C for serum samples in published studies compared to -80°C in this study, might have prevented RNA degradation. Comparison with EqHV prevalence studies in other countries, as discussed in chapter 1 (Table 2), echoed the prevalence rates found in this study.

Analysis of prevalence data revealed distinctive seasonal variation in EqHV within the rescue population. Seasonal fluctuations of EqHV prevalence has not been documented before. Most existing studies assessing prevalence of EqHV in a country or region use samples of convenience from various time

periods. Currently no other published studies have collected samples from a specific population throughout the year. This seasonality could implicate biting flies as a potential route of transmission.

The wide variability of EqHV prevalence seen from study to study, especially within the same country, could be due to the time of year the samples were taken. Prevalence may also vary between areas depending on the landscape. If biting flies are the main vectors of EqHV, they have specific requirements for preferred habitat, such as lowlands and nearby bodies of water. Additionally, as age is also an identified risk factor this may incline Tb populations to have a seemingly higher prevalence rate as the majority of racehorses are under 6 years of age. It has been documented that younger horses are more likely to test positive for EqHV RNA than older horses.

Flies as a transmission route was explored in Chapter 3. A population of *Stomoxys* were fed on whole equine blood spiked with plasmid containing EqHV DNA at a concentration found in infected horses. Several methods were employed to collect blood residue off the flies and enough of this contaminated blood was able to be detected by qPCR. Going forward this experiment should be repeated with a sample of equine blood infected with EqHV. This was outside the scope of this project, as all samples were 'convenience samples', obtained as an excess after necessary blood work was carried out, and arrived as sera. *Stomoxys* will only feed on whole blood, not sera.

Analysis of codon usage bias data contributed evidence to substantiate EqHV transmission via a mechanical vector rather than a biological vector. The RSCU profile of EqHV was more consistent with viruses that did not

use insects as an intermediate host or biological vector. Although this is not a conclusive study, it adds support to the likelihood of biting flies being responsible for mechanical transmission of EqHV.

Data from this study show EqHV infection affects clinical chemistry and haematological parameters differently in the two populations of horses. The lymphocyte count in the Bransby rescue horse population was significantly higher in the infected group, potentially due to an acute immune response to the virus, further supported by the ELISA results in chapter 4. In contrast, the Thoroughbred population showed some evidence of chronic infections as well as significantly elevated GLDH levels in the infected horses with no increased lymphocyte count.

While the Bransby population had a high level of EqHV infections, no obvious markers of disease were found other than increased lymphocyte count which is non-specific.

The positive Thoroughbred population also had significantly higher mean values of haemoglobin, MCH, PCV, and MCV, but a significantly lower MCHC value. This was only observed in the racehorse population more research is needed to identify the mechanism behind this.

Currently there are no published studies examining the effects of EqHV infections on haematology. This study serves as a starting point for future investigations into the impacts of this virus on various physiological systems in horses. If a link between EqHV and haematological parameters can be confirmed, it may have implications for the racehorse industry as well as other sports horse industries that rely heavily on the health and fitness of horses.

Further research is needed to understand the underlying mechanisms of chronic and acute infection and any relationship to clinical chemistry and haematological parameters and their implications for horse health and performance.

The ELISA developed in this study was reliable and repeatable as well as having less non-specific binding than all other methods tried. This ELISA may also be feasible to produce for veterinary use. This ELISA was used to assess EqHV antibody production in the Bransby horses. All horses positive for EqHV with PCR test showed an increase in EqHV antibodies, with decreasing levels over time in the limited repeat samples available for some horses. To further validate the EqHV ELISA assay, specificity against other viral strains and unrelated viruses should be assessed. Sensitivity should be evaluated with titration assays to assess the lower detection limit. Monitoring EqHV antibody levels may be useful for understanding infection dynamics and detecting outbreaks. A more accessible ELISA test could advance the prevalence and impact of EqHV infections in horses.

5.1 Summary

In summary, as well as providing new insight into EqHV, this thesis provides a working novel diagnostic assay, using viral proteins expressed in plants, that could be an asset to veterinary diagnostics for EqHV. A substantially higher level of prevalence than previously reported in the UK was detected in two different UK equine populations. A seasonal link was found among EqHV cases with a much higher prevalence of cases found in the warmer months which also correlates with the seasonal activity of biting flies. Chronic infections with no change in lymphocytes were more common in the younger Thoroughbreds and the Bransby welfare population had acute outbreaks with raised lymphocytes and antibody responses. Haematology data showed Thoroughbred horses positive for EqHV had blood parameters consistent with changes seen as a result of hypoxia. Creatine kinase was significantly lower in the Bransby population positive for EqHV and GLDH was significantly higher in the EqHV positive Thoroughbred population.

5.2 Future Research

To be validated as a diagnostic assay the ELISA would need to conform to the 'fitness for purpose' as per the World Organisation for Animal Health (OIE) guidelines. This includes submitting a validation report for review containing detailed information on the assay design, validation methods, performance data, and supporting evidence and approval by the relevant national or international authority. The assistance of an existing veterinary diagnostics manufacturing company would be most helpful for this.

The development of a more accessible and reliable ELISA for EqHV antibodies could provide options for use in further research into the dynamics of EqHV infection in equine populations. Such as the dynamics of the antibody responses to EqHV infection in different equine populations, and the distribution of EqHV in different geographic regions and time periods.

Further studies should be done to confirm the role biting flies play in EqHV transmission. More fly trapping should be conducted to identify the species of flies that carry the virus and the locations where they are most prevalent. In addition, feeding flies with infected blood could provide valuable information about the efficiency of EqHV transmission by flies. Such studies could contribute to the development of targeted control strategies to limit the spread of EqHV.

The clinical impacts of EqHV should be further assessed. Research should be done assessing the long-term effects of EqHV infection on equine liver health and function, as well as identifying the potential cause for chronic

infections and associated complications. Potential impact on performance of horses while infected with EqHV should be assessed. These studies would be valuable for veterinarians, trainers, and owners in managing the health and welfare of equine athletes, as well as the general equine population.

Appendix

6.1 EqHV E1/E2 sequence

Full EqHV E1/E2 sequence codon optimised for expression in plants including his tags and Kozak sequence:

accggtgccaccatgggattcgttttgttttctcaacttccatcatttcttttggtttctacacttttgctttttcttgt
tatttctcattcatgtagagcttgcgattcagattacattaatgtttctctttcatgtaactttactgttaagcaaat
gtggggttggactttctttcctaaatgggctttgcttaatggtcaaagattgaattgtacagaaggttctccat
acaatcctaagtgcaagggacctatggatttcaacatcactacagatccttttattgcttattctggtactaggt
cacatccaccttgtccataccatgtttctagaccttgctcagttcttaatgcttcaagggtttgtggaaagccaa
cttgctttggtccagctcctattgaggttggagttacagatagagatggtaatttggcttcttggaacgatact
ggacaatttttctttgatcttagatcacctcataggccacctagaggaaggtggtatggttgtgtttggttgaa
ttctactggatgggttaagcaatgtggtgctccaccttgcaatatgaagcttatgtctaacacttcaaaaacat
tcgtttgtccatctgattgctttaggcaaaatcctaaagctacatatcaattgtgtggacaaggtccatggatc
tcacataactgcttgatcgattacactgatagataccttcatttcccatgtactgaaaacttcacagtttaccctg
ttaggatgattttgggagatggtgctagagatgttagggttgcttgcaagtttaatagatctatctcatgcag
gacagaggatagacttagggcttctatcgtttcattgctttactctgttactacagctgctgttccaccttgcca
tttttcaccattgcctgcttttactacaggtttgatccatcttgatagaaatctttctgatgttcaatacgtttggg
ctatgactccttcagctgttaacatttttcttaggcatcatcatcatcatcattctgaaaaagatgagctttgact
cgag

6.2 EqHV Core Gene sequence

EqHV Core Gene sequence used for Plant expression before plant codon-optimization:

GGCAACAACTCTAAAAACCAAAAACCAAAACCCCAAAGAGGACC
ACGGAATAGGGTCAGGGGCCAGTCGCGGTCTGGCCCTGTAGTATT
CCCGTCCGGCGCTGTTCTCGTGGGAGGAAGGTATATTCCTCCCCCA
AAGAAGGCCATCCGCGGGCCTCGTAGAGGTTTGGTGCAGGCTCCT
AAGTCATCGGAGCGTGGTTCCCCCCGGAAAAAGCGCCAGCCTCCG
CCACAAACTGACAGCTCGTGGCGAAAGTATTTTCCAAAATTCTGG
GGGGATAGAGGATACCCATGGCCATATGTTGATCCCGTTCTCCAGT
GGAGTGCCTGGGGTACCTCTCCTGGTGCTTATCGCACTAGGTGGGG
ACCCCGTGACCCTCGACACAAGTCGCGCAATCTTGGGCGCGTCATT
GACACTCTCACCTGTGGGGTTGCCGATCTTGCCGGATATGTTCCGG
TTCTCGGCGCTCCCGCAGGTGCGTTGTGTCGTGGCGCTGCTCATCTT
GTGCGGTTTGTTGAGGACGGTGCCAATTTCATCACTGGCAACATTC
CTGGCATGGGTTTCTCTATCTTCTTGCTTGCTCTCCTCTCAGCTGTGT
CTTTCGGCGAGGCCT

6.3 NS3 Gene sequence

NS3 gene before codon optimisation for plants :

atacacttcgcagacatgcggacgcgtagtgtttcctcttgccccccaagtatacggatctggactctccTc
ctgcagttccttcttcataccaggtttcttttctccatgctcctactggcagtggcaaatccaccaagatgccgtt
gtcttatgtggagttgggttatcatgtcctagttcttaatccatcggttgcctcgactctcagttttgggccatac
atggataagacctatggtgaatgtcctaacatcaggacaggtgctagttgcaaaactacaggctctaaact
Tacttattccacctatgggaagttcctagcggatggtggggtttctgctggcgcttatgacataatcatttgt
gatgagtgccatagcacagactccacgtccgttttaggaattggctctgtccttgacggggcagagtctaaa
ggtgtaaagcttgttgttcttgctactgccaccccaccagggtctcagactgttcctcatcctaacatcgatga
ggaggcccttactcagagtggagacatccccttttacgggaagatgttgaagtcgtccttgctccttagtgg
gaggcatcttatcttctgccattcaaagaagaagtgcgaggaagtcgcgctccttctgagaaaggccgga
gctaatgctgtcacctactatcggggcttggatgtttccgtcatcccgaatgaggggaatgttgtcgtcgttg
ccacggatgcccttatgacagggtattctggcaacttcgacactgttactgactgcaaca

6.4 Bransby Horses Blood Parameter Plots

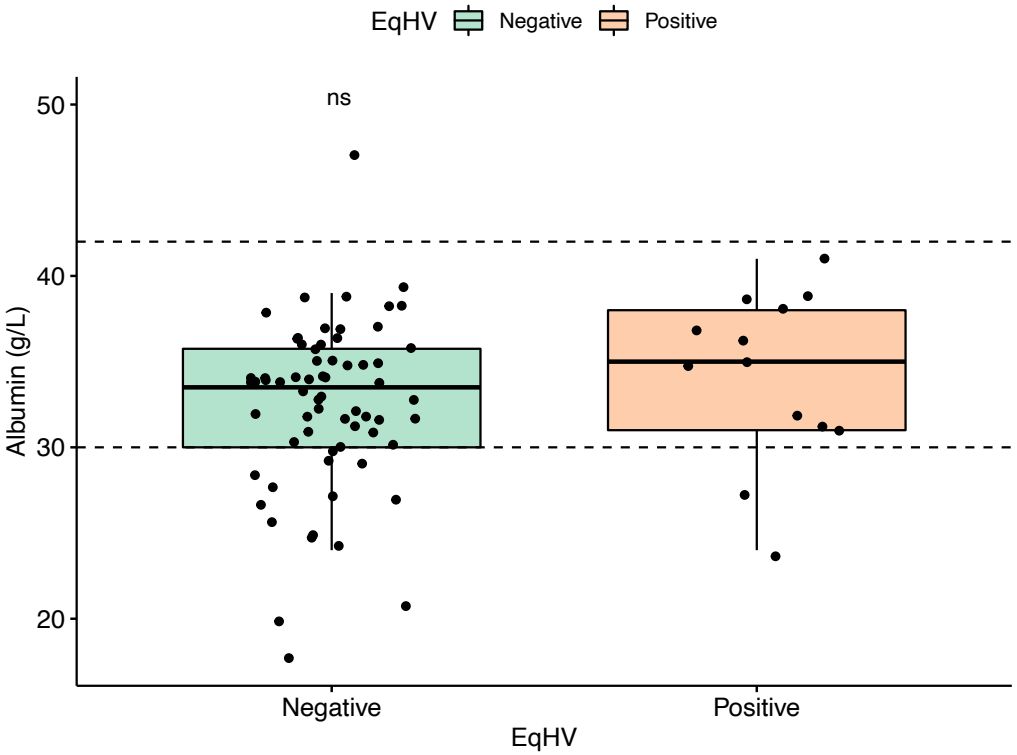


Figure 54 Individual values and mean average levels of albumin for sera either positive or negative for equine hepacivirus. Values within the dashed line indicates 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns)

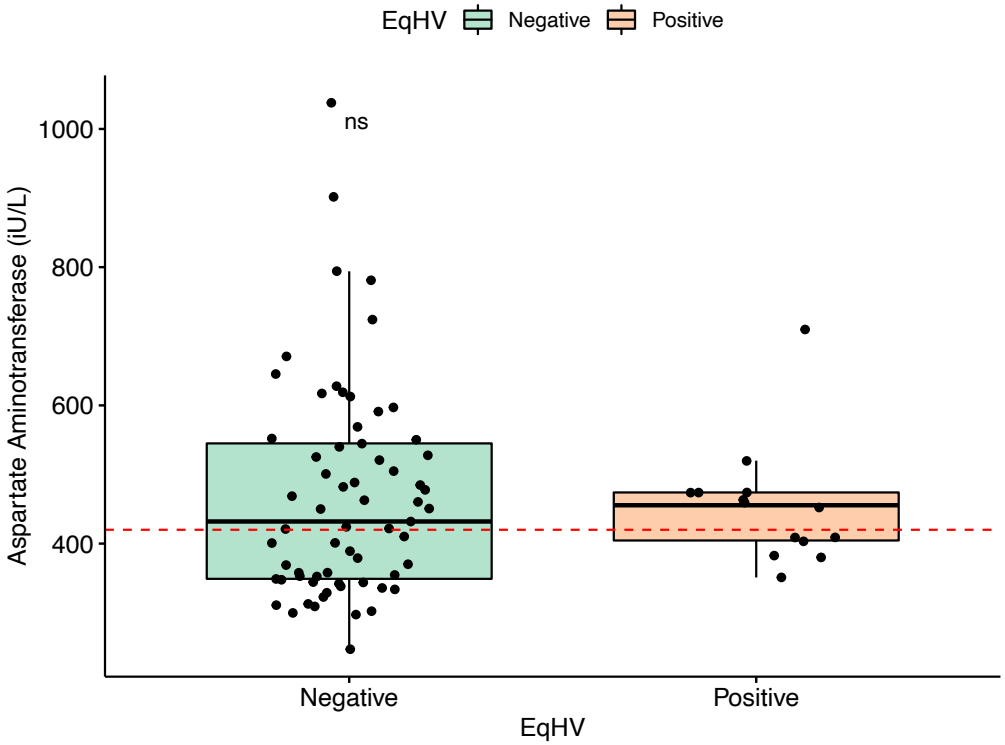


Figure 55 Individual values and mean average levels of aspartate aminotransferase for sera either positive or negative for equine hepacivirus. Values below the dashed red line are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns)

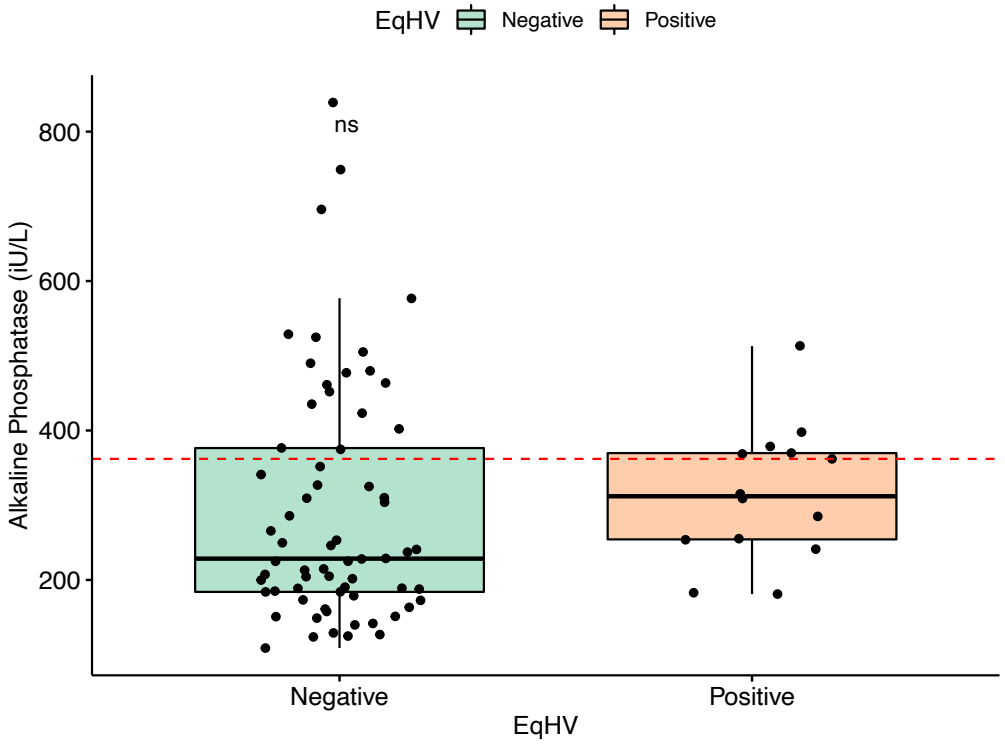


Figure 56 Individual values and mean average levels of alkaline phosphatase for sera either positive or negative for equine hepacivirus. Values below the dashed red line are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns).

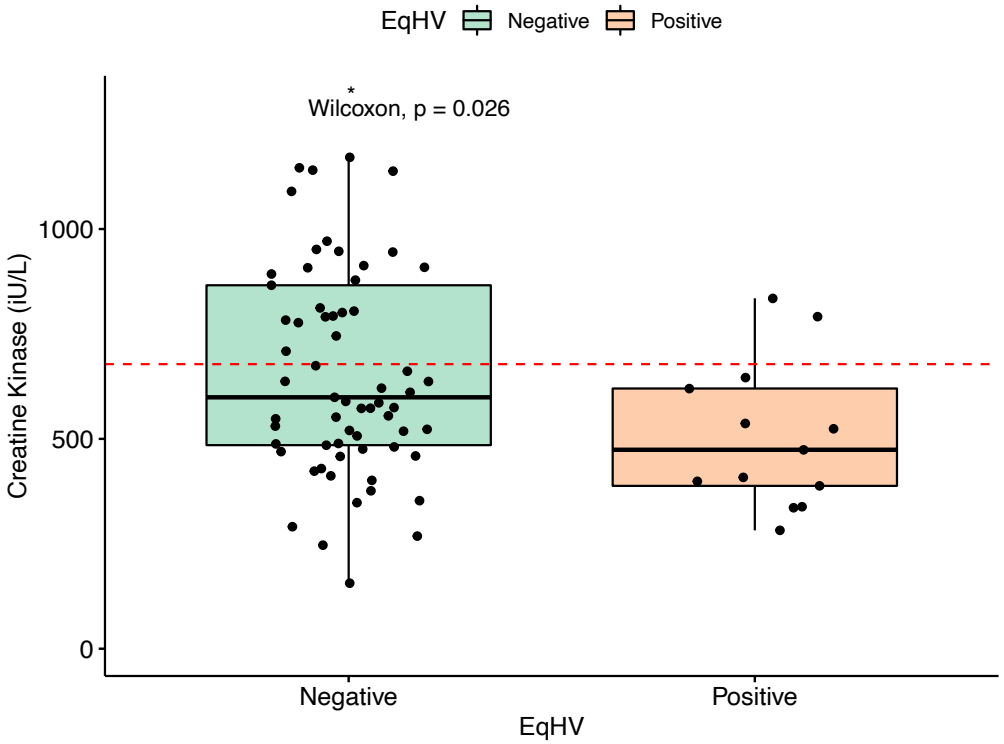


Figure 57 Individual values and mean average levels of creatine kinase for sera either positive or negative for equine hepacivirus. Values below the dashed red line are considered to be in 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means using the Wilcoxon test showed a significant difference between means with a p value of 0.026.

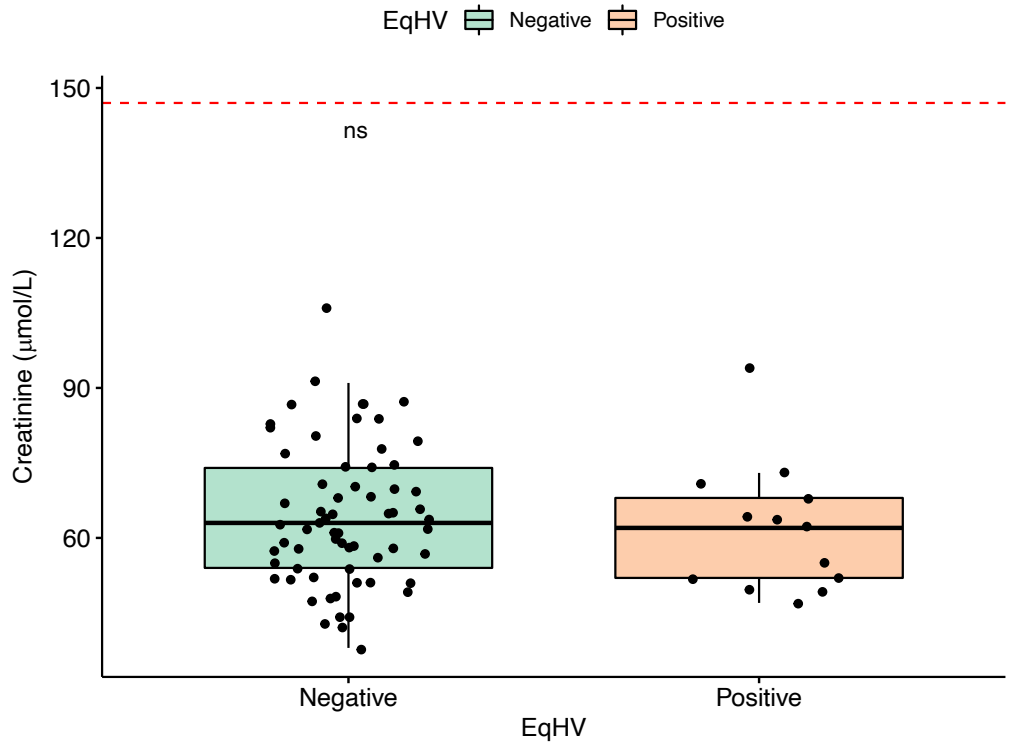


Figure 58 Individual values and mean average levels of creatinine for sera either positive or negative for equine hepacivirus. Values below the dashed red line are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns).

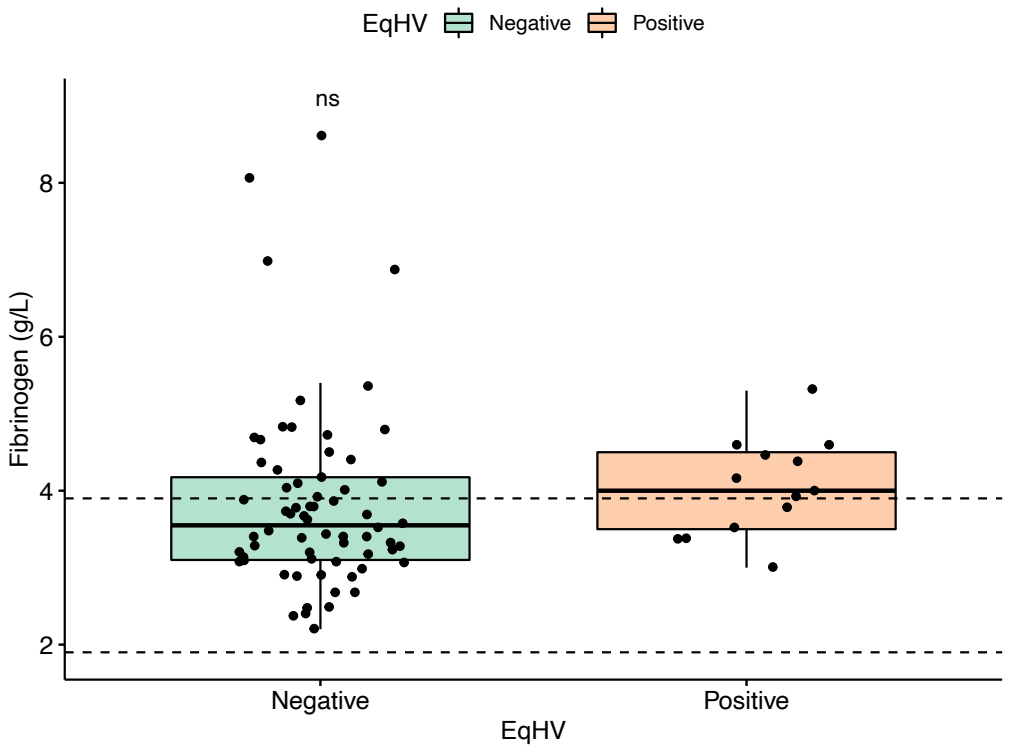


Figure 59 Individual values and mean average levels of fibrinogen for sera either positive or negative for equine hepacivirus. Values in between the dashed black lines are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns).

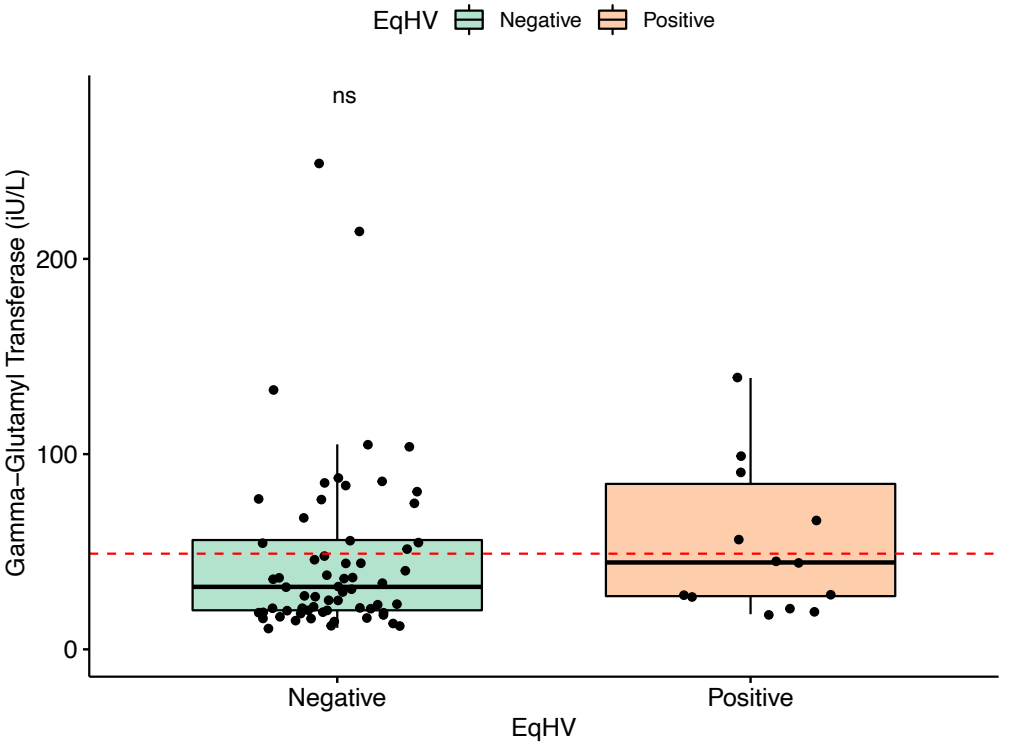


Figure 60 Individual values and mean average levels of gamma-glutamyl transferase for sera either positive or negative for equine hepacivirus. Values below the dashed red line are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns).

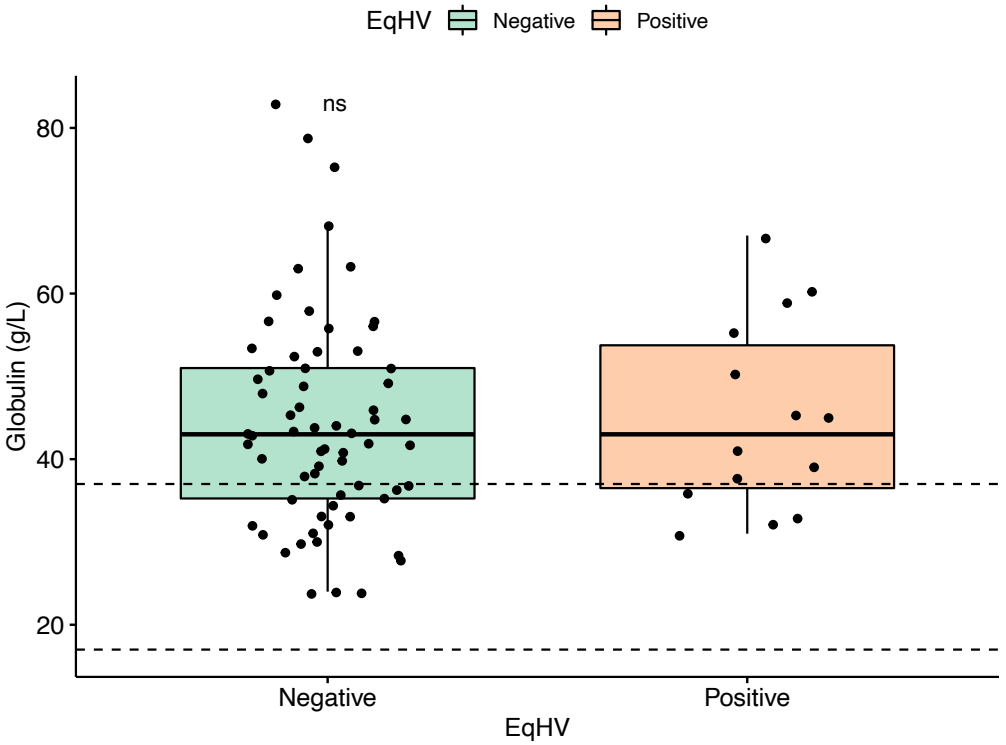


Figure 61 Individual values and mean average levels of globulin for sera either positive or negative for equine hepacivirus. Values below the dashed red line are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns).

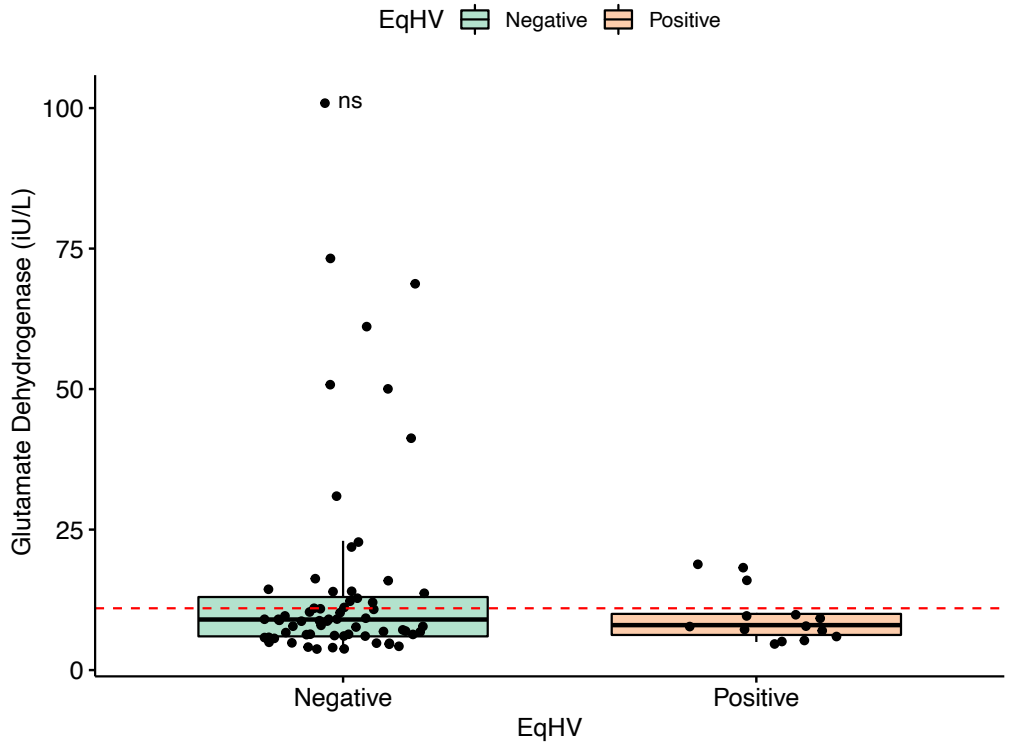


Figure 62 Individual values and mean average levels of glutamate dehydrogenase for sera either positive or negative for equine hepacivirus. Values below the dashed red line are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns).

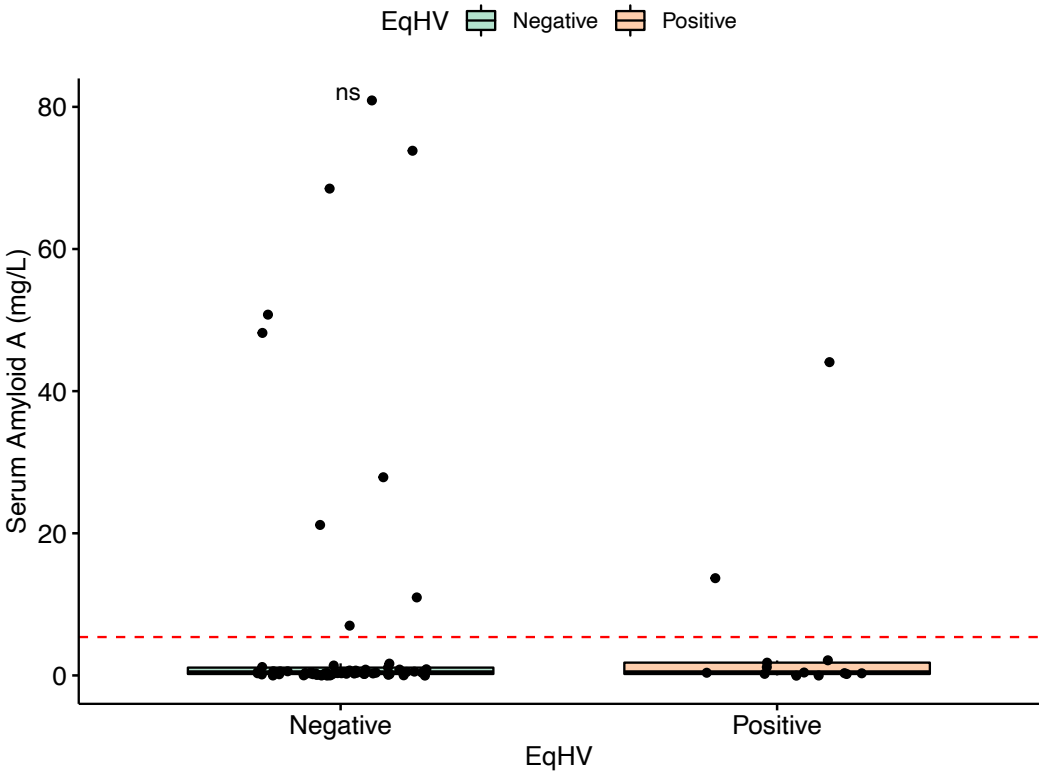


Figure 63 Individual values and mean average levels of serum amyloid A for sera either positive or negative for equine hepacivirus. Values below the dashed red line are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns).

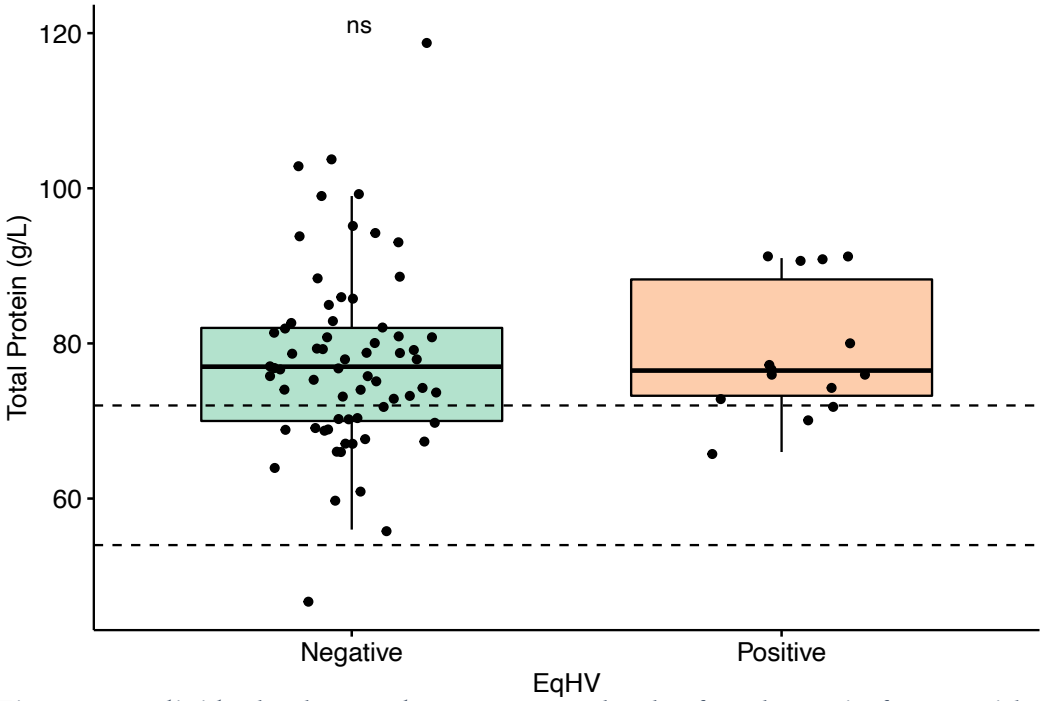


Figure 64 Individual values and mean average levels of total protein for sera either positive or negative for equine hepacivirus. Values within the dashed black lines are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns).

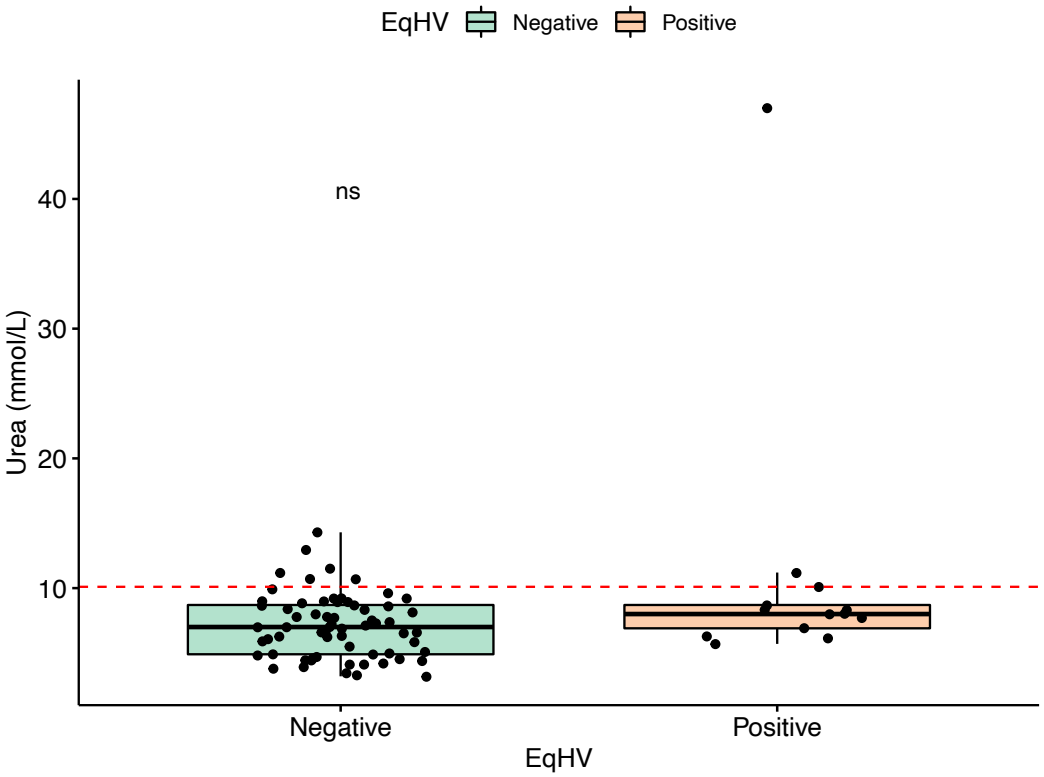


Figure 65 Individual values and mean average levels of urea for sera either positive or negative for equine hepacivirus. Values below the dashed red line are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns).

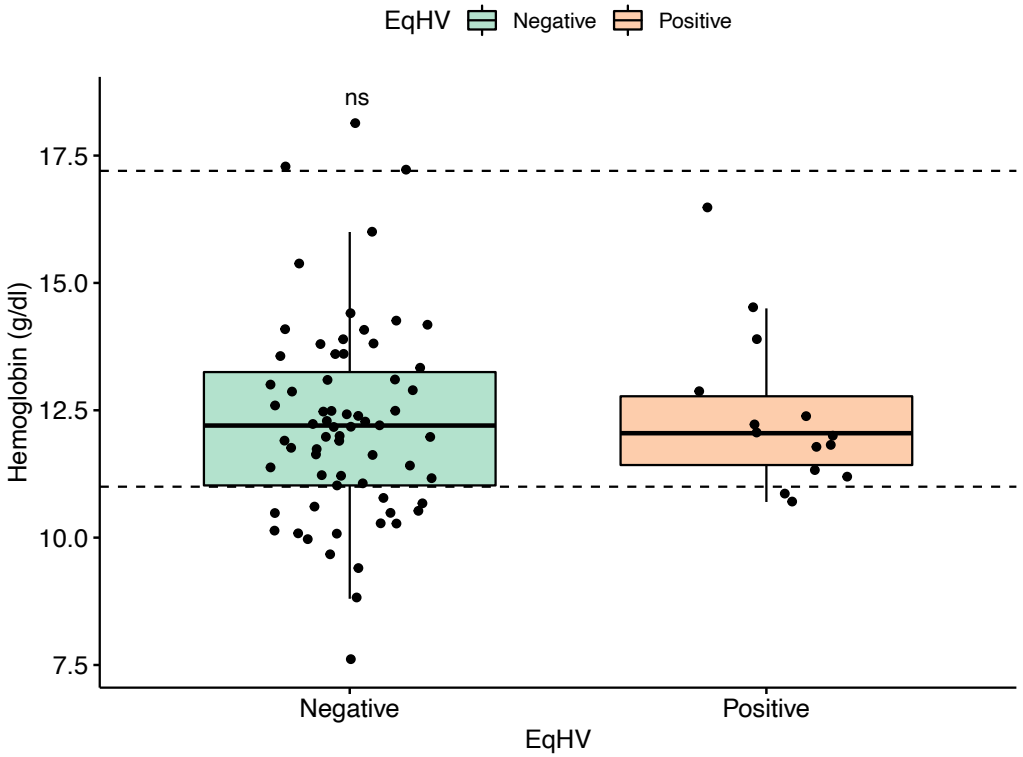


Figure 66 Individual values and mean average levels of haemoglobin for samples either positive or negative for equine hepacivirus. Values between the dashed line are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns)

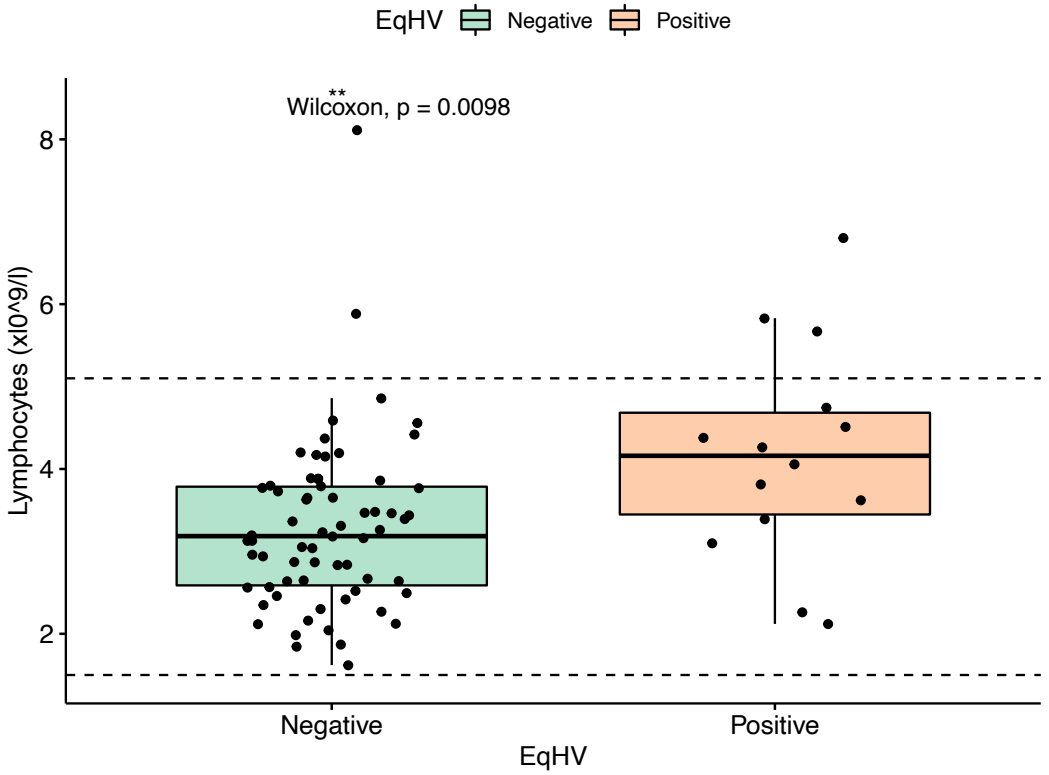


Figure 67 Individual values and mean average levels of lymphocytes for samples either positive or negative for equine hepacivirus. Values between the dashed lines are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means using the Wilcoxon test showed a significant difference between means with a p value of 0.00098

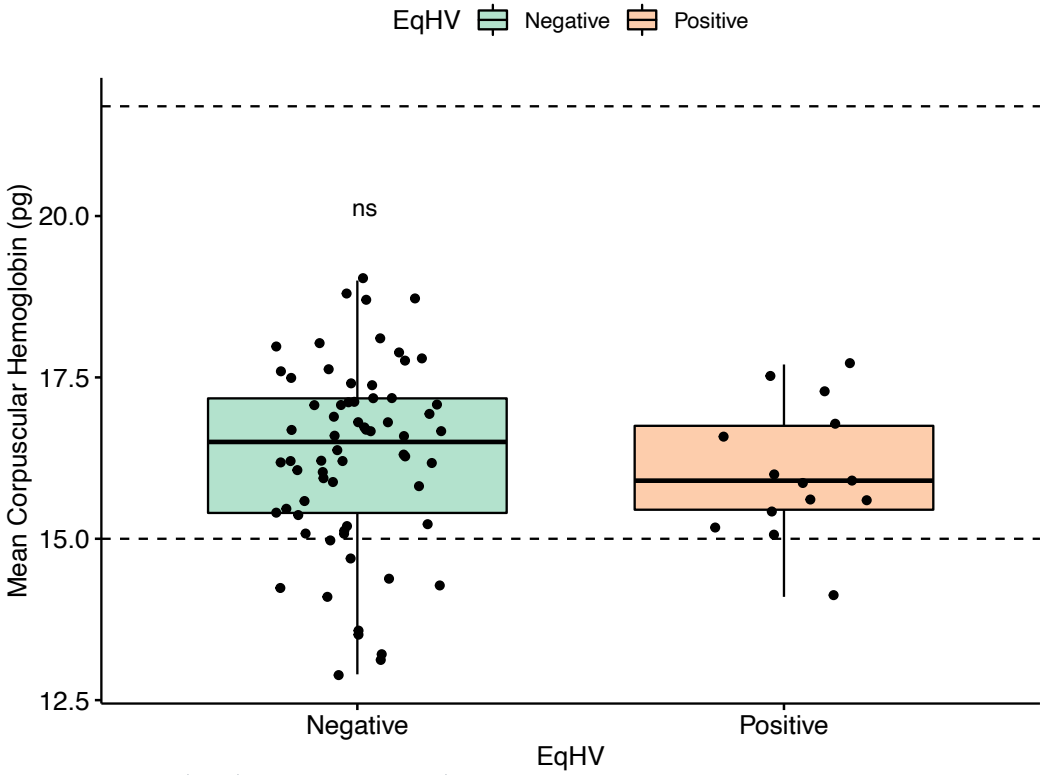


Figure 68 Individual values and mean average levels of mean corpuscular haemoglobin for samples either positive or negative for equine hepacivirus. Values between the dashed lines are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns)

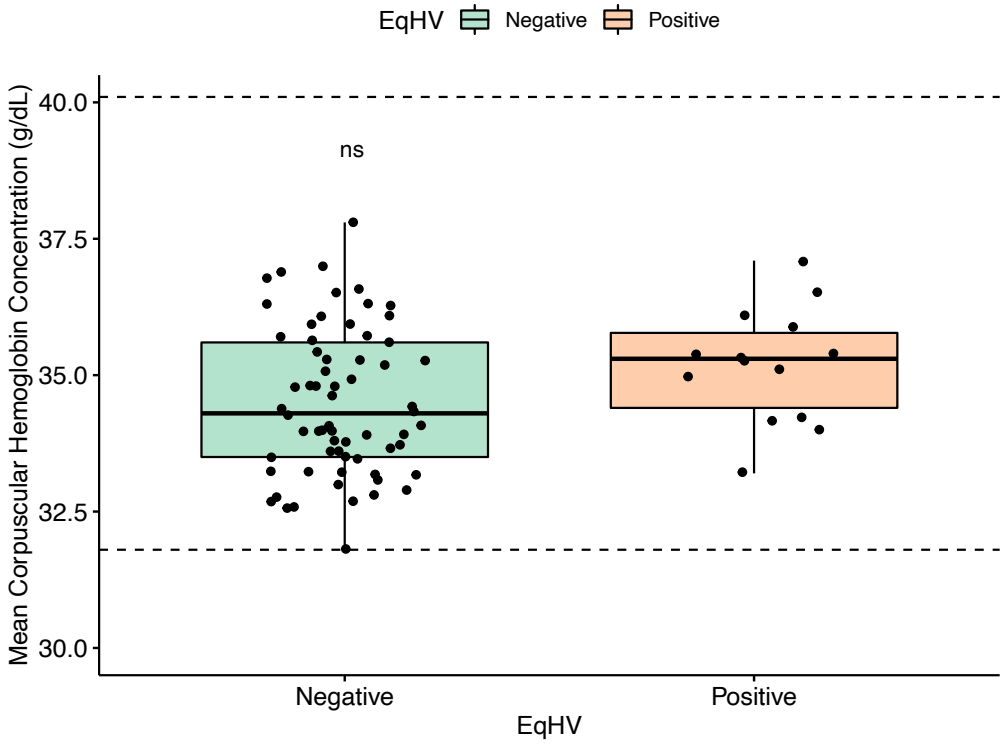


Figure 69 Individual values and mean average levels of mean corpuscular haemoglobin concentration for samples either positive or negative for equine hepacivirus. Values between the dashed lines are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns)

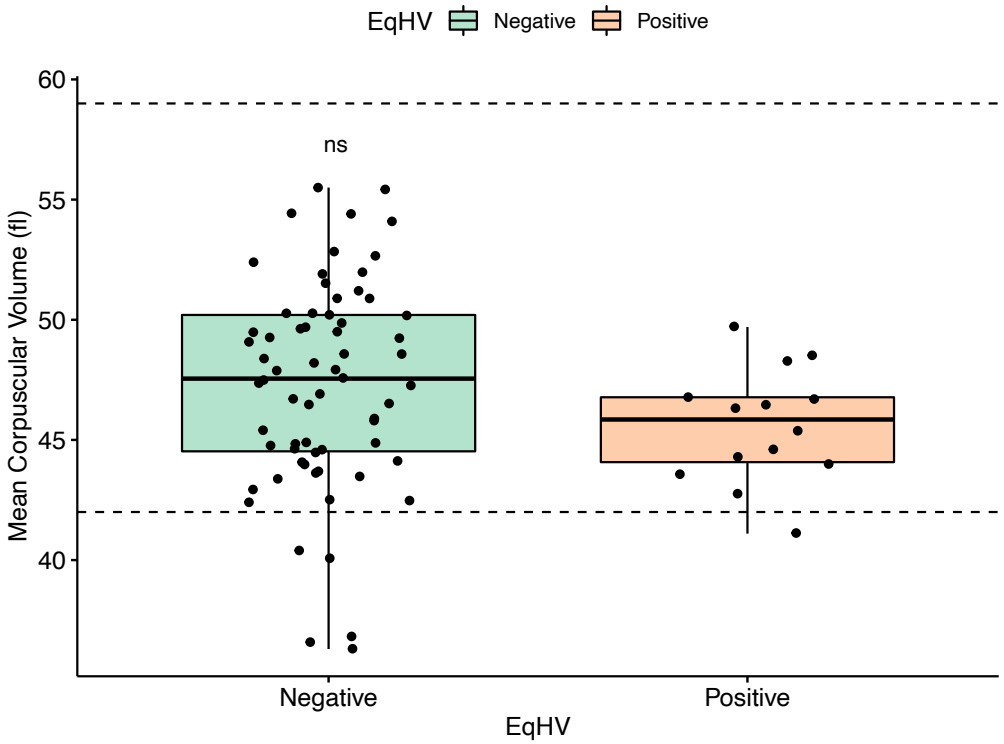


Figure 70 Individual values and mean average levels of mean corpuscular volume for samples either positive or negative for equine hepacivirus. Values between the dashed lines are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns)

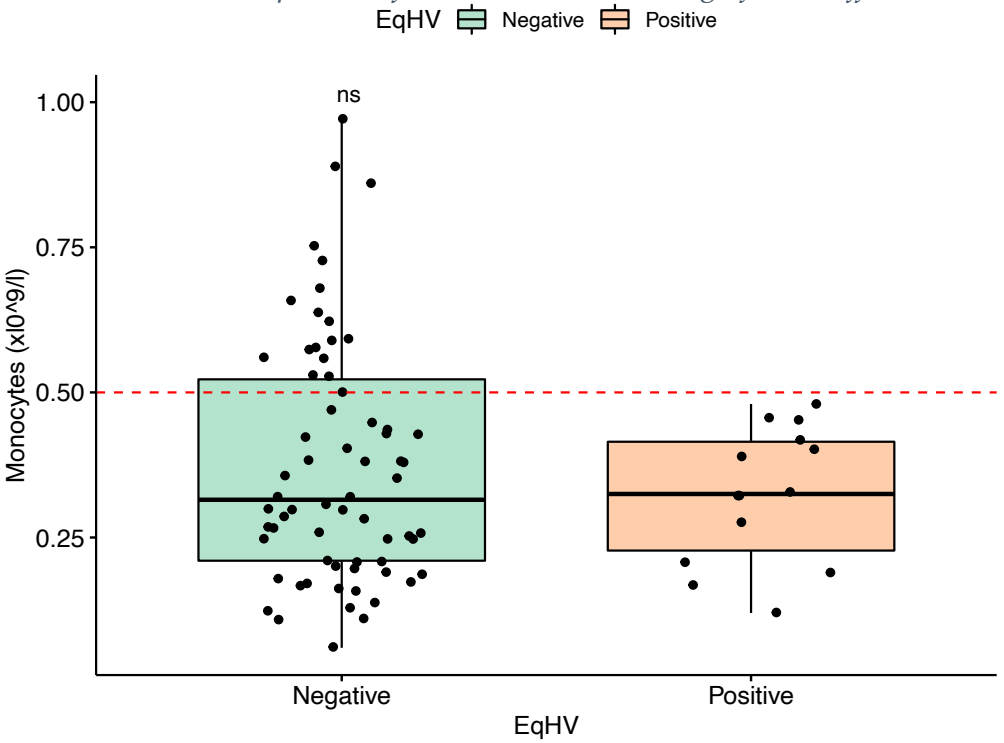


Figure 71 Individual values and mean average levels of monocytes for samples either positive or negative for equine hepacivirus. Values below the dashed line are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns)

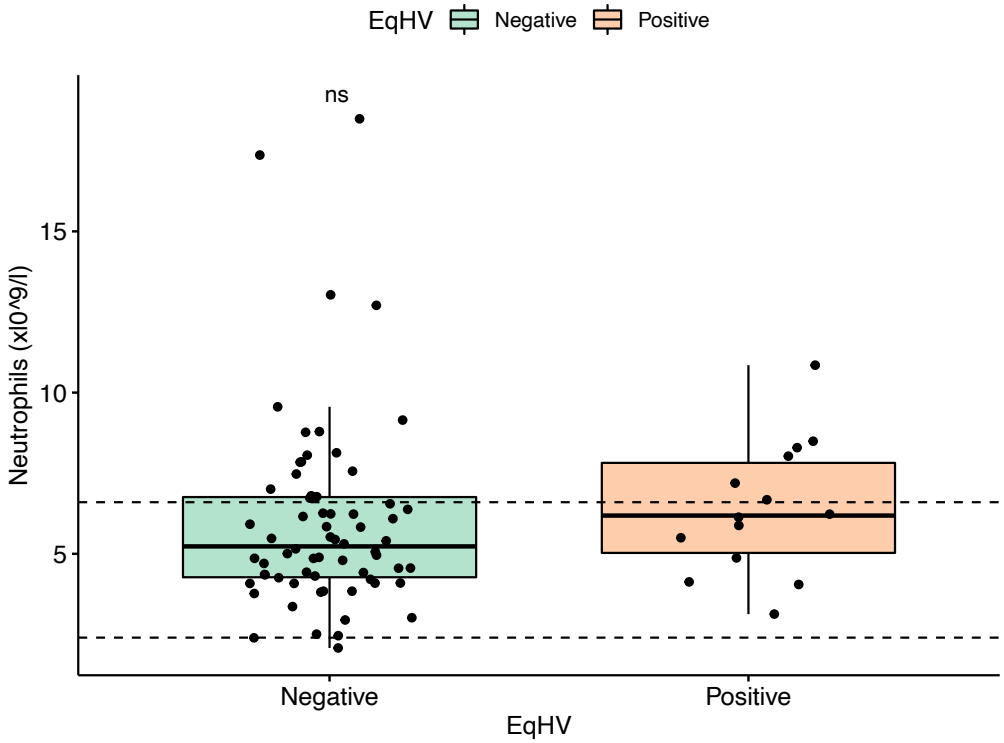


Figure 73 Individual values and mean average levels of mean neutrophils for samples either positive or negative for equine hepacivirus. Values between the dashed lines are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns)

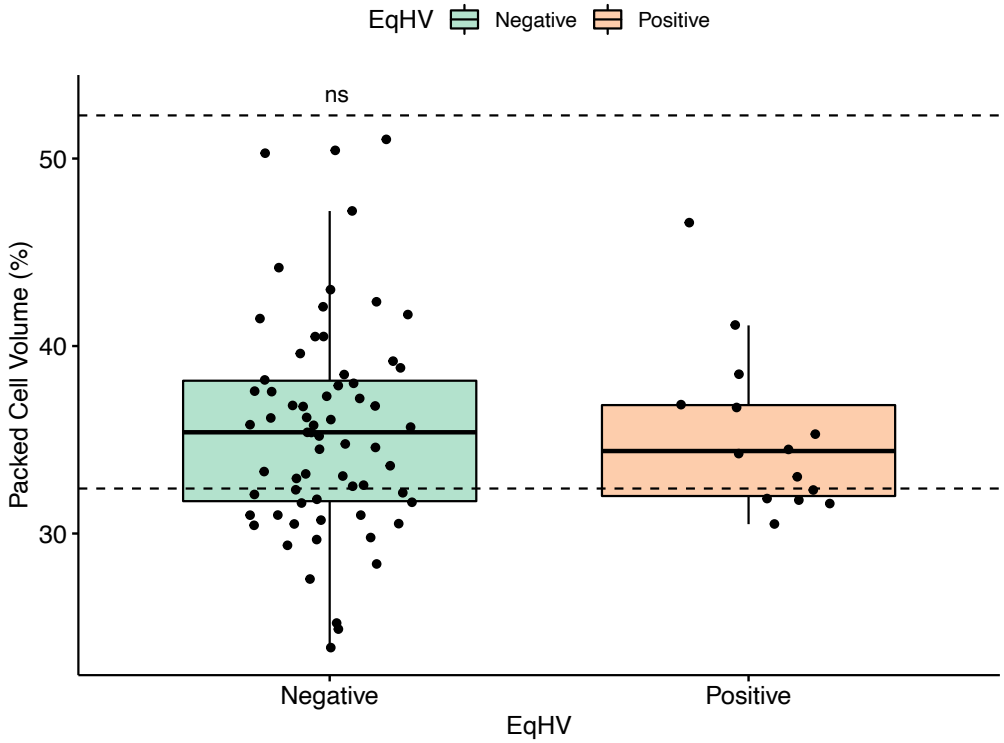


Figure 72 Individual values and mean average levels of packed cell volume for samples either positive or negative for equine hepacivirus. Values between the dashed lines are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns)

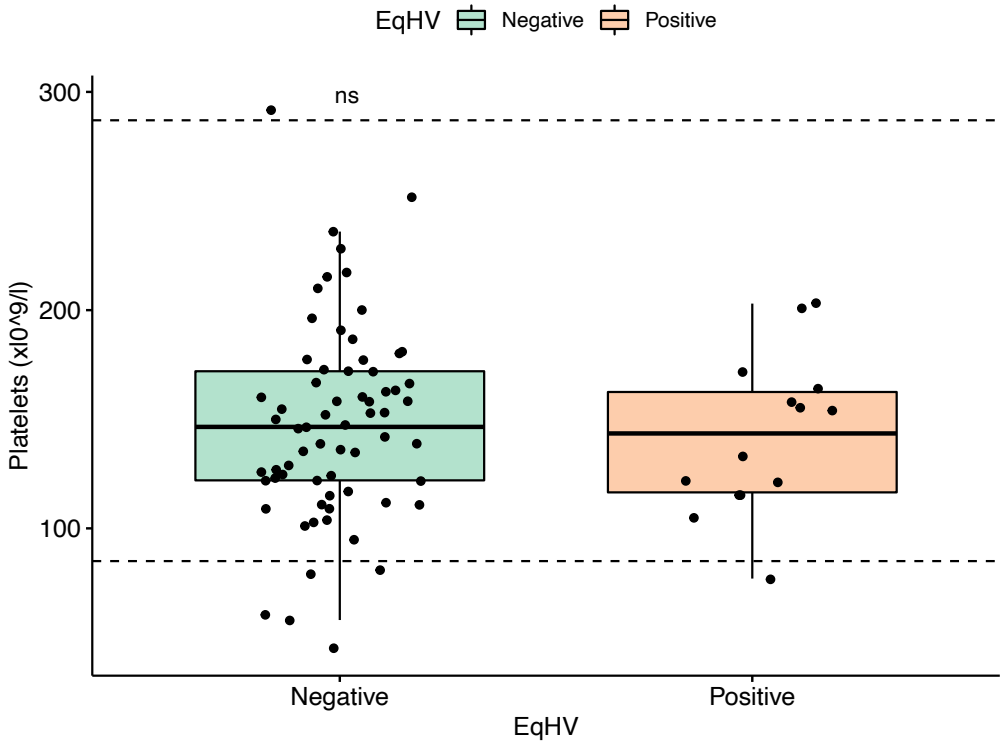


Figure 74 Individual values and mean average levels of platelets for samples either positive or negative for equine hepacivirus. Values between the dashed lines are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns)

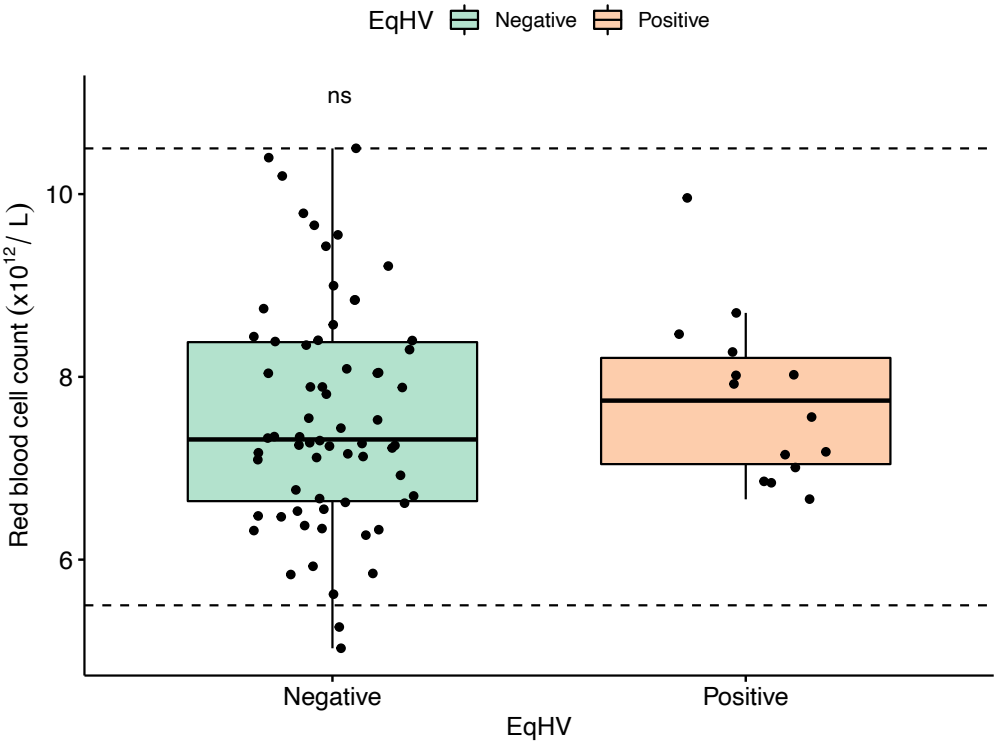


Figure 75 Individual values and mean average levels of red blood cell counts for samples either positive or negative for equine hepacivirus. Values between the dashed lines are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns)

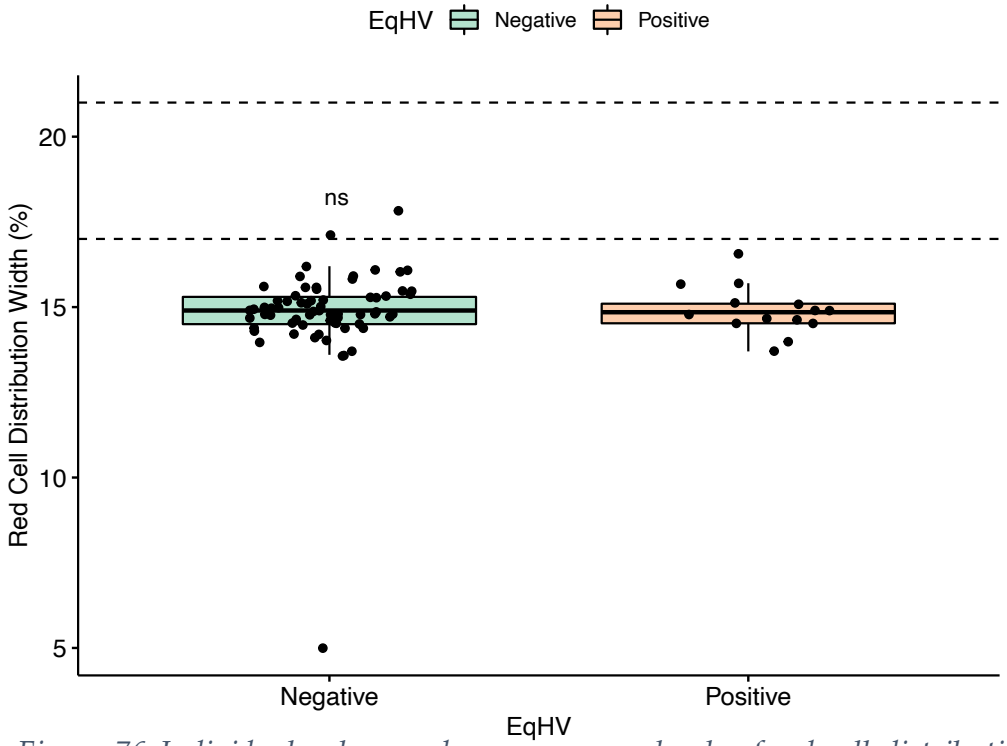


Figure 76 Individual values and mean average levels of red cell distribution width for samples either positive or negative for equine hepacivirus. Values between the dashed lines are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns)

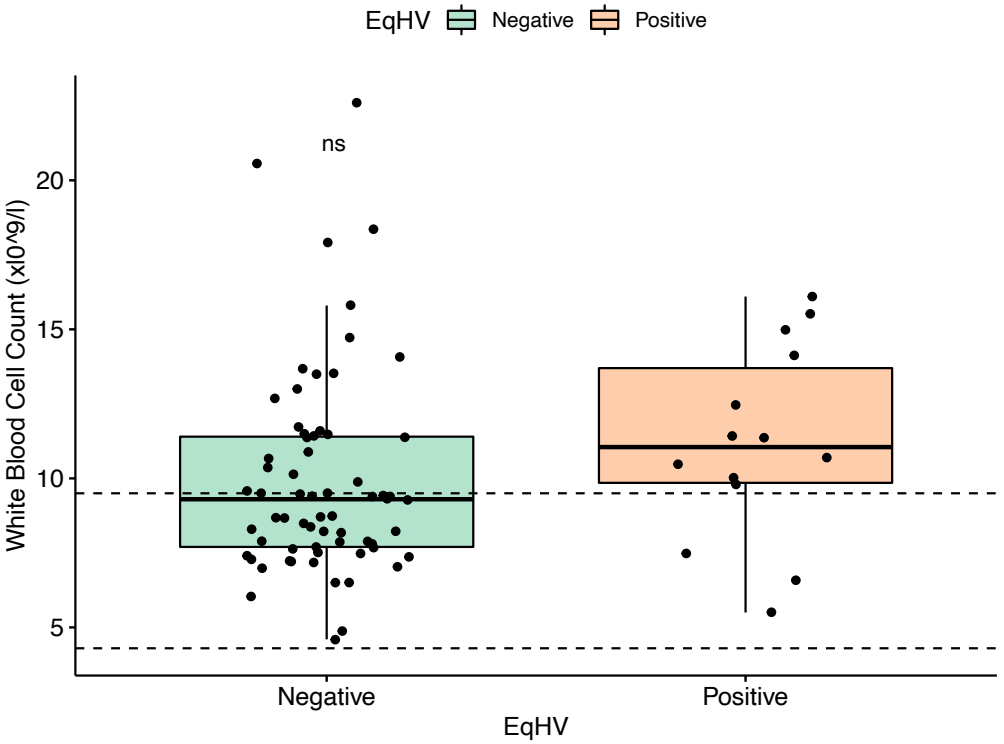


Figure 77 Individual values and mean average levels of white blood cell count for samples either positive or negative for equine hepacivirus. Values between the dashed lines are classed as 'normal range'. Blood samples from Bransby rescue horses. Statistical comparison of means concluded no significant difference (ns)

6.5 Thoroughbred Horse Blood Parameter Plots

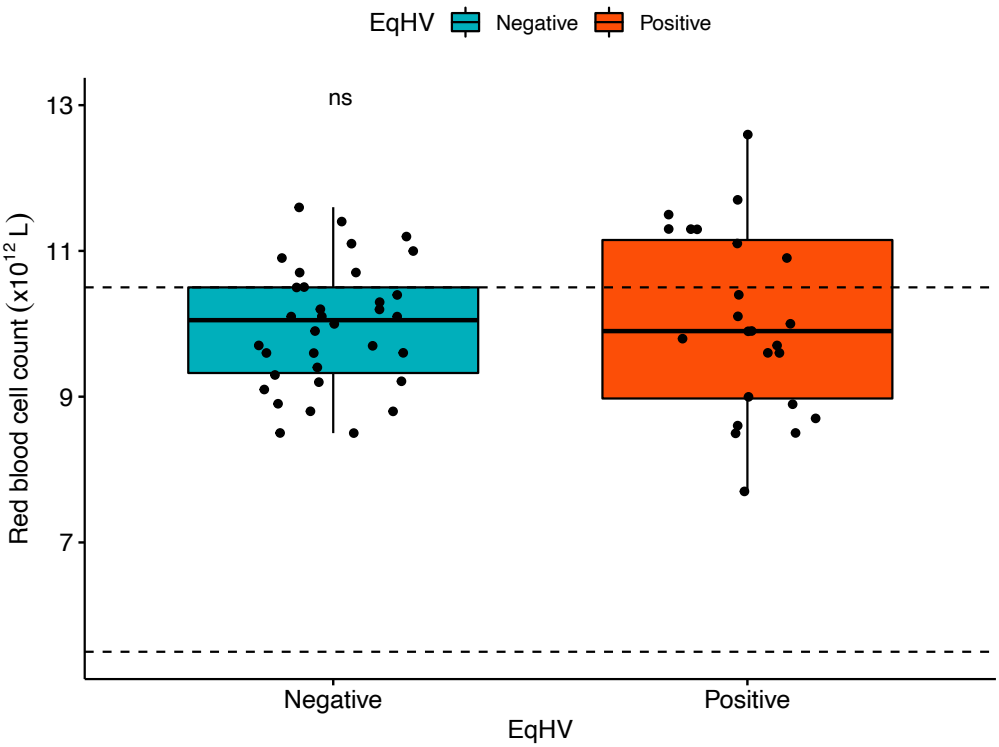


Figure 78 Individual values and mean average levels of red blood cell counts for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means concluded no significant difference (ns).

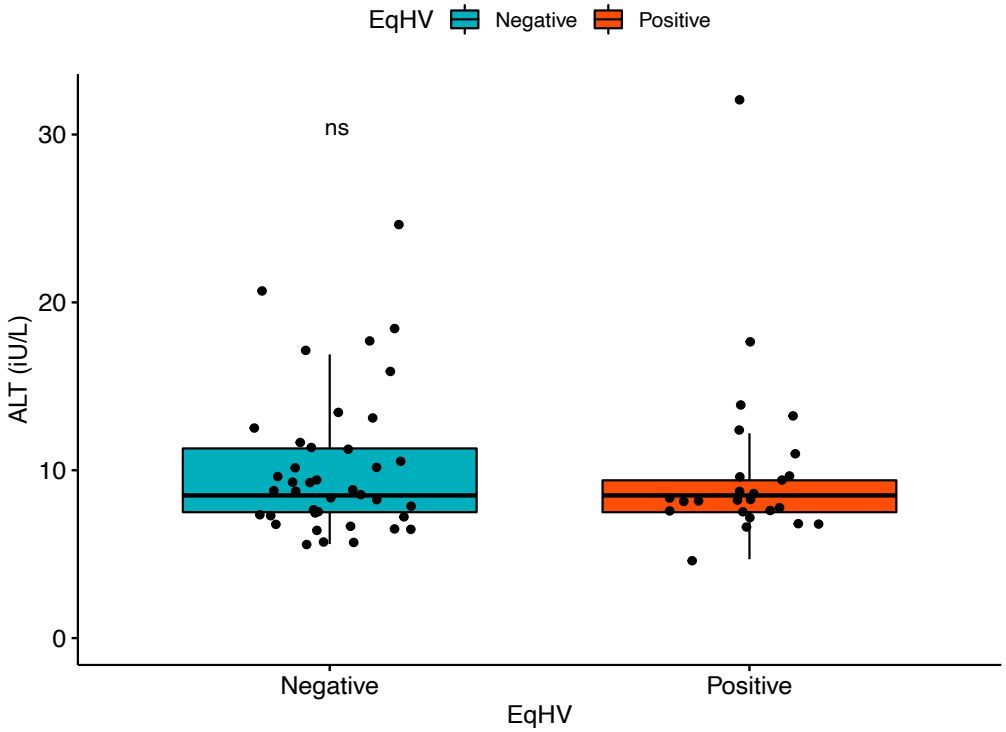


Figure 79 Individual values and mean average levels of alanine transaminase (ALT) for sera either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means concluded no significant difference (ns).

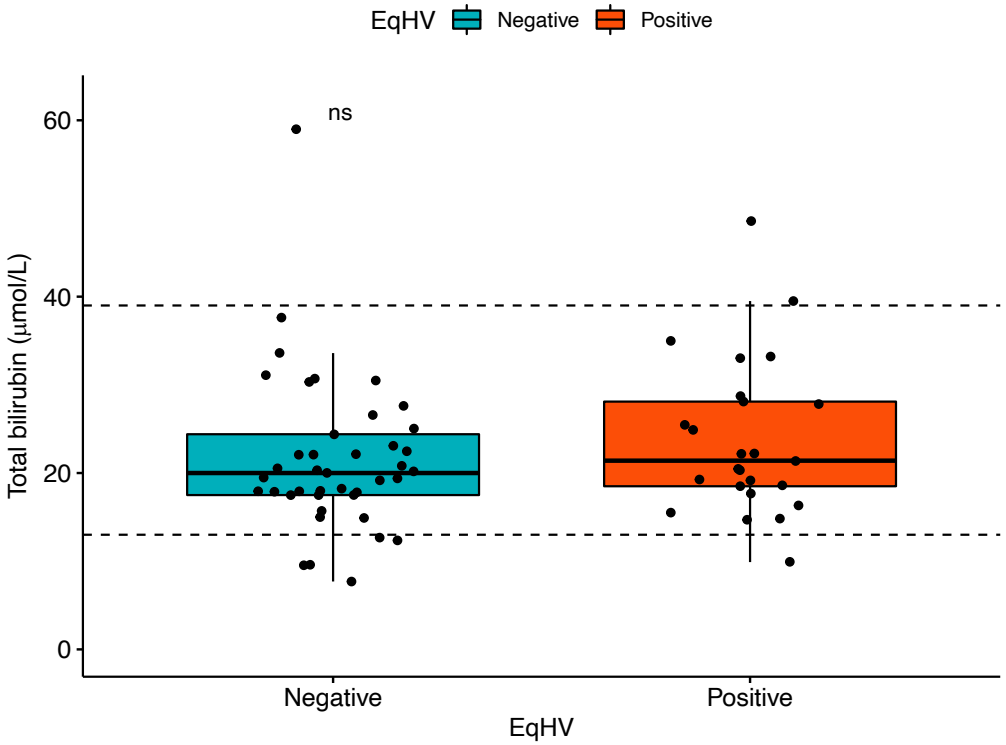


Figure 80 Individual values and mean average levels of total bilirubin for sera either positive or negative for equine hepacivirus. Dashed line indicates 'normal range'. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means concluded no significant difference (ns). Log transformation of data resulted in normal distribution but no statistical significance in means comparison.

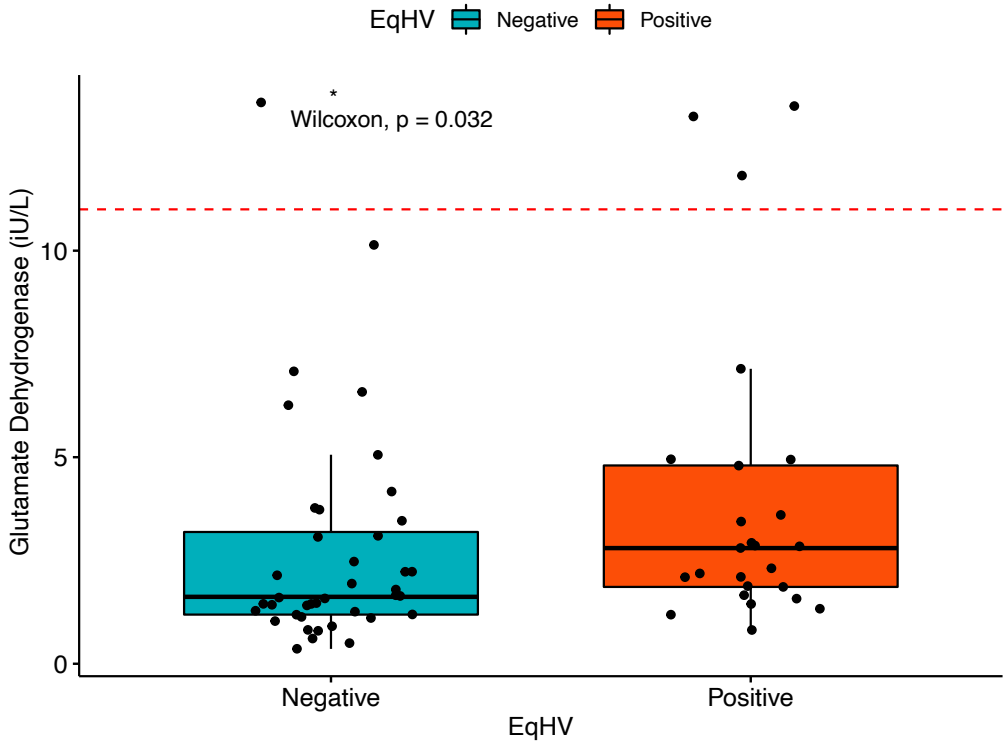


Figure 81 Individual values and mean average levels of glutamate dehydrogenase (GLDH) for sera either positive or negative for equine hepacivirus. Values under red dashed line are considered 'normal'. Horse blood from Thoroughbreds in training. Statistical comparison of means using the Wilcoxon signed rank test found a significant difference with a p value of 0.032. Log transformation resulted in normal distribution and means comparison using T-test gave a significant result and p value of 0.035.

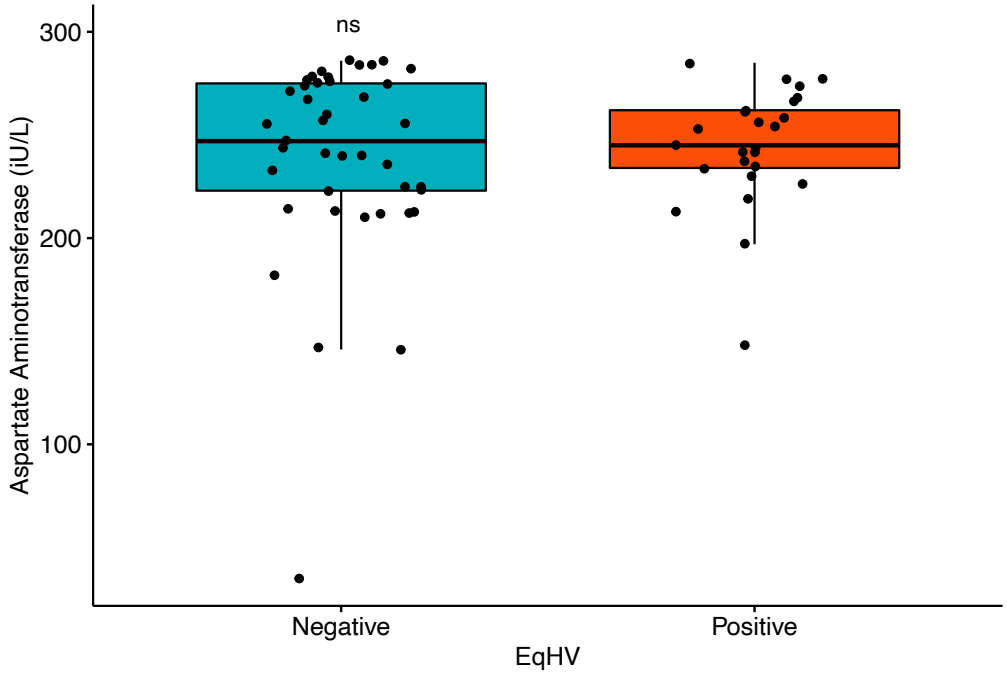


Figure 82 Individual values and mean average levels of aspartate aminotransferase (AAT) for sera either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means concluded no significant difference (ns).

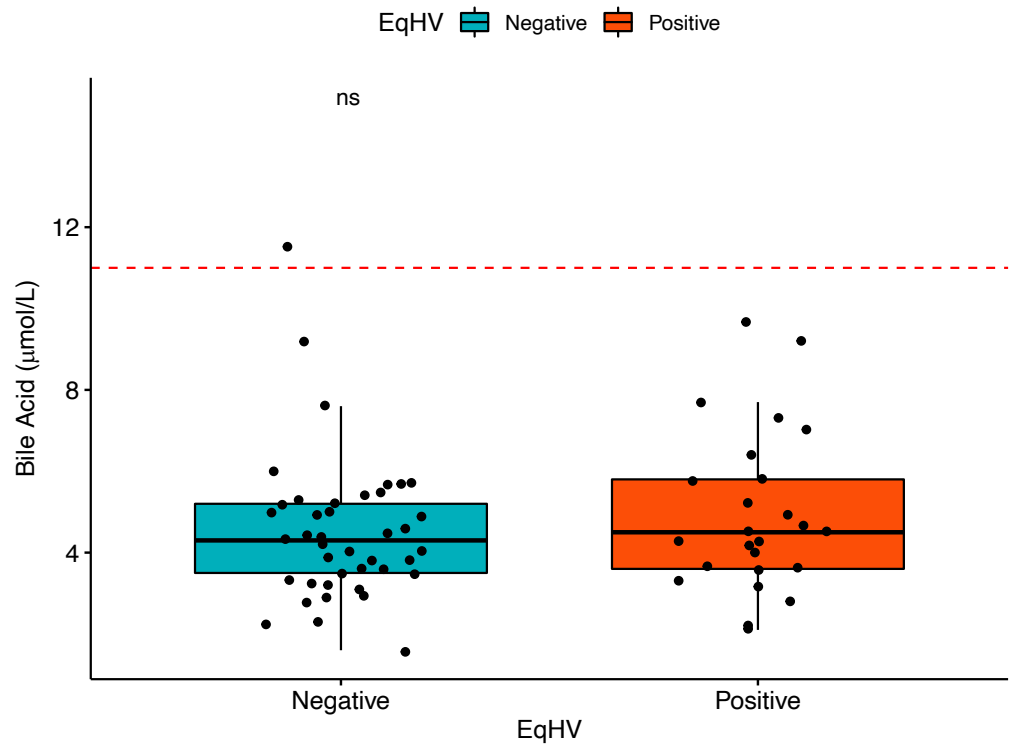


Figure 83 Individual values and mean average levels of bile acids for sera either positive or negative for equine hepacivirus. Values under red dashed line are considered 'normal'. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means concluded no significant difference (ns).

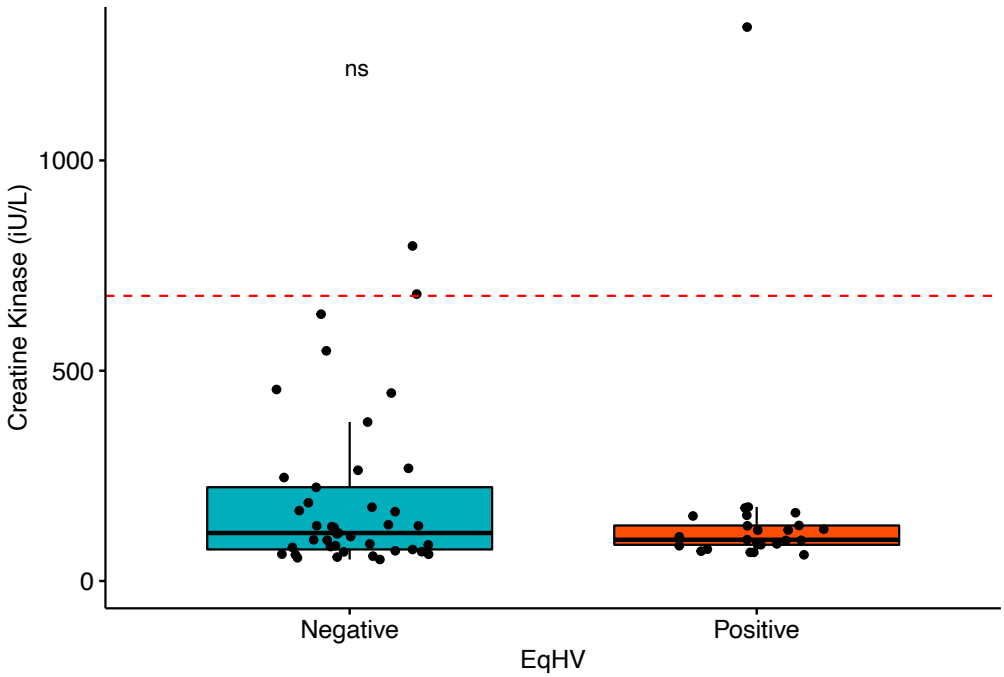


Figure 84 Individual values and mean average levels of creatine kinase (CK) for sera either positive or negative for equine hepacivirus. Values under red dashed line are considered 'normal'. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means concluded no significant difference (ns).

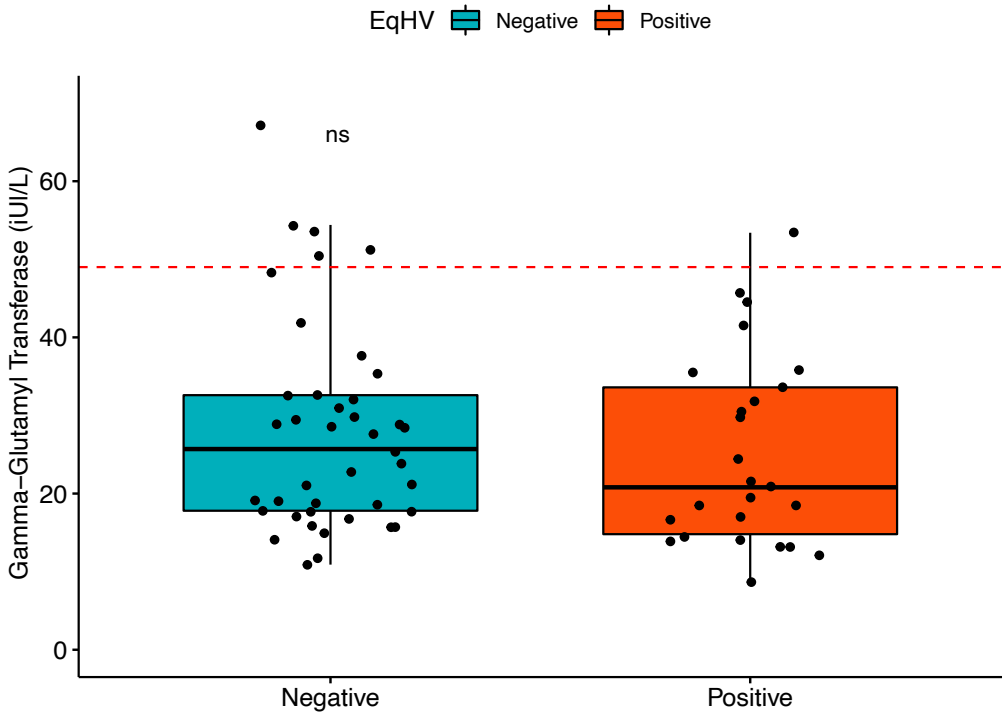


Figure 85 Individual values and mean average levels of gamma-glutamyl transferase (GGT) for sera either positive or negative for equine hepacivirus. Values under red dashed line are considered 'normal'. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means concluded no significant difference (ns).

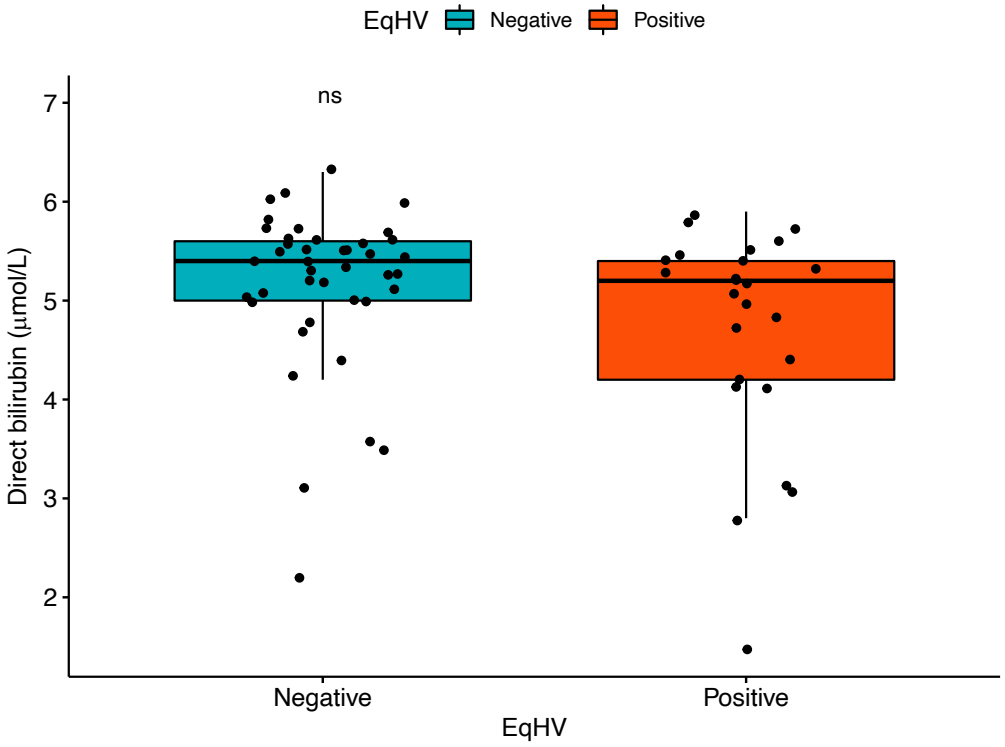


Figure 86 Individual values and mean average levels of direct bilirubin for sera either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means concluded no significant difference (ns).

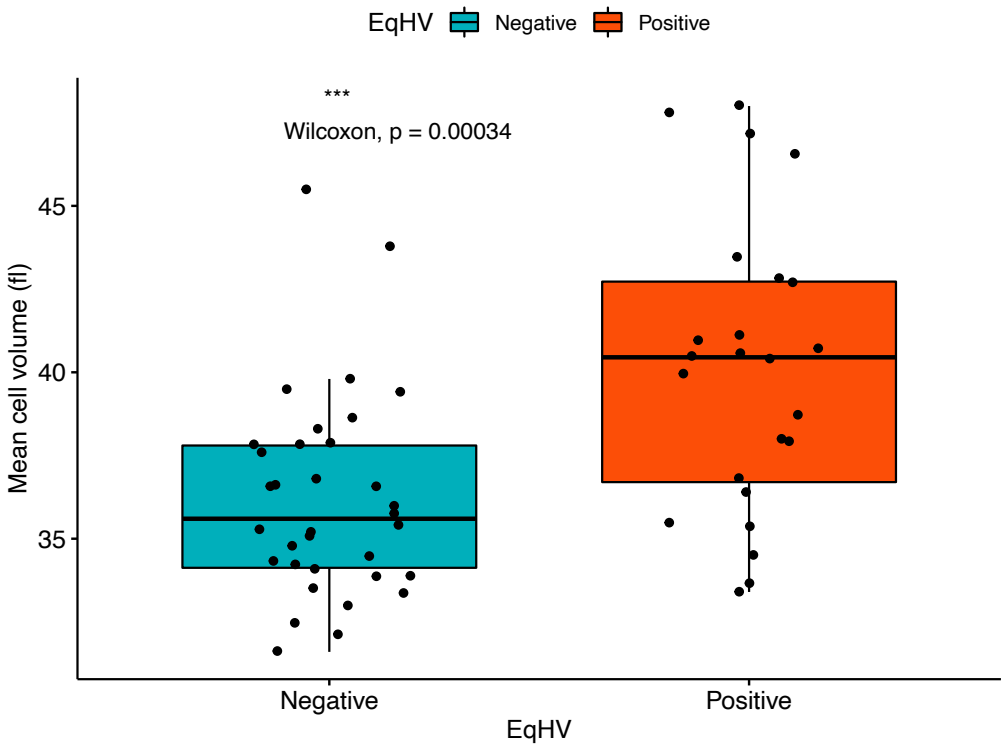


Figure 87 Individual values and mean average levels for mean cell volume for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Area in between dashed line indicates 'normal range'. Statistical comparison of means using the Wilcoxon test found a significant difference with a p value of 0.00034

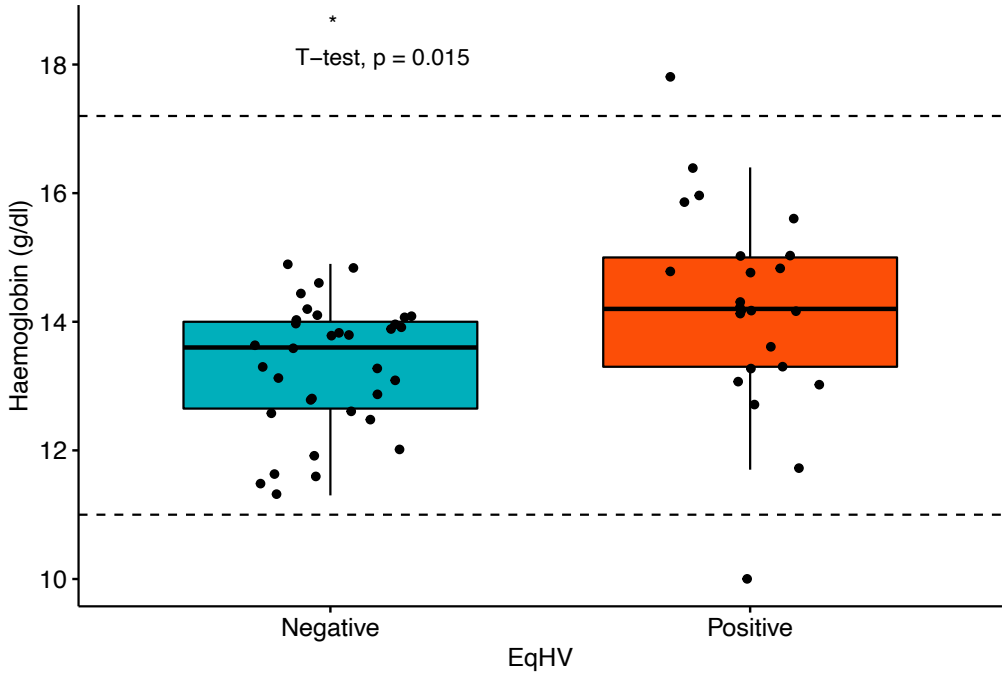


Figure 88 Individual values and mean average levels of haemoglobin for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Dashed line indicates 'normal range'. Statistical comparison of means using the T- test found a significant difference with a p value of 0.015.

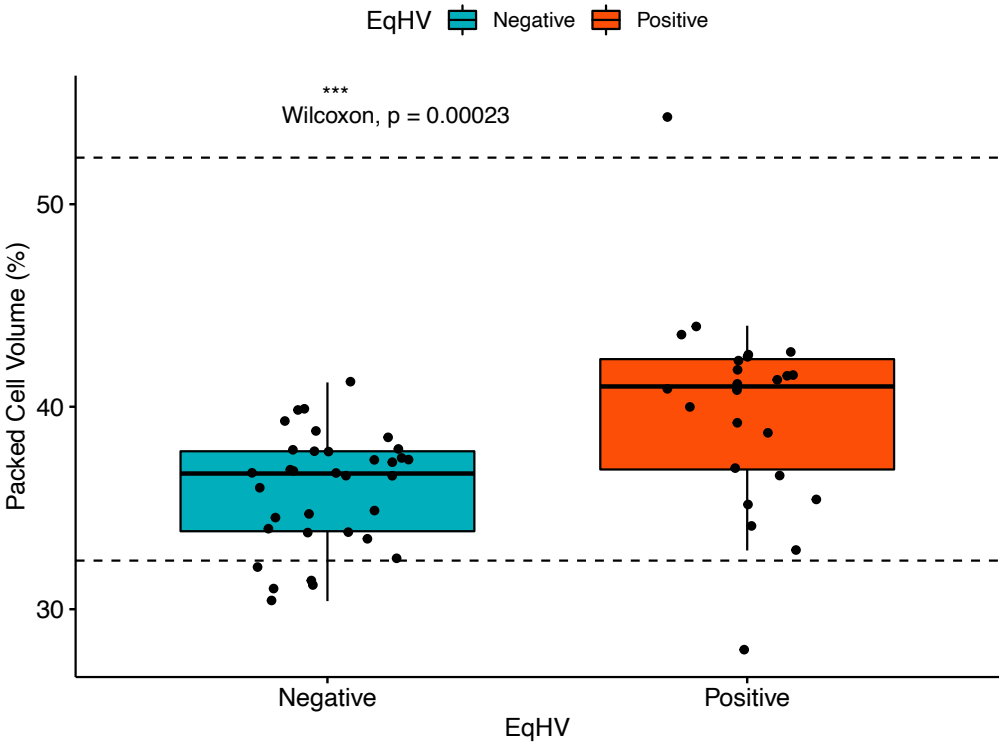


Figure 89 Individual values and mean average levels for % packed cell volume for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Area in between dashed line indicates 'normal range'. Statistical comparison of means using the Wilcoxon test found a significant difference with a p value of 0.00023.

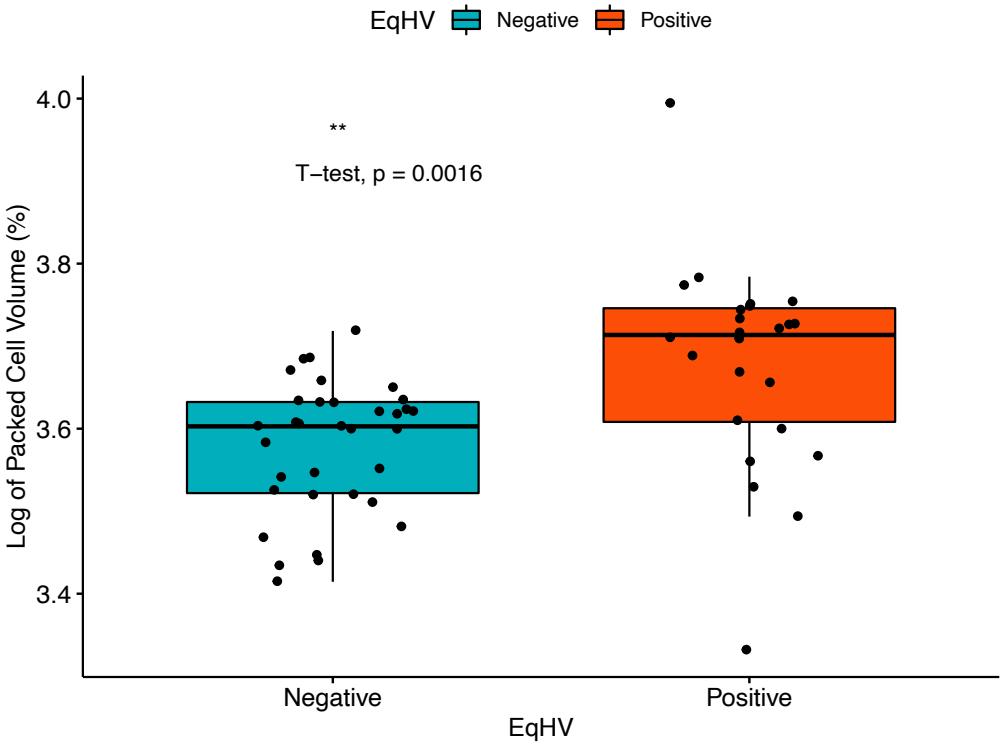


Figure 90 Individual values and mean average levels for Log of % packed cell volume for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means using a T-test found a significant difference with a p value of 0.0016.

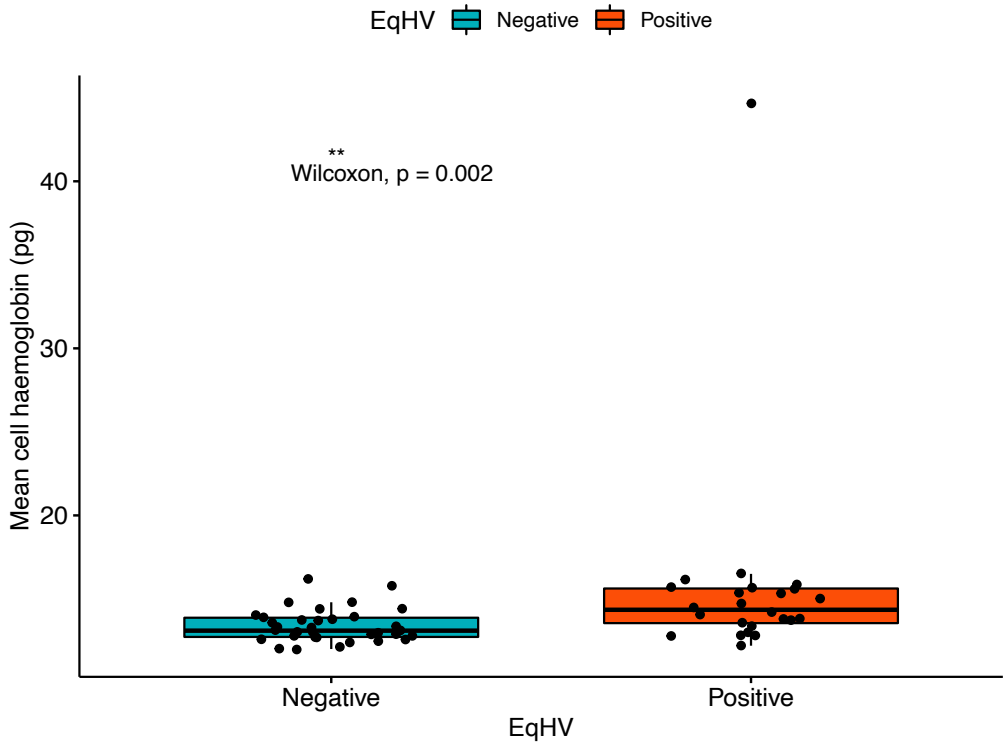


Figure 92 Individual values and mean average levels mean cell haemoglobin for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Comparison of means using the Wilcoxon test found a significant difference with a p value of 0.0016

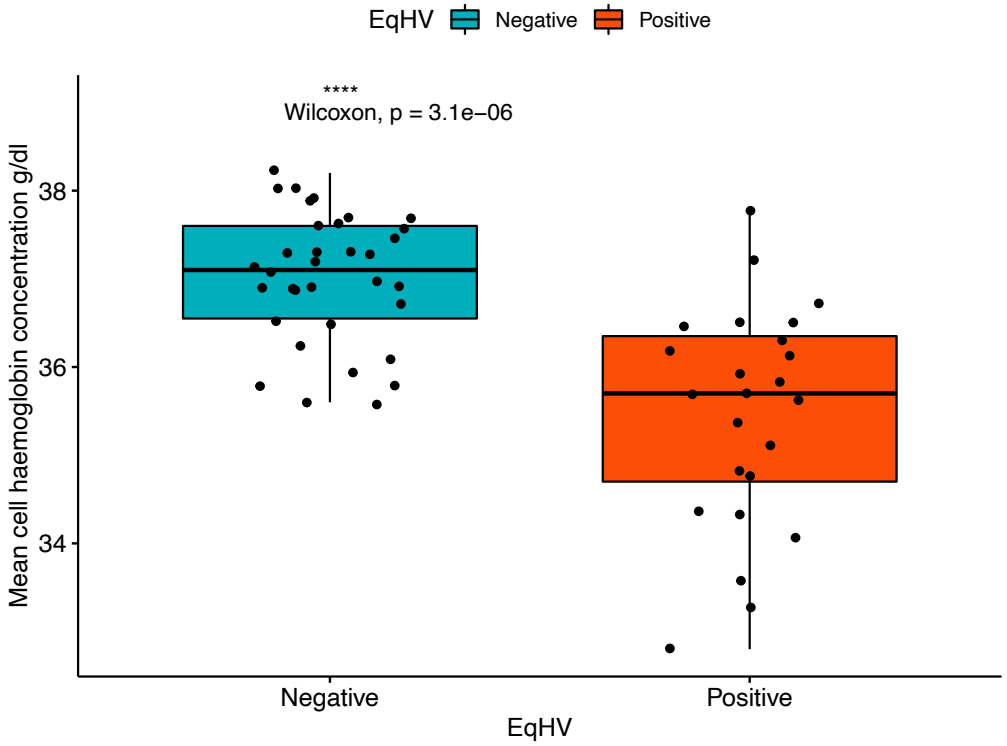


Figure 91 Individual values and mean average levels of mean cell haemoglobin concentration for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Comparison of means using the Wilcoxon test found a significant difference with a p value of 3.1e-06

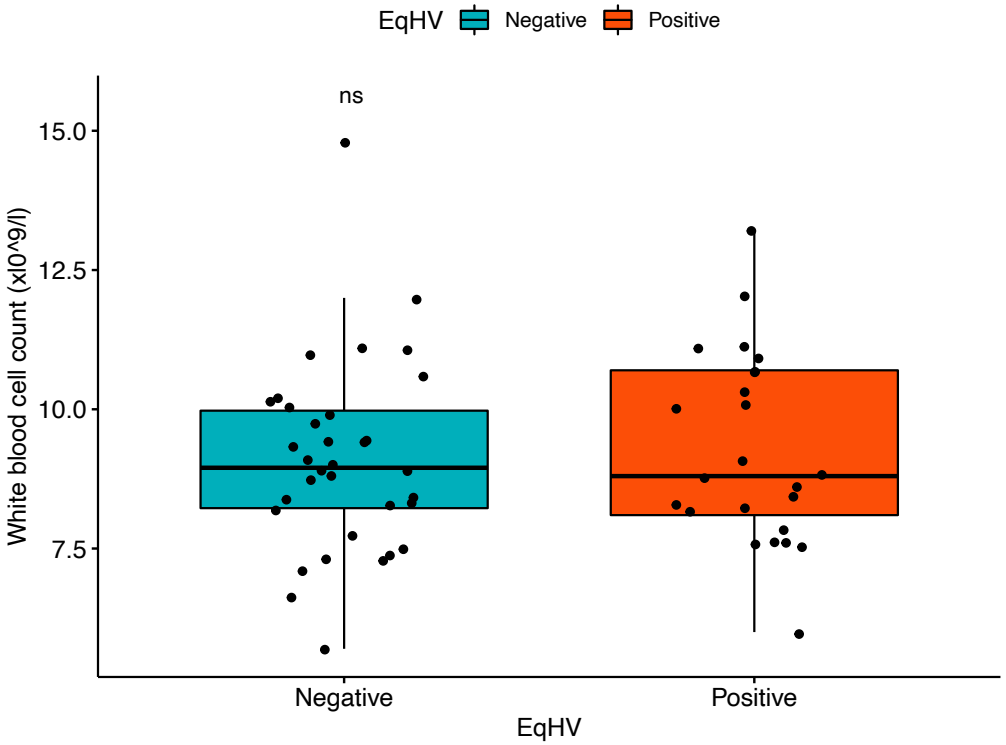


Figure 93 Individual values and mean average levels of white blood cell count for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means found no

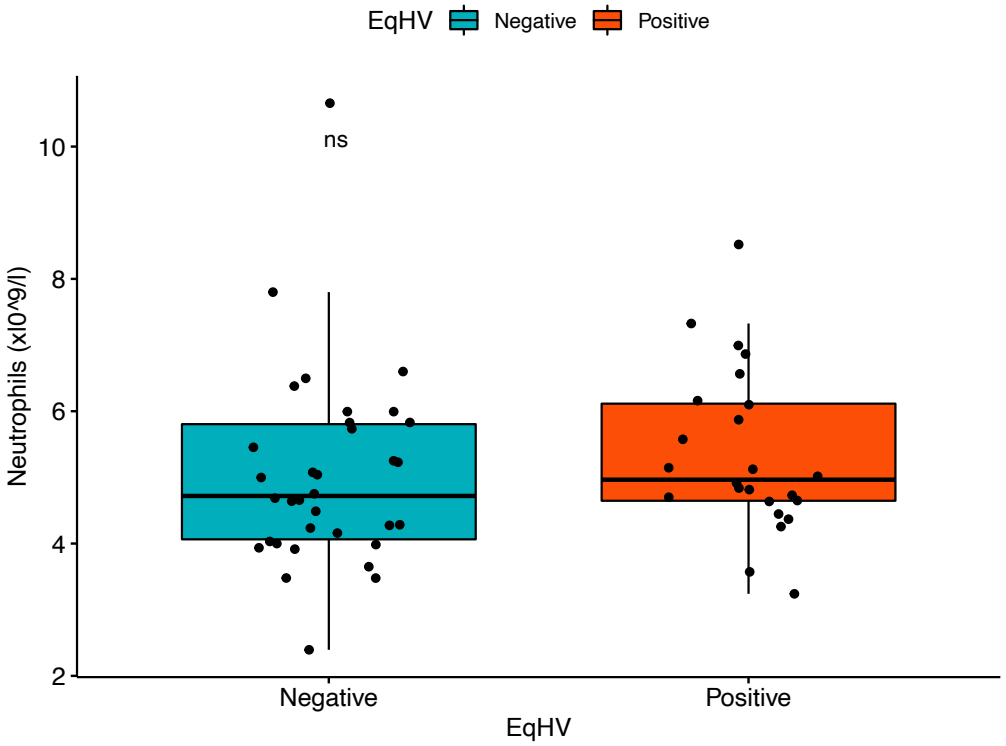


Figure 94 Individual values and mean average levels of neutrophils, for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means found no significance

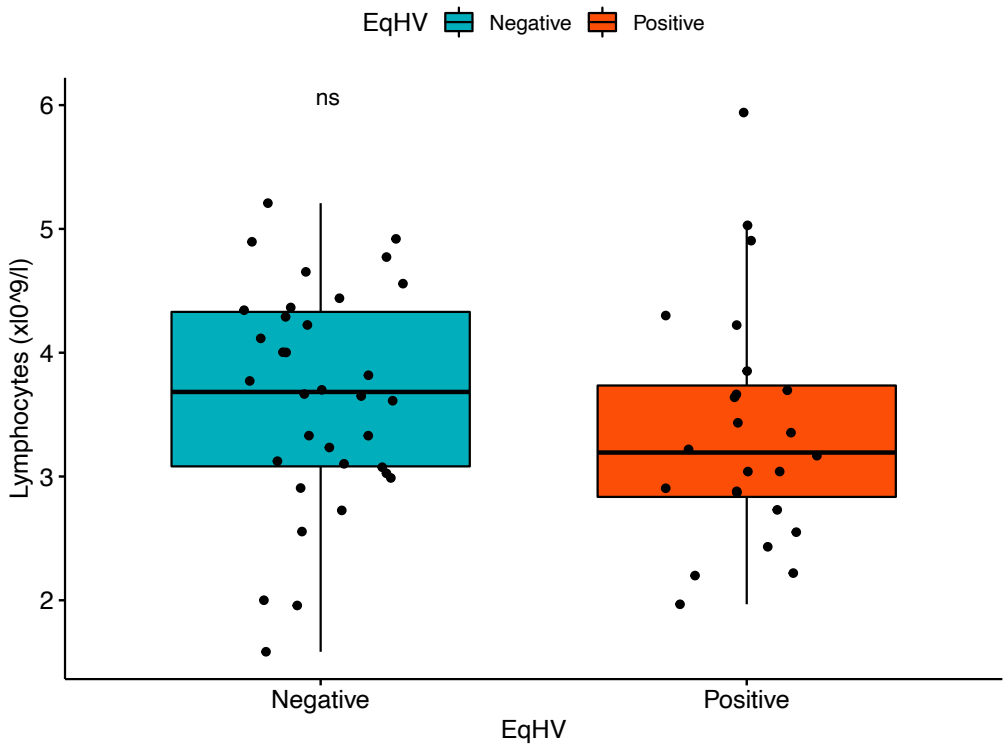


Figure 95 Individual values and mean average levels of lymphocytes for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means found no significance.

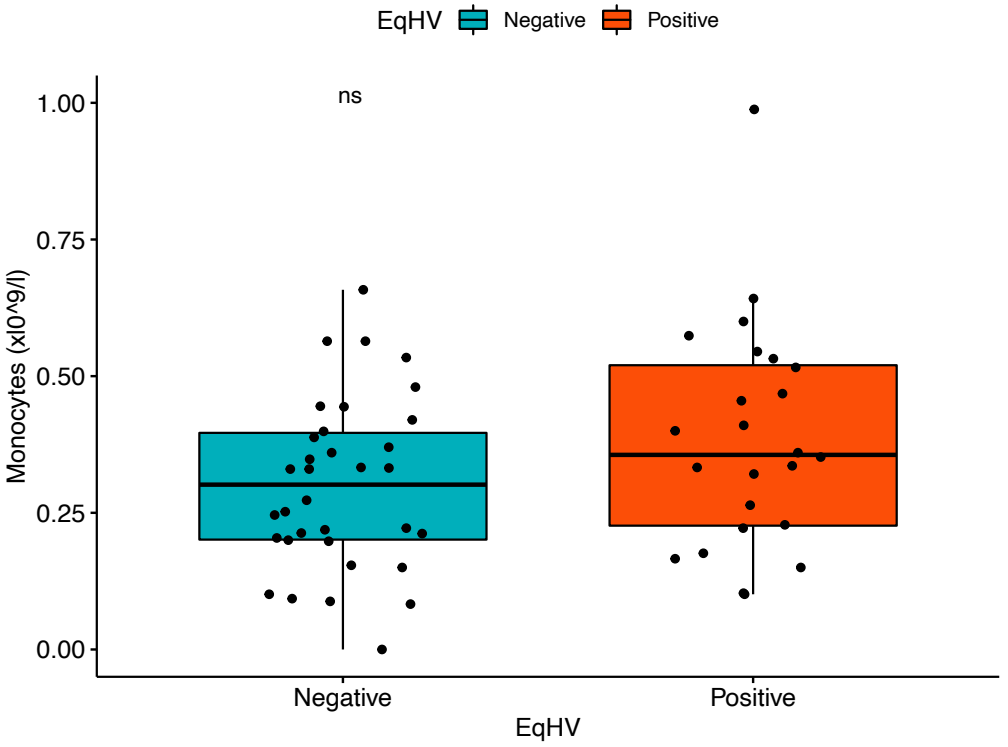


Figure 96 Individual values and mean average levels of monocytes for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means found no significance.

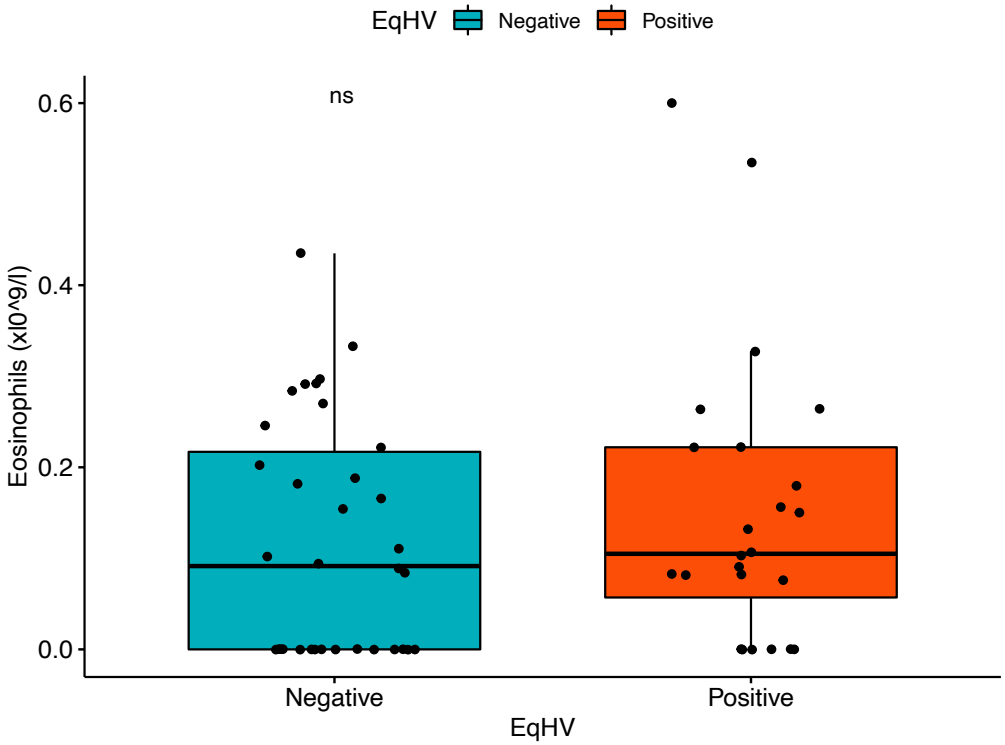


Figure 97 Individual values and mean average levels of eosinophils for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means found no significance.

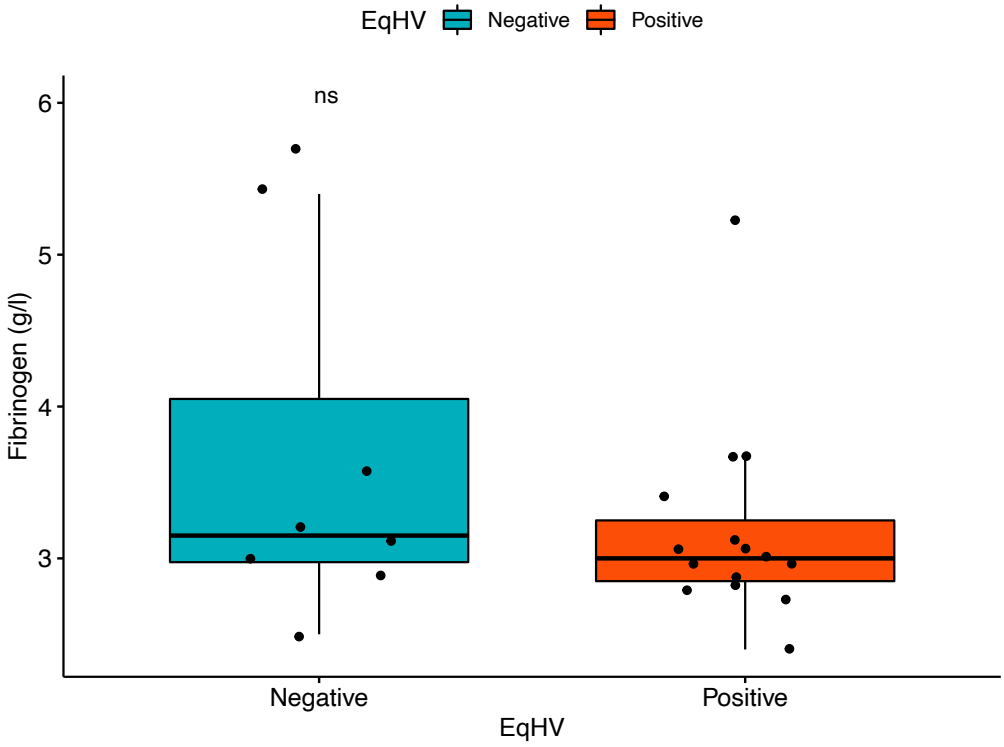


Figure 98 Individual values and mean average levels of fibrinogen for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means found no significance.

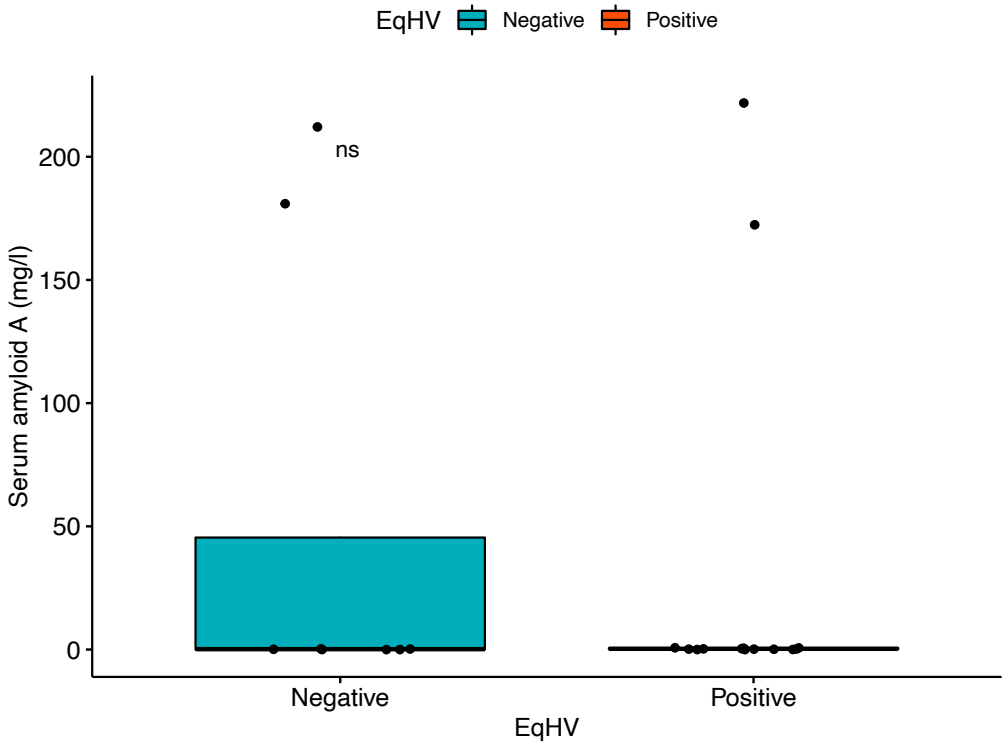


Figure 99 Individual values and mean average levels of serum amyloid A for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means found no significance.

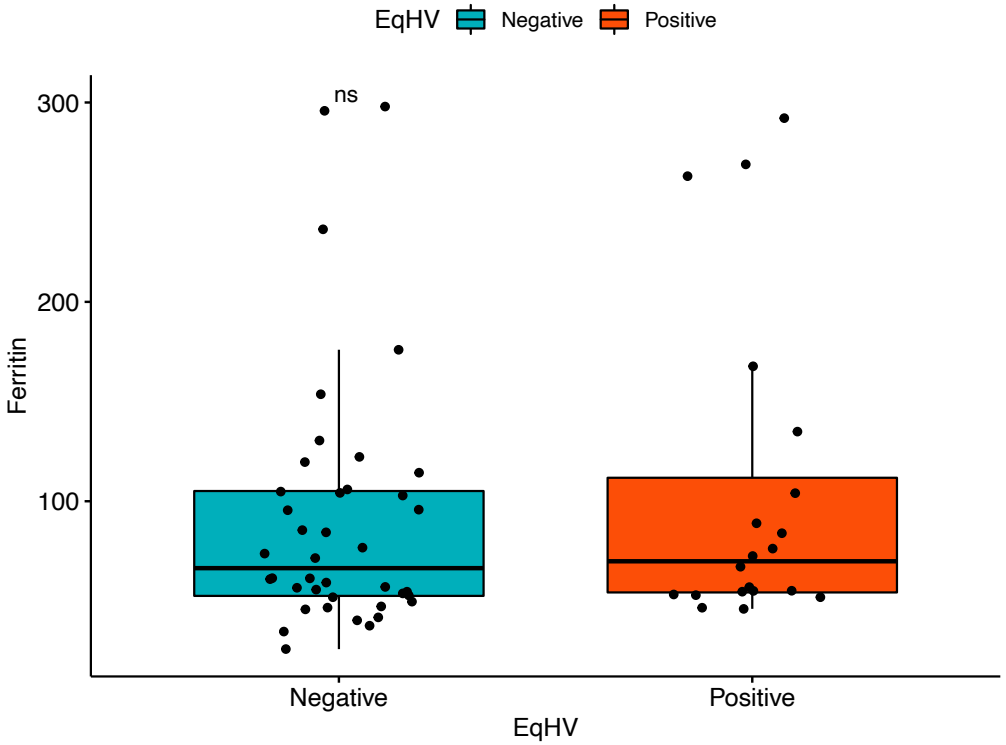


Figure 100 Individual values and mean average levels of ferritin for blood either positive or negative for equine hepacivirus. Horse blood was collected from Thoroughbred horses in training. Statistical comparison of means found no significance.

6.6 Blood sample data analysis

If the Sig. value of the Shapiro-Wilk Test is greater than 0.05, the data is normal. If it is below 0.05, the data significantly deviate from a normal distribution.

Table 20 Distribution normality of clinical chemistry variables from a Thoroughbred population using the Shapiro-Wilk normality test. Variables with a p-value of >0.05 are considered not significantly different from normal distribution

Variable name	statistic	p.value	Data distribution
%Eosinophils%	0.85	5.25E-06	Not normally distributed
%Lymphocytes%	0.96	0.08	Normally distributed
%Monocytes%	0.9	1.58E-04	Not normally distributed
%Neutrophils%	0.96	0.05	Not normally distributed
ALTUL	0.51	1.46E-13	Not normally distributed
AST	0.79	0.06	Normally distributed
ASTGOTUL	0.8	5.50E-08	Not normally distributed
BILEACIDSumoll	0.9	6.57E-05	Not normally distributed
BILIRUBINumoll	0.89	2.20E-05	Not normally distributed
CreatineKinaseUL	0.57	1.15E-12	Not normally distributed
DIRECTBILIRUBINumoll	0.82	1.98E-07	Not normally distributed
Eosinophils	0.85	5.58E-06	Not normally distributed
Ferritin	0.74	5.97E-09	Not normally distributed
Fibrinogen	0.75	6.55E-05	Not normally distributed
GGTUL	0.91	1.44E-04	Not normally distributed
GLDHUL	0.71	5.30E-10	Not normally distributed
Haemoglobin	0.98	0.35	Normally distributed
Log_Age	0.87	6.26E-06	Not normally distributed
Log_ALT	0.85	1.64E-06	Not normally distributed
Log_AST	0.99	0.98	Normally distributed
Log_ASTGOT	0.53	2.91E-13	Not normally distributed
Log_BileAcid	0.99	0.75	Normally distributed
Log_Bilirubin	0.98	0.25	Normally distributed
Log_CreKinase	0.88	1.12E-05	Not normally distributed
Log_DirectBiliRubin	0.7	3.10E-10	Not normally distributed
Log_Eosinophils	0.86	3.02E-04	Not normally distributed
Log_Ferritin	0.93	1.40E-03	Not normally distributed
Log_Fibrinogen	0.83	1.25E-03	Not normally distributed

Log_GGT	0.98	0.27	Normally distributed
Log_GLD	0.97	0.15	Normally distributed
Log_Haemoglobin	0.97	0.21	Normally distributed
Log_Lymphocytes	0.92	6.12E-04	Not normally distributed
Log_MCH	0.54	2.91E-12	Not normally distributed
Log_MCHC	0.93	1.94E-03	Not normally distributed
Log_MCV	0.95	0.02	Not normally distributed
Log_monocytes	0.94	5.05E-03	Not normally distributed
Log_Neutrophils	0.98	0.37	Normally distributed
Log_PCV	0.97	0.25	Normally distributed
Log_RedBloodCells	0.99	0.76	Normally distributed
Log_SerAmA	0.7	1.07E-04	Not normally distributed
Log_WhiteBCcount	0.99	0.89	Normally distributed
Lymphocytes	0.99	0.91	Normally distributed
MCH	0.33	8.79E-15	Not normally distributed
MCHC	0.94	4.51E-03	Not normally distributed
MCV	0.93	2.54E-03	Not normally distributed
Monocytes	0.96	0.03	Not normally distributed
Neutrophils	0.92	9.01E-04	Not normally distributed
PCV	0.95	0.02	Not normally distributed
Red blood cells	0.99	0.72	Normally distributed
SerumamyloidA	0.49	6.30E-08	Not normally distributed
Whitecellcount	0.97	0.12	Normally distributed

Table 21 Results of means comparisons of parameters between groups of horses either positive or negative for EqHV from a population of 66 Thoroughbred racehorses.

Parameter	obs.+	obs.-	obs.tot	mean.+	mean.-	mean.diff	Test	P value	Signif
Age	25	41	66	3.32	3.10	0.22	Wilcox Test	4.34 E-01	NS
ALTUL	25	41	66	10.02	11.61	-1.60	Wilcox Test	6.08 E-01	NS
BILIRUBINumoll	25	41	66	23.82	21.61	2.21	Wilcox Test	2.75 E-01	NS
ASTGOTUL	25	41	66	244.12	239.78	4.34	Wilcox Test	8.64 E-01	NS
GLDHUL	25	40	65	3.97	2.72	1.26	Wilcox Test	3.20 E-02	Signif
Creatine KinaseUL	25	41	66	157.80	193.39	-35.59	Wilcox Test	5.70 E-01	NS
BILEACIDSumoll	25	41	66	4.96	4.52	0.44	Wilcox Test	4.01 E-01	NS
GGTUL	25	41	66	25.16	27.91	-2.75	Wilcox Test	4.43 E-01	NS
DIRECTBILIRUBINumoll	25	41	66	4.72	5.15	-0.44	Wilcox Test	6.46 E-02	NS
redbloodcells	24	34	58	10.03	9.97	0.06	T-Test	8.37 E-01	NS
Haemoglobin	24	34	58	14.25	13.31	0.94	T-Test	1.51 E-02	Signif
PCV	24	34	58	39.92	35.96	3.96	Wilcox Test	2.27 E-04	Signif
MCV	24	34	58	40.13	36.21	3.92	Wilcox Test	3.38 E-04	Signif
MCH	24	34	58	15.64	13.39	2.25	Wilcox Test	2.01 E-03	Signif
MCHC	24	34	58	35.46	37.01	-1.55	Wilcox Test	3.12 E-06	Signif
Whitecellcount	24	34	58	9.28	9.11	0.17	T-Test	7.14 E-01	NS
%Neutrophils%	24	34	58	57.67	54.88	2.78	Wilcox Test	1.21 E-01	NS

Neutrophils	24	34	58	5.35	5.04	0.31	Wilcox Test	2.09 E-01	NS
%Lymphocytes%	24	34	58	36.38	40.41	-4.04	T-Test	4.61 E-02	Signif
Lymphocytes	24	34	58	3.39	3.65	-0.26	T-Test	3.02 E-01	NS
%Monocytes%	24	34	58	4.33	3.35	0.98	Wilcox Test	1.72 E-01	NS
Monocytes	24	34	58	0.39	0.30	0.08	Wilcox Test	1.11 E-01	NS
%Eosinophils%	24	34	58	1.63	1.35	0.27	Wilcox Test	4.52 E-01	NS
Eosinophils	24	34	58	0.15	0.12	0.03	Wilcox Test	5.23 E-01	NS
Fibrinogen	15	8	23	3.19	3.68	-0.48	Wilcox Test	3.63 E-01	NS
Serum amyloidA	15	8	23	26.52	49.20	-22.68	Wilcox Test	5.13 E-01	NS
CPK	2	3	5	200.50	323.67	-123.17	T-Test	1.25 E-01	NS
AST	2	3	5	561.00	743.00	-182.00	T-Test	6.19 E-01	NS
Ferritin	20	40	60	104.61	91.05	13.56	Wilcox Test	7.42 E-01	NS
Log_Age	24	41	65	1.13	1.07	0.06	Wilcox Test	4.34 E-01	NS
Log_ALT	25	41	66	2.22	2.30	-0.08	Wilcox Test	6.08 E-01	NS
Log_Bilirubin	25	41	66	3.11	3.00	0.11	T-Test	2.57 E-01	NS
Log_ASTGOT	25	41	66	5.49	5.44	0.05	Wilcox Test	8.64 E-01	NS
Log_GLD	25	40	65	1.08	0.66	0.42	T-Test	3.49 E-02	Signif
Log_CreKinase	25	41	66	4.75	4.93	-0.18	Wilcox Test	5.70 E-01	NS
Log_BileAcid	25	41	66	1.53	1.44	0.08	T-Test	3.93 E-01	NS
Log_GGT	25	41	66	3.11	3.23	-0.12	T-Test	3.42 E-01	NS
Log_DirectBiliRubin	25	41	66	1.51	1.62	-0.11	Wilcox Test	6.46 E-02	NS

Log_RedBloodCells	24	34	58	2.30	2.30	0.00	T-Test	9.44 E-01	NS
Log_Haemoglobin	24	34	58	2.65	2.59	0.06	T-Test	2.31 E-02	Signif
Log_PCV	24	34	58	3.68	3.58	0.10	T-Test	1.56 E-03	Signif
Log_MCV	24	34	58	3.69	3.59	0.10	Wilcox Test	3.38 E-04	Signif
Log_MCH	24	34	58	2.71	2.59	0.12	Wilcox Test	2.01 E-03	Signif
Log_MCHC	24	34	58	3.57	3.61	-0.04	Wilcox Test	3.12 E-06	Signif
Log_WhiteBCcount	24	34	58	2.21	2.19	0.02	T-Test	7.06 E-01	NS
Log_Neutrophils	24	34	58	4.05	3.99	0.05	T-Test	1.56 E-01	NS
Log_Lymphocytes	24	34	58	3.58	3.67	-0.10	Wilcox Test	2.01 E-02	Signif
Log_monocytes	24	33	57	1.29	1.10	0.19	Wilcox Test	2.29 E-01	NS
Log_Eosinophils	18	19	37	0.59	0.76	-0.17	Wilcox Test	3.27 E-01	NS
Log_Fibrinogen	15	8	23	1.14	1.26	-0.12	Wilcox Test	3.63 E-01	NS
Log_SerAmA	12	5	17	-0.11	1.09	-1.20	Wilcox Test	1.00 E+00	NS
Log_CPK	2	3	5	5.29	5.76	-0.47	T-Test	1.47 E-01	NS
Log_AST	2	3	5	5.29	5.76	-0.47	T-Test	1.47 E-01	NS
Log_Ferritin	20	40	60	4.44	4.34	0.11	Wilcox Test	7.42 E-01	NS

Table 22 Distribution normality of clinical chemistry variables from a population of rescue horses from Bransby horses, using the Shapiro-Wilk normality test. Variables with a p-value of >0.05 are considered not significantly different from normal distribution.

Variable	Shapiro-Wilk test	W	P value	Passed normality test (alpha=0.05)?	P value summary
Age		0.91	<0.0001	No	****
Total Protein		0.958	0.0103	No	*
Albumin		0.961	0.0156	No	*
Globulin		0.956	0.0079	No	**
Fibrinogen		0.831	<0.0001	No	****
Ser Amy A		0.303	<0.0001	No	****
AST		0.89	<0.0001	No	****
GGT		0.369	<0.0001	No	****
CK		0.289	<0.0001	No	****
GLDH		0.545	<0.0001	No	****
Alk Phos		0.884	<0.0001	No	****
Urea		0.48	<0.0001	No	****
Creatinine		0.969	0.0574	Yes	ns
Bile Acids		0.813	0.1461	Yes	ns

6.7 Ethical Review

This project was approved by the School of Veterinary Medicine and Science's Ethical Review panel (number 2156 171120).

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