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PROJECT TITLE: An exploration of antigen expression of hepatitis C entry receptors on equine cells in relation to equine hepacivirus A

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Abstract

Equine hepacivirus A (EqHV) belongs to the family *Flaviviridae* and has been identified as the hepacivirus phylogenetically most closely related to Hepatitis C virus (HCV). Like HCV, EqHV is a hepatotropic virus that has been reported in over seventeen countries from six continents. However, much is still largely unknown about EqHV, such as its entry, pathogenesis and exact mode of transmission, although some cases of vertical transmission have been reported. The main four receptors used by HCV for viral entry to human hepatocytes are cluster of differentiation-81 (CD-81), occludin (OCLN), claudin-1 (CLDN-1) and scavenger receptor class B member 1 (SR-B1). This study investigated the presence of HCV entry receptors on equine cells and tissues, including equine hepatocytes, peripheral blood mononuclear cells (PBMCs), lymph node, placenta and liver using flow cytometry, immunofluorescence, immunocytochemistry and immunohistochemistry. CD81 was present in equine liver tissue, equine term placenta, equine lymph nodes and equine PBMCs, in particular on equine lymphocytes. OCLN was present in some neutrophils, hepatocytes as well as the nuclei of endothelial cells in the equine liver and some lymphoblasts within equine lymph nodes. This study has provided preliminary insights into the expression of putative EqHV receptors on equine tissues and cells and thus highlighted potential mechanisms of viral entry of EqHV into cells, which will hopefully aid in the understanding of its transmission.

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Declaration

I hereby declare that this thesis has only been composed by me and unless otherwise stated all the work presented in this thesis is my own work and has not been submitted towards another degree.

Abbreviations

Aa	Amino acid
Ab	Antibody
AST	Aspartate transaminase
bp	Base pairs
BVDV	Bovine viral diarrhoea virus
CD81	Cluster of differentiation 81
CHV	Canine Hepacivirus
CLDN-1	Claudin 1
DAB	3, 3-diaminobenzidine
DAPI	4',6-Diamidine-2'-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
E.Derm	Equine dermal cells
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's Minimum Essential Medium
EPgV	Equine pegivirus
EqHV	Equine Hepacivirus
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FHK	Fetal horse kidney cells
GBV-A	GB virus A
GBV-B	GB virus B
GBV-C	GB virus C
GBV-D	GB virus D
GGT	Gamma-glutamyl transferase
GLDH	Glutamate dehydrogenase
GLIPS	Gaussia luciferase immunoprecipitation system
HCl	Hydrochloric acid
HCV	Hepatitis C virus

Hep G2	Hepatoma G2 cells
HPgV	Human pegivirus
HRP	Horseradish peroxidase
Huh7	Human hepatoma cells
ICTV	International Committee on Taxonomy of Viruses
IF	Immunofluorescence
IHC	Immunohistochemistry
ISH	In-situ hybridization
Kb	Kilo bases
Kbp	Kilo base pairs
kDa	kilo Daltons
L-glu	L-glutamine
LIPS	Luciferase immunoprecipitation system
NGS	Normal goat serum
Nm	Nanometres
NPHV	Non-primate hepacivirus
Nt	Nucleotides
OCLN	Occludin
OCT	Optimal cutting temperature
P/S	Penicillin and Streptomycin
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PV	Parvovirus
RdRp	RNA-dependent RNA polymerase
RO	Reverse Osmosis
rpm	Rotations per minute
RT-PCR	Reverse transcription polymerase chain reaction
SCID	Severe combined immunodeficiency disease
SDH	Sorbitol dehydrogenase

SR-B1	Scavenger receptor class B type 1
TBS	Tris-buffered saline
TDAV	Theiler’s disease associated virus
TE	Trypsin EDTA
USA	United States of America
UTR	Untranslated region
vWF	Von Willebrand factor
α	Anti-

1. INTRODUCTION

Since its initial domestication approximately 2000 BCE in Mesopotamia, the horse, which has ranged from wild horses to the currently known domesticated horses, has formed an integral part of society. Selective breeding has led to the development of a large variety of breeds for different purposes, such as the Thoroughbred's selective development as a racehorse. The Thoroughbred is one of the most valued breeds of horse in the world and its history reaches back to 17th century Britain (Cassidy, 2016). In 2018 in the UK alone, the Thoroughbred industry contributed approximately £427 million to the economy (The Thoroughbred Association, 2018). The Thoroughbred population registry includes about 9000 broodmares who foaled 4778 offspring in 2018. However, this large population of horses and their frequent movement, both nationally and internationally, makes this population highly susceptible to infectious diseases. Infectious disease has a major impact on a horse's welfare and performance and can have an economic impact on the horse's owner/breeder. One type of pathogen that commonly infects horses are viruses, which can cause acute and/or chronic infections that have an adverse impact on a horse's health. Many are well studied but new viruses are constantly emerging or being discovered and research is warranted to identify adequate measures to protect horses from these viruses, such as producing effective vaccinations, developing treatment or enhancing biosecurity measures. Without suitable information about the nature of a virus and its potential pathogenicity, it can create a threat to animal welfare. A common family of viruses that infects a variety of animal species is the family *Flaviviridae*.

1.1. Flaviviridae

There are currently four genera grouped under the *Flaviviridae* family: *Flavivirus, Hepacivirus, Pegivirus* and *Pestivirus* (Simmonds *et al.*, 2017). Members of this family are small, enveloped viruses with approximately 9.0 to 13 kilo bases (kb) positive-strand RNA genomes. Virions are spherical and have a diameter of 40–60 nm and a lipid envelope that contains two or three virus-encoded membrane proteins (Simmonds *et al.*, 2017).

1.1.1.Flavivirus

The majority of flaviviruses are transmitted by arthropod vectors, such as mosquitoes or ticks, which also support viral replication (Simmonds *et al.*, 2017). The primary hosts are usually birds and mammals, and they cause a variety of economically significant diseases in domestic animals, wild animals, and humans. Human pathogens of high significance include yellow fever virus, Zika virus, dengue virus, West Nile virus, Japanese encephalitis virus and tick-borne encephalitis virus. Clinical signs in mammals and birds range from asymptomatic to neurological disease or severe and potentially fatal haemorrhagic fever (Simmonds *et al.*, 2017).

1.1.2.Pestivirus

Pestiviruses infect ruminants, pigs, bats, and rats and include economically important viruses such as bovine viral diarrhoea virus and classical swine fever virus. These viruses tend to be transmitted through infected secretions and infection causes a range of diseases from subclinical to haemorrhagic, enteric or wasting (Simmonds *et al.*, 2017).

1.1.3.Pegivirus

Pegiviruses form a genus of viruses that readily infect humans, as well as a variety of other mammalian species, although their mode of transmission is not well described (Simmonds *et al.*, 2017). GB virus-A (GBV-A), GB virus C (GBV-C, formerly known as hepatitis G virus) and GB virus D (GBV-D) were reclassified recently into the pegivirus genus from the hepacivirus genus, as there is no evidence that these viruses cause hepatitis (Stapleton *et al.*, 2011). Human pegivirus (HPgV) and pegivirus E (EPgV) also belong to this genus (Kapoor *et al.*, 2013; Smith *et al.*, 2014). Equine pegivirus and Theiler's disease associated virus (TDAV; a pegivirus) were thought to be associated with hepatitis in equids when they were first identified (Chandriani *et al.*, 2013). It has since been shown that in fact equine parvovirus is associated with the onset of Theiler's disease and should therefore be taken into account when investigating equine liver disease (Tomlinson *et al.*, 2020).

1.1.4.Hepaciviruses

The most commonly known hepacivirus is Hepatitis C virus (HCV), which infects humans and is known to cause acute and chronic hepatitis, hepatocellular carcinoma and cirrhosis (Smith *et al.*, 2014; Simmonds *et al.*, 2017). GB virus-B (GBV-B) is another hepacivirus that primarily infects tamarins and other non-human primates (Simons *et al.*, 1995). There are several other hepaciviruses that are infectious to bats, rodents, monkeys, cattle, donkeys and horses (Kapoor *et al.*, 2011, 2013; Burbelo *et al.*, 2012; Quan *et al.*, 2012; Drexler *et al.*, 2013; Firth *et al.*, 2014; Sibley *et al.*, 2014; Baechlein *et al.*, 2015; Corman *et al.*, 2015; Walter *et al.*, 2017). Hepaciviruses show distinct host ranges and have therefore been further classified into hepacivirus species (Table 1).

Table 1. *Subspecies of hepaciviruses and the species they infect (modified from Simmonds et al., 2017).*

Hepacivirus Species Name	**Host Range**	**Viruses in this species**	**Virus abbreviation**
Hepacivirus A	Horses, dogs (possibly)	Non-primate hepacivirus/Equine hepacivirus A	EqHV
Hepacivirus B	Tamarins, other New World Primates	GB virus-B	GBV-B
Hepacivirus C	Humans	Hepatitis C virus (all genotypes)	HCV
Hepacivirus D	Colobus monkeys	Guereza hepacivirus	GHV
Hepacivirus E-I	Rodents	Rodent hepacivirus	RHV-E to I
Hepacivirus J-M	Bats	Bat hepacivirus	BHV K to M
Hepacivirus N	Cows	Bovine hepacivirus	BoHV

1.2. Equine Hepacivirus A (EqHV)

Equine hepacivirus (EqHV) was first described in 2011, when it was discovered in respiratory and liver samples of dogs displaying signs of respiratory disease; it was therefore tentatively named canine Hepacivirus (CHV) (Kapoor *et al.*, 2011). High-throughput sequencing of an around 6500 nucleotides (nt) continuous sequence of CHV revealed a similarity to known flaviviruses, with HCV being the most closely related (Kapoor *et al.*, 2011). In this study six of nine dogs in one outbreak and three of five dogs in another outbreak in the same shelter were found to be positive for CHV by RT-qPCR, with assays yielding values as high as $>10^7$ copies of RNA per nasal swab (Kapoor *et al.*, 2011). Sixty healthy dogs were also screened for CHV in nasal swabs, none of which tested positive for CHV by RT-PCR. Liver samples were also tested for CHV, of which 5 of 19 were positive but contained low RNA levels of approximately $<10^3$ copies, on average.

In-situ hybridization (ISH) of livers from the infected dogs showed focal and dispersed infection of the liver. RNA was primarily found in the cytoplasm of hepatocytes (Kapoor *et al.*, 2011). Another study has since also detected the virus in dogs, and in both studies, the dogs in question were living in close contact with horses. This first suggested the possibility of potential cross species transmission between dogs and horses (Lyons *et al.*, 2014; Abbadi *et al.*, 2021). CHV NS3 antibodies were then tested for in a variety of different species but were only found to be highly prevalent in horses; this led to the virus being renamed non-primate Hepacivirus (NPHV).

Neither EqHV RNA nor EqHV antibodies were detected in deer, rabbits, sheep, goats, cats, non-human primates or humans (Burbelo *et al.*, 2012; Lyons *et al.*, 2012, 2014; Drexler *et al.*, 2013; Scheel *et al.*, 2015; Walter *et al.*, 2017). However, one cow (1/84) tested intermittently seropositive but was negative for EqHV RNA (Burbelo *et al.*, 2012). The results for the studies on donkey sera were inconclusive, with EqHV RNA and antibodies detected in some studies, but not others (Lyons *et al.*, 2014; Gabriella Elia *et al.*, 2017; Walter *et al.*, 2017). Another report describes a lack of detection of EqHV RNA in donkey sera but found a relatively high seroprevalence of up to 40.0% (Pfaender *et al.*, 2017). It was speculated that although donkeys can be infected with EqHV, they may have a more efficient viral clearance mechanism in comparison with horses, which would explain the presence of EqHV antibodies without an apparent EqHV RNA infection (Walter *et al.*, 2017). Therefore, horses appear to act as the primary host for EqHV, although other species, such as donkeys and dogs, may be incidentally infected. The virus has subsequently been officially classified as Hepacivirus A by the International Committee on Taxonomy of Viruses (ICTV; Simmonds *et al.*,

2017), with NZP1 as the exemplar isolate. For the purpose of this thesis, the nomenclature of equine Hepacivirus A (EqHV) will be used.

1.2.1. Viral Structure and Morphology

EqHV has an around 9.6 kb long, positive sense, single-stranded RNA genome (Kapoor *et al.*, 2011). The genome consists of a single large open-reading frame (ORF) encoding a 2942 amino acid polyprotein with a short 5' untranslated region (5'UTR; Kapoor *et al.*, 2011). The 5'-UTR contains 366 nucleotides and demonstrates a 66% nucleotide identity to HCV and a 57% nucleotide identity to GBV-B (Kapoor *et al.*, 2011). In addition, the 5'-UTR has been shown to contain internal ribosome entry sites (IRES) activity, similar to that of HCV (Lattimer *et al.*, 2019). Although the 3'-UTR was difficult to characterize, it appears to contain a poly-A stretch near the terminus. Hypothetical proteolytic cleavage of the polyprotein by host and viral proteinases generates ten viral proteins, which is in accordance with other hepaciviruses (Kapoor *et al.*, 2011). Structural proteins encompass the core, E1, E2 and p7 protein whereas the non-structural (NS) proteins are made up of NS2, NS3, NS4A, NS4B, NS5A and NS5B (Gabriella Elia *et al.*, 2017). The basic structure of most hepaciviruses, including EqHV, is shown in Figure 1.

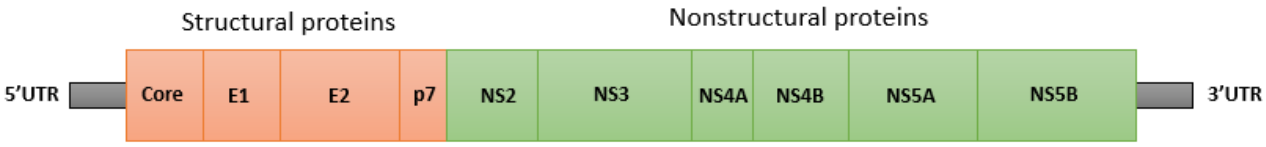


Figure 1. *Structure of the EqHV genome, including the ORF encoding for a polyprotein made up of structural (orange) and non-structural (green) proteins. Core = nucleocapsid; E1 and E2 = envelope glycoprotein subunits; p7 = ion channel protein for viral assembly; NS2 = transmembrane protein; NS3 = metalloprotease, serine protease, RNA helicase; NS4A and NS4B = cofactors; NS5A = IFN-resistance protein; NS5B = RNA polymerase. Adapted from* Kapoor *et al.* (2011).

The non-structural proteins of EqHV, namely NS3 and NS5B (also known as the RNA-dependent RNA polymerase, RdRp) have at least 55–65% amino acid identity with HCV, whereas the structural proteins E1, NS2 and NS5A have <35–45% amino acid identity, which makes EqHV the most closely related virus to HCV (Kapoor *et al.*, 2011). Noticeably, although the E2 protein tends to be highly variable within HCV, the C-terminal amino acid sequence of this protein has marked similarity between isolates of EqHV and HCV (Kapoor *et al.*, 2011).

1.2.2.Prevalence

The percentage of equids that test positively for viral RNA range from <1% to 34.1% and is thus highly variable (Lyons *et al.*, 2014; Tanaka *et al.*, 2014). Seroprevalence has been found to range from 22.2 up to 83.7% (Tanaka *et al.*, 2014; Badenhorst *et al.*, 2018). However, it should be noted that some of the studies tested a very limited number of animals, which may skew the data (see Table 2 for details).

Thus far, EqHV has been identified on 6 continents and in 17 countries (Table 2). Notably, within many of these countries there has been a variation of EqHV RNA positivity between different regions (Gemaque *et al.*, 2014; Figueiredo *et al.*,

2015; Kim *et al.*, 2017). One study by Date *et al.* (2020) used samples that were collected in 1993, demonstrating that EqHV has been around a long time before it was first identified.

Table 2. *Geographical distribution and prevalence of EqHV RNA and specific antibody detection in horses. (*n/a = not tested for*)*

Country	Year	RNA positive		Seropositive		Reference
		%	No. samples	%	No. samples	
AFRICA						
Morocco	2021	10.5	172	65.7	172	(Abbadi *et al.*, 2021)
S. Africa	2018	7.9	454	83.7	454	(Badenhorst *et al.*, 2018)
ASIA						
China	2016					(Lu *et al.*, 2016)
	2017	44.4	9	n/a		(Lu *et al.*, 2017)
Japan	2014	34.1	31	22.6	31	(Tanaka *et al.*, 2014)
	2015	13.7	453	33.6	453	(Matsuu *et al.*, 2015)
Korea	2017	18.9	74	n/a		(Kim *et al.*, 2017)
Mongolia	1993	56.1	139	n/a		(Date *et al.*, 2020)
	2015	39.4	160	n/a		
AUSTRALIA						
New Zealand	2012	100	1	n/a		(Burbelo *et al.*, 2012)
EUROPE						
Austria	2019	4.15	386	45.9	386	(Badenhorst *et al.*, 2019)
France	2017	6.2	1033	n/a		(Pronost *et al.*, 2017)
Germany	2013	3.3	210	n/a		(Drexler *et al.*, 2013)
	2015	2.5	433	31.4	433	(Pfaender, Cavalleri, *et al.*, 2015)
	2016	20.0	20	80.0	20	
	2016	8.4	119	n/a		(Gather, Walter, Todt, *et al.*, 2016)
	2017	18.2	733	61.8	733	(Postel *et al.*, 2016)
						(Reichert *et al.*, 2017)
Hungary	2014	100	1	n/a		(Reuter *et al.*, 2014)
Italy	2017	4.7	1932	n/a		(Gabriella Elia *et al.*, 2017)
Scotland	2012	2.1	142	n/a		(Lyons *et al.*, 2012)
	2014	<1.0	328	43.3	327	(Lyons *et al.*, 2014)
NORTH AMERICA						
Canada	2016	100	1	n/a		(Postel *et al.*, 2016)
USA	2011	64.2	14	n/a		(Kapoor *et al.*, 2011)
	2012	7.8	103	22.2	36	(Burbelo *et al.*, 2012)
	2015	93.3	15	n/a		(Scheel *et al.*, 2015)
	2018	14.3	14	n/a		(Tomlinson *et al.*, 2019)
SOUTH AMERICA						
Brazil	2014	8.3	300	n/a		(Gemaque *et al.*, 2014)
	2015	13.4	202	n/a		(Figueiredo *et al.*, 2015)
	2016	2.6	500	n/a		(Figueiredo *et al.*, 2019)
	2018	13.4	231	n/a		(Figueiredo *et al.*, 2018)
Chile	2016	100	2	n/a		(Postel *et al.*, 2016)

1.2.3.Virological and immune responses

The exact viral and immunological responses to EqHV infection are currently still unknown although some observations exist on the immunological responses. Pfaender *et al.* (2017) observed a higher level of interferon gamma (IFNγ)-expressing monocytes/granulocytes in EqHV-infected horses compared to non-infected horses, which indicates a moderate activation of the innate immune system in response to experimental EqHV infection (Pfaender *et al.*, 2017). EqHV viraemia can be detected for up to 15 weeks after initial EqHV detection (Lyons *et al.*, 2012; Ramsay *et al.*, 2015; Gather, Walter, Todt, *et al.*, 2016). After birth, EqHV antibodies can be detected in foal sera but appear to wane with age, which points to a short-term protective immune response through the transfer of antibodies in the maternal colostrum (Badenhorst *et al.*, 2018).

EqHV antibodies can be detected by ELISA for at least 12 months after their initial detection but further studies are needed to assess their longevity and neutralization potential (Lyons *et al.*, 2014; Matsuu *et al.*, 2015; Pfaender, Cavalleri, *et al.*, 2015). When two horses were experimentally challenged with EqHV RNA after they had previously been infected it appeared that an adaptive immune response was successful in interfering with EqHV infection as viral RNA levels remained low throughout the course of infection (Pfaender *et al.*, 2017).

1.2.4. Pathogenesis

The route of EqHV entry into the horse has not been established but may involve contact with infected animals, accidental passive transfer into the blood, or breach of the epithelium perhaps by biting insects and/or via ingestion. Viral RNA has been detected in the liver, specifically in hepatocytes and serum samples but not in mouth or nasal swabs, tracheal washes nor in peripheral blood

mononuclear cells (PBMCs) (Lyons *et al.*, 2012; Scheel *et al.*, 2015). This is in contrast to what has been found with HCV infection in humans, in which the virus is consistently found in PBMCs (Cavalheiro *et al.*, 2007; Abd Alla and El Awady, 2017). EqHV RNA has been detected primarily in serum, and as the liver is a vascular organ, the most likely way in which EqHV travels through the body is via the blood. It has also since been shown that the viral load in the equine liver is significantly higher than the viral load in plasma, which suggests hepatotropism of the virus (Ramsay *et al.*, 2015). Very low viral loads have also been detected in the spleen, lymph nodes, lung and kidney but this is likely due to blood contamination (Ramsay *et al.*, 2015; Scheel *et al.*, 2015; Pronost *et al.*, 2019).

Ramsay et al. (2015) first explored EqHV transmission experimentally by passive transfer of EqHV positive sera to two splenectomised and four normal horses and experimentally infected two normal foals and two foals with SCID (severe combined immunodeficiency). After initial inoculation, viraemia rapidly increased in the first 15 weeks and peaked at 10^6-10^7 RNA copies/mL. After 1-3 weeks post-infection the liver enzyme levels, namely GGT (gamma-glutamyl transferase) and sorbitol dehydrogenase (SDH), rose above normal levels in all horses. After 15 weeks the viral loads steadily decreased, until viral clearance by all horses in week 37 (Ramsay *et al.*, 2015). Seroconversion occurred at 6-8 weeks post inoculation in adult horses and 13 weeks post inoculation in foals and steadily increased in accordance with the elevations in liver enzymes (Ramsay *et al.*, 2015). Since then, two other studies also demonstrated the ability to experimentally infect horses by passive transfer of EqHV positive sera (Postel *et al.*, 2016; Pfaender *et al.*, 2017) It has been suggested that adaptive immunity at the time of seroconversion may contribute to liver damage by EqHV, which is

similar to what can be seen with HCV (Maasoumy and Wedemeyer, 2012; G Elia *et al.*, 2017).

Some studies have suggested that EqHV may cause an alteration in certain liver enzyme levels, which may correlate with the presence of subclinical hepatitis (Lyons *et al.*, 2012; Figueiredo *et al.*, 2015, 2018; Pfaender, Walter, *et al.*, 2015; Badenhorst *et al.*, 2019). Liver enzymes (and sometimes bile acids) were found to be either mildly elevated or at the upper end of the reference range (Lyons *et al.*, 2012; Figueiredo *et al.*, 2018; Badenhorst *et al.*, 2019). However, the specific enzymes that were found to be elevated have been inconsistent between different studies (Lyons *et al.*, 2012; Figueiredo *et al.*, 2015, 2018; Matsuu *et al.*, 2015; Pfaender, Cavalleri, *et al.*, 2015; Ramsay *et al.*, 2015; Gather, Walter, Pfaender, *et al.*, 2016; Badenhorst *et al.*, 2019). Elevations in gamma-glutamyl transferase (GGT) were significantly greater in seropositive horses over 11 years old than in younger seropositive horses (Gather, Walter, Pfaender, *et al.*, 2016; Figueiredo *et al.*, 2018). An elevation in liver enzymes, in particular of GGT and glutamate dehydrogenase (GLDH), was found at the time of seroconversion, which fell back to within reference levels around 4 weeks post-seroconversion (Pfaender, Cavalleri, *et al.*, 2015; Tomlinson *et al.*, 2021). In contrast, several reports have failed to detect any difference in liver enzyme levels between EqHV positive and EqHV negative animals (Burbelo *et al.*, 2012; Matsuu *et al.*, 2015; Gather, Walter, Todt, *et al.*, 2016; Pronost *et al.*, 2017; Badenhorst *et al.*, 2019). Thus, no definite conclusion can be drawn about the impact of EqHV infection on liver enzymes at this time.

The clinical signs associated with horses infected with EqHV are subtle, often sub-clinical and inconsistent. In some studies, EqHV has been associated with subclinical hepatitis (Gather, Walter, Pfaender, *et al.*, 2016). Liver biopsies show

evidence of mild necrotizing hepatitis, although this has not been a consistent finding (Ramsay *et al.*, 2015). In one case, a horse presented with severe jaundice, hepatitis and hepatic insufficiency and tested negative for common known pathogens (albeit not for EqPV-H) but tested positive for EqHV RNA by PCR (Reuter *et al.*, 2014). It cannot be confirmed whether these clinical signs are caused by EqHV, but EqHV appears to have a role in causing hepatopathies in horses although the pathogenesis remains unknown (Reuter *et al.*, 2014). In another study by Tegtmeyer *et al.* (2019), a 6-year-old gelding presented for anorexia, pyrexia and abnormal liver parameters. The gelding had elevated GLDH, ALP, AST and GGT and increased bile acid and plasma fibrinogen concentrations. After biopsy, intra- and interlobular hepatic fibrosis with bile duct hyperplasia and mixed inflammatory cell infiltrates was found (Tegtmeyer *et al.*, 2019). Serum was taken from the gelding and tested for EqHV using qRT-PCR, which was later also detected in numerous hepatocytes using fluorescence *in situ* hybridization (FISH).

The pathogenesis of EqHV was investigated further in a study by Ramsay *et al.* (2015) in which horses were experimentally infected with EqHV using whole blood transfusions. Two splenectomised horses presented with fulminant hepatitis approximately 2 months post-transfusion and on post-mortem examination the liver showed multifocal haemorrhage, multifocal lymphocytic hepatitis, hepatocellular vacuolation, mononuclear interface hepatitis, random individual hepatocyte necrosis, hepatocellular degeneration and regeneration, cholestasis and bridging biliary hyperplasia (Ramsay *et al.*, 2015). It needs to be noted that both of these horses also had EPgV RNA present (Ramsay *et al.*, 2015). In addition to the two splenectomised horses, four 'normal' adult horses were administered plasma or serum containing EqHV. All four horses developed a viraemia for 15

weeks post-inoculation with all horses clearing EqHV by 37 weeks post-inoculation. Again, EPgV was also present in all four horses, although it could only be consistently detected in two of the four horses with relatively low viral loads. Post-EqHV infection liver biopsies of these horses showed multifocal-random necrotizing hepatitis with individual necrotic hepatocytes surrounded by some mononuclear inflammatory cells and rarely neutrophils (Ramsay *et al.*, 2015). However, other studies have thus far found no consistent overt macroscopic or histological lesions in adults or fetuses (Pronost *et al.*, 2019). The pathogenicity of EqHV is currently unknown as it is still under debate whether EqHV is a causative agent in clinical and/or subclinical disease.

1.2.5.Acute and chronic infections

In general, when studying EqHV in horses, four groups with different RNA (ribonucleic acid) and Ab (antibody) states have been described: non-exposed [RNA-/Ab-], acutely-infected [RNA+/Ab-], previously, but currently non-infected [RNA-/Ab+], and chronically-infected horses [RNA+/Ab+] (see Table 3) (Badenhorst *et al.*, 2018, 2019).

Table 3. *The four categories of infection status seen with EqHV*

Group name	**Group characteristics**	**Abbreviation**
Non-exposed	RNA and EqHV antibody negative	[RNA-/Ab-]
Acutely infected	RNA positive and EqHV antibody negative	[RNA+/Ab-]
Previously infected	RNA negative and EqHV antibody positive	[RNA-/Ab+]
Chronically infected	RNA and EqHV antibody positive	[RNA+/Ab+]

Seronegative and RNA negative [RNA-/Ab-] horses are likely to not have been previously exposed to EqHV (Badenhorst *et al.*, 2018). Badenhorst *et al.* (2018)

found that in a population of 454 foals, 14.54% had never been exposed to EqHV. Seronegative but RNA positive [RNA+/Ab-] horses are likely to be acutely infected, which tends to be only a small portion of the population (1.8% in the foals studied by Badenhorst *et al.* (2018). Seropositive and RNA negative [RNA-/Ab+] horses have most likely undergone seroconversion and cleared the viral infection and represent a large part of the equine populations studied so far (Pfaender, Cavalleri, *et al.*, 2015; Badenhorst *et al.*, 2018; Abbadi *et al.*, 2021). RNA and antibody positive horses [RNA+/Ab+] may represent horses that have undergone recent seroconversion but not yet cleared the virus (Badenhorst *et al.*, 2018). Alternatively, chronic infection may have developed, which has previously been reported (Pfaender, Cavalleri, *et al.*, 2015; Gather, Walter, Pfaender, *et al.*, 2016; Gather, Walter, Todt, *et al.*, 2016; Badenhorst *et al.*, 2018; Tegtmeyer *et al.*, 2019). A chronic infection of EqHV is defined in the same way as for HCV, as a persistence of viral RNA in the blood that can be detected for at least 6 months (Scheel *et al.*, 2015). Some horses that are both RNA and EqHV antibody positive appear to be chronically infected despite having a high EqHV antibody titre. This suggests either the lack of neutralizing antibodies or an inefficiency of these antibodies in clearing the virus (Lyons *et al.*, 2014; Reuter *et al.*, 2014; Matsuu *et al.*, 2015; Pfaender, Cavalleri, *et al.*, 2015; Ramsay *et al.*, 2015; Tomlinson *et al.*, 2021). The infrequent occurrence of chronic infection in the horse population is in stark contrast to chronic infection of HCV, in which the majority (80%) of people infected with HCV remain infected for at least 6 months (Nelson *et al.*, 2011). Further investigation into the nature and course of acute and chronic infections and their spread throughout the equine population is needed.

1.2.6. Risk factors for infection

The risk factors associated with EqHV infection have thus far been rather inconclusive, with many conflicting results. Studies have found no correlation between age, sex, activity or breed and EqHV RNA status (Figueiredo *et al.*, 2018). Reichert et al. (2017) studied risk factors associated with EqHV including age, sex, chronic stress, state of reproduction, transport, and breeding history. There was an inverse relationship between animals testing EqHV RNA positive and age, and as horses aged, they were also more likely to be seropositive. Viraemia appears to occur more frequently in younger horses, which has been supported by other studies in which 1- to 2-year-old horses had a higher prevalence of EqHV RNA compared with older horses (Matsuu *et al.*, 2015; Kim *et al.*, 2017; Reichert *et al.*, 2017; Abbadi *et al.*, 2021). Some other studies however found no statistically significant associations between infection status and age (Pfaender, Cavalleri, *et al.*, 2015; Pronost *et al.*, 2017). Interestingly, viral loads seem to be significantly lower in horses over 2 years old compared to horses less than 2 years old (Figueiredo *et al.*, 2018). It appears that after birth the colostral antibody levels tend to be relatively high and start to wane between 3 and 6 months of age, after which point acute infection becomes more prevalent (Badenhorst *et al.*, 2018). Thus, with increasing age, a higher number of horses are EqHV RNA positive (Badenhorst *et al.*, 2018). Other risk factors that have been evaluated are the size of the stud as well as travel history of horses. Horses coming from large studs were more often RNA positive than horses coming from smaller studs. In addition, more horses with an international transportation history were acutely infected (EqHV RNA positive but antibody negative) than horses that had not travelled (Reichert *et al.*, 2017). Based on the information on prevalence and risk factors it can be predicted that the horses at highest risk of EqHV infection are young

maiden Thoroughbred mares from a large stud, with a recent international transport history. However, these findings require further confirmation.

The prevalence of horses infected with EqHV has been reported consistently as higher in Thoroughbreds than in other breeds (Pfaender, Cavalleri, *et al.*, 2015; Kim *et al.*, 2017; Pronost *et al.*, 2017; Reichert *et al.*, 2017; Badenhorst *et al.*, 2018). The high prevalence in Thoroughbreds may be due to a variety of reasons. This may be due to their genetic susceptibility, the use of solely live cover (artificial insemination is not allowed in UK racing Thoroughbreds), chronic stress, frequent national and international movement or passive transmission through equine-derived products (Pfaender, Cavalleri, *et al.*, 2015; Gather, Walter, Todt, *et al.*, 2016; Kim *et al.*, 2017; Pronost *et al.*, 2017; Reichert *et al.*, 2017).

Two studies (Kim *et al.*, 2017; Abbadi *et al.*, 2021) found the prevalence of EqHV RNA positive animals to be higher in stallions compared with mares. This was further confirmed by Reichert et al. (2017), who observed that males were more frequently EqHV RNA positive than females (29.2% *versus* 14.0%) (Kim *et al.*, 2017; Reichert *et al.*, 2017; Abbadi *et al.*, 2021). A higher number of maiden mares (mares that have never carried a foal) tested positive for EqHV RNA compared to brood mares (Reichert *et al.*, 2017). In another study, it was found that the prevalence of EqHV antibodies was higher in mares than in stallions (77.1% vs 46.3%, respectively) (Abbadi *et al.*, 2021). Other studies have found no significant differences in EqHV RNA or EqHV antibody prevalence based on sex (Matsuu *et al.*, 2015; Pfaender, Cavalleri, *et al.*, 2015; Pronost *et al.*, 2017). However, one study suggested that horses involved in breeding are more likely to be infected with EqHV than horses not involved in breeding (Figueiredo *et al.*, 2018). This may be through parenteral, vertical or sexual transmission but has not been conclusively demonstrated. Due to a decline in antibodies over time,

possibly below the point of detection, the actual population prevalence of horses exposed to EqHV may be underestimated (Lyons *et al.*, 2014). Further studies are needed to assess risk factors for EqHV infection in equids.

1.2.7.Phylogeny

Nucleotide sequences of EqHV isolates from 17 different countries reveal an overall nucleotide sequence diversity of 6.4 to 19.9% (Burbelo *et al.*, 2012; Gemaque *et al.*, 2014). In addition, molecular studies on EqHV demonstrate a very low genetic variation between isolates. This is unusual because hepaciviruses normally show considerable genetic variation (Simmonds, 2004; Gabriella Elia *et al.*, 2017). HCV has seven recognized genotypes. In comparison, EqHV sequences appear to be much more closely related, with low genetic variation internationally. This could be associated with a low selection pressure on EqHV, although this has not yet been shown (Pronost *et al.*, 2017; Badenhorst *et al.*, 2018).

Based on phylogenetic analyses of the NS5B and NS3 regions of EqHV, two separate clusters have been proposed, namely clade 1 and 2 (Figueiredo *et al.*, 2015, 2018; Lu *et al.*, 2016; Gabriella Elia *et al.*, 2017; Kim *et al.*, 2017; Pronost *et al.*, 2017). The distance value (24.2%) in the NS5B region between these clades is larger than the value used to separate HCV genotypes, which is why it has been proposed to assign these two clades (Smith *et al.*, 2014; Figueiredo *et al.*, 2015; Pronost *et al.*, 2017). It has been proposed that clade 1 comprises viruses that result in a viral load that is almost four times higher than clade 2 ($p=0.039$) (Pronost *et al.*, 2017). Clade 1 comprises sequences from Brazil, USA, Hungary, France, Italy and Scotland as well as all donkey isolates and appears to be the dominant clade in which most sequences are clustered (Figueiredo *et al.*, 2015, 2018; Pronost *et al.*, 2017). Clade 2 contains sequences from mid-west Brazil,

USA and Japan and also includes all EqHV strains isolated from dogs (Pronost *et al.*, 2017). There appears to be no geographical separation of clades by country, as strains from both clades are present in most countries. This may be explained by international trade and globalization of horses (Lu *et al.*, 2016; Figueiredo *et al.*, 2018). However, in comparison with clade 1, clade 2 appears to be more minor, containing fewer strains (Figueiredo *et al.*, 2015). However, differentiating EqHV sequences into different clades has not been supported by all studies, which may depend on the phylogenetic analysis method used (Badenhorst *et al.*, 2018).

1.2.8. Diagnosis

Many methods have been used to test for the presence of EqHV RNA as well as antibodies. RT-PCR and RT-qPCR methods have been used with primers specific to the NS3, NS5B, core-E1 and E1-E2 regions (Kapoor *et al.*, 2011; Burbelo *et al.*, 2012; Lyons *et al.*, 2012). In addition, a triplex RT-qPCR has been developed to test for the presence of and differentiate between EPgV 1 and TDAV in the same assay (Postel *et al.*, 2016).

An ELISA was developed using the NS3-helicase domain and core recombinant proteins. Samples were only considered positive if they were above the cut-off value and if they were positive for both NS3 and core antibodies. If samples only tested positive for one of the antibodies they were considered undetermined (Lyons *et al.*, 2014).

For detecting antibodies, a LIPS (luciferase immunoprecipitation system) assay was developed by Burbelo *et al.* (2012) that is highly effective in detecting anti-NS3 and anti-NS5B regions. GLIPS (*Gaussia* luciferase immunoprecipitation system) was then developed by Matsuu *et al.* (2015) due to the higher sensitivity of using the highly stable *Gaussia* luciferase. Sensitivity and specificity of the

GLIPS assay were determined to be 84.21% and 71.43%, respectively (Matsuu *et al.*, 2015). Other studies have reported sensitivity and specificity of a GLIPS assay to be 70% and 97%, respectively (Abbadi *et al.*, 2021).

1.2.9. Co-infections of EqHV

In recent years, a variety of new, apparently hepatotropic viruses have been described in horses, including EqHV, equine Parvovirus hepatitis (EqPV-H), EPgV and TDAV. It has been suggested that a co-infection between EqHV and one or more of these other viruses may lead to an increase in the observed frequency of hepatopathies and there is some evidence to support this (Lyons *et al.*, 2014). Although TDAV has been detected in commercial equine sera it has not yet been detected in the horse population (Lyons *et al.*, 2014; Lu *et al.*, 2016; Postel *et al.*, 2016; Tomlinson *et al.*, 2019). In contrast, EPgV has thus far not been associated with Theiler's disease, but has been shown to elevate serum liver enzymes as well as cause biochemical alterations (Chandriani *et al.*, 2013; Kapoor *et al.*, 2013; Lyons *et al.*, 2014; Lu *et al.*, 2016; Mealey and Ramsay, 2016; Zehetner *et al.*, 2021).

In addition to EPgV and TDAV, another virus that should be taken into consideration is the recently discovered parvovirus (EqPV-H) associated with serum hepatitis and fulminant hepatic necrosis (Divers *et al.*, 2018). Lu *et al.* (2018) demonstrated that serum samples can contain both EqPV-H and EqHV RNA, highlighting the possibility of a coinfection. As both EqHV and EqPV-H are associated with some form of liver disease, EqPV-H should be considered when investigating a possible hepatopathy in horses.

1.2.10. Modes of Transmission

EqHV appears to be transmitted both vertically and horizontally although the precise routes and mechanisms remain unclear.

1.2.10.1. Horizontal transmission

1.2.10.1.1. Blood products

One route of transmission that has been proposed is horizontal transmission, mainly through infected plasma or serum, including serum products. These commercially available sera are used for a variety of purposes, including as virus propagation supplements in the development of modified live vaccines, treatments such as anti-snake venoms and botulism antitoxins for both humans and animals, cell culture, and even tetanus prophylaxis (Paim *et al.*, 2019). Commercially-available horse sera used for tissue culture contained viral EqHV RNA as well as EqHV antibodies (Pfaender, Cavalleri, *et al.*, 2015; Postel *et al.*, 2016; Lu *et al.*, 2017; Paim *et al.*, 2019). Other viral RNA, such as that of EPgV could also be detected in the serum samples (Pfaender, Cavalleri, *et al.*, 2015; Postel *et al.*, 2016; Lu *et al.*, 2017). Untreated sera could pose a potential risk to susceptible hosts that may lead to infection with EqHV or other equine viruses whereas veterinary products and commercial anti-sera are highly unlikely to pose a risk as heat inactivation appears to eliminate the presence of viral RNA (Postel *et al.*, 2016). It is interesting to note that within individual pools of commercial sera there appear to be multiple different sequences of EqHV present (Postel *et al.*, 2016). One of the equine serum products contained fetal serum only and appeared to be uninfected with EqHV, which suggests infection post-parturition (Postel *et al.*, 2016). However, due to only one fetal serum sample being tested, no definitive conclusions can be drawn from this observation. In addition to commercial sera, six products containing pregnant mare serum gonadotrophin

(PMSG) were also negative for EqHV RNA (Lu *et al.*, 2017). Overall, these reports indicate that there is a low potential risk of transmitting EqHV infection using raw commercial equine blood products and the area needs more research to assess the potential implications.

1.2.10.1.2. Horizontal transmission through arthropods

A recent study by Badenhorst *et al.* (2019) investigated the presence of EqHV RNA in over 5000 mosquitoes by RT-qPCR in Austria and northern Italy. Although serum samples collected from horses from the same region as the mosquitoes had a high seroprevalence of EqHV (45.9%), EqHV RNA was not detected in any of the pools of mosquitoes that were analysed (Badenhorst *et al.*, 2019). Therefore, it is unlikely that mosquitoes play a role in the transmission of EqHV. Further investigations are needed to investigate the potential role of other arthropods in the transmission of EqHV, such as horse flies (Badenhorst *et al.*, 2019).

1.2.10.2. Vertical Transmission

Vertical transmission has been described in other infections, such as with equine arteritis virus infection (Timoney *et al.*, 1987). Twenty Thoroughbred mares and their foals were monitored by testing sera from prepartum mares, blood from the umbilical cord, post-suckling foal sera and colostrum samples for EqHV RNA from immediately before parturition until 6 months post-partum (Gather, Walter, Todt, *et al.*, 2016). Out of the 20 mares tested, 4 were positive for EqHV by RT-qPCR. Serum from one of the four foals, belonging to the mare with the highest viral titre, tested EqHV RNA positive after birth, indicating the possibility of vertical transmission, which may be facilitated through the increased viral load (Gather, Walter, Todt, *et al.*, 2016). When testing for antibodies, 16 out

of the 20 mares tested EqHV antibody positive by LIPS assay before parturition. Colostrum from the 16 seropositive mares all tested positive, whereas umbilical cord blood samples were all seronegative, except for those of the mare whose foal had tested positive for EqHV RNA after birth. This indicates the passive transfer of EqHV specific antibodies through the colostrum for 16 of the 20 foals. However, these colostral antibodies were not screened for neutralizing capacity as there was no assay available to test for this function at the time (Gather, Walter, Todt, *et al.*, 2016). Placentae from the seropositive mares were negative for EqHV RNA.

All mares and foals from the study investigating vertical transmission were followed up at 3- and 6-months post parturition. All four initially seropositive mares remained seropositive until at least 6 months post-partum. One mare cleared the infection whilst the other three mares continued to be viraemic at 6 months post-partum (Gather, Walter, Todt, *et al.*, 2016). The foal that was viraemic at birth remained both viraemic and seropositive until 6 months post-partum. Two of the other foals became viraemic at 3 months post-partum but were seronegative at this time point. The fourth foal remained EqHV RNA negative. No clinical signs associated with liver disease were observed and liver markers were within reference ranges for all mares and foals (Gather, Walter, Todt, *et al.*, 2016). Of the remaining horses, 2 mares and 7 foals became viraemic within 6 months after parturition. In the foals, a marked decline in antibody levels was observed at 6 months post-parturition. Overall, these data indicate horizontal transmission within the herd as all the isolates from the original and newly infected horses clustered together on phylogenetic analysis (Gather, Walter, Todt, *et al.*, 2016).

In a follow-up study, four of the foals from the vertical transmission study were monitored until 12–13 months post-partum, at which point EqHV RNA could

still be detected in all foals (Gather, Walter, Pfaender, *et al.*, 2016). Three of those foals were seropositive at birth (post-suckling); antibody levels decreased until 6 months post-partum but re-appeared when EqHV RNA could be detected again. The fourth foal was seronegative at birth but developed EqHV-specific antibodies at approximately 10 months of age (Gather, Walter, Pfaender, *et al.*, 2016).

Another study was conducted by Pronost *et al.* (2019) to investigate the prevalence of EqHV in perinatal foal deaths. Out of 394 samples, 3 (0.76%) were positive for EqHV by qPCR (Pronost *et al.*, 2019). In the first case, EqHV was detected in foal lung and liver without evidence of overt viral infection. In case 2, EqHV was found in lung, liver and allantochorion without overt lesions. The mare had a serum sample taken 10 months later, which was positive for EqHV RNA. The third case had an EqHV-positive allantochorion with no histopathological signs of viral infection. Interestingly, the viral loads in the allantochorion were much lower than those in foal liver and lung biopsies (Pronost *et al.*, 2019).

Overall, it appears that EqHV may be transmitted vertically, albeit at a low incidence. This may be due to the intricate six layer architecture of the equine epitheliochorial placenta or other, yet unexplored factors (Pronost *et al.*, 2019).

1.2.10.3. Potential alternative routes of transmission

In HCV, the seroprevalence is normally around 4–15% (Miller and Abu-Raddad, 2010; Averhoff, Glass and Holtzman, 2012). In contrast, the seroprevalence of EqHV-specific antibodies in horses is currently estimated to be at 40–70%. Horses are not exposed by sharing of EqHV contaminated needles during intravenous drug abuse, so this difference has led to the proposal that a non-parenteral route of EqHV transmission may exist. In the case of HCV, low levels of HCV RNA and antibodies are shed in saliva and urine and have also been detected in seminal

fluid, which suggest that there may be potential routes of non-parenteral infection that has not yet been tested with EqHV positive horses (Liou *et al.*, 1992; Elsana *et al.*, 1998; Sosa-jurado *et al.*, 2014). This is supported by the presence of EqHV antibodies in horses that were sharing stables with EqHV RNA positive horses, with no possibility of intravenous passive transfer, indicating a previous exposure through a non-parenteral route (Lyons *et al.*, 2014). It has been suggested that the transmission of EqHV between these horses may have occurred by close contact caused by exposure to urine, saliva, wounds, or contaminated surfaces (Lyons *et al.*, 2014). It is currently unknown whether equine saliva or urine contains sufficient levels of EqHV to be infective. Further studies into the possible routes of transmission of EqHV are warranted. Although EqHV has been frequently found in broodmares and breeding stallions it is still unclear whether EqHV can be transmitted venereally (Reichert *et al.*, 2017; Badenhorst *et al.*, 2019).

1.3. Receptors for viral entry

Due to the genetic similarities between EqHV and HCV, it has been proposed that EqHV may use the same receptors for viral entry as HCV, namely cluster of differentiation 81 (CD81), scavenger receptor class B type 1 (SR-B1), occludin (OCLN), and claudin-1 (CLDN-1). It appears that for HCV, human cells require all four receptors in order to be permissive for HCV entry (Ploss *et al.*, 2009)

1.3.1. Cluster of Differentiation 81 (CD-81)

CD81 belongs to the tetraspanin family, which is made up of relatively small (26 kilo Daltons [kDa]), type IV glycoproteins that contain four transmembrane domains, a large extracellular loop and intracellular N- and C-terminal domains, which can be seen in Figure 2 (Levy, Todd and Maecker, 1998; Boucheix and Rubinstein, 2001; Levy and Shoham, 2005).

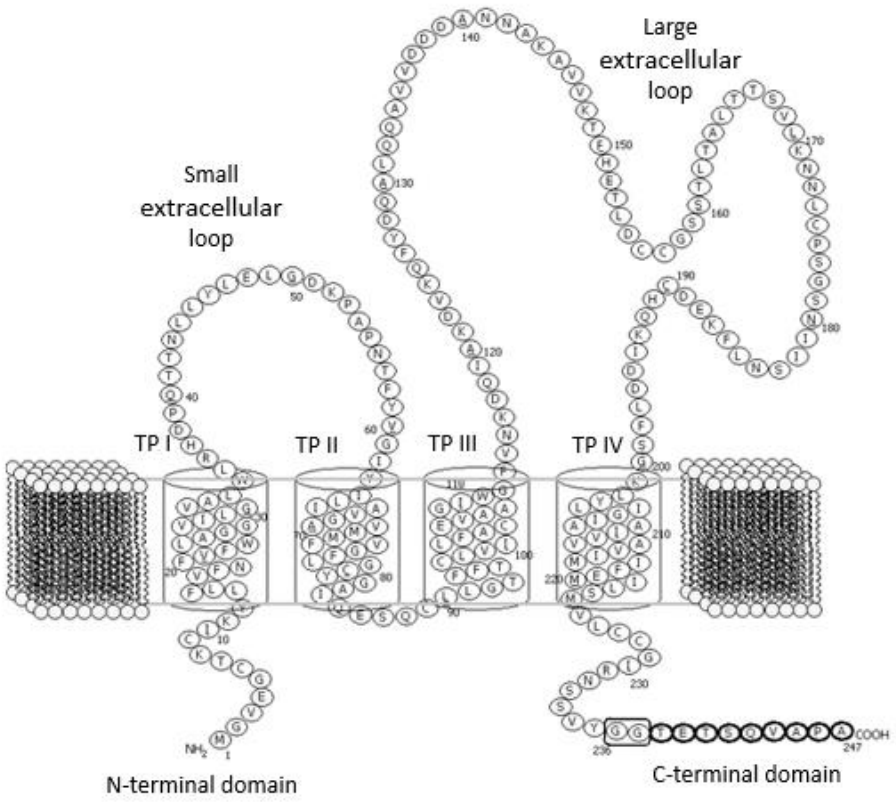


Figure 2. *Illustration of a two-dimensional model of the CD81 membrane receptor, including its extracellular loops, terminal domains and transmembrane domains (TP I – IV). Figure adapted from* Takayama *et al.* (2008)*.*

CD81 is expressed on a variety of human cell types, including hepatocytes, fibroblasts, epithelial and endothelial cells and white blood cells (Levy, Todd and Maecker, 1998; Boucheix and Rubinstein, 2001; Zona *et al.*, 2014). CD81 forms large complexes with membrane proteins (such as integrins, immunoglobulins and signalling molecules) that enable cell processes such as adhesion, proliferation, migration, signal transduction and differentiation, as well as enabling pathogen infection (Claas, Stipp and Hemler, 2001; Hemler, 2003, 2005; Martin *et al.*, 2005; Zona *et al.*, 2014). Notably, CD81 is one of the major receptors that enables HCV entry into the host cell through the specific interaction between the large

extracellular loop of CD81 with the HCV E2 glycoprotein (Cormier *et al.*, 2004; Zhang *et al.*, 2004).

1.3.2. Scavenger receptor type B class 1 (SR-B1)

SR-B1 is the other receptor that also interacts with the HCV E2 glycoprotein. It is an 82 kDa glycoprotein containing two cytosolic (N- and C-terminal) domains (Rhainds and Brissette, 2004). It is very highly expressed in liver hepatocytes and steroidogenic tissue but is also expressed in some other tissues, albeit not as highly (Rhainds and Brissette, 2004). Similar to CD81, SR-B1 enables HCV attachment to the cell but it is insufficient to enable HCV entry into cells on its own. In combination with CD81 however, it has been shown that the formation of the HCV E2 heterodimer with SR-B1 and CD81 efficiently mediates HCV entry into cells. In addition, liver-specific factors such as ephrin receptor A2, epidermal growth factor receptor, cholesterol transporter Niemann-Pick C1-like 1 and transferrin receptor 1 are also required (Bartosch *et al.*, 2003; Heo *et al.*, 2006; Lupberger *et al.*, 2011; Sainz *et al.*, 2012; Martin and Uprichard, 2013).

1.3.3. Occludin (OCLN)

Occludin is a 60 kDa tetraspan transmembrane protein situated at tight junctions made up of two extracellular loops, one intracellular loop and N- and C-cytoplasmic domains (Furuse *et al.*, 1993). OCLN, as well as CD81 and CLDN-1, is an essential receptor enabling HCV infection of host cells (Marceau *et al.*, 2016). Occludin is involved in signal transduction, epithelial-to-mesenchymal transition, controlling the actin cytoskeleton, and inhibiting apoptosis of cells (Barrios-Rodiles *et al.*, 2005; Murata *et al.*, 2005; Yu *et al.*, 2005; Chiba *et al.*, 2008).

1.3.4. Claudin-1 (CLDN-1)

Claudin-1 is also a tetraspan protein within the tight junction that is made up of two extracellular loops, a short N-terminus and a C-terminal cytoplasmic domain (Chiba *et al.*, 2008). It has the highest expression levels in the liver, but can also be found in many tissues of epithelial origin (Furuse *et al.*, 1998). The function of CLDN-1 is to regulate the paracellular permeability of endothelial and epithelial cells at tight junctions and establish polarity of the cell (Harris *et al.*, 2008).

1.4. Aims and objectives

Although EqHV is the most closely related hepacivirus to HCV, there are clearly some differences in the biology of the two viruses. Little is known about how EqHV is transmitted. The entry of HCV to human cells is well characterised and involves co-operation between four entry receptors. The aim of this MRes project was to examine the potential role of the four receptors associated with cell entry of HCV in EqHV, in particular in relation to potential vertical transmission from mares to foals. The presence of HCV receptors on equine liver cells and other cell lines as well as immunohistochemistry staining of these receptors were investigated.

2. MATERIALS AND METHODS

2.1. Cell lines

The cell lines used in all experiments are shown in Table 4.

***Table 4**. Cell lines and culture media used in this study*

Cells (abbreviation)	Format	Culture Medium	Source*
Human hepatoma cells (Huh7)	Line	DMEM	Dr Janet Daly
Equine dermal (E. Derm)	Line	EMEM	Dr Janet Daly
Foetal horse kidney (FHK)	Line	DMEM	Dr Janet Daly
Human hepatoma G2 (HepG2)	Line	EMEM	Dr Alex Tarr
Equine hepatocytes (EH)	Primary	William's E	Dr Stuart Paine

*all sources are part of the University of Nottingham

2.2. Cell culture

Huh7 cells (1 mL) and FHK cells (1 mL) were thawed from liquid nitrogen by placing in a 37°C water bath until only a fleck of ice remained then added in a drop wise fashion to Dulbecco's Modified Eagle Medium (Gibco®, ThermoFisher™ Scientific, Loughborough, UK) which had been brought to 37°C using a water bath. The medium was supplemented with 10% fetal calf serum (FCS; v:v; Biowest, Lutterworth, UK), 1% L-glutamine (200mm, Gibco™), 1% non-essential amino acids and 1% Penicillin and Streptomycin (5,000 U/mL Penicillin, 5,000 µg/mL Streptomycin; Gibco™).

Equine dermal cells and HepG2 cells were thawed from -80°C as described previously and added to Eagle's Minimum Essential Medium (EMEM; Biowest, Lutterworth, UK), supplemented with 10% FCS, 1% L-glutamine and 1% Penicillin /Streptomycin as above.

Primary equine hepatocytes were thawed from -80°C, added dropwise to pre-warmed William's E Medium (Gibco®; ThermoFisher Scientific™) supplemented with 10% FCS, 1% L-glutamine and 1% Penicillin/Streptomycin and incubated for 15 minutes at 37°C, 5% CO_2 before being used directly in assays.

2.3. Cell passage

All cells were passaged when they reached ~80–90% confluency and re-suspended in the appropriate media. Cells were washed with PBS (Gibco™) which was decanted off and then 0.25% trypsin EDTA (Gibco™) was added to cover the bottom of the flask and incubated at 37°C, 5% CO_2 for approximately 7 minutes or until the larger clumps of cells had separated and detached from the bottom of the flask. Cells were added dropwise into a 15 mL tube and diluted in a large volume of supplemented medium. A volume of 50 µL was removed and cells counted using a haemocytometer. Cells were then centrifuged for 5 minutes and re-suspended in supplemented medium. Cells were split between 1:2 and 1:5 dependent on how quickly confluence had been reached.

2.3.1. Cryopreservation of cells

Cells were routinely frozen in 1 mL cryovials at approximately 1 x 10^6 cells/cryovial. The freezing medium consisted of 50% (v:v) DMEM/EMEM, 40% FCS and 10% DMSO (ThermoFisher Scientific™). Cells were then put into a Mr. Frosty at -80°C then transferred into liquid nitrogen the next day.

2.4. Receptor alignments

The amino acid sequences of the human (*Homo sapiens*), equine (*Equus caballus*) and murine (*Mus musculus*) CD-81, OCLN, CLDN-1 and SR-B1 proteins were taken from GenBank® Protein database and aligned using Clustal Omega 12.1.

Differences between the equine and murine sequences compared to the human sequence were highlighted. These were then assessed as to whether they were conservative (no major change in structure or polarity; yellow) or non-conservative (major change in structure or polarity; pink) changes to the amino acids.

2.5. Flow cytometric analysis

A 96-well U-bottomed plate was prepared by adding 50 µL of monoclonal antibody per well, appropriately diluted in FACS buffer (PBS with 1% BSA [ThermoFisher Scientific™]; Table 5).

Table 5. *Primary antibodies used for flow cytometry analysis. Abbreviations: M = mouse, CD Cluster of differentiation, OCLN occludin, MHC Major Histocompatibility Complex, vWF von Willebrand factor.*

Use of antibody	Antibody	Clone	Class of Ig	Concentration of Ab (mg/ml)	Final concentration of antibody added/well (µg)
Test	M α human CD81	JS-81	IgG1	0.5	5
Test	M α human OCLN	1G7	IgG1	0.5	5
+ve control	M α equine MHC class I	CVS-22	IgG2a	1.0	5
-ve control	M α human vWF	F8/86	IgG1	0.2	1

The medium was removed from cells, and they were gently washed with PBS. Cells were detached from the flask by adding 5 mM EDTA diluted in PBS to

the flask and incubating the plate at 37°C for approximately 5 minutes, until cells had detached. Cells were then re-suspended in PBS, counted using a haemocytometer and then spun down using a centrifuge at 2500 rpm for 5 minutes. Cells were then re-suspended in FACS buffer at 2 x 10^6 cells/mL prior to adding 25 µl to each well. A plate sealer was attached, and the plate incubated at 4°C for 45 minutes on a plate shaker. The plate was then centrifuged for 3 minutes, and the supernatant removed. Cells were washed 3 times with 200 µL of FACS buffer with the plate being centrifuged after each wash for 3 minutes. Each well had 50 µL of Alexa-Fluor conjugated secondary antibody (dilution of 1:2000; goat anti-mouse IgG, AlexaFluor488®) added and the plate was wrapped in foil prior to incubation at 4°C for 45 minutes in the dark using the shaker. Cells were washed 3 times as before, and then re-suspended in 200 µL of FACS buffer. The plate sealer was re-attached, and the plate was wrapped in foil and stored in the dark at 4°C until analysis using the Beckman Coulter FC500 flow cytometer, usually within one day. Using the FlowJo™ software, the Overton positive value and the Chi Squared value were determined from the histograms created by the flow cytometer. This was achieved by gating the populations of cells in the test group, subtracting the gated population from the control group and counting the number of events that remain per bin, labelling them positive.

2.6. Immunofluorescence (IF)

2.6.1. Cell fixation

2.6.1.1. Culture of cells in chamber slides

Corning® BioCoat™ and Falcon® CultureSlides (Corning®, Wiesbaden, Germany) were used to culture cells for immunofluorescence. Cells were added to each of the 8 chambers at a pre-determined optimal density of approximately 5 x 10^5 cells/well, and 0.5 mL were added to each chamber. Cells were incubated in

a humidified atmosphere at 37°C, 5% CO_2 overnight for approximately 20 hours. When cells reached ~80% confluence, the medium was removed, and cells were washed twice with PBS. At room temperature, 4% paraformaldehyde (PFA; 100µL) was added to each chamber for 20 minutes. This was followed by three consecutive 5-minute washes with PBS. Chamber slides containing cells were then stored in PBS at 4°C until further use, usually the following day.

2.6.1.2. Culture of cells on a coverslip

Coverslips (Ø 13 mm) were first incubated at 50°C overnight in hydrochloric acid (Acros, ThermoFisher Scientific™, Loughborough, UK) and then washed in double distilled water for 30 minutes using a sonicator, followed by rinsing in absolute ethanol, after which coverslips were air dried and stored in an airtight container.

Cells were seeded at a density of approximately 5 x 10^5 cells/mL (1.0 mL per well) and incubated at 37°C overnight on 13 mm etched coverslips placed in a 24-well-plate (Corning®). Cells were then fixed in 4% PFA. Following immunofluorescence staining within the wells of the 24-well plate, the coverslips were carefully removed using a curved needle and fine tweezers and placed on a slide using the ProLong™ Gold AntiFade Mountant with DAPI (Life Technologies™). The borders of the coverslips were sealed with clear fast dry nail polish to prevent drying out and incubated over night at 4°C for stabilisation before viewing on an epifluorescence imaging system (Leica DM 5000B; Leica Microsystems Ltd., Milton Keynes, UK).

2.6.2. Immunofluorescent staining or Indirect immunofluorescence

Fixed cells were washed in PBS, then incubated with 100 µL of 2% goat serum (NGS; Sigma-Aldrich, Darmstadt, Germany) in PBS for 45 minutes at room

temperature as a blocking step and then washed three times with PBS. Cells were incubated with 100 µL of the respective, appropriately diluted primary antibody in 1% NGS-PBS, for 1 hour at room temperature. Cells were then washed three times with PBS. This was followed by incubation in the dark with the respective, appropriately diluted fluorescently labelled secondary antibody, for 1 hour. The excess solution was removed from the chamber and the slide was detached using the applicators provided with the culture slide kit. A small drop of ProLong™ Gold AntiFade Mountant with DAPI (Life Technologies™) was added to each chamber and a coverslip was placed on top of the slide. The borders of the coverslips were sealed with nail polish to prevent drying out and incubated overnight at 4°C for stabilisation before viewing on an epifluorescence imaging system (Leica DM 5000B; Leica Microsystems Ltd., Milton Keynes, UK).

2.6.3. Antibodies used for indirect immunofluorescence

Primary and secondary antibodies used for indirect immunofluorescent staining of the different cell lines are shown in Tables 6 and 7.

Table 6. *Details of the antibodies specific for human hepatitis C virus entry receptors used for indirect immunofluorescent staining of cell lines. M = mouse, Hu = human, Rb = rabbit.*

Target antigen Receptor	**Antibody (name)**	**Isotype**	**Concentration (mg/mL)**	**Working Dilution**	**Source**
Cluster of Differentiation (CD) 81	M α human CD81	IgG1	0.5	1:200	BD Pharmingen™
Scavenger receptor class B type 1	Rb α SRB1(C16 7)	Unknown	2.0	1:200	(Catanese *et al.*, 2007)
Claudin 1	Hu α CLDN1 (C8)	Unknown	0.3	1:60	(Paciello *et al.*, 2016)
Occludin	M α OCLN	IgG1	2.0	1:400	Sigma-Aldrich

Table 7. Details of *secondary antibodies used for immunofluorescent staining of cell lines.*

Fluorescent molecule (emission spectrum)	Antibody specificity	Concentration (mg/ml)	Working Dilution	Source
Alexa Fluor® (488)	Goat α mouse IgG (H+L)	2.0	1:2000	Life technologies™
Alexa Fluor® (488)	Goat α human IgG (H+L)	2.0	1:1000	Life technologies™

2.7. Immunohistochemistry (IHC) of formalin fixed paraffin embedded (FFPE) tissue

Equine liver samples (1 cm^3) were collected from horses with an unknown history and age immediately after slaughter from Potters abattoir (Royal Wootton Bassett, Wiltshire). Samples were placed in neutral buffered formol saline for approximately 40 hours, then trimmed and dehydrated through a series of alcohols of increasing concentration (Leica TP1020 Tissue Processor; Leica Biosystems). The samples were then placed into xylene replacement prior to impregnation with paraffin wax by immersion. Tissues were embedded in paraffin wax and attached to pre-labelled histology cassettes. Sections were cut on a microtome (RM 2235, Leica Biosystems) at a thickness of 5 µm, placed on to poly L-lysine slides (ThermoFisher Scientific™) in a water bath (45°C) and then placed on a hot plate (60°C) for 30 minutes until the paraffin wax had melted. Slides were then de-paraffinized in xylene, rehydrated using serial alcohol dilutions and then placed in tap water for 5 minutes prior to further steps.

Antigen retrieval was conducted using a 10 mM citrate buffer of pH 6. This was prepared by adding 2.94 g of sodium citrate tribasic dehydrate (ThermoFisher Scientific™) to 1000 mL of reverse osmosis purified water. The pH was decreased

until it reached 6.0 using hydrochloric acid. Tween 20 (0.5 mL; ThermoFisher Scientific™) was mixed in thoroughly prior to storage at 4°C until further use. For antigen retrieval, the buffer was heated on medium heat in the microwave for 5 minutes. The slides were then immersed in the buffer and heated in the microwave again on low heat for 10 minutes, then left to cool for 30 minutes.

Slides were washed in tris-buffered saline (TBS) for 5 minutes and then blocked for 20 minutes with diluted normal horse serum at room temperature. The slides were incubated for 30 minutes with primary antibody appropriately diluted in TBS buffer and then washed twice in TBS for 5 minutes. Sections were incubated with the secondary antibody (provided by the VECTASTAIN® Elite® ABC HRP Kit) for 30 minutes at room temperature and then washed again twice for 5 minutes using TBS. The VECTASTAIN® ABC Reagent (Maravai LifeSciences, Peterborough, UK) was re-constituted according to the manufacturer's instructions and then added to the sections and incubated for 30 minutes before washing twice more in TBS. Freshly made up diaminobenzidine (DAB) with peroxidase (HRP) substrate (Maravai LifeSciences) solution was then applied for 3 to 5 minutes until the desired stain intensity developed. Sections were thoroughly rinsed in tap water, counterstained with haematoxylin and then mounted under coverslips using DPX (Sigma-Aldrich, Darmstadt, Germany).

2.7.1. Antibodies used for FFPE sections

Primary antibodies used in immunohistochemistry are shown in Table 8. The secondary antibody used for IHC was a horse anti-mouse IgG (VECTASTAIN® Elite® ABC HRP Kit, Vector Laboratories) conjugated to biotin I.

Table 8. *Details of antibodies used for IHC of FFPE tissues. M=mouse antibody, Hu = human antibody*

Antigen	Antibody (name)	Isotype	Concentration (mg/ ml)	Working Dilution	Source
Von Willebrand factor	M α vWf	IgG	0.2	1:20	Abcam
Isotype -ve control	M isotype	IgG	2.5	1:50	ThermoFisher Scientific™
Human MHC class I	M α Hu HLA-ABC (W6/32)	IgG2a	0.5	1:100	eBioscience™
CD81	M α CD81 (JS-81)	IgG1	1.0	1:100	ThermoFisher Scientific™
CD81	M α CD81 (1D6)	IgG1	1.0	1:10	Thermo Scientific™

2.8. Immunohistochemistry (IHC) of frozen tissue sections

For snap freezing, 1 cm^3 blocks of liver were placed in full into OCT embedding medium (VWR™, Lutterworth, UK) within the cut off end of a plastic pastette. These were then held with forceps within a beaker of isopentane (ThermoFisher Scientific™) that had been cooled by sitting in a polystyrene box containing liquid nitrogen for about 1–2 minutes. When the tissue was frozen, the sample was placed in a small polythene bag and stored at -80°C prior to sectioning. Before sectioning, the edge of the plastic pastette was cut with a scalpel blade and the block placed onto either dry ice or directly into the cryostat chamber at -20°C. The block was mounted on a metal chuck using OCT medium.

Sections (7 µm) were cut and placed on poly L-lysine coated slides (ThermoFisher Scientific™), air dried for 20–30 minutes and then fixed in ice-cold acetone in the fume cupboard for 10 minutes. Slides were removed, wrapped gently back-to-back in foil and stored at -20°C prior to staining.

Before use, frozen sections were thawed, and slides were placed in a humidity chamber. The sections were incubated for 30 minutes at room

temperature with a primary antibody diluted in TBS before washing for 5 minutes in TBS. Primary antibodies are shown in Table 9. The diluted biotinylated secondary antibody was applied, and sections incubated at room temperature for 30 minutes. Slides were washed for 5 minutes in TBS again and then incubated for 30 minutes using the VECTASTAIN® ABC Reagent according to protocol before a final wash in TBS for 5 minutes. The chromogen (DAB) and peroxidase substrate solution was added to the sections for 3 minutes until the desired stain intensity developed. Sections were then rinsed in tap water and incubated with haematoxylin for 2 minutes. Slides were cleared by washing them in tap water and then mounted using molten glycerol gelatine on a hotplate (60°C). The glycerol was allowed to dry before imaging the sections under a microscope.

Table 9. *Details of the primary antibodies used for IHC of frozen tissues. M=mouse antibody, Hu = human antibody*

Antigen	Antibody (name)	Isotype	Concentration / mg per ml	Working Dilution	Source
Negative control	M –ve control	IgG1	0.1	1:50	Bio-Rad Laboratories
MHC class I	M α horse MHC class I (CVS 22)	IgG	1.0	1:100	Bio-Rad Laboratories
CD81	M α Hu CD81 (JS-81)	IgG1	1.0	1:100	ThermoFisher Scientific™
CD81	M α Hu CD81 (1D6)	IgG1	1.0	1:10	Thermo Scientific™
OCLN	M α Hu OCLN (1G7)	IgG1	1.0	1:100	Sigma-Aldrich

2.9. Immunocytochemistry (ICC) of PBMC cytospins

EDTA blood samples were processed and spun at 2500 rpm for 5 minutes, followed by carefully removing the buffy coat using a glass pipette and transferring the cells into a V-bottom tube. The rest of the volume was made up to the 10 mL mark using RPMI medium supplemented with 10% (v:v) FCS, 1% L-glutamine and 1% Penicillin/Streptomycin. The glass pipette was then used to add half the diluted buffy coat to the top of 4 mL of Ficoll-Paque™ in another V-bottom tube by slowly releasing it at the side of the tube, allowing it to layer on top of the Ficoll-Paque™. The Ficoll-Paque™ tubes were spun at 475 x g for 15 minutes and deceleration was decreased to 0 in order to avoid disruption of the cells. The interface between the Ficoll-Paque™ and the RPMI medium (now made up of primarily PBMCs) was carefully removed with a glass pipette, added to a new tube and made up with RPMI medium to the 10 mL mark once again. The PBMCs were counted using a haemocytometer and the volume needed to make up 1 x 10^6 cells/mL was calculated. Poly-L-lysine slides were labelled and set up in the cytospin according to the manufacturer's instructions (Shandon Cytospin 4 [Thermo Electron Corporation]). Each slide had 50 µL FCS and 50 µL cell solution added to it and was then spun at 700 rpm for 3 minutes. Once spun, the slides were air dried for 20 minutes, then fixed in ice-cold acetone for 10 minutes (as for the snap frozen tissue).

2.10. Cytospins and IHC of peripheral blood mononuclear cells (PBMCs)

Blood samples collected from Thoroughbred horses that were sent to the university were processed by the University's lab technicians. The buffy coat was removed from the EDTA tubes using a glass pipette to gently aspirate the layer

of cells and placed in a V-bottom 10 mL tube. The removed cells were then made up to 10 mL by adding RPMI supplemented medium. Then the diluted cells were layered over 4 mL of Ficoll-Paque™ and spun at 435 x g for 15 minutes. After spinning, the layer of cells on top of the Ficoll-Paque™ was carefully removed using a glass pipette, put into another V-bottom tube and made up again until 10 mL using 10% RPMI medium. The cells were counted using a haemocytometer, spun down at 435 x g for 5 minutes and then re-suspended with medium at a concentration of approximately 1 x 10^6 cells/mL. Then the cytospin machine was set up to spin at 700 rpm for 3 minutes. Each well had 50 µL of pure FCS added to it as well as 25 µL of the re-suspended cell solution (equals to approximately 2.5 x 10^4 cells). Two sets of cells were added to each poly-L-lysine slide, air-dried for 20 minutes, then fixed in ice-cold acetone for 10 minutes and subsequently wrapped in tinfoil and stored at -4°C.

3. RESULTS

3.1. Receptor Alignments

In order to assess the likely cross-reaction between antibodies to the human proteins and equine antigens, protein sequence alignments were conducted for the four major entry receptors used by HCV.

3.1.1. CD81

Amino acid alignments were compared between human, equine and murine sequences of CD81, a receptor made up of 236 amino acids (Figure 3). The percent identity matrix created by Clustal 12.1 indicated that the human and equine sequences were 96.61% identical. The sequence alignment also revealed that compared to the human sequence, the equine sequences had 8 aa substitutions whereas the murine had 19 substitutions. Out of the eight aa differences between the human and equine sequences there were two non-conservative substitutions in the region thought to be targeted by the JS81 antibody (A. Tarr, personal communication). These 2 non-conservative substitutions (D155N and N172I) account for a 0.8% (2/236) difference in amino acid sequence between the human and equine CD81 receptor sequences. Due to the relatively high degree of conservation between the sequences, it was predicted that the human-specific antibody against CD81 was highly likely to cross-react with the equine antigen. The CD81 antibody used here targeted the large extracellular loop of CD81 although the epitope mapping was not detailed by the manufacturer.

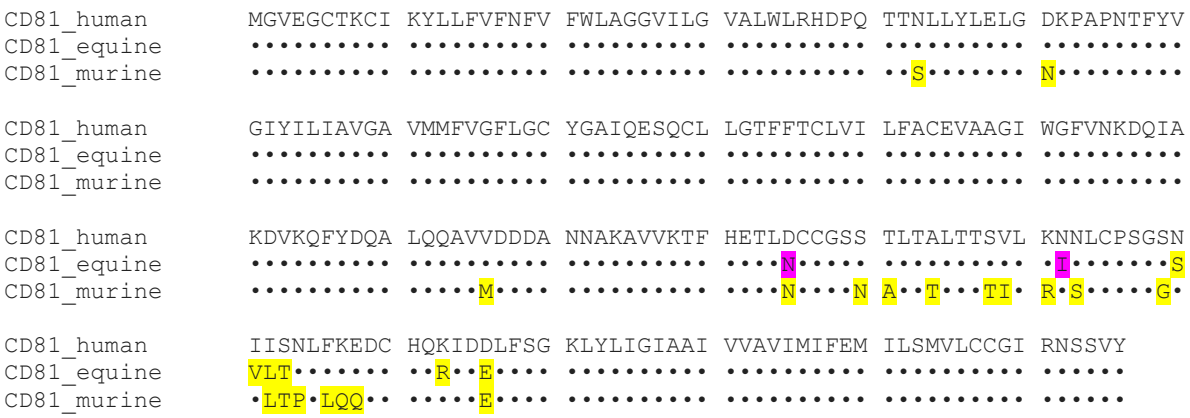


Figure 3*. CD81 amino acid alignments for human (AAH93047.1), murine (AAH11433.1) and equine (XP_023510477.1) sequences. All amino acids that were different to the reference sequence (human) are highlighted in yellow. For the comparison between human and equine sequences, non-conservative changes are highlighted in pink and conservative changes are highlighted in yellow.*

3.1.2. OCLN

Amino acid alignments were compared between human, equine and murine sequences of OCLN, a receptor made up of 522 (human), 521 (mouse) or 520 (horse) amino acids (Figure 4). The percent identity matrix created by Clustal 12.1 indicated that the human and equine sequences were 92.69% identical (Figure 4). The sequence alignment also revealed that compared to the human sequence, the equine sequences had 38 aa substitutions, whereas the murine sequence had 53 substitutions. Out of the 38 aa differences between the human and equine sequences there were 16 non-conservative substitutions, four of which were present in the N-terminus targeted by the antibody used in the experiments (A. Tarr, personal communication). These 16 non-conservative substitutions account for a 3.1% (16/522) difference in amino acid sequence between the human and equine OCLN receptor sequences. Due to the relatively low amino acid sequence variation between the human and equine sequence it

was predicted that this human specific antibody against OCLN would also cross-react with the equine antigen.

Figure 4*. OCLN amino acid alignments for human (AAL47094.1), murine (AAI38681.1) and equine (XP_023474007.1) sequences. All amino acids that were different to the reference sequence (human) are highlighted in yellow. For the comparison between human and equine sequences, non-conservative changes are highlighted in pink and conservative changes are highlighted in yellow.*

3.1.3. CLDN-1 and SR-B1

Amino acid alignments were compared between human, equine and murine sequences of CLDN-1, a receptor made up of 211 amino acids. The percent identity matrix created by Clustal 12.1 indicated that the human and equine sequences were 95.26% identical. The sequence alignment also revealed

that compared to the human sequence, the equine sequences had 10 aa substitutions whereas the murine sequence had 20 substitutions (Figure 5). Out of the 10 aa differences between the human and horse sequences, there was one non-conservative substitution. This one non-conservative substitution accounts for a 0.5% (1/211) difference in amino acid sequence between the human and equine CLDN-1 receptor sequences. Although the region of the protein bound by the anti-human CLDN-1 antibody used is not known, the relatively few non-conservative aa changes suggest that the antibody may cross-react with the equine protein.

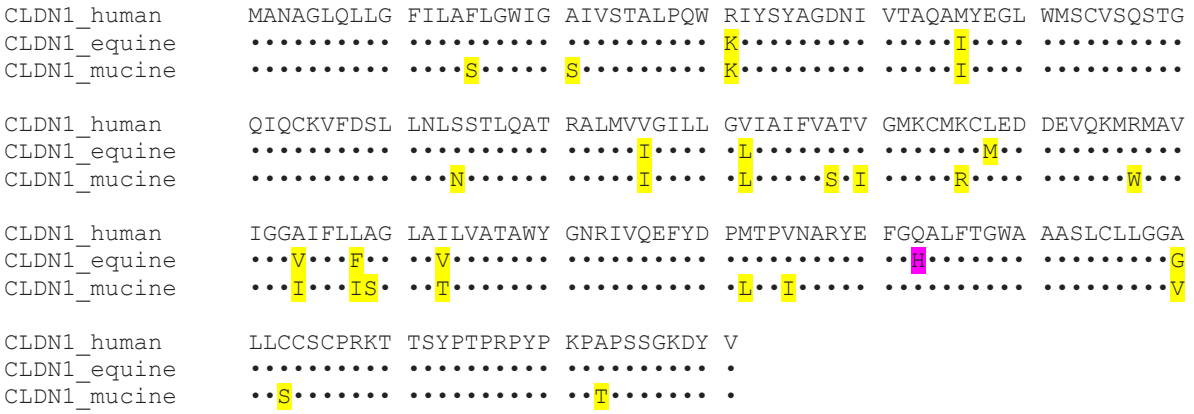


Figure 5*. CLDN-1 amino acid alignments for human (AAH12471.1), murine (AAH02003.1) and equine (XP_001500138.1) sequences. All amino acids that were different to the reference sequence (human) are highlighted in yellow. For the comparison between human and equine sequences, non-conservative changes are highlighted in pink and conservative changes highlighted in yellow.*

Amino acid alignments were compared between human, equine and murine sequences of SR-B1, a receptor made up of 509 amino acids. The percent identity matrix created by Clustal 12.1 indicated that the human and equine sequences were 85.27% identical. The sequence alignment also revealed that compared to the human sequence, the equine sequences had 75 aa substitutions, whereas the murine sequence had 104 substitutions (Figure 6). Out of the 75 aa differences between the human and horse sequences there

were 41 non-conservative substitutions. These 41 non-conservative substitutions account for an 8.1% (41/509) significant difference in amino acid sequence between the human and equine receptor sequences. Although the region targeted by the anti-human SR-B1 antibody used is not known, the large number of non-conservative amino acid changes suggest that the antibody may be poorly cross-reactive with the equine protein.

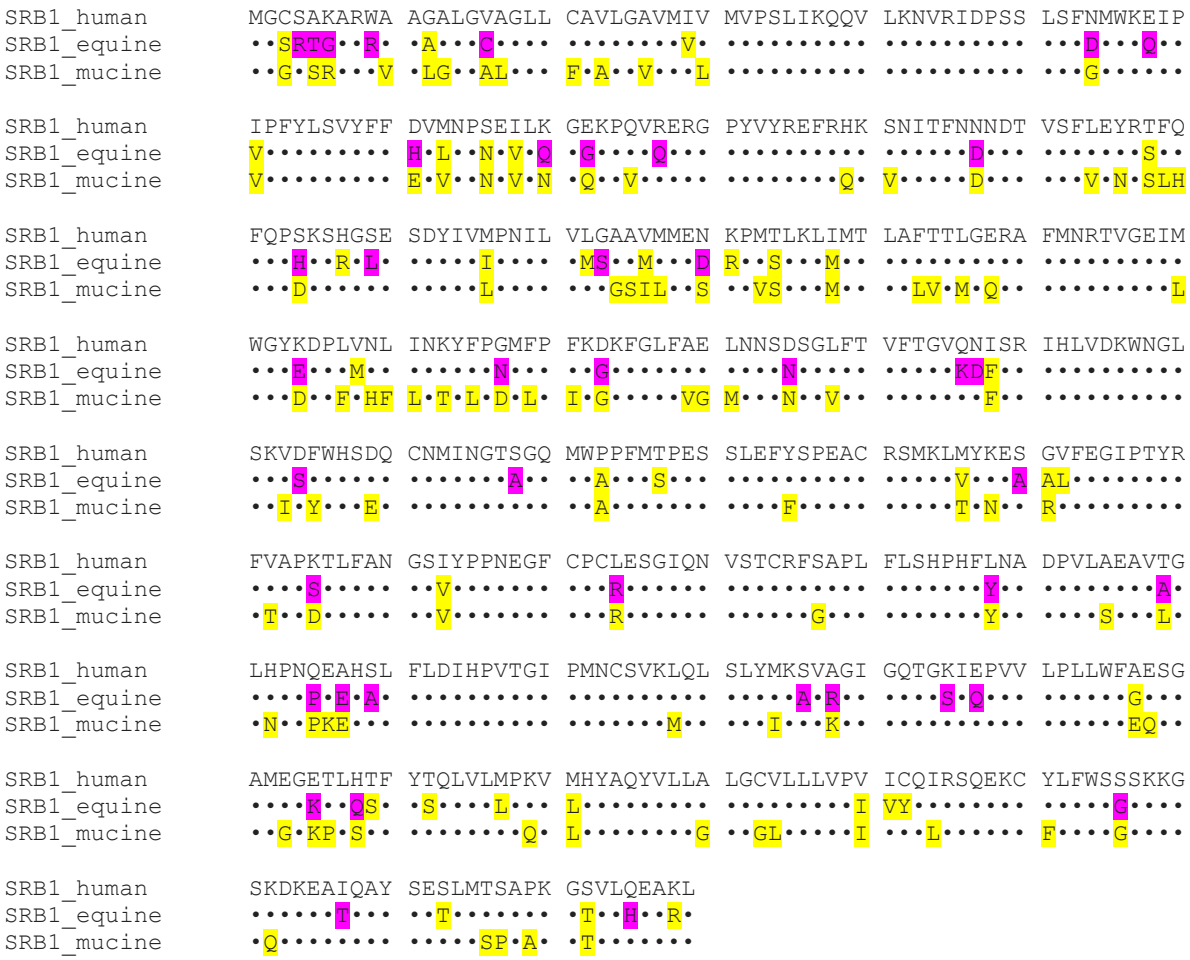


***Figure 6**. SR-B1 amino acid alignments for human (AAH80647.1), murine (AAH04656.1) and equine (XP_023503082.1) sequences. All amino acids that were different to the reference sequence (human) are highlighted in yellow. For the comparison between human and equine sequences, non-conservative changes are highlighted in pink and conservative changes highlighted in yellow.*

3.1.4. Summary of receptor alignments

A summary of the percentage identities and the differences between human and equine sequences is presented in Table 13. The mouse anti-human CD81 antibody and the mouse anti-OCLN were commercially available. The anti-CLDN1 and anti-SR-B1 were provided by collaborators and therefore the exact sequences were not known.

Table 13. *Summary of the percent identity matrix and percent of non-conservative substitutions of amino acid sequences when comparing the horse sequence with the human sequence*

Details of receptor identities	*Receptors*			
	CD81	*OCLN*	*CLDN-1*	*SR-B1*
% identity matrix between human and equine receptors	96.61	92.69	95.26	85.27
% non-conservative substitutions between human and equine receptors	0.8	4.7	0.5	8.1

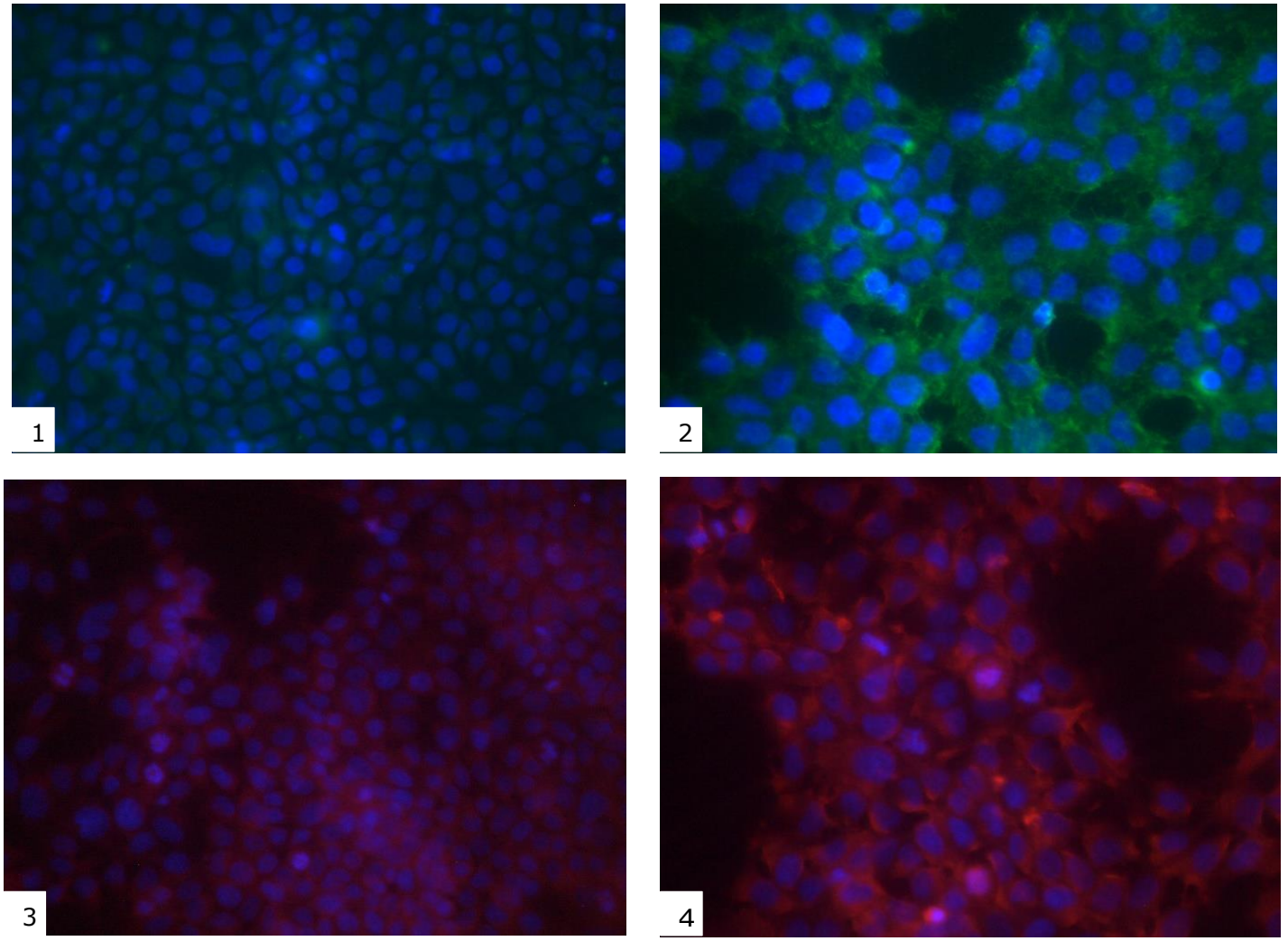
Due to the results of the sequence alignments, it appears that the likelihood of cross-reaction between human antibodies and equine receptors is relatively high for CD81, CLDN-1 and possibly OCLN. The SR-B1 antibody is less likely to cross react with the equine antigen due to the higher number of non-conservative changes.

3.2. Expression of EqHV receptors on Huh7 and equine dermal cells (E.Derm) as detected by immunofluorescence (IF)

Detection of HCV receptors on Huh7 cells was attempted using antibodies against all four receptors but immunofluorescent staining was generally

unsuccessful. CD81 appeared to be expressed on Huh7 cells but the staining was not clear and not specific to the hepatocyte membranes (Figure 7). IF was attempted on equine dermal (E.Derm) cells but this was unsuccessful due to non-specific binding by the secondary antibody. This approach was not continued for several reasons; namely the lack of availability of an equine liver cell line, the poor viability of liver cells collected *ex vivo,* the presence of some non-specific binding by the secondary antibody and the poor quality of specific staining (Figure 7) with some of the receptor reagents.

***Figure 7**. Immunofluorescent staining of Huh7 cells. 1) Presence of non-specific binding (NSB) of the secondary antibody (goat anti-human) at 1:2000. 2) Staining with anti-human CD81 (JS81) at 1:100 and the secondary antibody (goat anti-human) at 1:2000. 3) Presence of NSB of the secondary antibody (goat anti-human) at 1:1000. 4) Staining with anti-human CLDN1 at 1:60 and the secondary antibody (goat anti-human) at 1:1000. All cells were counterstained with DAPI (blue cell nuclei).*



3.3. Flow cytometric analysis of CD81 and OCLN expression on Huh7 and EH cells

The expression of two receptors commonly used by HCV to enter human hepatocytes, namely CD81 and OCLN, was measured on equine hepatocyte (EH) cells using flow cytometric analysis. Due to the lack of commercially available equine hepatocyte (EH) cell lines, dissociated frozen/thawed EH cells derived from equine livers collected at an abattoir (a kind gift from Dr Stuart Paine, University of Nottingham) were used. Huh7 cells were used as a control as they express CD81 as well as OCLN (Akazawa *et al.*, 2007; Koutsoudakis *et al.*, 2007). Experiment 1 was done without any cell permeabilization, and all steps used TBS. Experiment 2 tested whether cell permeabilization with 0.2% Tween 20 facilitated the detection of intracellular and surface antigen expression by flow cytometry.

3.3.1. Non-specific binding (NSB) and autofluorescence controls

Both cell types were screened for the presence of any non-specific binding (NSB) by the secondary antibody alone, a goat anti-mouse IgG conjugated to AlexaFluor® 488. In addition, autofluorescence of the Huh7 and EH cells was assessed in the absence of any antibodies (black or hereafter referred to as unstained cells). No autofluorescence or NSB by the secondary antibody could be detected in any of the samples and there were no significant differences detected between unstained and NSB control samples (Figure 8).

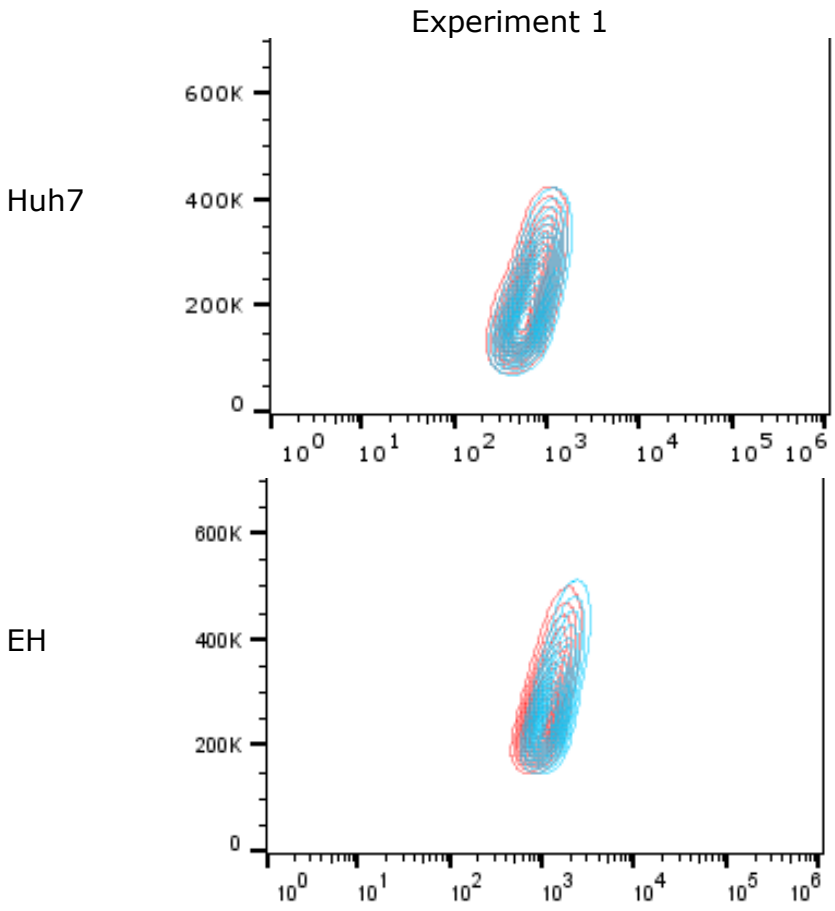


Figure 8. *Contour plot of Huh7 and EH cells to show absence of both autofluorescence and non-specific binding by the secondary antibody. For each population, its emission spectrum of unstained cells (no antibodies; red) and cells stained only with secondary antibody (NSB control; light blue) were compared. Y-axis: SS::SS Lin, X-axis: Comp-FL1::FL1 log (488nm).*

3.3.2. Negative control antibody

In addition to testing for the presence of autofluorescence and NSB the cells were also incubated with a negative control antibody of the same isotype, in this case a mouse anti-human von Willebrand factor (vWF) IgG1 antibody. Two runs were conducted with this antibody in order to account for inter-assay variation. There was no significant difference detected between unstained cells and cells that had been incubated with the negative control antibody. No

variation could be seen between experiments 1 and 2 for either the Huh7 or EH cells (Figure 9).

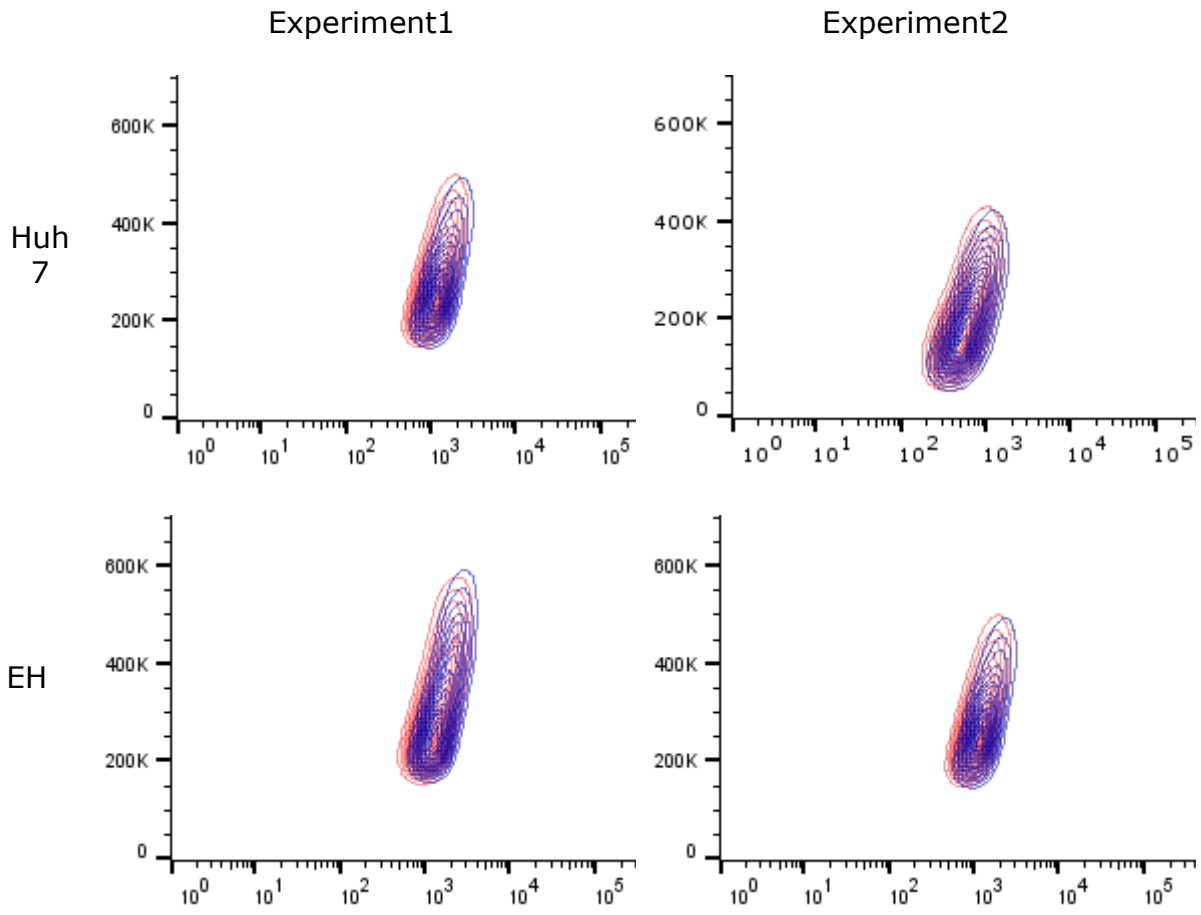


Figure 9. *Contour plot of Huh7 and EH cells to compare the emission spectrum of unstained cells (no antibodies; red) and cells stained only with the negative isotype primary antibody control (m anti-vWF; dark blue). Y-axis: SS::SS Lin, X-axis: Comp-FL1::FL1 log.*

3.3.3. CD81

Both cell types were tested for the expression of CD81 receptor using the anti-human CD81 (JS81) antibody together with the AlexaFluor® 488 conjugated secondary antibody. Samples were run in duplicate to account for intra-assay variation and two experiments were performed to account for inter-assay variation. Huh7 and EH cells expressed CD81 at similarly high levels

(Figure 10). Interestingly, there was some overlap between the unstained and CD81-positive populations within the Huh7 cells, whereas there was no overlap between the populations with the EH cells. There also appeared a wider variation in the expression of CD81 in Huh7 cells compared to EH cells. Permeabilization of all cells with Tween 20 in experiment 2 resulted in no change to the staining pattern. Figure 11 demonstrates the clear fluorescent shift between the unstained and CD81-positive populations of cells.

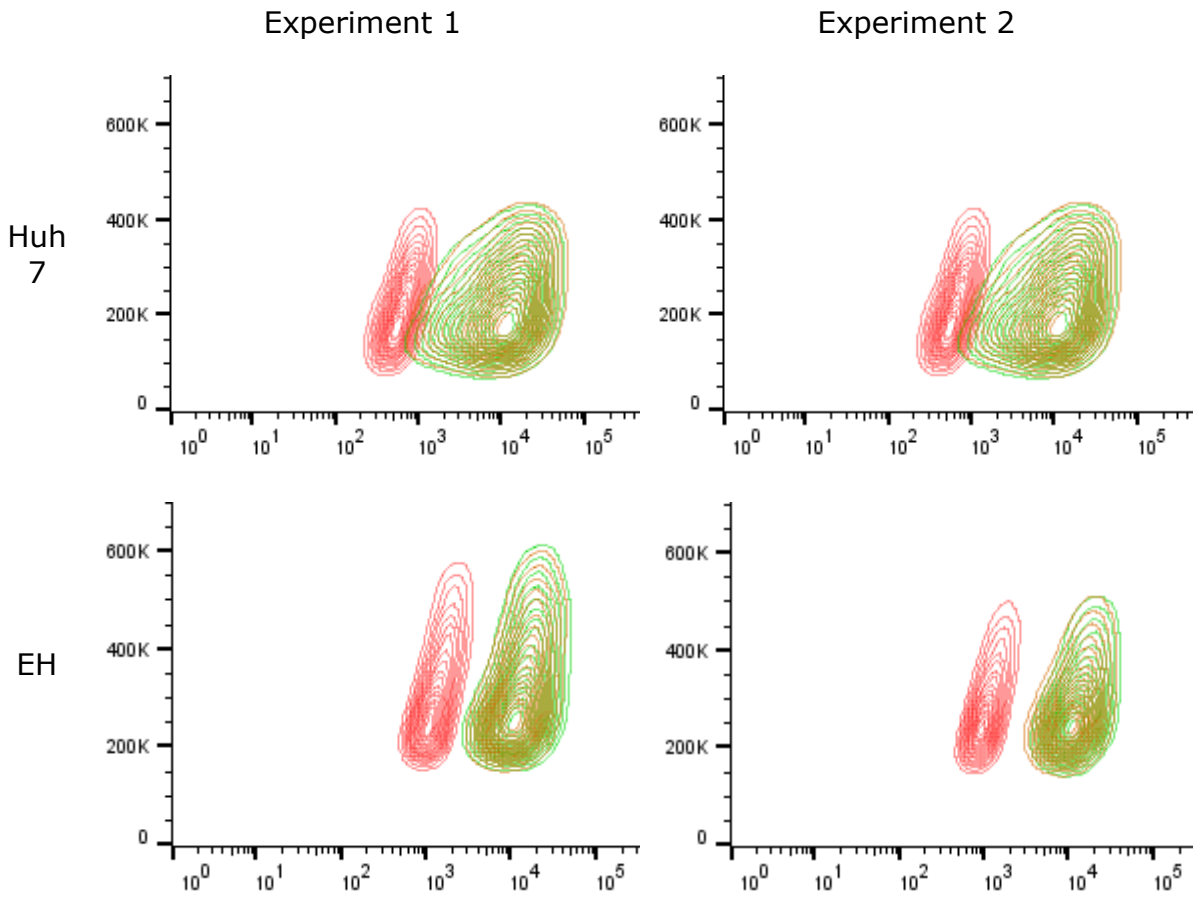


Figure 10. *Contour plot of the Huh7 and EH cell to compare between the emission spectrum of unstained cells (red) and cells stained with the anti-CD81 (JS81) antibody (green and orange). Within the CD81 positive populations, the results of two duplicate samples are superimposed to show replicate 1 (orange) and replicate 2 (light green); there was no intra-assay variation. Two runs (experiments 1 and 2) were included to check for inter-assay variation. Y-axis: SS::SS Lin, X-axis: Comp-FL1::FL1 log.*

In addition to EH cells and to assess the expression of CD81 receptors on some other non-permeabilised equine cells, flow cytometry of E.Derm and FHK cells was also performed. In one experiment, CD81 was present on 96.0% of E.Derm cells but only on 37.2% of FHK cells (data not shown).

On top of the contour plots of the different cell populations, histograms were also included to obtain the Overton % Positive value as well as the Chi Squared T(x) values (Figure 11). The mean Overton % Positive value for EH cells labelled with the anti-CD81 reagent was 98.88%, which demonstrates that nearly all EH cells express the CD81 receptor, regardless of permeabilization status. The mean T(x) value between both samples was 1501.29, which indicates that the two populations are distinct.

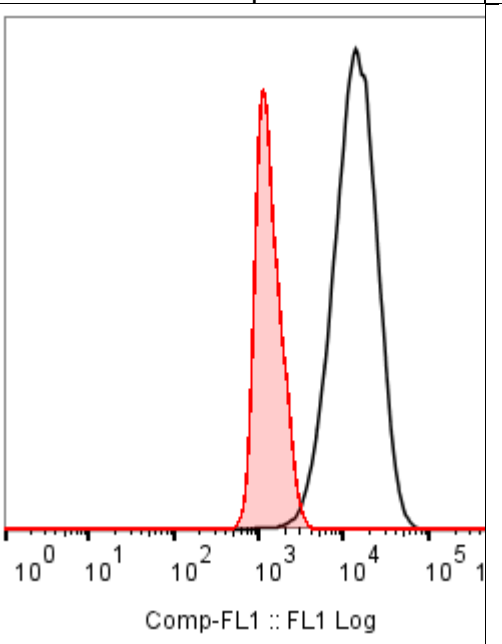
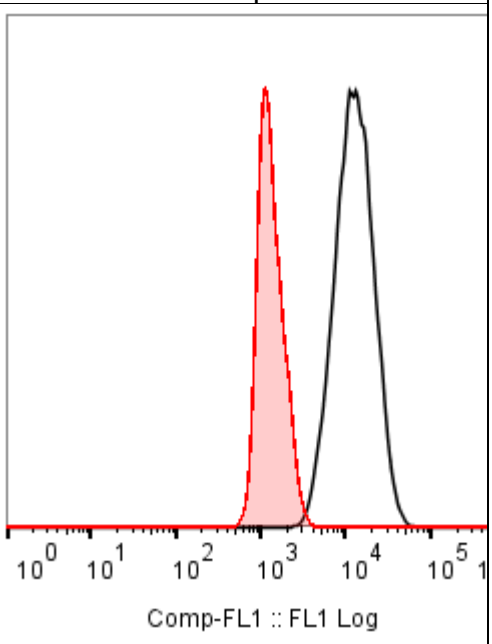
	Anti-CD81 Experiment 1	Anti-CD81 Experiment 2
Histogram		
Overton % Positive	98.52	99.24
T(x) value	1540.78	1461.80

Figure 11. *Histograms to determine the CD81 positive EH cell populations. Unstained (red) and anti-CD81 (JS81) antibody (black). Statistics were run using the FlowJo v10 software to obtain the Overton % Positive value and Chi Squared T(x) values.*

3.3.4. OCLN expression

Using flow cytometry, Huh7 and EH cells were also tested for the expression of OCLN receptors through the use of anti-human OCLN antibody combined with the AlexaFluor® 488 secondary antibody. Samples were tested in duplicate to evaluate intra-assay variation and two identical experiments were run to determine inter-assay variation. A large proportion (87–90%) of Huh7 and EH cells expressed OCLN (Figure 12), although this was lower than for CD81.The fluorescence intensity of OCLN at approximately 10^3 was lower than that for CD81 at 10^4. Overlap between the unstained and OCLN-positive populations could be detected within both the Huh7 and EH cell populations (Figure 12). No significant differences could be detected between experiments 1 and 2.

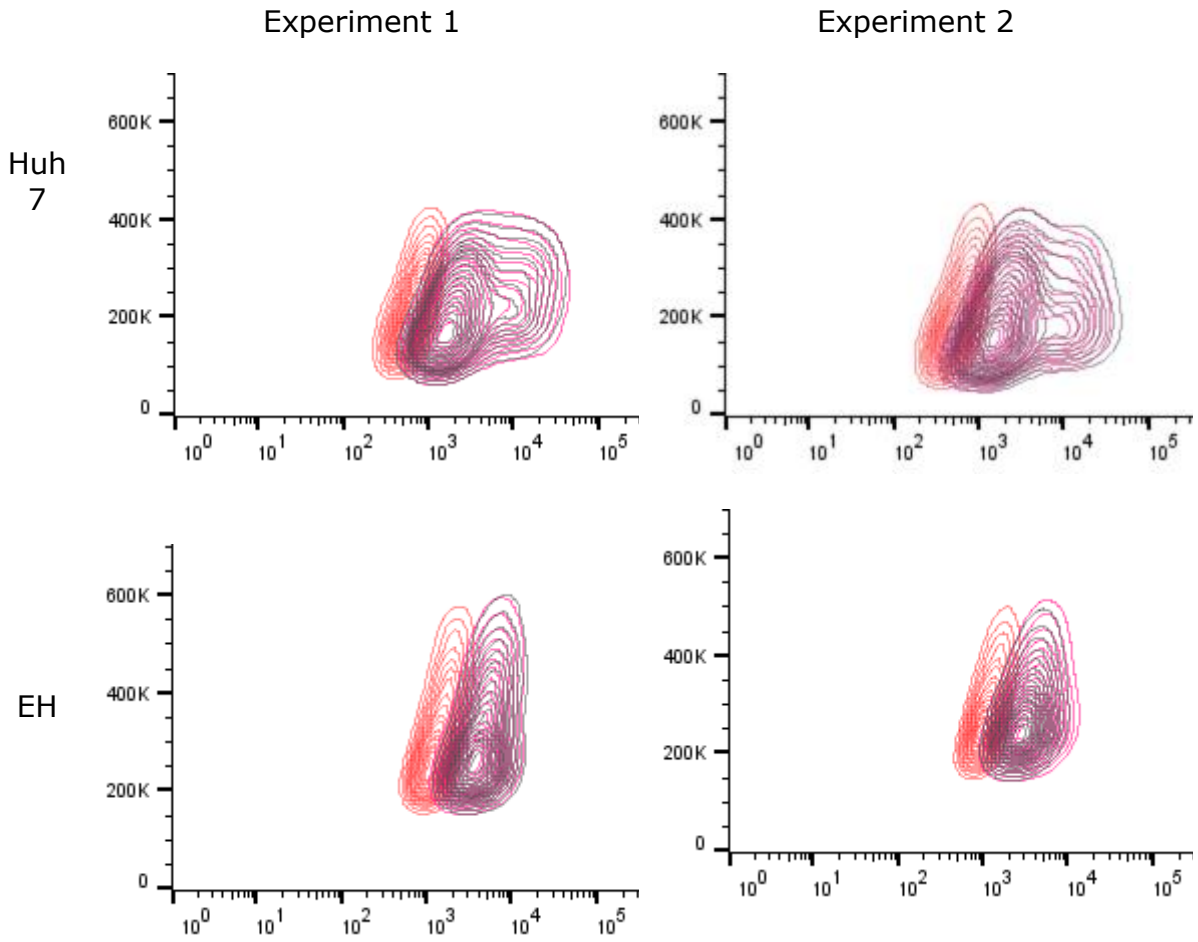


Figure 12. *Contour plot of the Huh7 and EH cells to compare the emission spectrum of unstained cells (red) and cells stained with the anti-OCLN antibody (dark red). Within the OCLN positive populations, the results of two duplicate samples are superimposed to show duplicate 1 (grey) and duplicate 2 (pink); there was no intra-assay variation. Two runs were included to determine any inter-assay variation. Y-axis: SS::SS Lin, X-axis: Comp-FL1::FL1 log.*

In addition to EH cells and to assess the expression of OCLN receptors on some other non-permeabilised equine cells, flow cytometry of E.Derm and FHK cells was also performed. OCLN was not expressed in E.Derm cells (1.7%) but was found in 16.0% of FHK cells (data not shown).

In addition to contour plots of the different populations, histograms were also included in order to obtain the Overton % Positive value as well as the Chi Squared T(x) values (Figure 13). Two unstained samples were used as the

control for autofluorescence. The average Overton % Positive value for anti-OCLN expression on EH cells was 88.37%, which demonstrates that the majority express the OCLN receptor. The mean T(x) value between the unstained and stained samples was 1314.13, which indicates that the two populations are distinct.

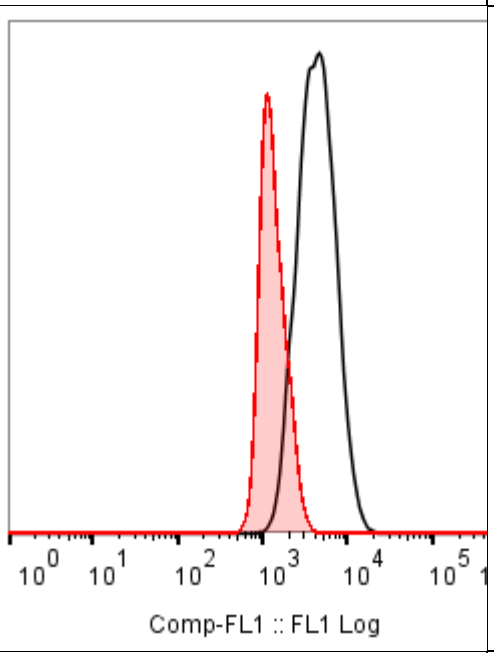
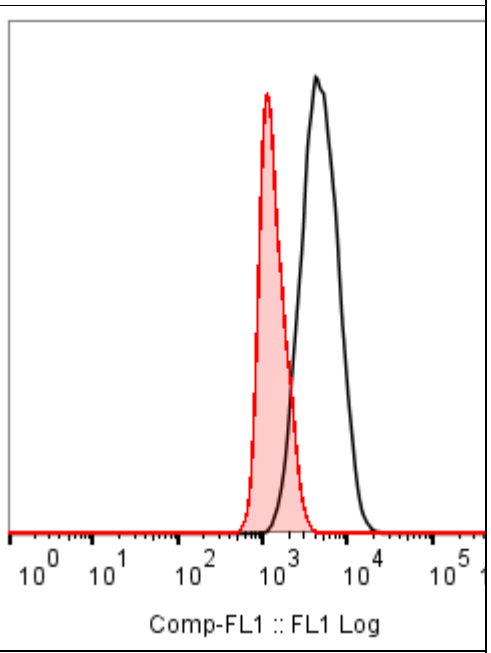
	Anti-CD81 Run 1	Anti-CD81 Run 2
Histogram		
Overton % Positive	86.88	89.86
T(x) value	1269.45	1358.81

Figure 13. *Histograms to compare the EH cell populations. EH cells were either unstained (red) or stained using a mouse anti-OCLN antibody (black). Statistics were run using the FlowJo v10 software to obtain the Overton % Positive value and Chi Squared T(x) values.*

Overall, the flow cytometric analysis indicated that CD81 as well as OCLN are expressed on the surface membrane of the majority of equine hepatocytes, at similar proportions and levels as Huh7 cells. Permeabilization made no difference, suggesting that the majority of CD81 and OCLN are expressed on the cell membrane and not intracellularly. CD81 was expressed on other equine cell

lines of skin and kidney origin to varying degrees, but many fewer cells expressed OCLN.

3.4. Expression of CD81 and OCLN receptors using immunohistochemistry (IHC)

IHC was attempted on FFPE sections of equine liver to assess expression of CD81 and OCLN but despite multiple attempts and alterations to the protocol, including antigen retrieval, no consistent staining could be detected. Therefore, staining was attempted on snap frozen tissue sections instead.

3.4.1. Controls

3.4.1.1. NSB

A non-specific binding control was included in each IHC experiment, which included the application of secondary antibody in the absence of any primary antibody (Figure 14.1). Non-specific binding was absent in all experiments.

3.4.1.2. Negative and positive controls

The negative control used for both liver and placenta was an anti-mouse IgG antibody at an Ig concentration that matched that of the positive control. As this negative control antibody was IgG1 isotype, it also served as an isotype control for the anti-CD81 antibody. Application of the negative control antibody to equine liver resulted in no detectable labelling (Figure 14.1). The positive control antibody used for the liver sections was CVS22 mouse anti-equine MHC class I monoclonal antibody at a 1:100 dilution. In the liver, equine MHC class I was expressed on endothelial cells and sinusoidal cells as indicated by the presence of the brown chromagen, as seen in figure 14.2. Equine hepatocytes were negative for MHC class I.

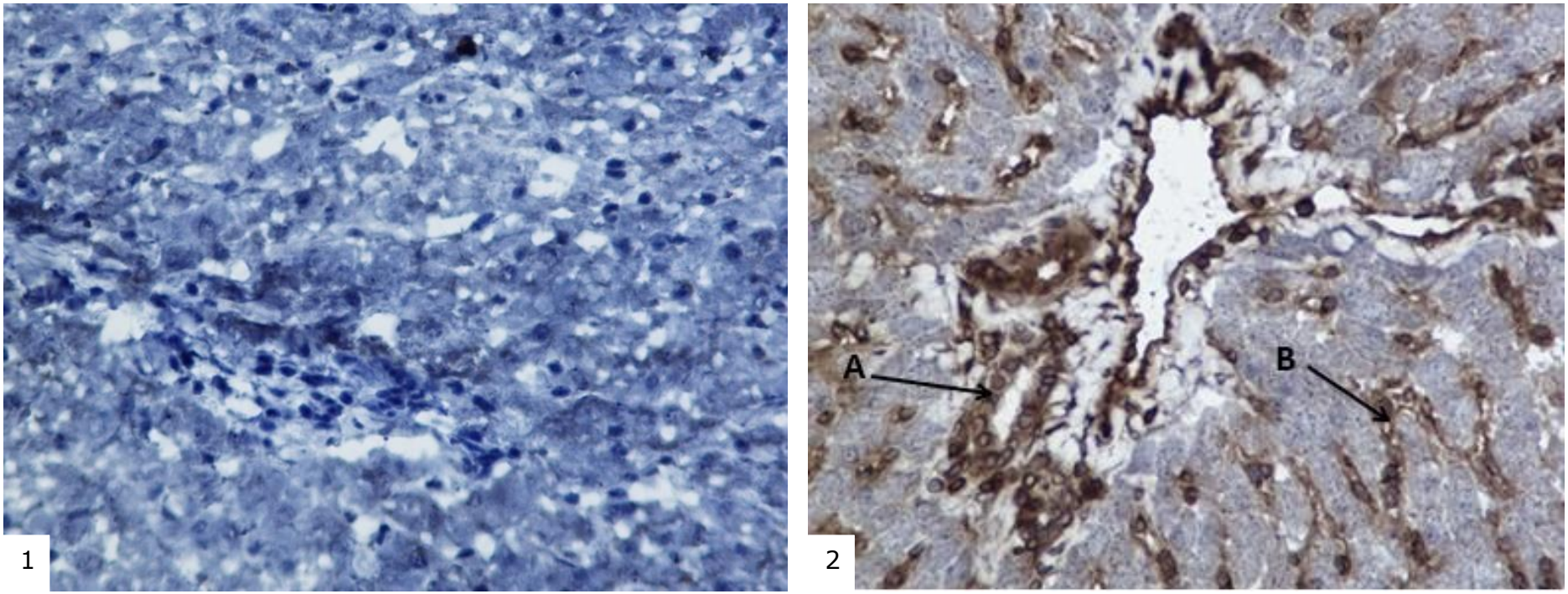


Figure 14. *Immunohistochemistry on snap frozen equine liver to show 1) absence of non-specific binding by secondary antibody and 2) expression of MHC class I (positive control antibody; brown) on cells in the portal triad of the equine liver. A = endothelial cells, B = sinusoidal cells. Counterstain with haemalum.*

3.4.2. Expression of putative EqHV receptors in the equine liver

IHC was used to detect expression of CD81 and OCLN in equine liver. Expression of CLDN1 and SR-B1 was not attempted due to the low level of expression on equine liver cells as detected by flow cytometry (data not shown). For the equine liver, three different experiments were performed. Two experiments were a titration of the anti-human CD81 (JS81 and 1D6) antibodies and the anti-OCLN antibody to find an endpoint titration. The third experiment included the permeabilization of the frozen tissue using either: only acetone; acetone and 0.2% Tween 20; or 0.1% Triton X to assess if cell permeabilization would improve the sensitivity and specificity of the staining.

3.4.2.1. Experiment 1: JS81 and 1D6 titrations and CD81 expression

The titration of the JS81 antibody (Figure 15) demonstrated a reduced ability to detect CD81 expression with increasing dilutions to 1:1000. According to this experiment, it was decided that the best concentration to use of the JS81 anti-human CD81 antibody was 1:100 and this was used for all other experiments. No additional permeabilization methods were used.

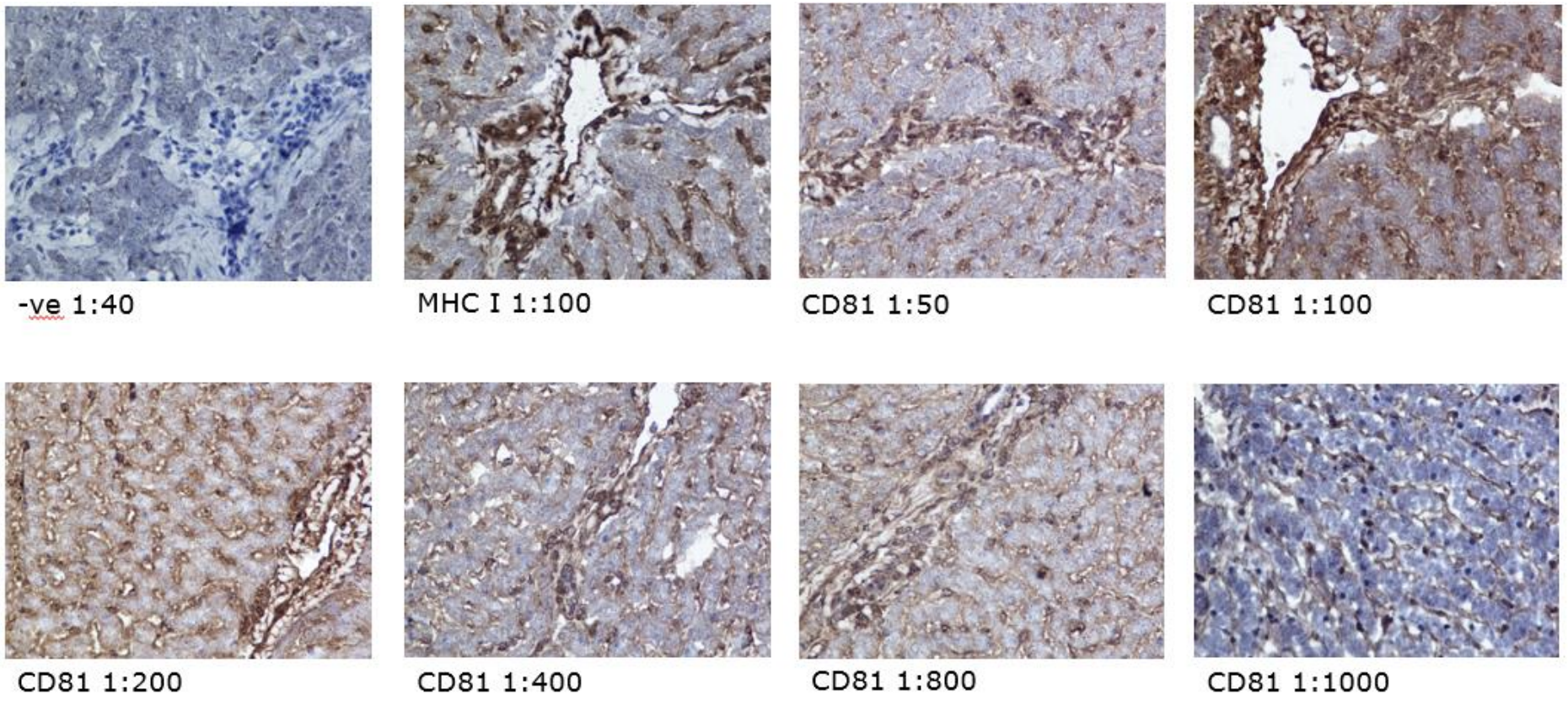


Figure 15. *Immunohistochemistry of snap frozen equine liver to titrate the anti-human CD81 (JS 81) antibody. Dilutions of 1:50 to 1:1000 were attempted. Negative isotype control antibody: anti-mouse IgG. Positive control antibody: anti-equine MHC class I.*

Two antibodies were used to stain for the expression of CD81 receptors in the equine liver and placenta, namely 1D6 and JS81. Both antibodies showed similar staining patterns in liver sections, but the endpoint for JS81 was a dilution of 1:100 compared to 1:10 for the 1D6 antibody. Thus, JS81 was used for the majority of IHC.

CD81 was mainly expressed in endothelial cells in portal veins, arteries and bile ducts as well as sinusoidal cells and connective tissue surrounding these vessels (Figure 16). Hepatocytes appear to be CD81 negative, and no staining could be detected in hepatocyte membranes or nuclei using this method. The pattern was very similar to expression of the MHC class I positive control.

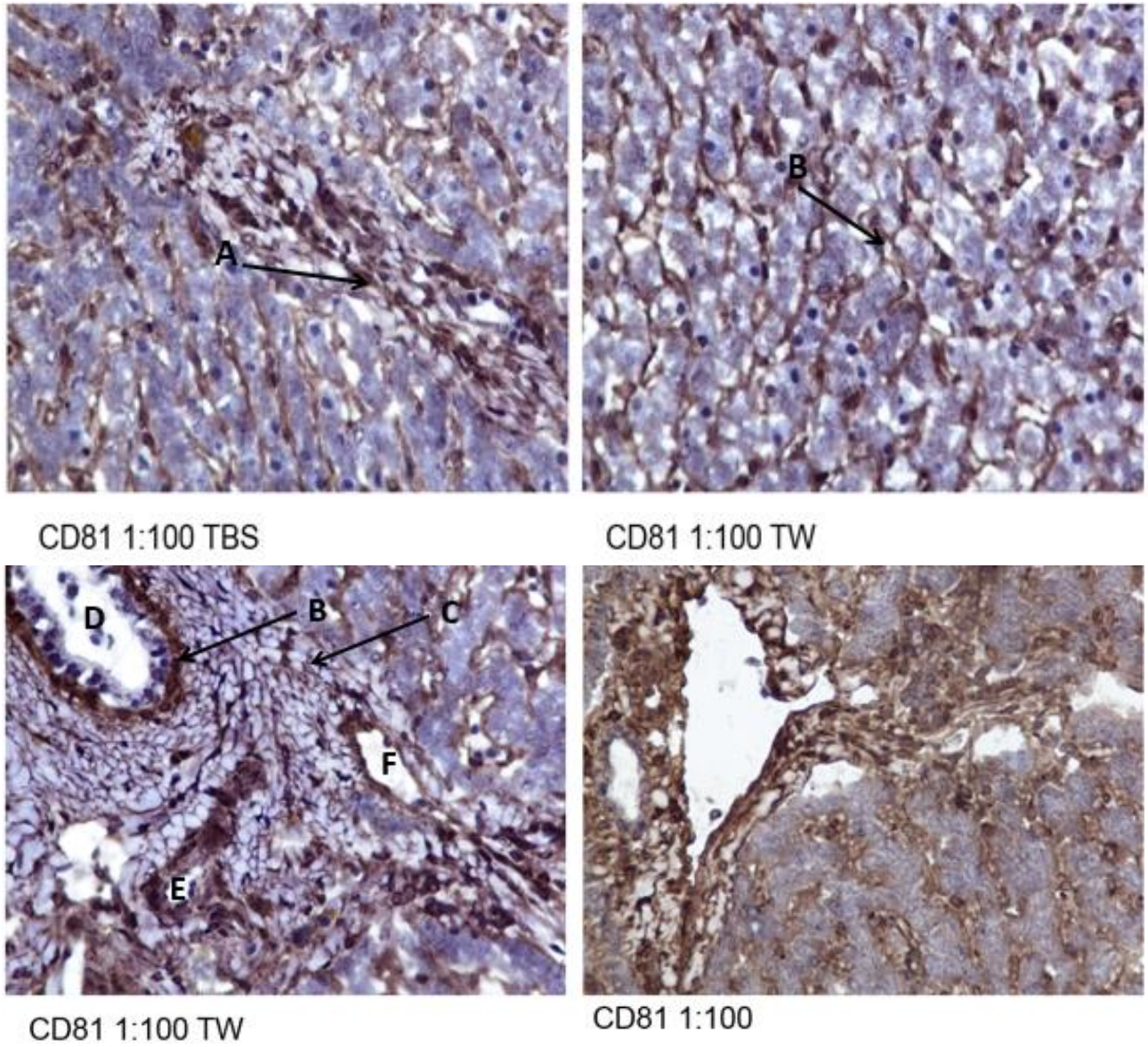


Figure 16.
Immunohistochemistry of snap frozen equine liver to show CD81 expression in the portal triad of the equine liver. CD81 is expressed by endothelial cells, sinusoidal cells, and connective tissue surrounding the bile duct, portal artery and portal veins. A = endothelial cells, B = sinusoidal cells, C = connective tissue, D = portal vein, E = bile duct, F = portal artery, TW = Tween 20, TBS = Tris buffered saline

3.4.2.2. Experiment 2: OCLN expression and titration

A mouse anti OCLN antibody was used to detect expression of OCLN on the equine liver and placenta. The antibody was titrated at the manufacturer's recommendation of 1:200 but also at a dilution of 1:100 for comparison. As the antibody was specific for a cytoplasmic domain of this receptor, a comparison was made using different methods for permeabilization. The antibody was tested on tissues that had been fixed in acetone followed by either TBS alone for 10 minutes, with 0.2% Tween 20 in TBS for 10 minutes, or with 0.1% Triton X added for 10 minutes. The results showed that simple permeabilization with acetone alone and then adding TBS only was the most effective method to show clear OCLN expression in equine liver (Figure 17). This expression was mainly in the nuclear envelope and the nucleosome of hepatocytes, as well as in the nuclei of endothelial cells (Figure 18). In comparison to CD81, no OCLN receptors could be detected in sinusoidal cells.

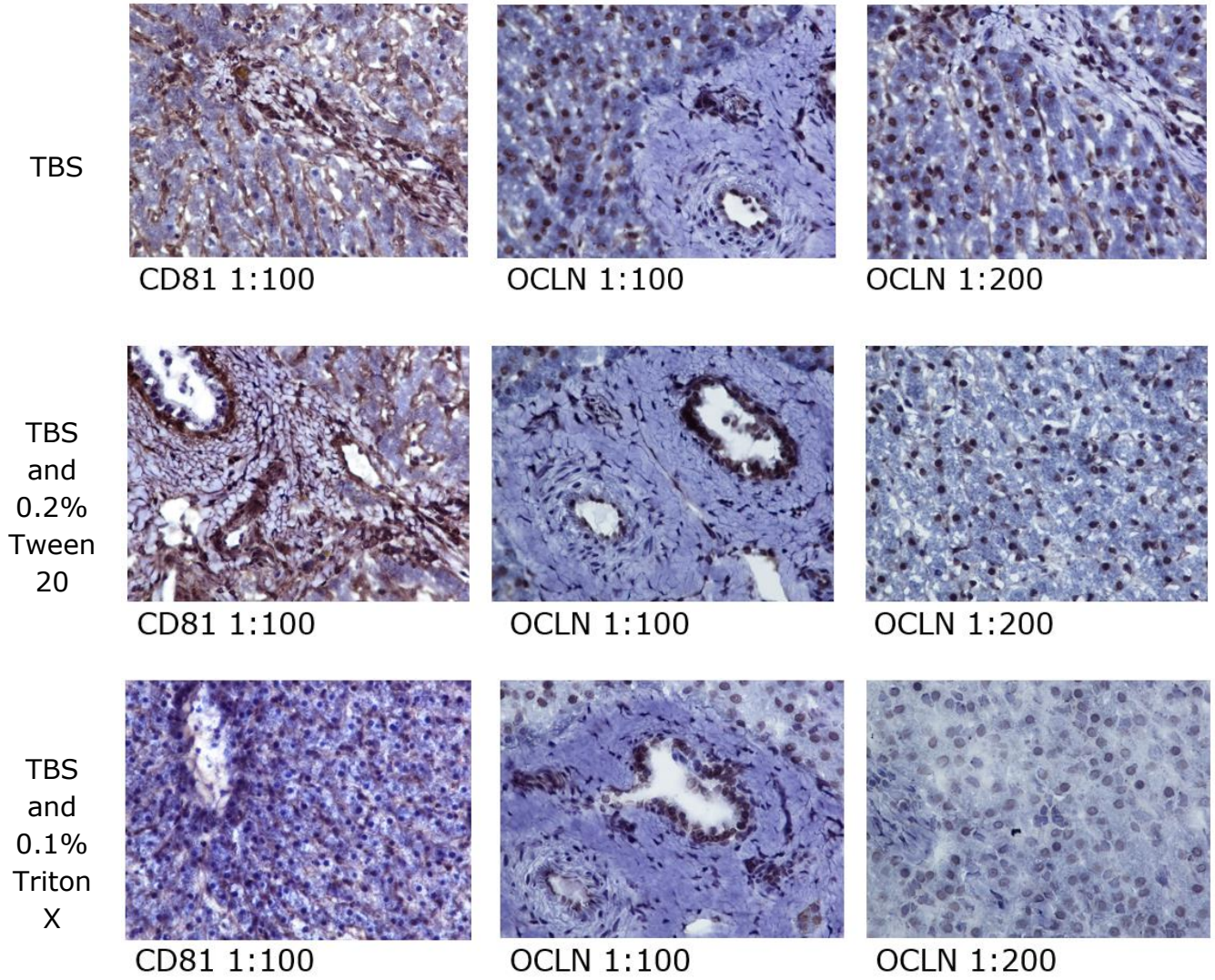


Figure 17.
Immunohistochemistry of snap frozen equine liver stained with antibodies to human OCLN and CD81 receptors using either TBS only, TBS with 0.2% Tween 20 and TBS with 0.1% Triton X as permeabilization methods. The mouse anti-human CD81 antibody was used at the previously determined optimal dilution of 1:100. The mouse anti-human OCLN antibody was tried at a dilution of 1:100 and 1:200. TBS = Tris buffered saline

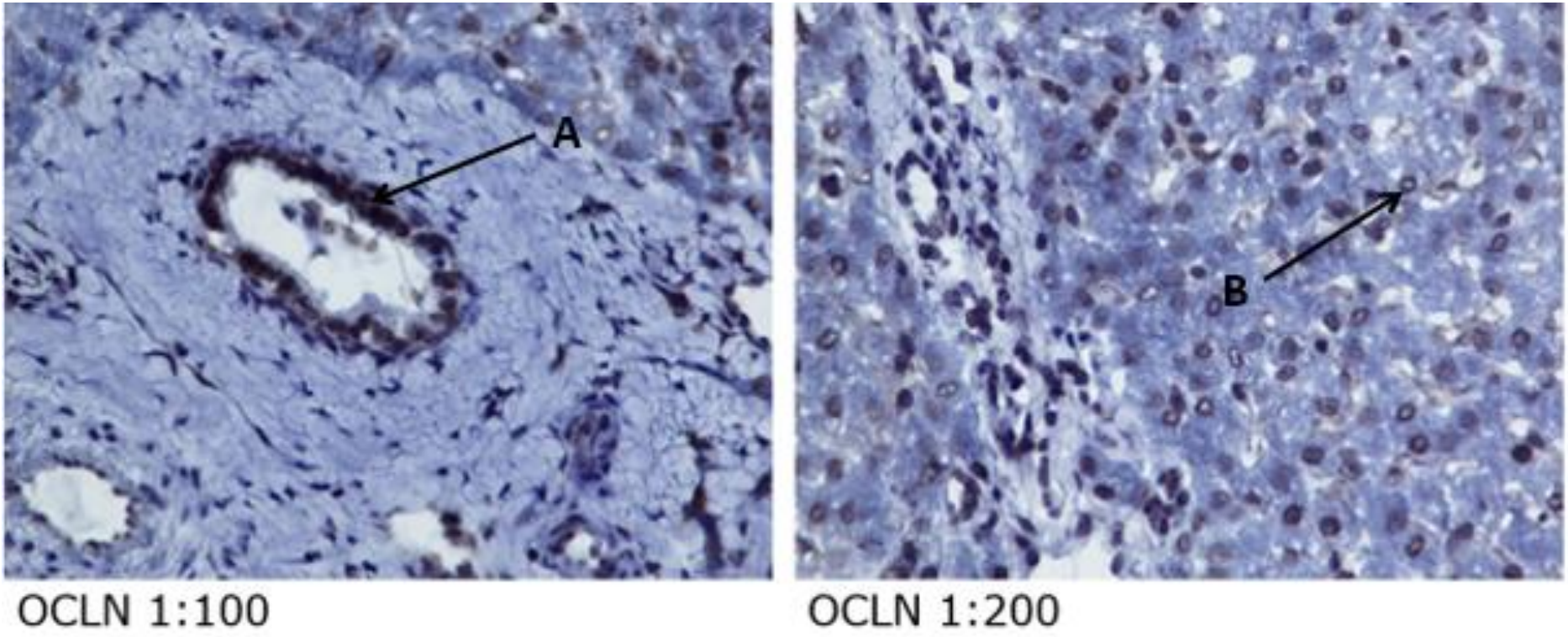


Figure 18. *Immunohistochemistry of snap frozen, acetone fixed equine liver stained with anti-human OCLN at the dilution shown to demonstrate expression in the portal triad of the equine liver. OCLN was present in the nuclei of endothelial cells as well as in the nuclei and nucleosomes of hepatocytes. A = endothelial cells, B = hepatocytic nucleus*

The results showed no differences in staining pattern between sections treated with only TBS, 0.2% Tween 20 and 0.1% Triton X. Triton X made the hepatocytes appear paler and actually made the staining pattern less intense and was therefore not used again. Use of the anti-human OCLM antibody at dilutions of 1:100 and 1:200 appeared to make no significant differences to the staining pattern (Figure 18).

3.5. Expression of CD81 and OCLN on snap frozen equine placenta as detected by immunohistochemistry

Using the optimised antibody concentrations and experimental conditions, IHC was performed on frozen sections of term equine placenta to determine the expression of CD81 and OCLN, the putative EqHV receptors.

3.5.1. Controls

3.5.1.1. Non-specific binding (NSB)

A NSB control, the secondary antibody (goat anti mouse immunoglobulin conjugated to horseradish peroxidase at 1:50 dilution) was applied alone to equine placenta then the remainder of the IHC completed as normal. Non-specific binding was not detected in any of the sections (Figure 19).

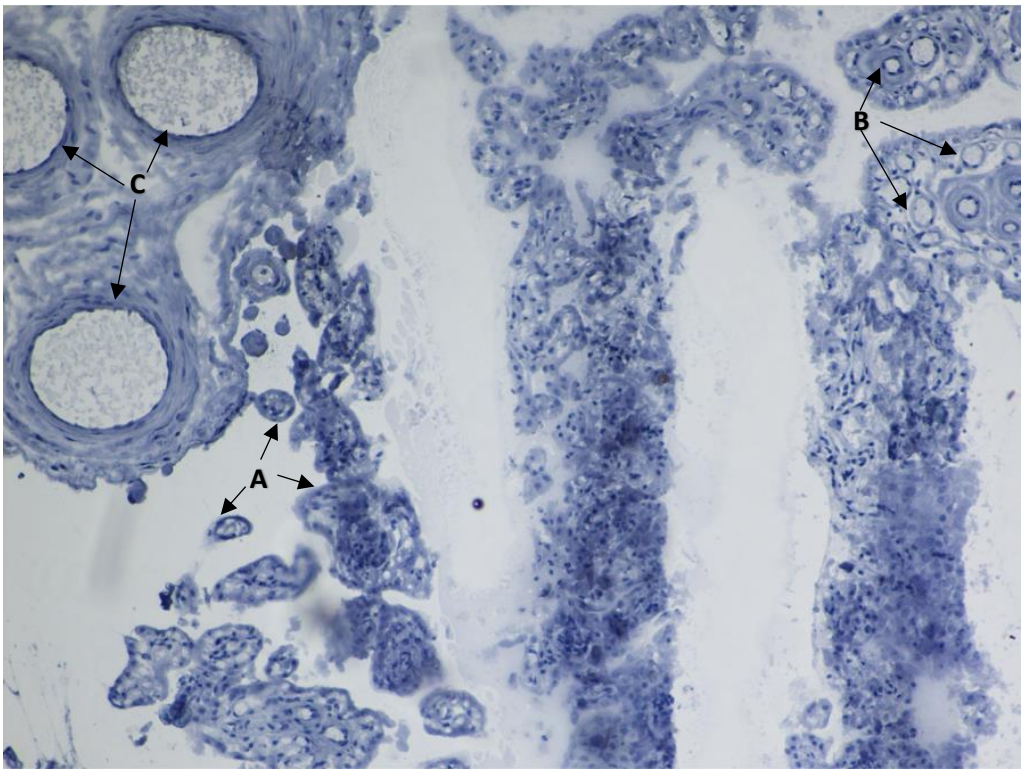


Figure 19. *Non-specific binding (NSB) control (goat anti-mouse immunoglobulin conjugated to horse radish peroxidase at 1:50) on frozen equine placenta. A = microcotyledons, B = capillaries, C = endothelial cells of blood vessels*

3.5.1.2. Negative and positive controls

The majority of placental tissue stained with the negative control of anti-murine immunoglobulin antibody was negative. Some small artefacts showed evidence of brown staining resembling haematoidin crystals (Figure 20.1 and 20.2).

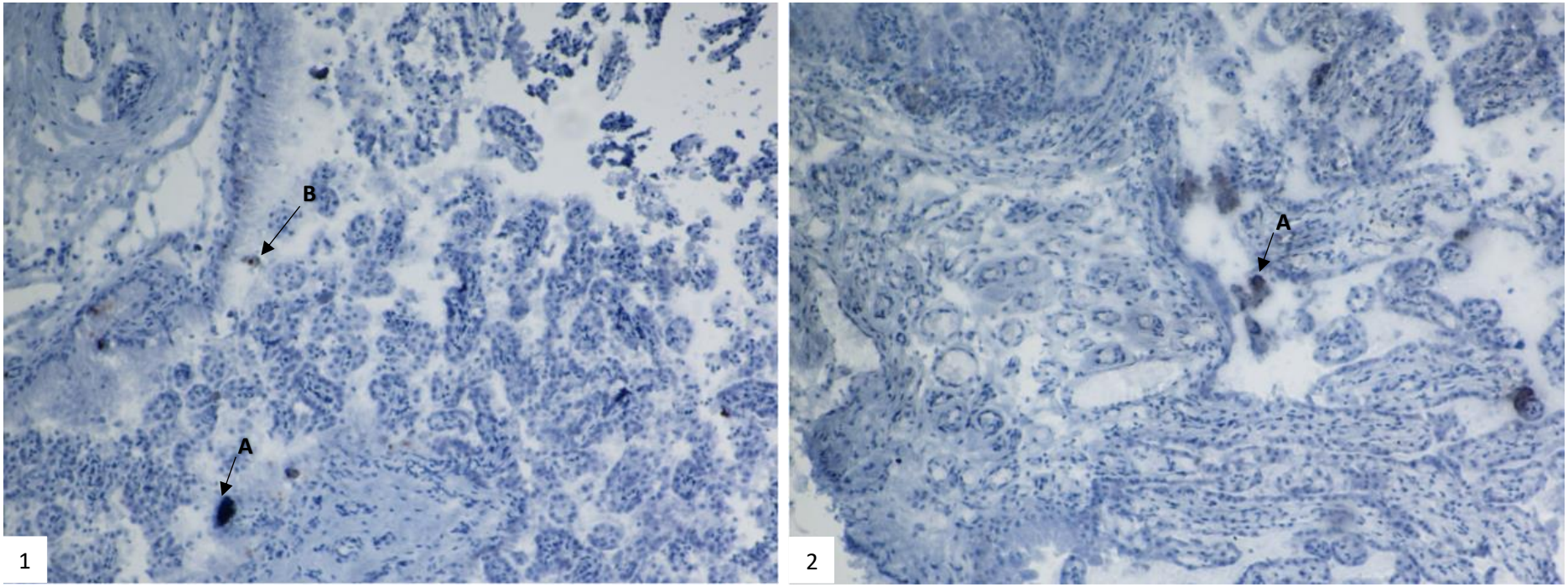


Figure 20. *Negative control antibody (anti murine IgG) showing absence of any staining of the equine placenta (1 and 2). A few haematoidin crystals were present (brown). A = artefacts, B = haematoidin crystals*

The positive control anti-equine MHC class I antibody was expressed consistently on the allantochorionic membranes of the microcotyledons, areolae, connective tissue cells, and endothelial cells, as seen in Figure 21.

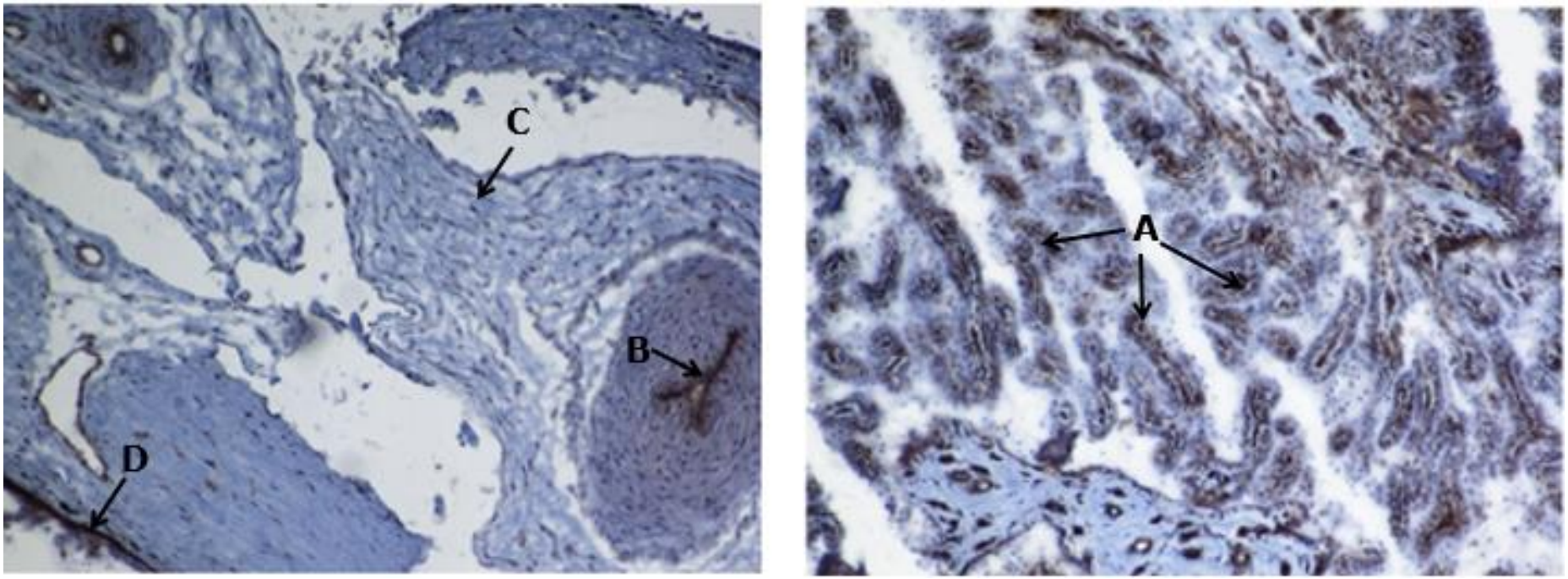


Figure 21. *Positive control antibody (mouse anti-equine MHC class I) showing expression of MHC class I on the equine placenta (brown staining) A = microcotyledons, B = endothelial cells, C = connective tissue, D = areolar membrane*

3.5.2. Immunohistochemical detection of putative EqHV receptors CD81 and OCLN expression on the equine placenta

3.5.2.1. CD81 staining

For study of the equine placenta, only the JS81 anti-human CD81 (at 1:100) was used. The staining pattern was similar to that of the positive control mouse anti-equine MHC class I antibody and indicated the presence of CD81 on the epithelial cells of the allantochorionic microcotyledons and areolar areas, the connective tissue surrounding blood vessels and also on the endothelial cells and smooth muscle cells surrounding these vessels (Figure 22). The expression pattern was similar to that of equine MHC class I.

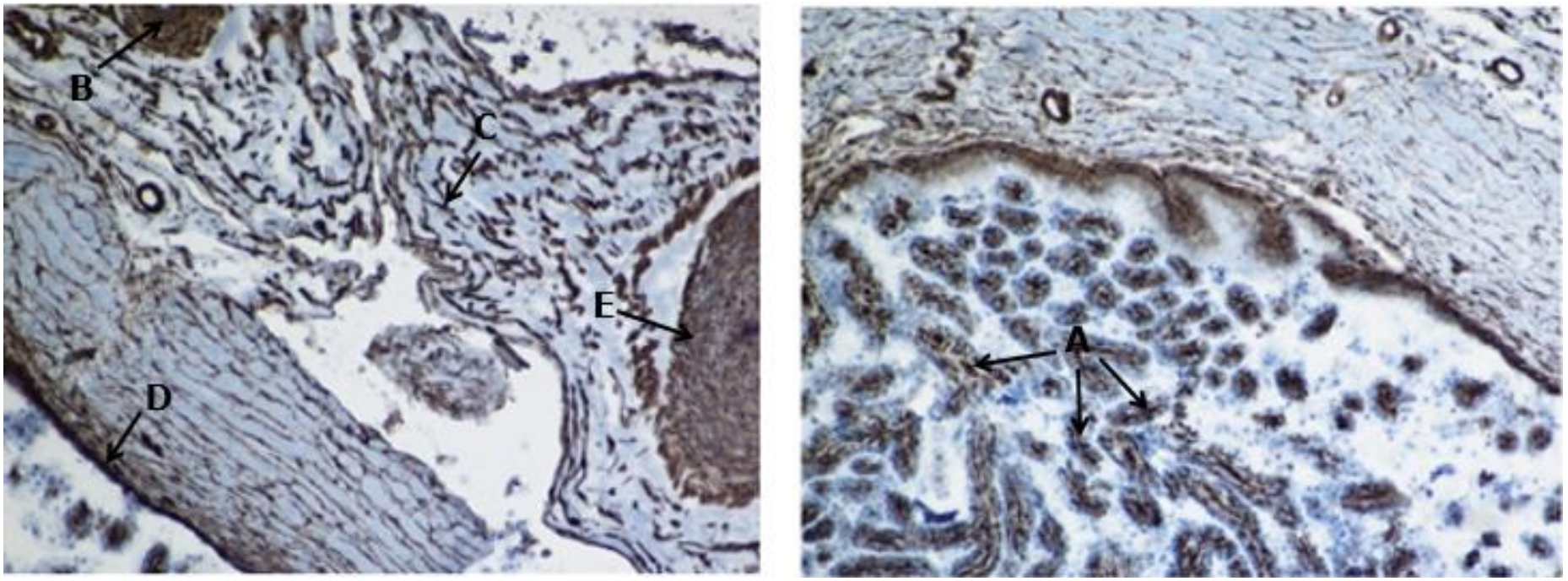


Figure 22. *Anti-human CD81 staining in the equine term placenta. A = microcotyledons, B = endothelial cells, C = connective tissue, D = areolar membrane, E = smooth muscle cells surrounding the blood vessels*

3.5.2.2. OCLN staining

OCLN expression was absent from all sections of equine placenta using a dilution of 1:100 (Figure 23).

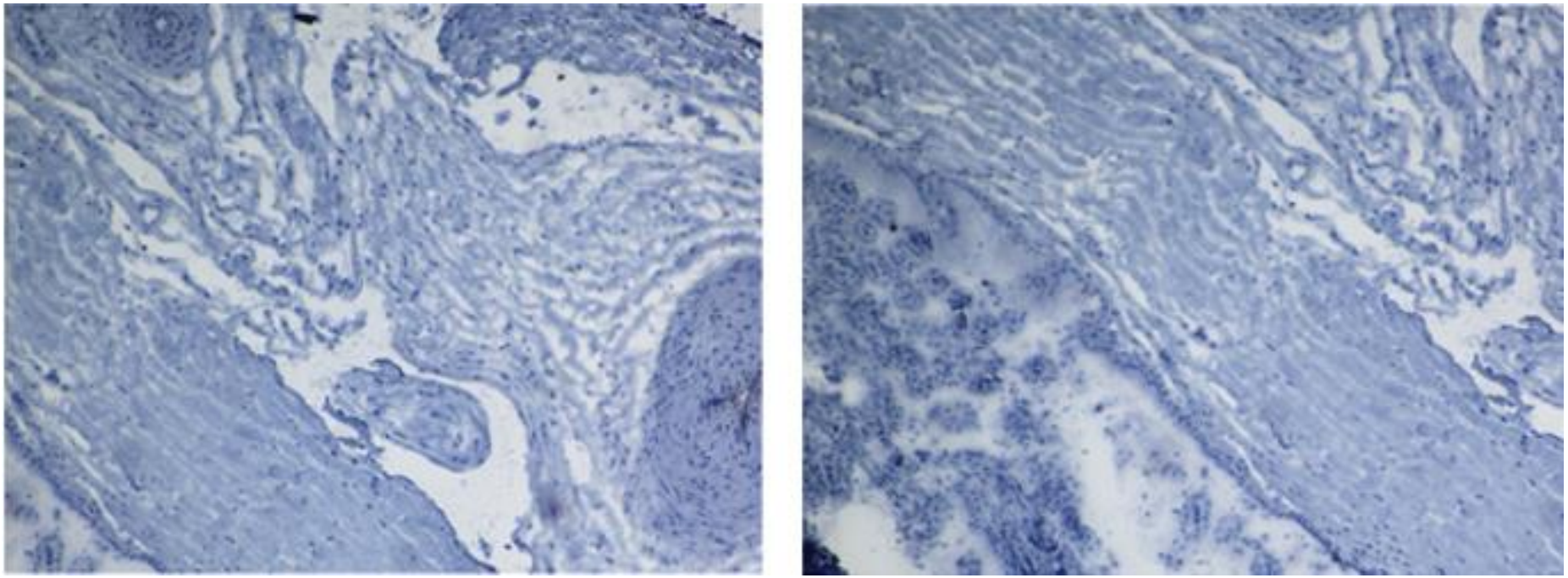


Figure 23. *Anti-human OCLN (1:100) staining showing absence of occludin in the equine term placenta*

3.6. Expression of CD81 and OCLN on equine peripheral blood mononuclear cells (PBMC)

Due to the potential expression of CD81 and OCLN on sections of equine liver and for CD81 on equine placenta, further questions arose surrounding the pathogenesis and potential replication of EqHV within the host. Previous experiments revealed some expression of CD81 and OCLN in some white blood cells present in the vessels of the portal triad in the equine liver. Therefore, expression of CD81 and OCLN on white blood cells, in particular in PBMCS was investigated using an excess diagnostic sample.

The same controls and dilutions were used for ICC staining of the PBMCs as the ones used previously for the IHC staining. The negative control, anti murine IgG antibody (dilution of 1:50) and the positive control mouse anti-equine MHC class I antibody (dilution of 1:100) were applied to PBMC which had been spun in the cytospin machine and acetone fixed. The NSB control showed endogenous peroxidase in the granules of eosinophils which results in activation of the chromogen substrate and production of the brown colour (Figure 24). It was taken into account in the interpretation of the following experiments. No staining was evident in the negative control (mouse IgG1 negative control) and in the positive control (mouse anti-equine MHC class I). MHC class I was detected in most of the PBMCs, including on lymphocytes, neutrophils and eosinophils, with particularly intense expression on the cell membrane (Figure 24).

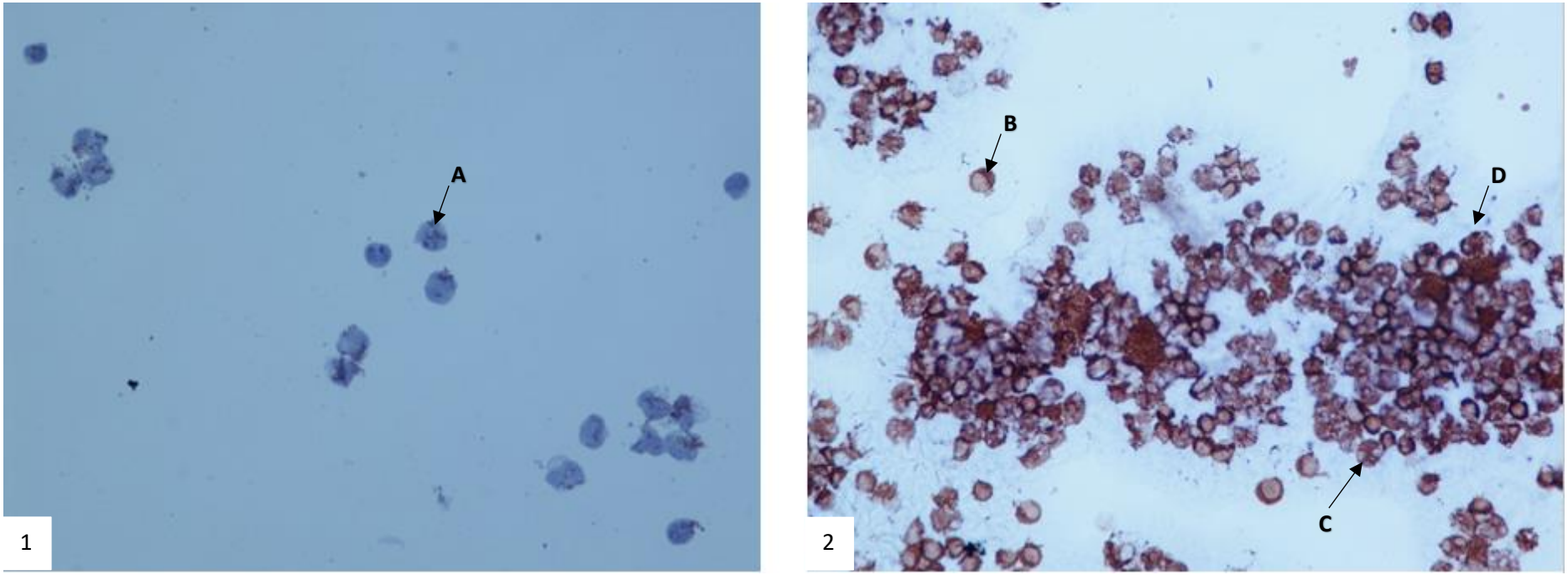


***Figure 24**. Staining using negative and positive control antibodies on acetone fixed peripheral blood mononuclear cells (PBMC) cytospins. 1) Negative (mouse IgG1 negative control) and 2) positive control (anti-equine MHC class I) for PBMCs. Counterstained with haemalum. A = endogenous peroxidase in eosinophils, B = lymphocyte, C = neutrophil, D = eosinophil*

Expression of CD81 was confirmed in lymphocytes, neutrophils and eosinophils (Figure 24). Further studies to elucidate the expression of CD81 on sub-populations equine of PBMCs are necessary using a more discriminating method, such as flow cytometry. Flow cytometry was then used to confirm more precisely what proportion of leucocytes expressed CD81 and whether intensity of expression differed between leucocyte subpopulations. All PBMCs were negative for OCLN expression.

3.7. Expression of CD81 and OCLN on equine lymph nodes as detected by IHC

After CD81 and OCLN expression was tested in PBMCs, IHC was used to detect any differences in CD81 and OCLN expression between circulating and resident lymphocytes within lymph nodes. Non-specific binding was not detected using the mouse IgG1 negative control. The anti-MHC class I positive control showed staining of endothelial cells, some connective tissue, nuclei of the lymph node capsule and the majority of the lymphocytes (Figure 25).

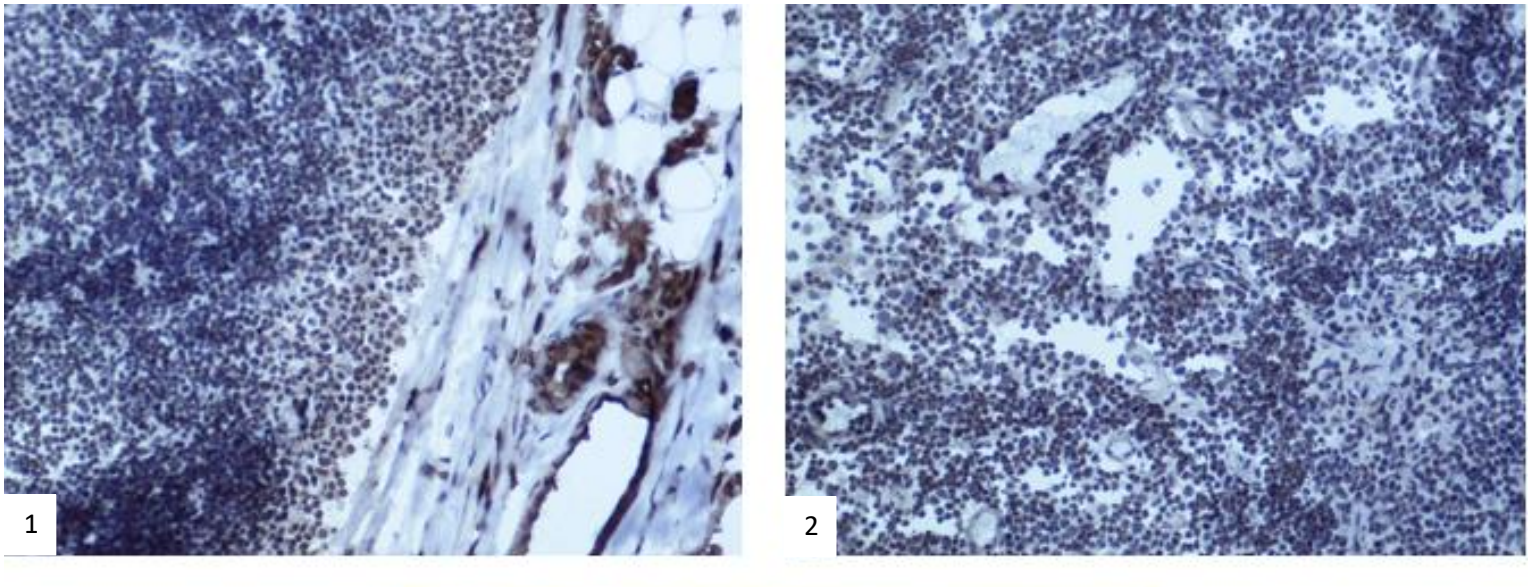
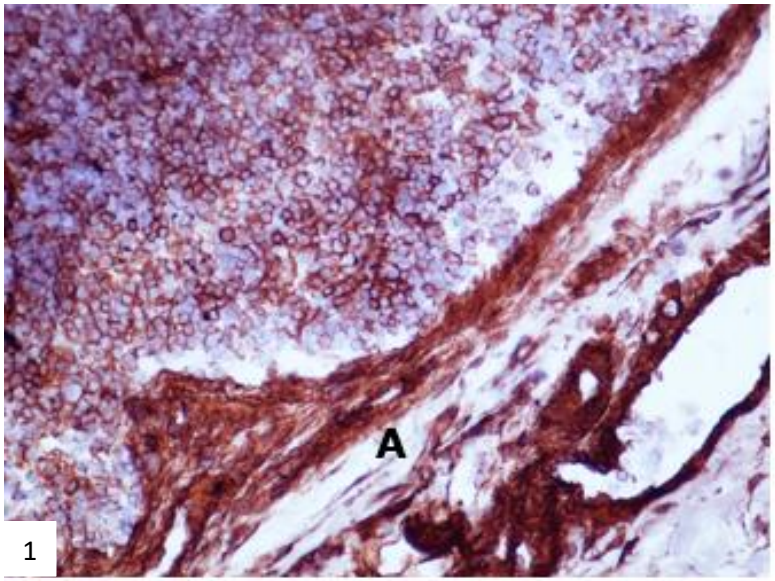
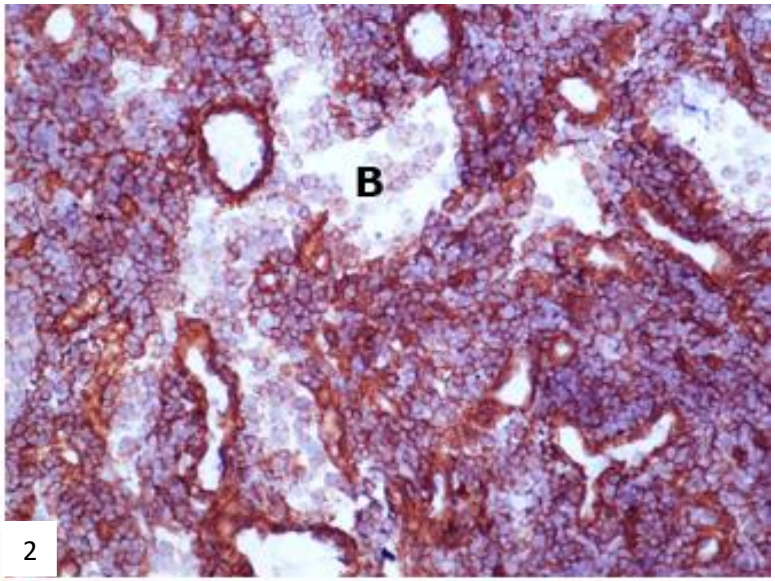


Figure 25.
Immunohistochemistry on equine lymph nodes 1 and 2) Anti-MHC class I positive control. 3) Mouse IgG1 negative control. Counterstained using haemalum.

Sections of equine lymph node were stained for CD81 and OCLN. CD81 expression was very intense in the equine lymph node and the staining pattern was very similar to that of MHC class I (positive control). Staining could be detected in the endothelial cells of the lymph node capsule and within the connective tissue and the lymph node capsule itself (Figure 26). The staining pattern portrayed that germinal centres within the lymph node were more CD81 positive than non-germinal centres. In addition, lymphocytes within the medullary cords also appeared to have a lower intensity of CD81 expression or alternatively weaker staining due to lesser apposition of cell membranes between cells. The majority of lymphocytes within the lymph node were CD81 positive but some areas (the connective tissue within the non-germinal centre) appeared CD81 negative.

Although OCLN could not be detected in equine PBMCs, OCLN expression was detected on some cells within the LN, in particular larger cells, likely to be monocytes or lymphoblasts (Figure 27). OCLN was not detected on endothelial cells or within the capsule of the LN. To specifically identify which subpopulations of mononuclear cells were CD81 and/or OCLN positive, it was decided to run flow cytometry of PBMCs.





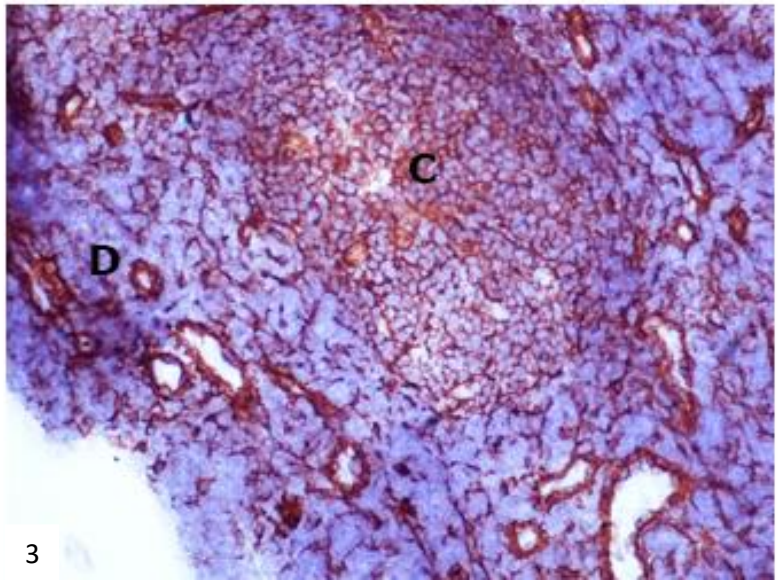
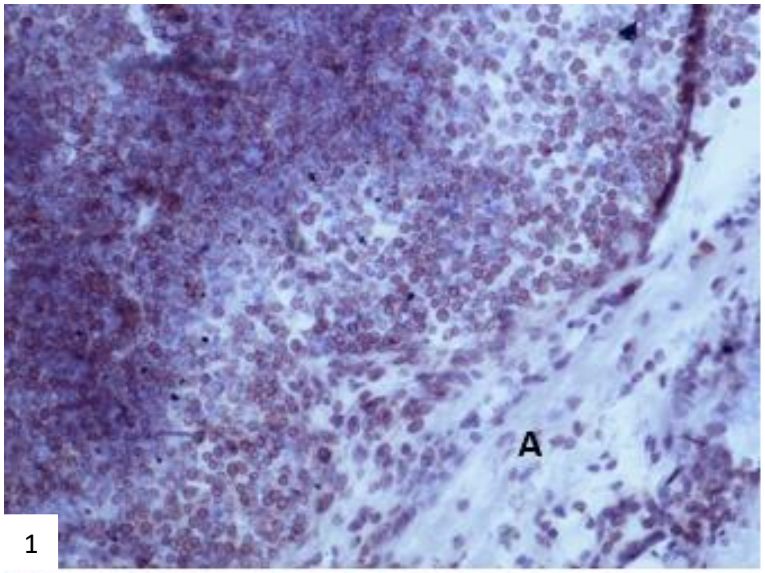
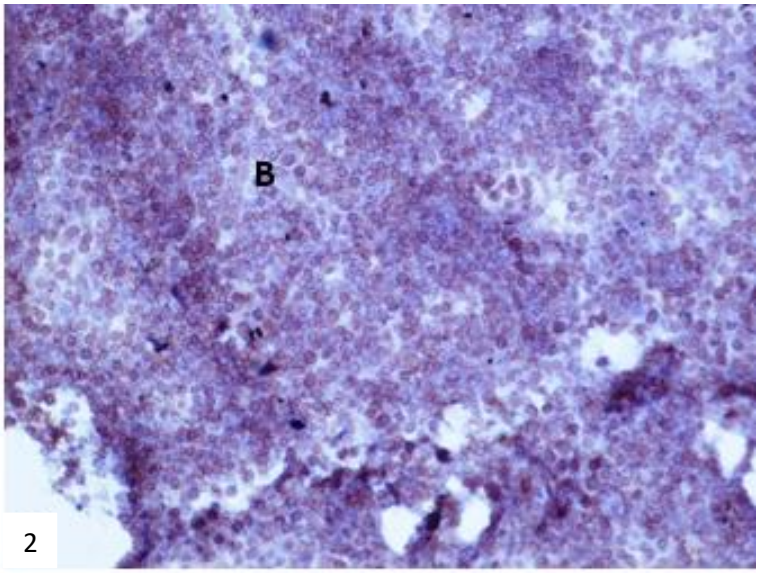


Figure 26.
Immunohistochemistry staining using anti-CD81 (JS81) antibody (with NovaRed) on frozen equine lymph node. 1 = capsule, 2 = medullary cord region, 3 = germinal and non-germinal centres of LN, A = LN capsule, B = medullary cords, C = germinal centre, D = non-germinal centre





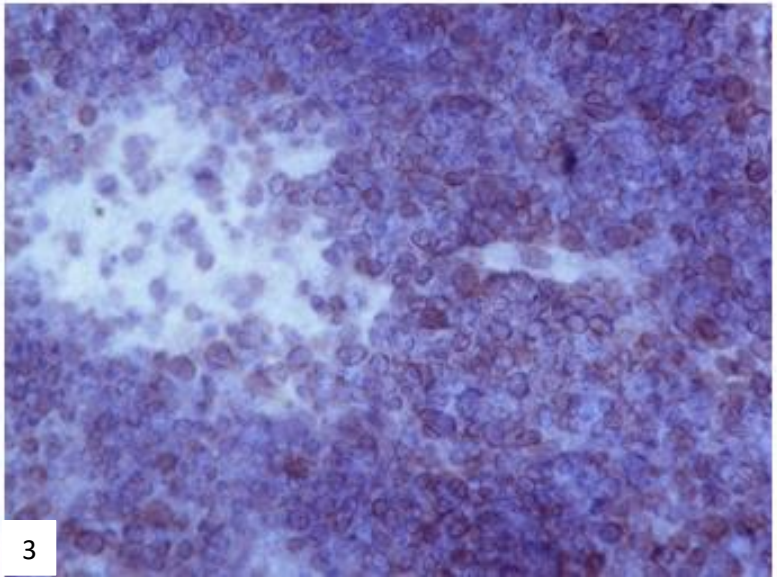


Figure 27. *Immunohistochemistry staining using the anti-OCLN antibody (with NovaRed) on frozen sections of equine lymph nodes. 1) capsule, 2) medullary cord region, 3) germinal and non-germinal centres of lymph node. A = lymph node capsule, B = medullary cords.*

3.8. Expression of CD81 and OCLN on equine PBMCS as detected by flow cytometry

Flow cytometry was conducted to gain a better understanding of the CD81 and OCLN receptor expression levels of different immune cell types, namely neutrophils, lymphocytes and monocytes. Two types of anti-coagulated blood samples were obtained, namely lithium heparin or EDTA.

The EDTA samples had been stored for 72 hours prior to flow cytometry, therefore the majority of the monocytes had degraded and there was no distinguishable monocyte population. Therefore, monocytes were ruled out of flow cytometry analysis in this group. The gating of lymphocytes and neutrophils is displayed in figure 28.

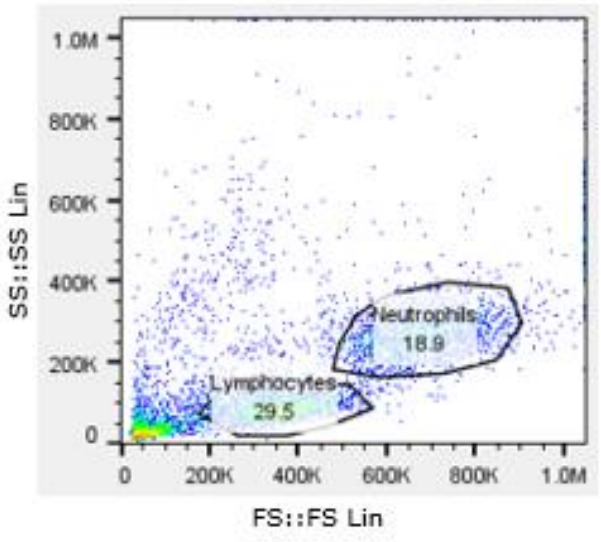


Figure 28. *Flow cytometry of cell populations from the EDTA anti-coagulant blood sample (72 hours storage) to show gates for lymphocytes and neutrophils separated by size (forward scatter, FS) and granularity (side scatter, SS)*

The lithium heparin samples were stored for 24 hours prior to analysis; therefore the monocytes were less degraded and three subpopulations were gated: lymphocytes, neutrophils and monocytes (Figure 29).

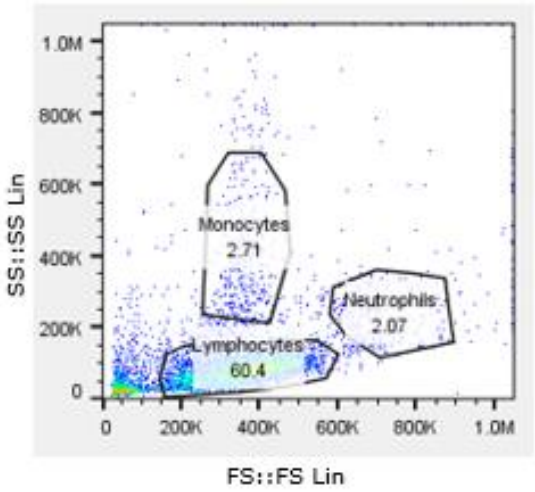


Figure 29. *Flow cytometry of cell populations from the lithium heparin anti-coagulant blood sample (24 hours storage) to show gates for lymphocytes, neutrophils and monocytes separated by size (forward scatter, FS) and granularity (side scatter, SS)*

Some non-specific binding of the secondary antibody alone existed; EDTA 2.6% and lithium heparin 3.1%. These percentages were subtracted from any results of receptor staining in order to get to the true value of percent expression. The negative control antibody for the flow cytometry was a mouse IgG1 negative control and the positive control was a mouse anti-equine MHC class I antibody. Expression of MHC class I on cells in the lymphocyte gate from both types of anti-coagulant can be seen in Figure 30. Approximately 82.45% of EDTA lymphocytes and 57.88% of lithium heparin lymphocytes expressed MHC class I. The results for the positive control (anti-equine MHC class I) for neutrophils from both groups can be seen in Figure 31.

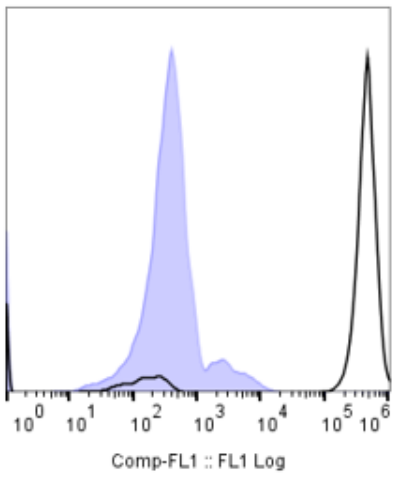
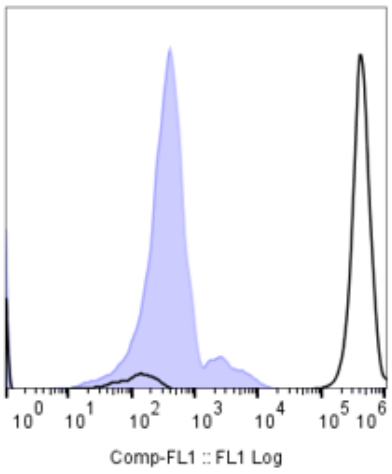
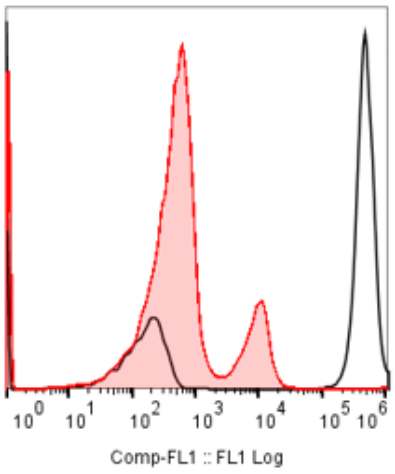
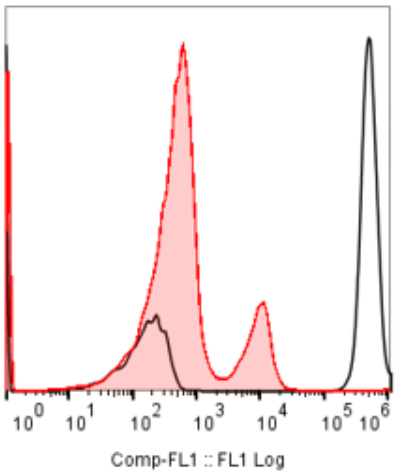
Lymphocyte	Anti-MHC I Experiment 1 EDTA	Anti-MHC I Experiment 2 EDTA
Histogram		
Overton % Positive	81.87	83.12
T(x) value	883.10	852.06
Lymphocyte	Anti-MHC I Experiment 1 Lithium Heparin	Anti-MHCI Experiment 2 Lithium Heparin
Histogram		
Overton % Positive	58.89	56.87
T(x) value	1328.68	1380.92

Figure 30. *Histograms for the population comparison between lymphocytes from EDTA samples (EDTA group; blue) and lymphocytes from lithium heparin samples (Lithium Heparin Group; red). The shading represents the control group of unstained lymphocytes compared with the unshaded black line stained with the positive control anti-equine MHC class I antibody. The cut off marker for the negative control was placed adjacent to the right shoulder of the smaller red peak. All cells to the right of this marker were deemed positive. Statistics were run using the FlowJo v10 software to obtain the Overton % Positive value and Chi Squared T(x) values.*

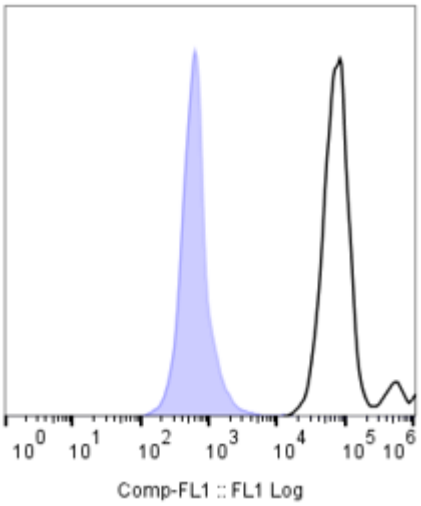
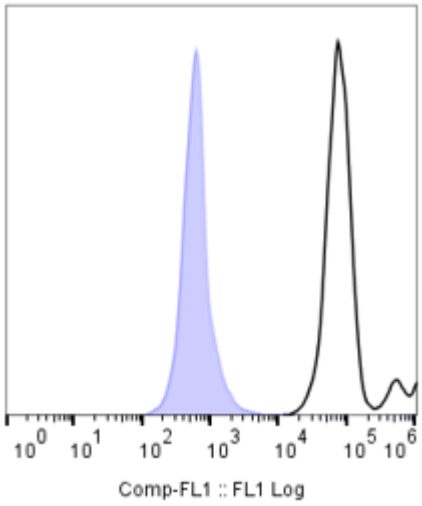
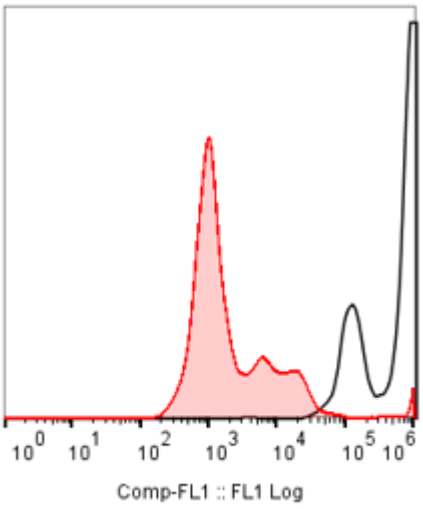
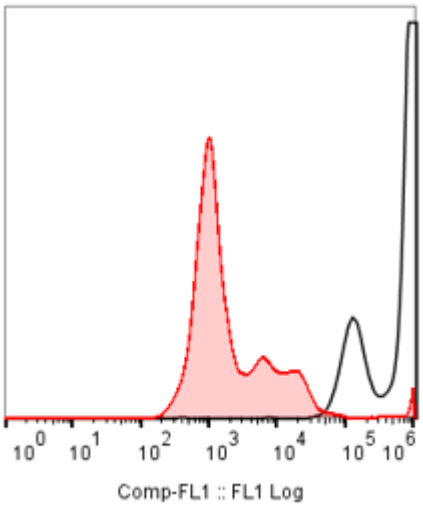
Neutrophil	Anti-MHC I Experiment1 EDTA	Anti-MHC I Experiment2 EDTA
Histogram		
Overton % Positive	96.67	96.63
T(x) value	697.33	682.79
Neutrophil	Anti-MHC I Experiment1 Lithium Heparin	Anti-MHC I Experiment2 Lithium Heparin
Histogram		
Overton % Positive	90.53	90.79
T(x) value	121.39	92.83

Figure 31. *Histograms for the population comparison between neutrophils from EDTA samples (EDTA group; blue) and neutrophils from lithium heparin samples (Lithium Heparin Group; red). The shading represents the control group of unstained neutrophils, versus the unshaded group stained with the positive control anti-equine MHC class I antibody. The cut off marker for the negative control was placed adjacent to the right shoulder of the right-hand red peak. Statistics were run using the FlowJo v10 software to obtain the Overton % Positive value and Chi Squared T(x) values.*

The results for the positive control for neutrophils from both groups can be seen in Figure 30. Neutrophil expression of MHC class I in the EDTA group was 96.65% and in the lithium heparin group was 90.66%. In addition to MHC class I, both cell subpopulations were analysed to determine the percent expression of CD81 (Figures 30 and 31).

Compared with the negative control, lymphocytes stained with the CD81 specific antibody, consisted of three populations: negative, weak expression and stronger expression although details of the patterns differed with each anti-coagulant.

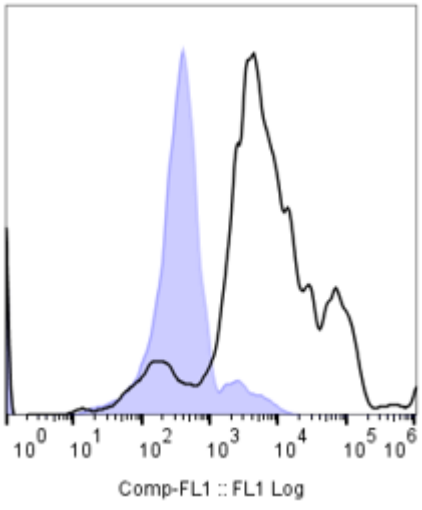
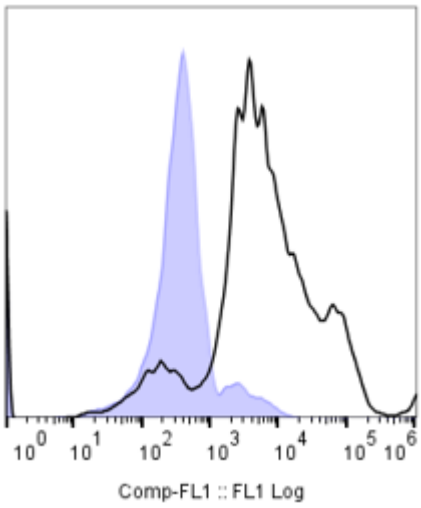
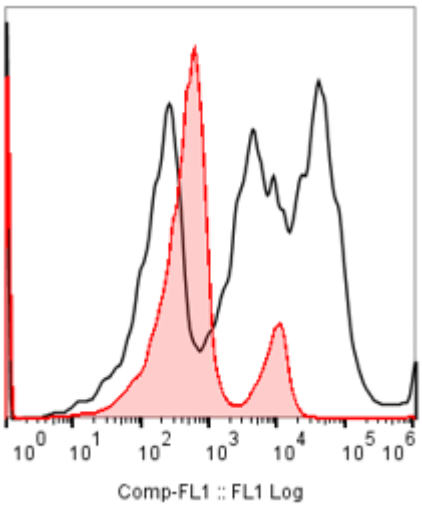
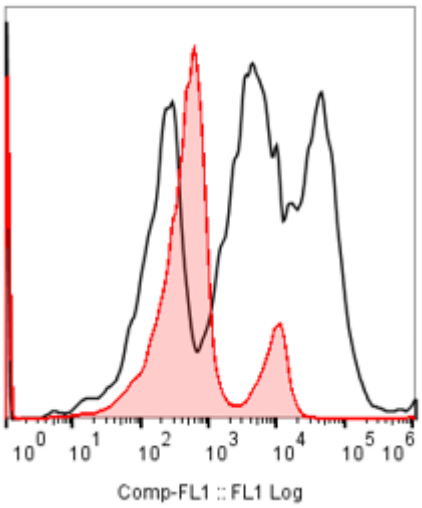
Lymphocyte	Anti-CD81 Experiment1 EDTA	Anti-CD81 Experiment2 EDTA
Histogram		
Overton % Positive	71.86	71.59
T(x) value	686.19	675.64
Lymphocyte	Anti-CD81 Experiment1 Lithium Heparin	Anti-CD81 Experiment2 Lithium Heparin
Histogram		
Overton % Positive	41.81	44.25
T(x) value	799.88	807.97

Figure 32. *Histograms for the population comparison between lymphocytes from EDTA samples (EDTA group; blue) and lymphocytes from lithium heparin samples (Lithium Heparin Group; red). The shaded cell population represents the control group of unstained lymphocytes, versus the group stained with anti-human CD81 (JS81) antibody (no shading). The cut off marker for the negative control was placed adjacent to the right shoulder of the right-hand red peak. Statistics were run using the FlowJo v10 software to obtain the Overton % Positive value and Chi Squared T(x) values.*

Three distinct populations of lymphocytes (two populations negative for CD81 and one population that was positive for CD81) were also seen for the lithium heparin samples. Fresher samples (lithium heparin) appear to have more clearly distinct populations, indicated by the appearance of three peaks. Overall percentage of CD81 expression for the EDTA group was 71.73% and the percentage for the lithium heparin group was 43.03%. In addition to lymphocytes, CD81 expression was also evaluated in equine neutrophils, as seen in figure 32.

Overall, it appears that the majority of lymphocytes and neutrophils express CD81 although the patterns differed depending on the anti-coagulant used. Therefore, further work is required to determine the precise expression patterns.

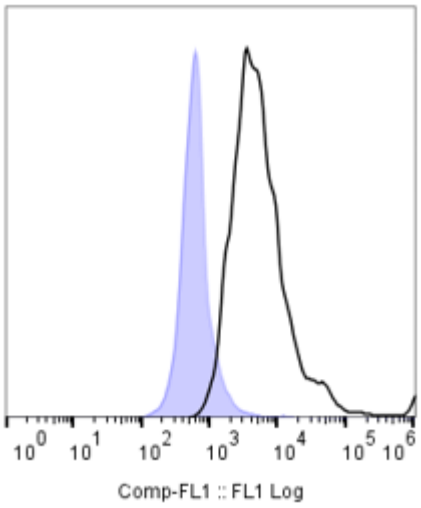
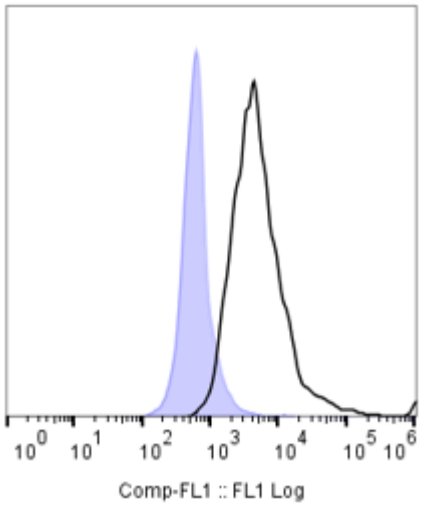
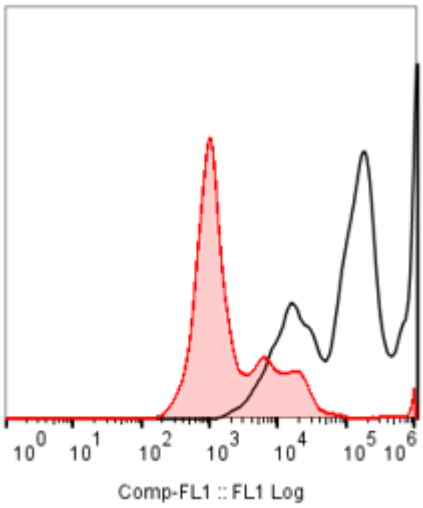
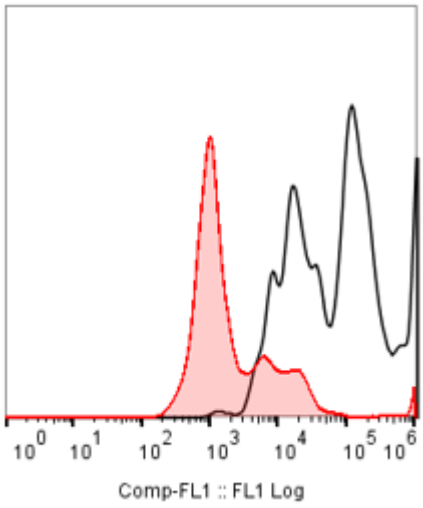
Neutrophil	Anti-CD81 Experiment1 EDTA	Anti-CD81 Experiment2 EDTA
Histogram		
Overton % Positive	86.88	86.01
T(x) value	605.27	597.06
Neutrophil	Anti-CD81 Experiment1 Lithium Heparin	Anti-CD81 Experiment2 Lithium Heparin
Histogram		
Overton % Positive	72.36	71.87
T(x) value	53.77	60.69

Figure 33. *Histograms for the population comparison between neutrophils from EDTA samples (EDTA group; blue) and neutrophils from lithium heparin samples (Lithium Heparin Group; red). The shading represents the control group of unstained neutrophils, versus the unshaded group stained with anti-human CD81 (JS81) antibody. The cut off marker for the negative control was placed adjacent to the right shoulder of the red peak. Statistics were run using the FlowJo v10 software to obtain the Overton % Positive value and Chi Squared T(x) values.*

In neutrophils, CD81 was expressed in an average of 86.45% in the EDTA group and in 72.12% in the lithium heparin group.

In addition to CD81 expression, OCLN expression was also investigated in both groups. The expression of OCLN in lymphocytes can be seen in Figure 32. OCLN was expressed on a small number of lymphocytes namely an average of 4.99% of lymphocytes in the EDTA group and 9.05% of the lithium heparin group. Neutrophils expressed OCLN on an average of 29.27% of EDTA and 29.8% of lithium heparin samples (Figure 33).

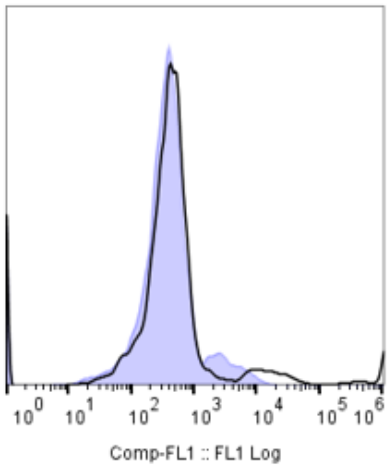
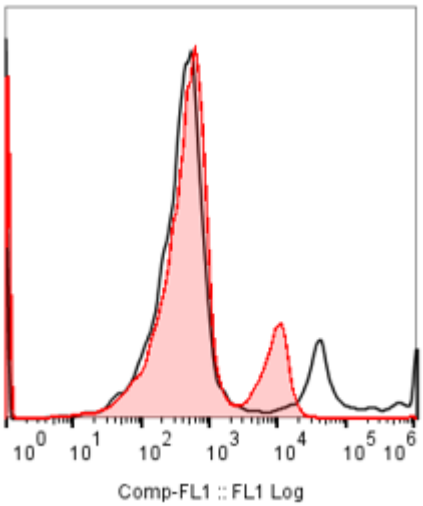
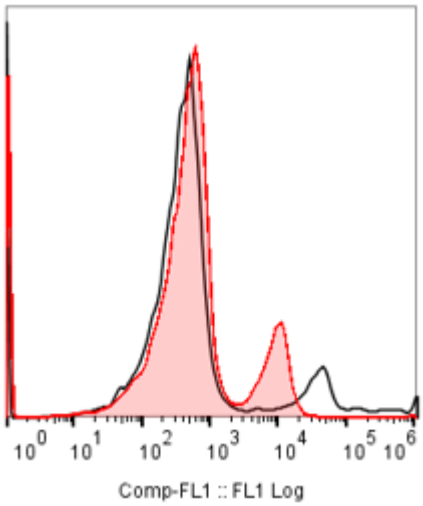
Lymphocyte	Anti-OCLN Experiment1 EDTA	
Histogram		
Overton % Positive	4.99	
T(x) value	46.33	
Lymphocyte	Anti-OCLN Experiment1 Lithium Heparin	Anti-OCLN Experiment2 Lithium Heparin
Histogram		
Overton % Positive	11.05	7.05
T(x) value	248.97	206.74

Figure 34. *Histograms for the population comparison between lymphocytes from EDTA samples (EDTA group; blue) and lymphocytes from lithium heparin samples (Lithium Heparin Group; red). The shading represents the control group of unstained lymphocytes, versus the unshaded group stained with anti-human OCLN antibody. The cut off marker for the negative control was placed adjacent to the right shoulder of the right-hand red peak. Statistics were run using the FlowJo v10 software to obtain the Overton % Positive value and Chi Squared T(x) values.*

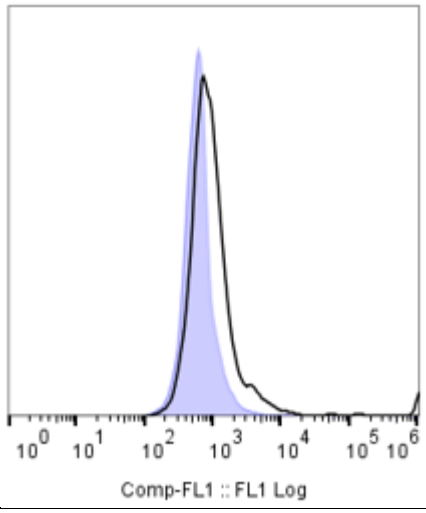
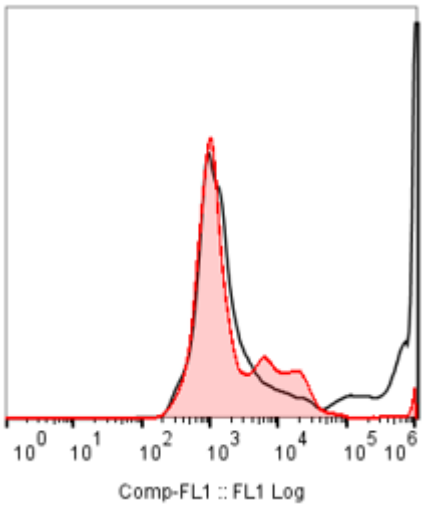
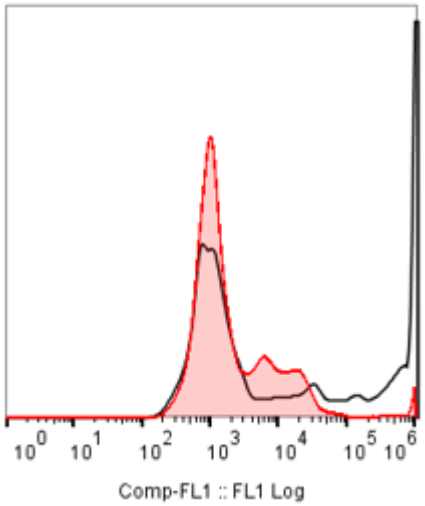
Neutrophil	Anti-OCLN Experiment1 EDTA	
Histogram		
Overton % Positive	29.27	
T(x) value	83.93	
Neutrophil	Anti-OCLN Experiment1 Lithium Heparin	Anti-OCLN Experiment2 Lithium Heparin
Histogram		
Overton % Positive	27.56	32.04
T(x) value	11.39	10.83

Figure 35. *Histograms for the population comparison between neutrophils from EDTA samples (EDTA group; blue) and neutrophils from lithium heparin samples (Lithium Heparin Group; red). The shading represents the control group of unstained neutrophils, versus the unshaded group stained with anti-human OCLN antibody. The cut off marker for the negative control was placed adjacent to the right shoulder of the right-hand red peak. Statistics were run using the FlowJo v10 software to obtain the Overton % Positive value and Chi Squared T(x) values.*

4. Discussion

The entry, pathogenesis and transmission of HCV, the virus phylogenetically most closely related to EqHV, has been studied in detail and it is known that the four entry receptors most studied and used by HCV for cell entry are CD81, OCLN, CLDN-1 and SR-B1 (Ploss *et al.*, 2009). Unlike HCV, the entry, pathogenesis and transmission of EqHV is still largely unknown since it was first identified in 2011 (Kapoor *et al.*, 2011). Although some cases of vertical transmission have been described, the exact mechanism is still unknown. A better understanding of the entry, pathogenesis and transmission of EqHV is needed to understand and assess the impact of this emerging flavivirus on the wider equine population (Scheel *et al.*, 2015). In addition, due to some of the similarities shared by both viruses, there is hope that vaccine studies of EqHV may inform future HCV research.

Given that EqHV is the virus most closely related to HCV it was hypothesized that EqHV may use some of the same receptors for cell entry. This study investigated the presence of HCV entry receptors on equine cells and tissues, including equine hepatocytes, PBMCs, lymph node, placenta and liver. A multimodal approach was conducted and investigated the expression of these receptors through the use of flow cytometric analysis, immunofluorescence, immunohistochemistry, and immunocytochemistry. Using IHC and ICC, CD81 was present in equine liver tissue, equine term placenta, equine PBMCs, in particular lymphocytes, and equine lymph nodes. OCLN was present in some neutrophils and within the nuclear envelope and nucleosome of hepatocytes, as well as the nuclei of endothelial cells in the equine liver and some lymphoblasts within equine lymph nodes. In comparison, in humans OCLN is mostly expressed in squamous epithelial cells of tonsil tissue, with low levels of expression present in human hepatocytes

(The Human Protein Atlas, 2022). Flow cytometry allowed differentiation of the different classes of PBMCs (namely monocytes, neutrophils, lymphocytes) and platelets.

4.1. Receptor alignments

Receptor alignments of the four receptors most commonly used by HCV for cell entry revealed marked similarities between the equine and human proteins. CD81 and CLDN-1 had the highest per cent identity at 96.61 and 95.26%, respectively, followed by OCLN at 92.69%. SR-B1 had a large number of non-conservative amino acid substitutions and therefore was found to only have an 85.27% identity matrix. Although CLDN-1 had less non-conservative substitutions, OCLN and CD81 were recently identified as the two key species-specific factors necessary for entry of HCV (Dorner *et al.*, 2011, 2013). This, combined with both antibodies being commercially available, led us to focus more closely on OCLN and CD81 in further experiments. As expected from previous studies, the murine sequences contained more amino acid substitutions than the equine sequences. These differences were previously shown to prevent entry of HCV into murine cells (Dorner *et al.*, 2011, 2013). The similarities of the equine and human receptor sequences suggests that further research into interspecies transmission of the human and equine viruses is warranted.

4.2. Immunofluorescence detection of HCV receptors

Detection of all four receptors using immunofluorescence staining proved problematic due to the lack of an established equine liver cell line, the poor viability of the liver cells collected *ex vivo* at abattoirs as well as the presence of non-specific binding of the secondary antibody and the lack of specific staining with some of the receptor reagents. It is likely that some of the difficulties

encountered were due to technical problems with the immunofluorescent microscope. Further work is necessary in this area, ideally using an established equine liver cell line and with more time to optimise the experiments.

4.3. Flow cytometric analysis

Flow cytometry was used to attempt to establish the expression levels of the different receptors on a variety of different equine cells, including E.Derm, FHK and EH cells. CD81 was present on 96.0% of E. Derm cells, 37.2% of FHK cells and 98.88% of EH cells, which was similar to the levels of CD81 expression found on Huh7 cells. OCLN was expressed on only 1.7% of E.Derm cells, 16.0% of FHK cells and 88.37% of EH cells. These differing expression patterns were expected as CD81 is known to be a ubiquitous receptor in humans, and has been found on the majority of human cells (Proteomics DB, 2019). OCLN on the other hand, is not as widely expressed on connective tissue, nor adipocytes nor epithelial cells, which supports our findings (Proteomics DB, 2019).

One of the limitations of the flow cytometry experiments was the lack of an equine liver cell line. Although livers harvested from abattoirs were used, these are not composed purely of hepatocytes but will include a variety of other cells, such as Kupfer cells, sinusoidal cells, and endothelial cells. Therefore a direct comparison with Huh7 cells on the exact levels of expression cannot be made as *ex vivo* harvested equine hepatocytes do not replace a culture of pure equine hepatocytes. In addition, the viability of cells was only approximately 60% after 2-4 hours and as dead cells were excluded from the gate, not all of the cells present in liver were analysed. Therefore, any conclusions drawn from these experiments should be interpreted with care and the experiment repeated if an equine liver cell line is established.

4.4. IHC of FFPE and Frozen tissue

Immunohistochemistry staining of FFPE tissues was unsuccessful. This is likely due to lack of effective antigen retrieval from the FFPE sections. On the other hand, immunohistochemistry staining of frozen tissue appeared to be more successful. Within snap frozen sections of equine liver, CD81 was mainly expressed on endothelial cells of portal veins, arteries and bile ducts as well as on sinusoidal cells and connective tissue surrounding these vessels. Hepatocytes appeared largely CD81 negative, which is in stark contrast to the findings of CD81 expression in equine hepatocytes using flow cytometry. This may be due to the sensitivity of immunohistochemistry being much lower than that of flow cytometry but should be investigated in further studies.

In the equine placenta, CD81 was found to be mainly expressed on the epithelial cells of the allantochorionic microcotyledons and areolar areas, the connective tissue, the endothelial cells and the smooth muscle cells surrounding the blood vessels within the placenta. To the author's knowledge, this is the first time that CD81 has been shown to be expressed in aspects of the equine allantochorion. In other recent studies, EqHV RNA was detected in the allantochorion of two foals that died during or shortly after birth, albeit at a viral load much lower (10^3) than found within the fetal liver and lung (Pronost *et al.*, 2019). Gather, Walter, Todt, *et al.* (2016) found EqHV genomic material in maternal serum, umbilical cord blood and serum from the mare's foal. This suggests that vertical transmission does occur with EqHV, with the exact mechanism still unknown. It appears that transmission through blood from the mare to the foal, perhaps through microhaemorrhage at the feto-maternal interface, may play a role, with CD81 receptors found on endothelial cells and epithelial cells of allantochorionic microcotyledons within the equine term

placenta. Given the small number of cases of vertical transmission reported and the complex six-layer architecture of the equine epitheliochorial placenta separating maternal and fetal blood circulations, transmission directly through the placenta is likely to be uncommon. Although the anatomy of the human discoid placenta is vastly different to that of the equine epitheliochorial placenta, it has been described that vertical transmission with HCV may occur at different stages (intra-uterine, intra-partum and post-natal) (Benova *et al.*, 2014). Further studies are needed to further investigate the presence of CD81 in the equine term placenta using more sensitive methods, such as flow cytometry. This also includes the stage at which the fetus may be infected and whether this occurs intra-uterine, as previously suggested by Pronost *et al.* (2019), or whether infection actually or also occurs intra-partum or post-natally.

In the equine lymph node, CD81 was expressed on the endothelial cells of the lymph node capsule as well as within the lymph node capsule itself. Germinal centres expressed a higher proportion of CD81 than non-germinal centres. These results were expected as CD81 is present commonly in human lymph nodes, in particular in the cytoplasm and membranes of germinal centre lymphocytes (Luo *et al.*, 2010).

OCLN staining was present on the nuclear envelope and nucleosome of hepatocytes and in the nuclei of endothelial cells. No OCLN could be detected in sinusoidal cells using this method. OCLN expression was also absent from all sections of snap frozen equine placenta. OCLN seemed to be expressed on monocytes and/or lymphoblasts within the equine lymph node.

There were a large number of processing artefacts in both FFPE and snap frozen sections, leading to loss of liver architecture. This was made worse as the

sections were a few years old and the cryostat sections were not ideal to fully assess the full extent of the tissues. Not all areas of the lymph nodes were present (e.g. medulla) and the microcotyledons of the placenta were sometimes difficult to observe on the sections because of their orientation. This may have decreased the antibody's ability to access these cell membrane specific receptors, as cell membranes are readily disrupted and altered through any tissue processing. In addition to the processing artefacts, the staining pattern of CD81 also appeared to be almost identical to that of the positive control MHC class I antibody. It has been suggested that MHC class I and CD81 are often located adjacently to each other on cell membranes, which would explain the similarity in staining patterns for both of these antibodies (Pata *et al.*, 2011).

4.3. Immunocytochemistry and flow cytometry of equine PBMCs

Immunocytochemistry analysis of equine PBMCs revealed that CD81 is expressed in most PBMCs, including neutrophils, lymphocytes and eosinophils. It has previously been shown that the HCV virus is consistently found in human PBMCs, and due to the similarity between both viruses it would be important to investigate whether this also holds true for EqHV, although to date it has not been detected in equine PBMCs (Cavalheiro *et al.*, 2007; Scheel *et al.*, 2015; Abd Alla and El Awady, 2017) All PBMCs were negative for OCLN, which was expected, as OCLN is a liver specific receptor in comparison to CD81, which is considered a ubiquitous receptor. With HCV infection, it appears that its infection of lymphocytes is limited to particular strains, with evidence suggesting that different receptors are used on lymphocytes compared to hepatocytes (Sarhan *et al.*, 2012; Sarhan, Chen and Michalak, 2013; Chen *et al.*, 2017).

Flow cytometry of PBMCs revealed that lymphocytes seem to consist of three different populations, one of which was CD81 negative, one with weak CD81 expression and one with stronger CD81 expression, although details of this pattern differed between coagulant groups (lithium heparin and EDTA). Future work using dual labelling should explore expression of CD81 on lymphocyte subpopulations, including reagents to distinguish B or T cells, the latter involving subpopulations for which reagents are available e.g., CD4, CD8, CD5. CD81 was also expressed on the majority of neutrophils. OCLN was only expressed on less than 5% of lymphocytes (and was therefore considered to be negative) and only expressed on less than a third of neutrophils. The cells collected in EDTA appeared to have much more distinct staining compared with the cells obtained using lithium heparin, which made the latter more difficult to gate accurately using forward and side scatter. This is probably due to lithium heparin altering the morphology of the cell membrane and cytoplasm, which is why EDTA is recommended for haematology analysis in practice (Bowen and Remaley, 2014). Another limitation was the lack of double staining to clearly identify cells as all neutrophils or lymphocytes. It appears that the majority of neutrophils and lymphocytes express CD81, but due to the difficulties with the anti-coagulant, further work is required to determine the precise expression patterns. In addition, monocytes in the EDTA group had to be disregarded as this population was too disparate, due to a 72-hour delay in processing after sampling. In the future, samples should be processed as soon as possible to avoid eliminating populations of cells due to processing artefacts and/or degradation.

Overall, using a variety of methods to determine expression of potential EqHV receptors on equine tissues and cells, this project has given further insight into the potential routes of transmission for EqHV, with a focus on vertical

transmission. The anti-human CD81 and anti-human OCLN antibodies did cross-react with the equine proteins but in order to further investigate the distribution of the other co-receptors, in particular SR-B1, it may be necessary to develop equine specific reagents. As CD81 receptors were found on equine placenta, including on placental endothelial cells as well as the epithelium of allantochorionic microcotyledons, it may be that EqHV uses CD81 receptors to enter the allantochorion and this may be the route of vertical transmission in a small number of cases when the placenta is in direct contact with EqHV positive maternal blood. OCLN was absent from all sections of snap-frozen placenta, but due to the low sensitivity of immunohistochemistry, this will require further investigation using a more sensitive method, such as fluorescent *in* situ hybridization. If OCLN is not found using a more sensitive method, this may indicate that infection of the placenta involves a different combination of receptors or altogether different receptors than those used by HCV, as appears to be the case for HCV infection of PBMC (Chen *et al.*, 2017). This study has provided preliminary insights into the expression of putative EqHV receptors on equine tissues and cells and thus highlighted potential mechanisms of vertical transmission of EqHV between the pregnant mare and her foal *in utero*.

5. BIBLIOGRAPHY

Abbadi, I. *et al.* (2021) 'Non-primate hepacivirus transmission and prevalence: Novel findings of virus circulation in horses and dogs in Morocco', *Infection, Genetics and Evolution*, 93(June), p. 104975. doi: 10.1016/j.meegid.2021.104975.

Abd Alla, M. D. A. and El Awady, M. K. (2017) 'Hepatitis C Virus RNA Strands Detection in Peripheral Blood Mononuclear Cells Legitimizes Virus Eradication in Negative Serum PCR Naïve and Post-treatment Patients', *Journal of Clinical and Translational Hepatology*, XX(XX), pp. 1–8. doi: 10.14218/JCTH.2016.00054.

Akazawa, D. *et al.* (2007) 'CD81 Expression Is Important for the Permissiveness of Huh7 Cell Clones for Heterogeneous Hepatitis C Virus Infection ', 81(10), pp. 5036–5045. doi: 10.1128/JVI.01573-06.

Averhoff, F. M., Glass, N. and Holtzman, D. (2012) 'Global burden of hepatitis C: considerations for healthcare providers in the United States.', *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 55 Suppl 1(Suppl 1), pp. 10–15. doi: 10.1093/cid/cis361.

Badenhorst, M. *et al.* (2018) 'First detection and frequent occurrence of Equine Hepacivirus in horses on the African continent', *Veterinary Microbiology*, 223, pp. 51–58.

Badenhorst, M. *et al.* (2019) 'No evidence of mosquito involvement in the transmission of equine hepacivirus (flaviviridae) in an epidemiological survey of Austrian horses', *Viruses*, 11(11). doi: 10.3390/v11111014.

Baechlein, C. *et al.* (2015) 'Identification of a Novel Hepacivirus in Domestic

Cattle from Germany', *Journal of Virology*, 89(14), pp. 7007–7015. doi: 10.1128/JVI.00534-15.

Barrios-Rodiles, M. *et al.* (2005) 'High-Throughput Mapping of a Dynamic Signaling Network in Mammalian Cells High-Throughput Mapping of a Dynamic Signaling Network in Mammalian Cells', *Science*, 307(2005), pp. 1621–1625. doi: 10.1126/science.1105776.

Bartosch, B. *et al.* (2003) 'Cell Entry of Hepatitis C Virus Requires a Set of Co-receptors that Include the CD81 Tetraspanin and the SR-B1 Scavenger Receptor', *Journal of Biological Chemistry*, 278(43), pp. 41624–41630. doi: 10.1074/jbc.M305289200.

Benova, L. *et al.* (2014) 'Vertical Transmission of Hepatitis C Virus : Systematic Review and Meta-analysis', 59, pp. 765–773. doi: 10.1093/cid/ciu447.

Boucheix, C. and Rubinstein, E. (2001) 'Tetraspanins', in *Cellular and Molecular Life Sciences CMLS*, pp. 1189–1205.

Bowen, R. A. R. and Remaley, A. T. (2014) 'Interferences from blood collection tube components on clinical chemistry assays', *Biochemia Medica*, 24(1), pp. 31–44.

Burbelo, P. D. *et al.* (2012) 'Serology-Enabled Discovery of Genetically Diverse Hepaciviruses in a New Host', *Journal of Virology*, 86(11), pp. 6171–6178. doi: 10.1128/JVI.00250-12.

Cassidy, R. (2016) 'The Sport of Kings : Kinship , Class and Thoroughbred Breeding in Newmarket The Sport of Kings', (January 2003). doi: 10.1017/CBO9780511613760.

Catanese, M. T. *et al.* (2007) 'High-Avidity Monoclonal Antibodies against the

Human Scavenger Class B Type I Receptor Efficiently Block Hepatitis C Virus Infection in the Presence of High-Density Lipoprotein ', 81(15), pp. 8063–8071. doi: 10.1128/JVI.00193-07.

Cavalheiro, N. D. P. *et al.* (2007) 'Detection of HCV by PCR in serum and PBMC of patients with hepatitis C after treatment.', *The Brazilian journal of infectious diseases : an official publication of the Brazilian Society of Infectious Diseases*, 11(5), pp. 471–474. doi: 10.1590/S1413-86702007000500006.

Chandriani, S. *et al.* (2013) 'Identification of a previously undescribed divergent virus from the Flaviviridae family in an outbreak of equine serum hepatitis', *Proceedings of the National Academy of Sciences*, 110(15), pp. E1407–E1415. doi: 10.1073/pnas.1219217110.

Chen, C. *et al.* (2017) 'Hepatitis C virus has a genetically determined lymphotropism through co-receptor B7.2'. doi: 10.1038/ncomms13882.

Chiba, H. *et al.* (2008) 'Transmembrane proteins of tight junctions', *Biochimica et Biophysica Acta - Biomembranes*, 1778(3), pp. 588–600. doi: 10.1016/j.bbamem.2007.08.017.

Claas, C., Stipp, C. S. and Hemler, M. E. (2001) 'Evaluation of Prototype Transmembrane 4 Superfamily Protein Complexes and Their Relation to Lipid Rafts', *Journal of Biological Chemistry*, 276(11), pp. 7974–7984. doi: 10.1074/jbc.M008650200.

Corman, V. M. *et al.* (2015) 'Highly Divergent Hepaciviruses from African Cattle', *Journal of Virology*, 89(11), pp. 5876–5882. doi: 10.1128/JVI.00393-15.

Cormier, E. G. *et al.* (2004) 'CD81 is an entry coreceptor for hepatitis C virus.', *Proceedings of the National Academy of Sciences of the United States of*

America, 101(19), pp. 7270–4. doi: 10.1073/pnas.0402253101.

Date, T. *et al.* (2020) 'Prevalence of equine hepacivirus infection in Mongolia', *Virus Research*, 282(March), p. 197940. doi: 10.1016/j.virusres.2020.197940.

Divers, T. J. *et al.* (2018) 'A new parvovirus associated with serum hepatitis in horses following inoculation of a common equine biological', *Emerging Infectious Diseases*, 24(2), pp. 303–310. doi: 10.3201/eid2402.171031.

Dorner, M. *et al.* (2011) 'infection', 474(7350), pp. 208–211. doi: 10.1038/nature10168.A.

Dorner, M. *et al.* (2013) 'Completion of the entire hepatitis C virus life cycle in genetically humanized mice', *Nature*, 501(7466), pp. 237–241. doi: 10.1038/nature12427.

Drexler, J. F. *et al.* (2013) 'Evidence for Novel Hepaciviruses in Rodents', *PLoS Pathogens*, 9(6). doi: 10.1371/journal.ppat.1003438.

Elia, G *et al.* (2017) 'Equine hepacivirus persistent infection in a horse with chronic wasting', *Transboundary and Emerging Diseases*, 64(5), pp. 1354–1358. doi: 10.1111/tbed.12679.

Elia, Gabriella *et al.* (2017) 'Identification and genetic characterization of equine hepaciviruses in Italy', *Veterinary Microbiology*, 207(July), pp. 239–247. doi: 10.1016/j.vetmic.2017.07.004.

Elsana, S. *et al.* (1998) 'HCV Antibodies in Saliva and Urine', 27(November 1997), pp. 24–27.

Figueiredo, A. S. *et al.* (2015) 'Identification of two phylogenetic lineages of equine hepacivirus and high prevalence in Brazil', *Veterinary Journal*, 206(3), pp.

414–416. doi: 10.1016/j.tvjl.2015.10.015.

Figueiredo, A. S. *et al.* (2018) 'Epidemiological investigation and analysis of the NS5B gene and protein variability of non-primate hepacivirus in several horse cohorts in Rio de Janeiro state, Brazil', *Infection, Genetics and Evolution*, 59(January), pp. 38–47. doi: 10.1016/j.meegid.2018.01.017.

Figueiredo, A. S. *et al.* (2019) 'First description of Theiler's disease-associated virus infection and epidemiological investigation of equine pegivirus and equine hepacivirus coinfection in Brazil', *Transboundary and Emerging Diseases*, 66(4), pp. 1737–1751. doi: 10.1111/tbed.13210.

Firth, C. *et al.* (2014) 'Detection of zoonotic pathogens and characterization of novel viruses carried by commensal rattus norvegicus in New York city', *mBio*, 5(5), pp. 1–16. doi: 10.1128/mBio.01933-14.

Furuse, M. *et al.* (1993) 'Occludin: A novel integral membrane protein localizing at tight junctions', *Journal of Cell Biology*, 123(6 II), pp. 1777–1788. doi: 10.1083/jcb.123.6.1777.

Furuse, M. *et al.* (1998) 'Claudin-1 and -2: Novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin', *Journal of Cell Biology*, 141(7), pp. 1539–1550. doi: 10.1083/jcb.141.7.1539.

Gather, T., Walter, S., Pfaender, S., *et al.* (2016) 'Acute and chronic infections with nonprimate hepacivirus in young horses', *Veterinary Research*, 47(1), pp. 1–5. doi: 10.1186/s13567-016-0381-6.

Gather, T., Walter, S., Todt, D., *et al.* (2016) 'Vertical transmission of hepatitis C virus-like nonprimate hepacivirus in horses', *Journal of General Virology*, 97(10), pp. 2540–2551. doi: 10.1099/jgv.0.000561.

Gemaque, B. S. alame *et al.* (2014) 'Hepacivirus infection in domestic horses, Brazil, 2011-2013', *Emerging infectious diseases*, 20(12), pp. 2180–2182. doi: 10.3201/eid2012.140603.

Harris, H. J. *et al.* (2008) 'CD81 and Claudin 1 Coreceptor Association: Role in Hepatitis C Virus Entry', *Journal of Virology*, 82(10), pp. 5007–5020. doi: 10.1128/JVI.02286-07.

Hemler, M. E. (2003) 'Tetraspanin Proteins Mediate Cellular Penetration, Invasion, and Fusion Events and Define a Novel Type of Membrane Microdomain', *Annual Review of Cell and Developmental Biology*, 19(1), pp. 397–422. doi: 10.1146/annurev.cellbio.19.111301.153609.

Hemler, M. E. (2005) 'Tetraspanin functions and associated microdomains', *Nature Reviews Molecular Cell Biology*, 6(10), pp. 801–811. doi: 10.1038/nrm1736.

Heo, T. H. *et al.* (2006) 'Hepatitis C virus E2 links soluble human CD81 and SR-B1 protein', *Virus Research*, 121(1), pp. 58–64. doi: 10.1016/j.virusres.2006.04.002.

Kapoor, A. *et al.* (2011) 'Characterization of a canine homolog of hepatitis C virus', *Proceedings of the National Academy of Sciences*, 108(28), pp. 11608–11613. doi: 10.1073/pnas.1101794108.

Kapoor, A. *et al.* (2013) 'Identification of a Pegivirus (GB Virus-Like Virus) That Infects Horses', *Journal of Virology*, 87(12), pp. 7185–7190. doi: 10.1128/JVI.00324-13.

Kim, H. S. *et al.* (2017) 'First identification and phylogenetic analysis of equine hepacivirus in Korea', *Infection, Genetics and Evolution*, 49, pp. 268–272. doi:

10.1016/j.meegid.2017.01.030.

Koutsoudakis, G. *et al.* (2007) 'The Level of CD81 Cell Surface Expression Is a Key Determinant for Productive Entry of Hepatitis C Virus into Host Cells ', 81(2), pp. 588–598. doi: 10.1128/JVI.01534-06.

Lattimer, J. *et al.* (2019) 'Structure-function analysis of the equine hepacivirus 5' untranslated region highlights the conservation of translational mechanisms across the hepaciviruses', *Journal of General Virology*, 100(11), pp. 1501–1514. doi: 10.1099/JGV.0.001316.

Levy, S. and Shoham, T. (2005) 'The tetraspanin web modulates immune-signalling complexes', *Nature Reviews Immunology*, 5(2), pp. 136–148. doi: 10.1038/nri1548.

Levy, S., Todd, S. C. and Maecker, H. T. (1998) 'CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system.', *Annual review of immunology*, 16, pp. 89–109. doi: 10.1146/annurev.immunol.16.1.89.

Liou, T. *et al.* (1992) 'Detection of HCV RNA in Saliva , Urine , Seminal Fluid , and Ascites', 202, pp. 197–202.

Lu, G. *et al.* (2016) 'First description of hepacivirus and pegivirus infection in domestic Horses in China: A study in guangdong province, heilongjiang province and Hong Kong district', *PLoS ONE*, 11(5), pp. 1–12. doi: 10.1371/journal.pone.0155662.

Lu, G. *et al.* (2017) 'Identification and genetic characterization of hepacivirus and pegivirus in commercial equine serum products in China', *PLoS ONE*, 12(12), pp. 1–11. doi: 10.1371/journal.pone.0189208.

Lu, G. *et al.* (2018) 'Identification and genetic characterization of a novel

parvovirus associated with serum hepatitis in horses in China', *Emerging Microbes and Infections*, 7(1). doi: 10.1038/s41426-018-0174-2.

Luo, R. F. *et al.* (2010) 'CD81 protein is expressed at high levels in normal germinal center B cells and in subtypes of human lymphomas ☆', *Human Pathology*, 41(2), pp. 271–280. doi: 10.1016/j.humpath.2009.07.022.

Lupberger, J. *et al.* (2011) 'EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy', *Nature Medicine*, 17(5), pp. 589–595. doi: 10.1038/nm.2341.

Lyons, S. *et al.* (2012) 'Nonprimate hepaciviruses in domestic horses, United Kingdom', *Emerging Infectious Diseases*, 18(12), pp. 1976–1982. doi: 10.3201/eid1812.120498.

Lyons, S. *et al.* (2014) 'Viraemic frequencies and seroprevalence of non-primate hepacivirus and equine pegiviruses in horses and other mammalian species', *Journal of General Virology*, 95(PART 8), pp. 1701–1711. doi: 10.1099/vir.0.065094-0.

Maasoumy, B. and Wedemeyer, H. (2012) 'Natural history of acute and chronic hepatitis C', *Best Practice and Research: Clinical Gastroenterology*, 26(4), pp. 401–412. doi: 10.1016/j.bpg.2012.09.009.

Marceau, C. D. *et al.* (2016) 'Genetic dissection of Flaviviridae host factors through genome-scale CRISPR screens', *Nature*, 535(7610), pp. 159–163. doi: 10.1038/nature18631.

Martin, D. N. and Uprichard, S. L. (2013) 'Identification of transferrin receptor 1 as a hepatitis C virus entry factor', *Proceedings of the National Academy of Sciences*, 110(26), pp. 10777–10782. doi: 10.1073/pnas.1301764110.

Martin, F. *et al.* (2005) 'Tetraspanins in Viral Infections : a Fundamental Role in Viral Biology ?', *J. Virol.*, 79(17), pp. 10839–10851. doi: 10.1128/JVI.79.17.10839.

Matsuu, A. *et al.* (2015) 'Genetic and serological surveillance for non-primate hepacivirus in horses in Japan', *Veterinary Microbiology*, 179(3–4), pp. 219–227. doi: 10.1016/j.vetmic.2015.05.028.

Mealey, R. H. and Ramsay, J. D. (2016) 'Journal of Equine Veterinary Science', *Journal of Equine Veterinary Science*, 39, p. S20. doi: 10.1016/j.jevs.2016.02.042.

Miller, F. D. and Abu-Raddad, L. J. (2010) 'Evidence of intense ongoing endemic transmission of hepatitis C virus in Egypt', *Proceedings of the National Academy of Sciences*, 107(33), pp. 14757–14762. doi: 10.1073/pnas.1008877107.

Murata, M. *et al.* (2005) 'Down-regulation of survival signaling through MAPK and Akt in occludin-deficient mouse hepatocytes in vitro', *Experimental Cell Research*, 310(1), pp. 140–151. doi: 10.1016/j.yexcr.2005.07.017.

Nelson, P. *et al.* (2011) 'The epidemiology of viral hepatitis among people who inject drugs: Results of global systematic reviews', *Lancet*, 378(9791), pp. 571–583. doi: 10.1016/S0140-6736(11)61097-0.The.

Paciello, R. *et al.* (2016) 'Novel human anti-claudin 1 mAbs inhibit hepatitis C virus infection and may synergize with anti-SRB1 mAb', pp. 82–94. doi: 10.1099/jgv.0.000330.

Paim, W. P. *et al.* (2019) 'Characterization of the viral genomes present in commercial batches of horse serum obtained by high-throughput sequencing', *Biologicals*, 61(August), pp. 1–7. doi: 10.1016/j.biologicals.2019.08.005.

Pata, S. *et al.* (2011) 'Association of CD99 short and long forms with MHC class I , MHC class II and tetraspanin CD81 and recruitment into immunological synapses', *BMC Research Notes*, 4(293).

Pfaender, S., Walter, S., *et al.* (2015) 'Assessment of cross-species transmission of hepatitis C virus-related non-primate hepacivirus in a population of humans at high risk of exposure', *Journal of General Virology*, 96(9), pp. 2636–2642. doi: 10.1099/vir.0.000208.

Pfaender, S., Cavalleri, J. M. V., *et al.* (2015) 'Clinical course of infection and viral tissue tropism of hepatitis C virus-like nonprimate hepaciviruses in horses', *Hepatology*, 61(2), pp. 447–459. doi: 10.1002/hep.27440.

Pfaender, S. *et al.* (2017) 'Immune protection against reinfection with nonprimate hepacivirus', *Proceedings of the National Academy of Sciences*, 114(12), pp. E2430–E2439. doi: 10.1073/pnas.1619380114.

Ploss, A. *et al.* (2009) 'Human occludin is a hepatitis C virus entry factor required for infection of mouse cells', *Nature*, 457(7231), pp. 882–886. doi: 10.1038/nature07684.Human.

Postel, A. *et al.* (2016) 'Frequent presence of hepaci and pegiviruses in commercial equine serum pools', *Veterinary Microbiology*, 182, pp. 8–14. doi: 10.1016/j.vetmic.2015.10.032.

Pronost, S. *et al.* (2017) 'Prevalence of Equine Hepacivirus Infections in France and Evidence for Two Viral Subtypes Circulating Worldwide', *Transboundary and Emerging Diseases*, 64(6), pp. 1884–1897. doi: 10.1111/tbed.12587.

Pronost, S. *et al.* (2019) 'Further evidence for in utero transmission of equine hepacivirus to foals', *Viruses*, 11(12), pp. 1–12. doi: 10.3390/v11121124.

Proteomics DB (2019) *OCLN (Q16625).* Available at : https://www.proteomicsdb.org/proteomicsdb/#protein/proteinDetails/60966/expression (Accessed: 15 April 2019)

Proteomics DB (2019) *CD81 antigen (P60033)*. Available at : https://www.proteomicsdb.org/proteomicsdb/#human/proteinDetails/P60033/expression (Accessed: 15 April 2019)

Quan, P. *et al.* (2012) 'Bats are a major natural reservoir for hepaciviruses and pegiviruses', *Proceedings of the National Academy of Sciences*, 110, pp. 3–8. doi: 10.1073/pnas.1303037110/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1303037110.

Ramsay, J. D. *et al.* (2015) 'Experimental transmission of equine hepacivirus in horses as a model for hepatitis C virus', *Hepatology*, 61(5), pp. 1533–1546. doi: 10.1002/hep.27689.

Reichert, C. *et al.* (2017) 'Frequent occurrence of nonprimate hepacivirus infections in Thoroughbred breeding horses – A cross-sectional study for the occurrence of infections and potential risk factors', *Veterinary Microbiology*, 203(February), pp. 315–322. doi: 10.1016/j.vetmic.2017.03.030.

Reuter, G. *et al.* (2014) 'Non-primate hepacivirus infection with apparent hepatitis in a horse — Short communication', *Acta Veterinaria Hungarica*, 62(3), pp. 422–427. doi: 10.1556/AVet.2014.011.

Rhainds, D. and Brissette, L. (2004) 'The role of scavenger receptor class B type I (SR-BI) in lipid trafficking: Defining the rules for lipid traders', *International Journal of Biochemistry and Cell Biology*, 36(1), pp. 39–77. doi: 10.1016/S1357-2725(03)00173-0.

Sainz, B. *et al.* (2012) 'absorption receptor as a new hepatitis C virus entry factor', *Nat Med*, 18(2), pp. 281–285. doi: 10.1038/nm.2581.Identification.

Sarhan, M. A. *et al.* (2012) 'Hepatitis C Virus Infection of Human T Lymphocytes Is Mediated by CD5', pp. 3723–3735. doi: 10.1128/JVI.06956-11.

Sarhan, M. A., Chen, A. Y. and Michalak, T. I. (2013) 'Differential Expression of Candidate Virus Receptors in Human T Lymphocytes Prone or Resistant to Infection with Patient-Derived Hepatitis C Virus', 8(4), pp. 1–12. doi: 10.1371/journal.pone.0062159.

Scheel, T. K. H. *et al.* (2015) 'Characterization of nonprimate hepacivirus and construction of a functional molecular clone', *Proceedings of the National Academy of Sciences*, 112(7), pp. 2192–2197. doi: 10.1073/pnas.1500265112.

Sibley, S. D. *et al.* (2014) 'Discovery and characterization of distinct simian pegiviruses in three wild African old world monkey species', *PLoS ONE*, 9(6). doi: 10.1371/journal.pone.0098569.

Simmonds, P. (2004) 'Genetic diversity and evolution of hepatitis C virus - 15 years on', *Journal of General Virology*, 85(11), pp. 3173–3188. doi: 10.1099/vir.0.80401-0.

Simmonds, P. *et al.* (2017) 'ICTV Virus Taxonomy Profile: Flaviviridae', *Journal of General Virology*, (98), pp. 2–3.

Simons, J. N. *et al.* (1995) 'Identification of two flavivirus-like genomes in the GB hepatitis agent.', *Proceedings of the National Academy of Sciences of the United States of America*, 92(8), pp. 3401–3405. doi: 10.1073/pnas.92.8.3401.

Smith, D. B. *et al.* (2014) 'Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: Updated criteria and genotype assignment web

resource', *Hepatology*, 59(1), pp. 318–327. doi: 10.1002/hep.26744.

Sosa-jurado, F. *et al.* (2014) 'Detection of hepatitis C virus RNA in saliva of patients with active infection not associated with periodontal or liver disease severity'. doi: 10.1186/1471-2334-14-72.

Stapleton, J. T. *et al.* (2011) 'The GB viruses: A review and proposed classification of GBV-A, GBV-C (HGV), and GBV-D in genus Pegivirus within the family Flaviviridae', *Journal of General Virology*, 92(2), pp. 233–246. doi: 10.1099/vir.0.027490-0.

Takayama, H. *et al.* (2008) 'High-level expression, single-step immunoaffinity purification and characterization of human tetraspanin membrane protein CD81', *PLoS ONE*, 3(6). doi: 10.1371/journal.pone.0002314.

Tanaka, T. *et al.* (2014) 'Hallmarks of Hepatitis C Virus in Equine Hepacivirus', *Journal of Virology*, 88(22), pp. 13352–13366. doi: 10.1128/JVI.02280-14.

Tegtmeyer, B. *et al.* (2019) 'Chronic equine hepacivirus infection in an adult gelding with severe hepatopathy', *Veterinary Medicine and Science*, 5(3), pp. 372–378. doi: 10.1002/vms3.181.

The Human Protein Atlas (2022) *OCLN*. Available at:

https://www.proteinatlas.org/ENSG00000197822-OCLN/tissue (Accessed 18 January 2022)

The Thoroughbred Association 2018, *Economic impact study*, The Thoroughbred Association, Accessed 21 October 2018, <https://www.thetba.co.uk/british-thoroughbred-breeding-industry-economic-impact-study-2018/>

Timoney, P. J. *et al.* (1987) 'The carrier state in equine arteritis virus infection in

the stallion with specific emphasis on the venereal mode of virus transmission', *Journal of Reproduction and Fertility: Supplement*, (35), pp. 95–102.

Tomlinson, J. E. *et al.* (2019) 'Viral testing of 18 consecutive cases of equine serum hepatitis: A prospective study (2014-2018)', *Journal of Veterinary Internal Medicine*, 33(1), pp. 251–257. doi: 10.1111/jvim.15368.

Tomlinson, J. E. *et al.* (2020) 'Tropism, pathology, and transmission of equine parvovirus-hepatitis', *Emerging Microbes and Infections*, 9(1), pp. 651–663. doi: 10.1080/22221751.2020.1741326.

Tomlinson, J. E. *et al.* (2021) 'Pathogenesis, MicroRNA-122 Gene-Regulation, and Protective Immune Responses After Acute Equine Hepacivirus Infection', *Hepatology*, 0(0), pp. 1–16. doi: 10.1002/hep.31802.

Walter, S. *et al.* (2017) 'Differential Infection Patterns and Recent Evolutionary Origins of Equine Hepaciviruses in Donkeys', *Journal of Virology*, 91(1), pp. e01711-16. doi: 10.1128/JVI.01711-16.

Yu, A. S. L. *et al.* (2005) 'Knockdown of occludin expression leads to diverse phenotypic alterations in epithelial cells', *AJP: Cell Physiology*, 288(6), pp. C1231–C1241. doi: 10.1152/ajpcell.00581.2004.

Zehetner, V. *et al.* (2021) 'Equine Parvovirus-Hepatitis Screening in Horses and Donkeys with Histopathologic Liver Abnormalities', *Viruses*, 13(1599), pp. 1–18.

Zhang, J. *et al.* (2004) 'CD81 Is Required for Hepatitis C Virus Glycoprotein-Mediated Viral Infection CD81 Is Required for Hepatitis C Virus Glycoprotein-Mediated Viral Infection', *Journal of virology*, 78(3), pp. 1448–1455. doi: 10.1128/JVI.78.3.1448.

Zona, L. *et al.* (2014) 'CD81-receptor associations - Impact for hepatitis C virus

entry and antiviral therapies', *Viruses*, 6(2), pp. 875–892. doi: 10.3390/v6020875.