Imperial College London

Characterisation of enteric viruses in dogs

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Abstract

Human noroviruses (HuNoV) are a significant cause of viral gastroenteritis in man worldwide. Noroviruses are also associated with intestinal disease in multiple species, including dogs. Canine norovirus (CNV) was initially discovered in 2007 and the first aim of this thesis was to determine the prevalence of CNV in the UK dog population. qPCR screening of canine stool samples did not identify CNV RNA, but canine astroviruses (CaAstV) were serendipitously identified and subsequently characterized according to the second aim of this work. For serological screening, CNV virus-like particles (VLPs) to three CNV strains were produced. CNV circulation in the UK was confirmed by identification of CNV-specific antibodies in 60% of canine serum samples collected in 2012-2013.

The third aim of this thesis was investigate to CNV interactions with host cells by identifying the cellular attachment factor for CNV. Synthetic carbohydrates and canine tissue samples were used to assess the binding specificity of CNV VLPs, and it was shown that antigens of the HBGA family were recognized. Phenotyping studies then demonstrated expression of HBGAs in dogs. As HuNoV also uses HBGAs to attach to cells, this raised concerns that dogs may be susceptible to HuNoV.

Evaluating the zoonotic risk of enteric viruses in dogs was the final aim of this thesis. The susceptibility of dogs to HuNoV and hepatitis E virus (HEV) was determined by screening canine samples for the presence of HuNoV or HEV RNA and HuNoV or HEV-specific antibodies. Antibodies to both HuNoV and HEV were identified in dogs, and results confirmed HuNoV VLPs can bind to canine gastrointestinal samples. This data indicates that dogs are susceptible to HuNoV and HEV infections.

In conclusion, this thesis has provided epidemiological and molecular characterization of CNV and CaAstV, in addition to highlighting the zoonotic potential for CNV, HuNoV and HEV in dogs.

Declaration of Originality

The work presented in this thesis is my own and in no way forms part of any other thesis. This work was performed at the Section of Virology, Department of Medicine, Imperial College London, and at the Division of Virology, Department of Pathology, University of Cambridge, under the supervision of Professor Ian Goodfellow. The preliminary work on hepatitis E virus in dogs was performed jointly with Aoife McElroy, an undergraduate student from Trinity College Dublin, under my supervision.

Copyright Declaration

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Green Animal Shelter, Abington Boarding Kennels and Greenlow Kennels in Cambridgeshire. Additionally, Pet Blood Bank UK have been very generous with their donation of valuable canine blood samples. Finally my own special dog Benny has been very accommodating throughout, as well as providing much needed canine compassion.

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Abbreviations

BCA – bicinchoninic acid
BSA – bovine serum albumin
CaAstV – canine astrovirus
CECoV – canine enteric coronavirus
CHO – Chinese hamster ovary
CPE – cytopathic effect
CPV – canine parvovirus
CNV – canine norovirus
CVV – canine vesivirus
DNA – deoxyribonucleic acid
EAV – equine arteritis virus
ELISA – enzyme linked immunosorbant assay
EM – electron microscopy
FCS – fetal calf serum
FNV – feline norovirus
FUT – fucosyltransferase
FTB – fucosyltransferase B
Gal – galactose
GalNAc – N-acetylgalactosamine
GlcNAc – N-acetylglucosamine
HBGA – histo-blood group antigen
HAS – human serum albumin
HuNoV – human norovirus
IHC - immunohistochemistry
kDa - kilodalton
MOI – multiplicity of infection
MFI – mean fluorescent intensity
MNV – murine norovirus
NS – non structural
OD450 – optical density at 450nm
ORF – open reading frame
PAA – polyacrylamide
PBS – phosphate buffered saline
PBS-T – PBS-0.05% Tween 20
PCR – polymerase chain reaction
PFU – plaque forming unit
PTB - polypyrimidine tract-binding protein
qPCR – quantitative PCR
RACE – rapid amplification of cDNA ends
RdRp – RNA dependant RNA polymerase
RHDV – rabbit haemorrhagic disease virus
RNA – ribonucleic acid
RT – reverse transcription
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
TMB - tetramethylbenzidine
VLP – virus-like particle
VP1 – major capsid protein
VP2 – minor capsid protein
VPg – viral protein genome linked

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Chapter 1

Introduction

1.1 *Caliciviridae*

The *Caliciviridae* are a large and diverse family of single stranded, positive sense RNA viruses with an icosahedral capsid that can cause a variety of disease manifestations in a wide range of species. There are currently five genera of caliciviruses; *Lagovirus*, *Vesivirus*, *Nebovirus, Sapovirus and Norovirus* as presented in figure 1.1. Another four calicivirus genera have recently been proposed; *Recovirus* (Farkas *et al.*, 2008), *Valovirus* (L'Homme *et al.*, 2009), *Nacovirus* (Day *et al.*, 2010) and *Bavovirus* (Wolf *et al.*, 2011).

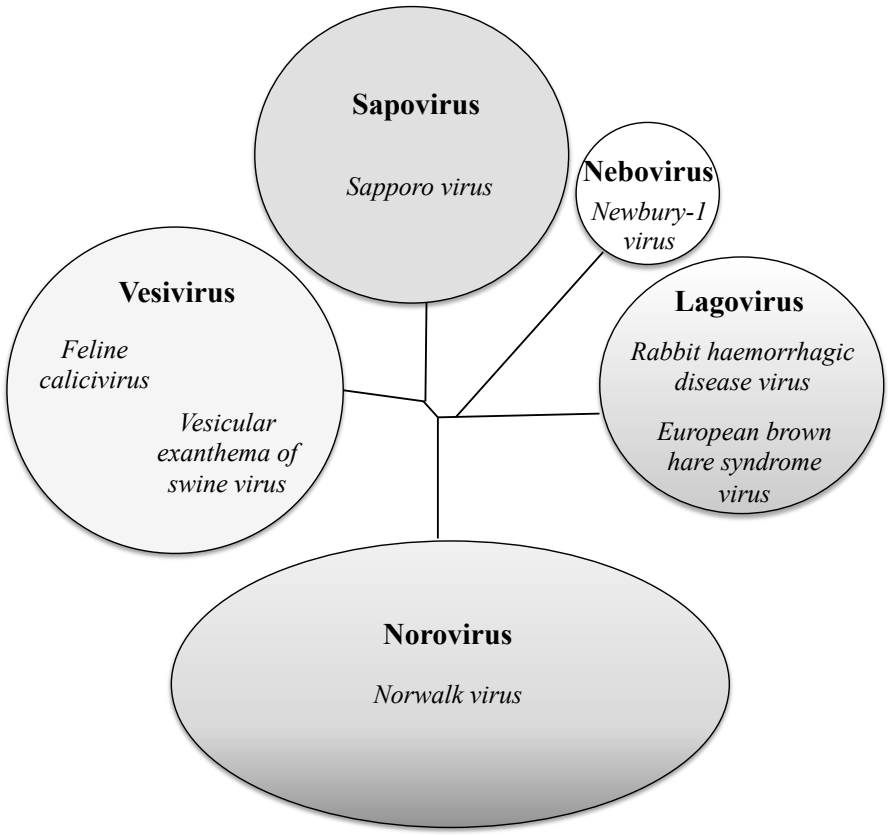


Figure 1.1 Phylogenetic relationship of the five approved calicivirus genera.
Genera are listed in bold, with italics specifying the species listed in each genera by the ninth report of the International Committee on Taxonomy of Viruses (King *et al.*, 2011). This unrooted tree was adapted from (Simmonds *et al.*, 2008), which compared the RNA dependant RNA polymerase of all caliciviruses for which a full genome sequence was available in GenBank, May 2007. The size of each genera branch is proportional to the number of sequences analysed and is not an indication of diversity.

Lagoviruses are typified by rabbit haemorrhagic disease virus (RHDV), which causes acute lethal systemic disease in rabbits (*Oryctolagus cuniculus*). The genus *Vesivirus* includes feline calicivirus, a pathogen of the upper respiratory tract in cats, and vesicular exanthema of swine virus, which results in development of vesicular lesions in infected pigs and has been associated with a related virus, San Miguel sea lion virus (Gelberg & Lewis, 1982). A single virus is classified within the *Nebovirus* genus; the bovine enteric virus Newbury-1 (Bridger *et al.*, 1984; Oliver *et al.*, 2006). The final two genera, *Sapovirus* and *Norovirus*, cause gastroenteric disease in a wide range of mammalian species (Bank-Wolf *et al.*, 2010).

1.2 Identification and classification of noroviruses

A small, round, structured virus was identified as the cause of a gastroenteritis outbreak in Norwalk, Ohio in 1968 (Kapikian *et al.*, 1972). Stool samples from infected individuals were incubated with convalescent sera from experimentally infected volunteers, and electron microscopy revealed the presence of aggregated 27nm sized particles (figure 1.2).

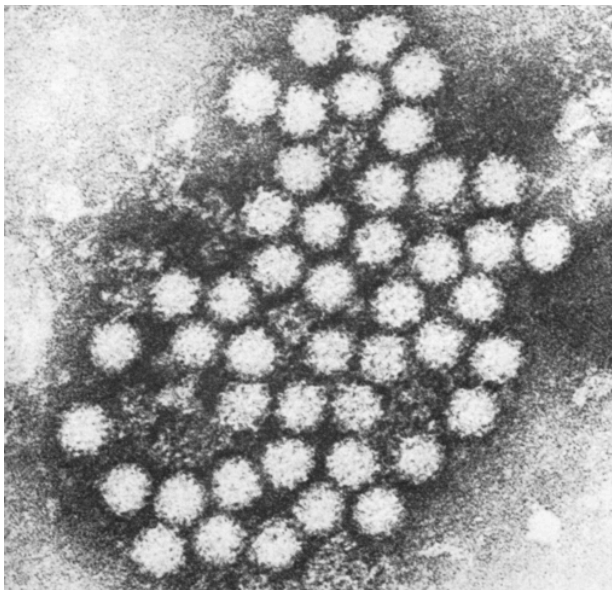


Figure 1.2 Electron micrograph of norovirus particles (Kapikian *et al.*, 1972)
A filtered stool sample from a human with clinical signs of gastroenteritis was shown to contain virus particles with a picornavirus-like morphology.

Pre-challenge sera did not induce aggregation of particles, whereas post-challenge sera did, indicating that antibodies specific for the particles were being generated. Particle morphology was similar to that of picornaviruses and parvoviruses, but the fine ultrastructure was undetermined. It was almost a decade later when Norwalk virus was classified as a member of the *Caliciviridae* family of viruses, based on protein structure and visualization of cup-shaped depressions (calyx is latin for cup) on the surface of virus particles (Greenberg *et al.*, 1981). Phylogenetic analyses later resulted in Norwalk virus being designated the type virus of a new calicivirus genera, 'Norwalk-like viruses'(Green *et al.*, 2000), renamed *Norovirus* in 2002 (Mayo, 2002).

The *Norovirus* genera is divided into at least six (Mesquita *et al.*, 2010), possibly seven (Vinjé, 2015), different genogroups based on capsid sequences, with strains of norovirus assigned to the same genogroup if the uncorrected pairwise distance of the major capsid protein is less than 44.9% (Zheng *et al.*, 2006). As shown by figure 1.3, human norovirus (HuNoV) strains fall into genogroups I, II and IV (GI, GII and GIV). The remaining genogroups are composed of noroviruses identified in other animal species; bovine noroviruses in GIII, murine noroviruses in GV and canine noroviruses in GIV/GVI and potentially GVII. Noroviruses are further subdivided into genotypes based on genetic similarities (>85% amino acid identity), with nine GI genotypes and at least 22 GII genotypes recognized at present (Vinjé, 2015).

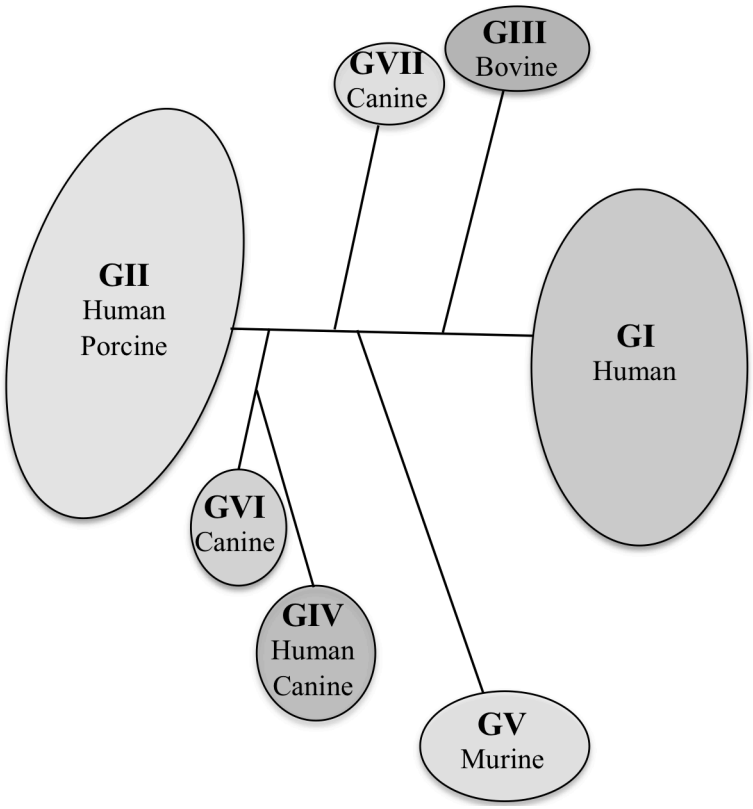


Figure 1.3 Classification of noroviruses into seven genogroups (GI-GVII).
This phylogenetic tree is based on the amino acid sequences of the capsid protein VP1, adapted from (Vinjé, 2015). The species names listed within each genogroup refer to the animal species from which noroviruses of the genogroup have been isolated. The size of each branch is proportional to the number of sequences analysed from each genogroup and is not an indication of diversity.

1.3 Genetic organization of noroviruses

Noroviruses are non-enveloped viruses with a positive sense single stranded RNA genome, approximately 7.7kb in length (Jiang *et al.*, 1990). A virally encoded VPg protein is covalently linked to the 5' end of the genome, which is polyadenylated at the 3' end. The genome has 3 open reading frames (ORFs) as shown by figure 1.4.

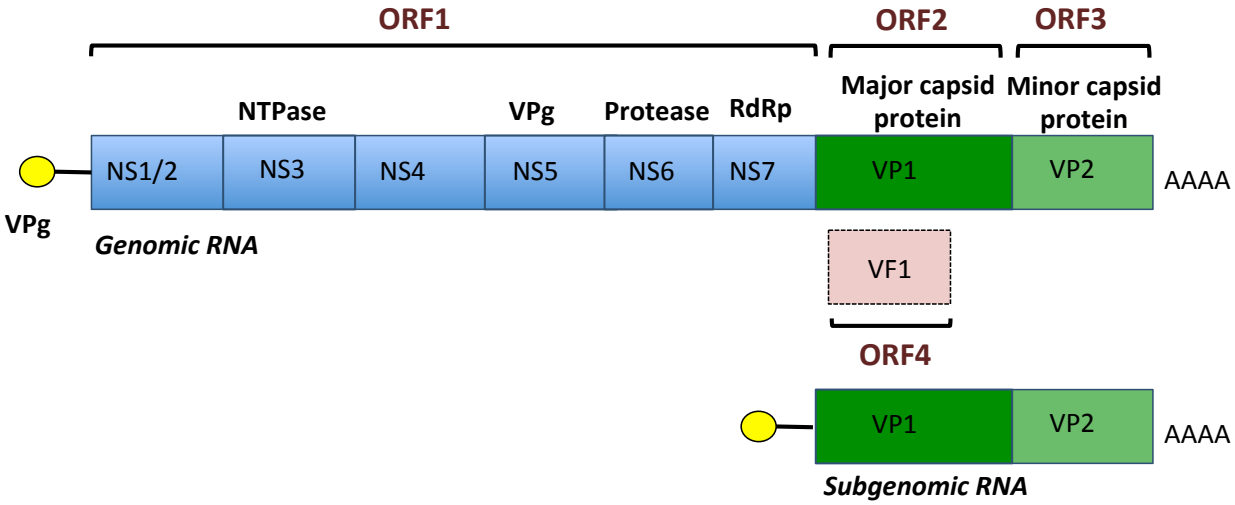


Figure 1.4 Organization of the norovirus positive-sense, single-stranded RNA genome.
The viral protein VPg is covalently attached to the 5' end of the RNA, and a polyA tail is at the 3' end. ORF1 is encoded by the genomic RNA, which consists of a polyprotein that is subsequently cleaved into six separate proteins by the NS6 protease. The structural proteins of ORF2 (VP1) and ORF3 (VP2) are encoded by the subgenomic RNA. ORF4 is an alternative reading frame only identified in MNV.

The first ORF comprises the non-structural proteins and the structural proteins of the capsid are encoded by ORFs 2 and 3. The polyprotein translated from ORF1 is cleaved into 6 proteins by the virus encoded protease (NS6) (Sosnovtsev *et al.*, 2006). Together these proteins function to replicate the positive sense genomic RNA into a negative sense copy, from which positive sense subgenomic RNA can be synthesized. ORFs 2 and 3 are then translated to produce the major capsid protein (VP1) and minor capsid protein (VP2) respectively from the subgenomic RNA.

A fourth ORF has been identified in murine norovirus (MNV), but not human norovirus (HuNoV), overlapping with ORF2 (Thackray *et al.*, 2007). A comparable ORF has been predicted in some human sapoviruses although its function is unknown (Oka *et al.*, 2015).

The product of MNV ORF4 is virulence factor 1 (VF1), which has been shown to co-localise with the mitochondria and have a role in induction of innate immunity and apoptosis in infected cells (McFadden *et al.*, 2011).

Noroviruses have an icosahedral capsid composed of 180 copies (90 dimers) of VP1. VP2 is understood to play a role in stabilization of the capsid structure and by binding to the interior surface of the capsid it has been proposed to mediate capsid assembly and genome encapsidation (Lin *et al.*, 2014; Vongpunsawad *et al.*, 2013). The 59kDa VP1 protein was first identified by purification of the virus from stools (Greenberg *et al.*, 1981). VP1 can be divided into 3 sub-domains, P1 and P2 (the protruding domains), and the S domain (shell domain) (figure 1.5) (Prasad *et al.*, 1999). The P2 domain extends above the viral surface, and consequently is the most diverse region of the genome, playing an important role in cellular interactions and immune recognition.

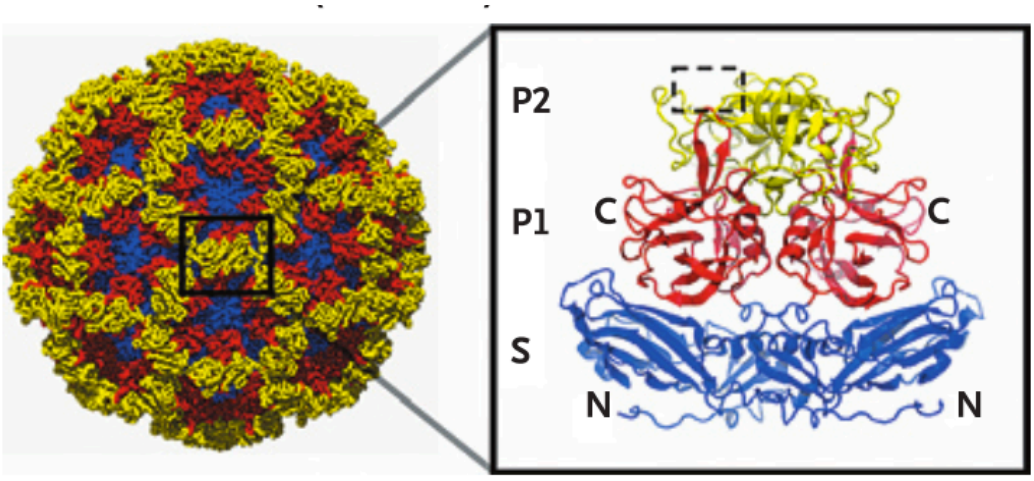


Figure 1.5 Structure of the Norwalk virus capsid (Glass & Parashar, 2009)
The image on the left shows the icosahedral capsid structure of Norwalk virus by cryo-EM. This is formed of 180 molecules (90 dimers) of the major capsid protein (VP1). A ribbon representation of a VP1 dimer is shown on the right, which is colour-coded to identify the three different sub-domains; P1 and P2 (protruding) domains and the S (shell) domain. The region highlighted by the dashed box represents the histo-blood group antigen binding region (see section 1.4.2)

1.4 Norovirus replication

1.4.1 Model systems for studying noroviruses

Characterisation of the norovirus life cycle has been significantly hampered by the inability to grow HuNoV in cell culture. Multiple attempts have been made to replicate the virus *in vitro* using purified stool sample isolates and a diverse range of cell lines and supplements (Duizer *et al.*, 2004; Lay *et al.*, 2010). Replication of HuNoV RNA has been demonstrated in human hepatoma Huh-7 cells following transfection of RNA, but even though viral particles are released into the medium, no subsequent cell entry is possible, suggesting the restriction may be at the level of receptor expression or entry pathways (Guix *et al.*, 2007). However, a possible breakthrough has recently been made using a Human Burkitt lymphoma B cell line (BJAB cells) (Jones *et al.*, 2014a). HuNoV was shown to replicate in B cells, with a 25-fold increase in viral genome copy number after five days. However this level of replication is limited compared to virus levels shed in human volunteer studies (Atmar *et al.*, 2008), and this system is yet to be replicated successfully in other laboratories.

In lieu of an efficient *in vitro* cell culture system for HuNoV, surrogate viruses have been widely utilized to probe the molecular details of this viral genus. Feline calicivirus (FCV), a member of the *Vesivirus* genus, has been a valuable model system with *in vitro* growth readily studied using a cell culture and reverse genetics system (Sosnovtsev & Green, 1995). Tulane virus, a *Recovirus,* has also been proposed as a model virus for HuNoV, capable of inducing gastroenteritis in non-human primates and with comparable genetic diversity and epidemiology to human noroviruses (Farkas *et al.*, 2014). However, the most relevant and widely used viral model for HuNoV is MNV, identified in 2003 and providing the field with the first cultivatable norovirus that can be easily studied in an *in vivo* model (Wobus *et al.*,

2004). MNV replicates efficiently in primary or immortalized murine dendritic and macrophage cells, and a range of reverse-genetics systems have been developed (Arias *et al.*, 2012; Chaudhry *et al.*, 2007; Ward *et al.*, 2007; Yunus *et al.*, 2010).

1.4.2 Binding and entry mechanisms

Many caliciviruses use carbohydrates as attachment factors to bind to cells prior to internalization. MNV and FCV recognize forms of sialic acids (Stuart & Brown, 2007; Taube *et al.*, 2009), bovine norovirus binds to alphaGal (Zakhour *et al.*, 2009) and a number of caliciviruses bind to specific carbohydrates known as histo-blood group antigens (HBGAs). The *Lagovirus* RHDV was the first virus identified as using HBGAs as attachment factors, (Ruvoën-Clouet *et al.*, 2000) and this was soon followed by the demonstration that human Norwalk virus also uses these carbohydrates (Marionneau *et al.*, 2002). Subsequent studies showed that the majority, if not all genogroup I and genogroup II HuNoVs recognize HBGAs. Most recently the Tulane virus of the recently proposed *Recovirus* genera was also shown to bind these carbohydrate structures (Farkas *et al.*, 2010).

HBGAs are terminal structures of glycan chains expressed on the surface of red blood cells in man and great apes, as well as being located on epithelial cells of the gastrointestinal, genitourinary and respiratory tracts in a wide variety of species. In addition, HBGAs can be secreted by these cells into bodily fluids, including saliva (Marionneau *et al.*, 2001). The biosynthesis of HBGAs requires the stepwise addition of monosaccharide units onto glycan chains, a process performed by specific glycosyltransferases (figure 1.6).

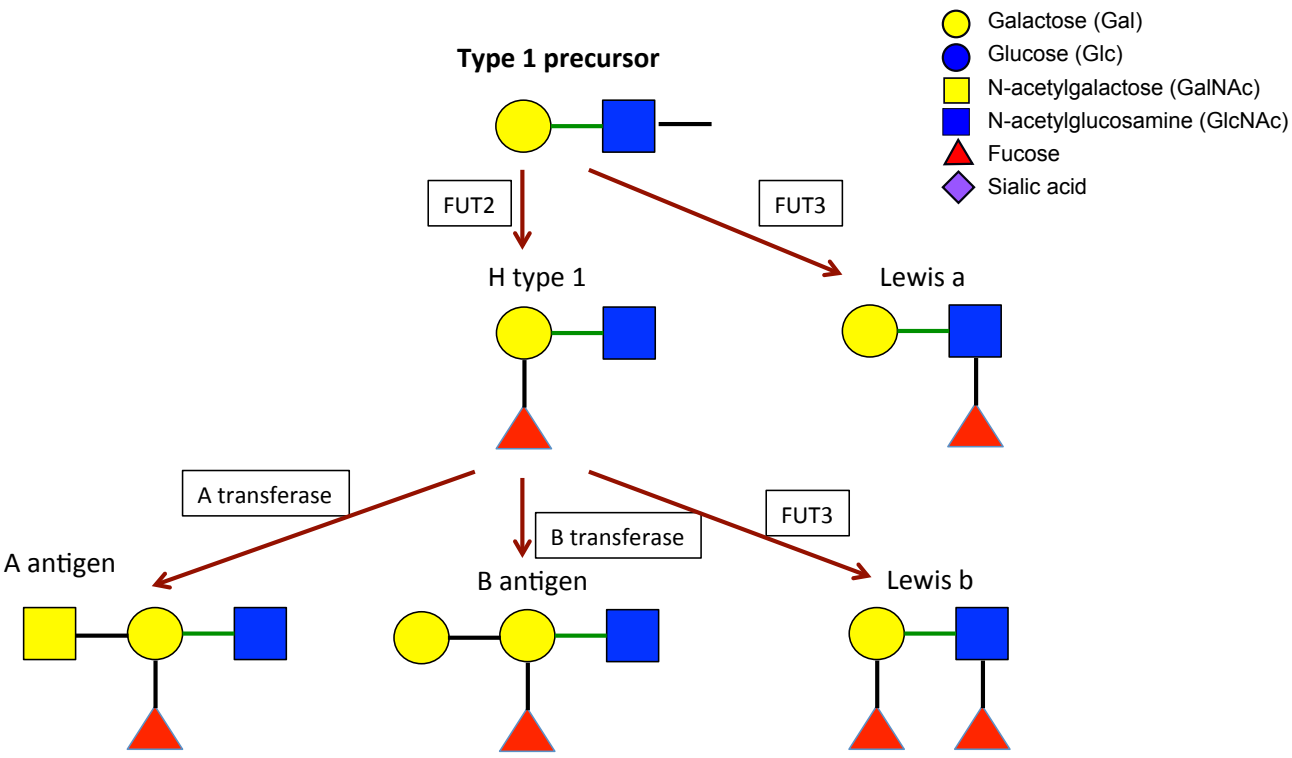


Figure 1.6 Biosynthesis of histo-blood group antigens (HBGAs) on epithelial cells.
Activity of different glycosyltransferases (labeled in boxes) results in generation of multiple oligosaccharides from the type 1 precursor. The symbol presentation of glycans is based on the nomenclature used in Essentials of Glycobiology textbook (Varki *et al.*, 2009).

HBGAs are derived from different types of precursor disaccharide structures; the type 1 precursor molecule is a galactose (Gal) joined to a N-acetylglucosamine (GlcNAc) via a β1,3 linkage. Type 2 is Galβ1,4GlcNAc. The type 3 precursor is a Galβ1,3GalNAc in α linkage to the subjacent structure (i.e. serine or threonine of the peptide chain) and the type 4 is also a Galβ1,3GalNAc, but in β linkage to the subjacent structure. Conversion of these structures into the H antigen requires action of α1,2-fucosyltransferases. On red blood cells and vascular endothelium fucosyltransferase 1 (FUT1) is active, whereas in epithelial cells FUT2 is active (Marionneau *et al.*, 2002).

Approximately 20% of Caucasians do not express a functional FUT2 gene, hence do not express HBGAs in their gastrointestinal tract and are termed 'non-secretors'. It was hypothesized that these individuals would have a significantly reduced susceptibility to infection with HuNoV (Marionneau *et al.*, 2002). Experimental challenge studies in humans later confirmed that susceptibility to HuNoV infection is related to expression of HBGAs in the gastrointestinal tract (Hutson *et al.*, 2005; Lindesmith *et al.*, 2003). However, it is worth noting that HBGA expression on cells *in vitro* is not sufficient to render the cells permissive to HuNoV infection, which suggests an extra factor or receptor is required (Duizer *et al.*, 2004).

1.4.3 Viral protein translation

Following entry of a norovirus particle into a cell, the viral RNA genome is released into the cytoplasm, and interacts with the host cell translation machinery. The genome of the *Caliciviridae* is covalently linked to the viral protein VPg, which acts as a cap substitute for translation initiation (Goodfellow *et al.*, 2005). This novel mechanism of directing the cellular translation apparatus is only confirmed for one other viral family, *Potyviridae* (Léonard *et al.*, 2000), although evidence suggests *Astroviridae* also require VPg for infectivity (Fuentes *et al.*, 2012). Norovirus VPg interacts with the translation initiation factor complex via eIF4E (Goodfellow *et al.*, 2005) and eIF3 (Daughenbaugh *et al.*, 2003), which aid in recruiting the 43S ribosomal pre-initiation complex.

The 5' and 3' ends of the norovirus genome contain highly conserved RNA structures that interact with host cell factors to drive viral replication and translation (Vashist *et al.*, 2012). A number of cellular proteins have been identified which bind to secondary structures in norovirus RNA, including La, polypyrimidine tract-binding protein (PTB) and poly(A)-

binding protein (PABP) (Gutiérrez-Escolano *et al.*, 2003). The exact roles of these proteins in controlling norovirus translation has not been defined, but La and PTB are known to drive internal ribosome entry site (IRES)-mediated translation of picornaviruses (Toyoda *et al.*, 1994), thus they may play a role in circularizing and stabilizing norovirus RNA to promote translation.

The subgenomic RNA of noroviruses (see figure 1.4) is required for translation of the viral structural proteins VP1 and VP2. It is hypothesized that this has evolved to enable production of much larger amounts of the major capsid protein than the non-structural proteins. The minor capsid protein (VP2) is translated by a termination-reinitiation mechanism, as the subgenomic RNA of norovirus is polycistronic (Napthine *et al.*, 2009). ORF4 of MNV is also translated from the subgenomic RNA; it is predicted that this is initiated by leaky scanning as the ORF4 start codon is positioned 13 bases downstream of the first AUG triplet of ORF2 in a strong Kozak context (McFadden *et al.*, 2011).

1.4.4 Genome replication

Translation of ORF1 yields a polyprotein that is co- and post-translationally cleaved by the viral protease NS6. Polyprotein cleavage generates viral non-structural proteins that play important roles in formation of the viral replication complex, although the molecular details of this process and the functions of several of the viral proteins involved (NS1-2, NS3 and NS4) have not yet been fully elucidated. Noroviruses, along with many other positive sense RNA viruses, replicate in association with host cell membranes in the cell cytoplasm (Wobus *et al.*, 2004). Studies with MNV have demonstrated membranes of the secretory pathway are involved in formation of the replication complex in the perinuclear region of

the cell, and that all of the ORF1 proteins are associated with double-stranded RNA (dsRNA), the viral RNA intermediate, during the course of infection (Hyde *et al.*, 2009).

Replication of the norovirus genome proceeds via a negative-sense RNA intermediate, which is generated by the viral RNA dependent RNA polymerase RdRp (NS7). It has been proposed that the mechanism by which RdRp activity is driven to generate the negative-sense RNA involves a *de novo* initiation strategy. The norovirus major capsid protein has been shown to enhance RdRp activity, which suggests that the early rounds of RNA synthesis utilize VP1 to drive RNA replication without requiring VPg (Subba-Reddy *et al.*, 2012). VPg-dependant RNA synthesis then follows after generation of dsRNA, from which positive sense genomic and subgenomic RNA is synthesized.

1.5 Clinical features and management of HuNoV infection

Following exposure to HuNoV, clinical signs develop after an incubation period of 10-51 hours (Glass & Parashar, 2009). Acute vomiting, abdominal cramps, watery diarrhoea and pyrexia typically occur, with symptoms lasting an average of 28-60 hours in the majority of cases. Disease can be of longer duration with greater severity in the young and elderly (Rockx *et al.*, 2002), and immunocompromised individuals (Kaufman *et al.*, 2005). In addition, HuNoV has also been associated with inflammatory bowel disease (Kolho, 2012), seizures (Bartolini *et al.*, 2011), and liver dysfunction (Nakajima *et al.*, 2012). Mortality rates in high-income countries are typically low, with an estimated 570-800 deaths in the US annually, of which a major proportion of people are over 65 years old (Hall *et al.*, 2013). However, an estimated 200,000 children die following norovirus infection in the developing world each year (Patel *et al.*, 2008).

1.5.1 HuNoV Diagnostics

Diagnosis of HuNoV infection is typically PCR based; many broad-spectrum PCR methodologies have been designed to allow amplification of small quantities of HuNoV RNA (Atmar & Estes, 2001). Serological testing is also possible to identify previous exposure to HuNoV. The lack of a cell culture system means that production of large quantities of HuNoV antigen is not possible by traditional methods, but generation of HuNoV virus-like particles (VLPs) can rapidly provide ample non-infectious antigen material for use in immunoassays. Expression of VP1 in a synthetic system results in the formation of virus-like particles as VP1 proteins spontaneously fold into the icosahedral form. This is most commonly achieved in insect cells using a recombinant baculovirus expression system (Belliot *et al.*, 2001; Jiang *et al.*, 1992). VLP production in mammalian cells is possible using a Venezuelan equine encephalitis virus replicon system in BHK cells (Baric *et al.*, 2002) or transfection of a VP1-expression vector into HEK-293T cells (Taube *et al.*, 2005). A yeast-based expression system has also shown effective VLP production (Xia *et al.*, 2007).

1.5.2 Treatment of HuNoV disease

Treatment of norovirus infections in humans is focused on maintaining hydration, either orally, or intravenously if severe. There are no licensed anti-viral drugs effective against human norovirus at present, although a number of *in vitro* studies have shown efficacy for anti-viral drugs against the Norwalk virus replicon. These include interferons, the guanosine analogue ribavirin, and the protease inhibitor rupintrivir (Chang & George, 2007; Rocha-Pereira *et al.*, 2014). Furthermore, recent animal studies have identified anti-viral drugs with efficacy against MNV *in vivo*. These include the viral polymerase inhibitor 2'-C-

methylcytidine (Rocha-Pereira *et al.*, 2013), small molecule deubiquitinase inhibitors (Perry *et al.*, 2012) and favipirivir, a broad-spectrum nucleoside analogue (Arias *et al.*, 2014).

1.5.3 HuNoV vaccines

There is currently no vaccine available to prevent HuNoV infection in humans, however two promising phase II vaccine trials have recently been published (Atmar *et al.*, 2011; Bernstein *et al.*, 2014). Generating suitable HuNoV vaccine candidates has been challenging due to the lack of a cell-culture system, unknown duration of immunity in man, and the significant heterogeneity of HuNoV strains (Debbink *et al.*, 2014). The vaccines under development are VLP-based, and whereas the first vaccine trial used a single HuNoV strain (GI.1), it is acknowledged that a multi-valent approach will be ideal and thus the second trial utilized GI.1 and GII.4 VLPs. Both studies demonstrated efficacy at reducing the severity of gastroenteritis ($p = 0.009$ / $p = 0.02$ as measured by the modified Vesikari score), although reduction in viral infection rates was only marginally significant in the first study using GI.1 as the challenge strain ($p = 0.05$), and not significant in the second study using GII.4 as the challenge strain ($p = 0.179$).

1.6 Epidemiology of HuNoV

HuNoV infections are most common in healthcare institutions such as hospitals and long term care facilities (Lopman *et al.*, 2003), but outbreaks are often reported in association with schools, restaurants, cruise ships and other settings such as military bases (Ahmed *et al.*, 2012). A seasonal distribution has been identified for HuNoV outbreaks, with an increase in reported cases each winter (figure 1.7) (Lopman *et al.*, 2003).

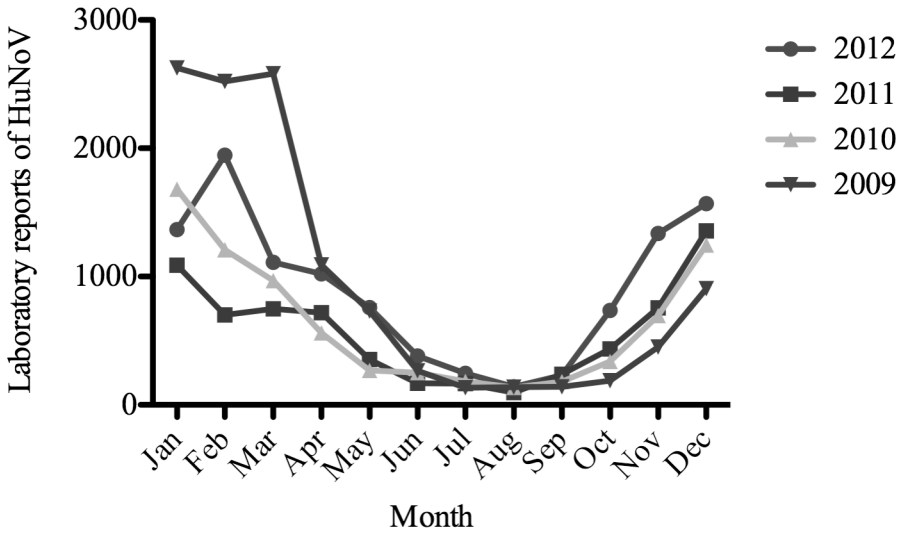


Figure 1.7 Seasonality of HuNoV infections from 2009-2012.
Data is from laboratory reports of HuNoV infections in England and Wales by Public Health England (PHE). This data comprises all faecal specimens that tested positive for HuNoV each month over the time periods listed (Public Health England, 2013).

Transmission of HuNoV is via contact with infected faeces or vomit which occurs predominantly through direct person-to-person contact, but contaminated surfaces and aerosolized vomit can transmit the virus as well (Mathijs *et al.*, 2012). Contaminated food and water are also known to play an important role in HuNoV spread, with 58% of food borne illnesses in the US attributed to HuNoV (Scallan *et al.*, 2011). As few as 18 viral particles are required for infection (Teunis *et al.*, 2008).

Although the GI Norwalk virus was the first member of the norovirus genera to be described, GII strains are now responsible for 96% HuNoV cases worldwide, with the GII.4 genotype being the most prevalent overall (Tran *et al.*, 2013). Over the past decade GII.4 strains have caused epidemics every 2-3 years, starting with emergence of the Farmington Hills strain in 2002 which was characterized following an unusual increase in HuNoV infections reported over the winter of 2002-2003 (Dingle *et al.*, 2004). Subsequent epidemic

strains include Hunter 2004, Den Haag 2007, New Orleans 2009 and most recently the Sydney 2012 strain (Beek *et al.*, 2013). Rapid nucleotide substitutions and homologous recombination are both factors contributing to the continued evolution of GII.4 strains (Eden *et al.*, 2013).

Serological surveys have shown a high frequency of HuNoV antibodies in human populations. Almost 100% seropositivity to GII.4 HuNoVs in India and UK has been reported (Menon *et al.*, 2013a) and there is a clear age-related trend in seropositivity; children less than 5 years of age have been shown to have significantly lower HuNoV antibody levels in numerous studies (Gray *et al.*, 1993; Menon *et al.*, 2013a).

1.7 Noroviruses of different species

Viruses in the norovirus genera have been identified in a range of non-human mammalian species. These have generally been associated with intestinal infection, although clinical symptoms are typically mild.

1.7.1 Bovine noroviruses

Bovine noroviruses were the first non-human norovirus to be identified (Woode & Bridger, 1978). Diarrhoea samples from calves were analysed by electron microscopy and shown to contain caliciviruses, with subsequent experiments with gnotobiotic calves confirming pathogenicity. This first bovine norovirus was designated Newbury-2 virus, (Newbury-1 calicivirus is a Nebovirus) and a second bovine norovirus, 'Jena virus' was later identified in Germany (Gunther & Otto, 1987). Bovine noroviruses have since been detected across Europe, US and in Asia (Mauroy *et al.*, 2009; Park *et al.*, 2007; Thomas *et al.*, 2014), with prevalence levels of approximately 10% of diarrhoeic calves.

Molecular characterization of bovine noroviruses resulted in formation of the third norovirus genogroup (GIII) (Oliver *et al.*, 2003). Jena virus was classified as GIII.1, and Newbury-2 virus as GIII.2. No human noroviruses are classified within GIII, and the only other species known to be susceptible to GIII noroviruses are sheep (Wolf *et al.*, 2009).

1.7.2 Porcine noroviruses

A norovirus of pigs was identified in Japan following screening of swine caecal contents for caliciviruses (4/1117 pigs positive) (Sugieda *et al.*, 1998). Porcine norovirus was classified as a GII norovirus alongside many human strains, but was shown to form a cluster separate from the HuNoV genotypes. Later studies have designated porcine noroviruses as GII.11, GII.18 and GII.19 genotypes, and these have not been associated with any clinical signs. However, a novel porcine norovirus was recently identified in piglets with diarrhoea, which caused disease in an experimental model and was shown by phylogenetic analysis to be distinct from previously identified genotypes (Shen *et al.*, 2012).

Viral RNA surveys in different regions have identified porcine norovirus at varying prevalence levels; 2% of farms in the Netherlands were positive (Poel *et al.*, 2000), whereas 9% porcine faecal samples from New Zealand (Wolf *et al.*, 2009), 18.9% samples in the US (Scheuer *et al.*, 2013), and almost 52% in Brazil were positive for viral RNA (Silva *et al.*, 2015).

Porcine norovirus VLPs have been generated for serological surveys and studies on binding preferences (Farkas *et al.*, 2005). Whereas 36% pigs sampled in Japan were seropositive, this figure rose to 71% in US pigs. Interestingly, porcine norovirus VLPs did not bind to a

range of HBGAs in human saliva samples, which is in direct contrast to human noroviruses within the same genogroup.

1.7.3 Murine norovirus

Murine norovirus (MNV) has already been discussed as a valuable model virus for studies of HuNoV. MNV was initially discovered incidentally in colony of RAG2/STAT1$^{-/-}$ mice, where it caused acute systemic disease characterized by encephalitis, meningitis, hepatitis and pneumonia (Karst *et al.*, 2003). Immunocompetent mice infected with MNV were subsequently shown to be asymptomatic, with innate immune responses limiting pathogenicity. Multiple strains of MNV have now been characterized, with up to 13% genetic variability identified and variation in the ability to persist in the murine host reported (Thackray *et al.*, 2007). Screening for this pathogen in immunocompetent mice colonies worldwide has identified this to be a highly prevalent and globally distributed pathogen, with over 20% research mice in the US seropositive for this virus (Hsu *et al.*, 2006). MNV has also been identified in non-laboratory mice, with 35% show mice in the UK testing positive by PCR (Smith *et al.*, 2012).

1.7.4 Feline norovirus

A lion (*Panthera leo*) was the first member of the mammalian family *Felidae* from which a norovirus was identified (Martella *et al.*, 2007). A 4-week old lion cub showing signs of severe haemorrhagic gastroenteritis was euthanased at a zoo in Italy, and in addition to testing positive for several pathogenic bacteria, a novel GIV norovirus was identified. A GIV norovirus with 97.9% capsid amino acid identity to the lion strain was identified five years later in domestic cats (*Felis catus*) in the US (Pinto *et al.*, 2012). A total of 6/14 cats aged 8-12 weeks from a New York shelter were positive for this feline norovirus. A second report from the US has confirmed that this virus is circulating in cats in the shelter

population (Zhang *et al.*, 2014), although at present there are no reports from other regions of the world. Both the lion and the domestic cat noroviruses have been classified as GIV.2 noroviruses based on sequence comparison with human GIV.1 noroviruses.

1.7.5 Canine norovirus

The first canine norovirus (CNV) was reported in a 2 month-old dog with a four day history of gastroenteritis in Italy in 2007 (Martella *et al.*, 2008). Sequence analysis of this novel norovirus showed it had the highest sequence identity to the GIV.2 lion norovirus (90.1% amino acid), hence it was designated a member of the GIV.2 genotype. Subsequent epidemiological studies have identified CNV in stools of dogs from Portugal (Mesquita & Nascimento, 2012b; Mesquita *et al.*, 2010), Greece (Ntafis *et al.*, 2010), the Far East (Soma *et al.*, 2014; Tse *et al.*, 2012) and the US (Azevedo *et al.*, 2012).

The pathology induced by CNV infection is very difficult to determine. CNV was detected in stool samples for 22 days in the first case reported, indicative of active infection in the gastrointestinal tract (Martella *et al.*, 2008). However, this case was co-infected with canine parvovirus (CPV), a virus proven to cause gastroenteritis by experimental infections (see section 1.9.1). Co-infections with CPV or canine enteric coronavirus (CECoV) appear to be relatively common in dogs diagnosed with CNV (Martella *et al.*, 2009; Ntafis *et al.*, 2010) thus it is difficult to attribute clinical signs to CNV alone. Despite this, CNV has been identified in association with gastroenteritis in the absence of other detectable pathogens (Martella *et al.*, 2009), but the majority of studies only screen for a limited number of other viruses and bacteria. Finally, it is important to consider the possibility of subclinical infections as CNV has been detected in the stools of healthy dogs (Mesquita *et al.*, 2010). To

definitively confirm the role of CNV in gastroenteritis in dogs, experimental infections will be required.

The prevalence of CNV in dogs with clinical signs of gastroenteritis across Europe has been estimated to be between 2.1% (Italy (Martella *et al.*, 2009)) and 40% (Portugal (Mesquita *et al.*, 2010)). A study in the US identified CNV at a prevalence of 11% in canine diarrhoea samples (Azevedo *et al.*, 2012). A seasonal distribution for CNV has been reported by Mesquita *et al*, who found significantly more CNV positive cases in the winter than in the spring and autumn months; 36% (33/91) compared to 25% (21/84) and 7% (6/81) respectively (Mesquita & Nascimento, 2012a). This is similar seasonal variation to that shown for human norovirus as discussed above and presented in figure 1.7.

Serological prevalence of CNV in Italy has been estimated using the genogroup IV.2 lion norovirus (strain Pistoia/387/06/ITA). A preliminary serological survey in Italy suggested less than 5% dogs were seropositive to GIV.2 viruses but the sample size was small (Di Martino *et al.*, 2010). As with HuNoV, CNV has yet to be cultivated in cell culture thus obtaining sufficient quantities of virus for serological screening is not possible. However, production of lion norovirus and CNV VLPs has previously been achieved and proven to be an efficient way of generating antigen for serological studies (Di Martino *et al.*, 2010; Pereira *et al.*, 2012).

The CNV strains identified and characterized to date have been genetically heterogeneous. CNV strains in Portugal had less than 65% amino acid identity to the CNV isolates in Italy, and hence the novel genogroup GVI was proposed, to also include the HuNoV strain Chiba/040502/2004/JP (Mesquita *et al.*, 2010). This diversity in CNV strains is likely to

have arisen from the ability for CNV strains to recombine with other noroviruses. Evidence for recombination between CNV strains has been reported (Martella *et al.*, 2009).

1.8 Noroviruses as potential zoonotic agents

1.8.1 Animal-specific noroviruses and man

The identification of animal-specific noroviruses with genetic similarities to HuNoV has raised questions regarding their zoonotic potential. The animal noroviruses within the same genogroups as the human strains theoretically have the greatest potential ability to infect humans, for example the GII human and swine strains, and the GIV human and feline or canine strains. However the swine, feline and canine norovirus strains are distinct from the human strains with less than 86% amino acid identity in their capsid sequences, thus the human and animal noroviruses are grouped into different genotypes. Nevertheless, noroviruses have been shown to undergo genetic recombination (Phan *et al.*, 2007) and there is concern that animal and human strains could recombine to generate novel strains. At present however, sequence data for several thousand norovirus strains obtained from infected humans have found no swine norovirus sequences (Palmer *et al.*, 2005) and no RNA of GIV canine or feline noroviruses has been detected in human samples..

Serological studies have been performed to determine whether antibodies for animal-specific noroviruses can be identified in man; humans in India had a 10.7% seroprevalence to bovine norovirus (Menon *et al.*, 2013b) and in the Netherlands a 20% seroprevalence to bovine noroviruses was detected. This proportion rose to 28% amongst Dutch veterinarians (Widdowson *et al.*, 2005). Antibody production against bovine norovirus suggests that humans can become infected with the virus, but whether bovine norovirus actually causes disease in humans has not yet been proven. No serological studies have been reported for

porcine norovirus antibodies in man. Given the close genetic relatedness between GII porcine and human norovirus strains, differentiating antibody responses to each virus is likely to be challenging.

To determine the zoonotic potential of canine norovirus, a recent survey screened a total of 493 people for anti-CNV antibodies (Mesquita *et al.*, 2013). Of the 120 participants who did not have regular contact with dogs, 5.8% were seropositive to canine norovirus. For the 373 study participants with regular contact with dogs (all were veterinarians), 22.3% people were seropositive. The authors concluded that CNV may infect humans and that veterinarians are at increased risk of exposure. However, the clinical significance of this is unknown and no active replication of CNV in man has been identified. The report by Mesquita *et al* has been followed by a second study, which focused on human seroprevalence to both human and carnivore GIV noroviruses in Italy (Martino *et al.*, 2014). Human serum samples were collected form 533 hospital patients, and samples were then screened in ELISAs for reactivity to a human GIV.1 strain VLP, and a lion GIV.2 VLP. In total 28% samples were reactive to GIV VLPs, with 20% reactive to both GIV.1 and GIV.2, and 0.9% samples reactive to GIV.2 alone. Again this is suggestive of zoonotic transmission of disease, and indicates there is likely to be a relationship between the evolution of human and animal noroviruses.

It has been hypothesized that the species specificity of noroviruses may be directed by the cell surface carbohydrates to which the virus particles bind. As discussed in section 1.4.1, HuNoVs bind to HBGAs on surface of cells, a process predicted to play a key role in cell entry. The types of carbohydrates expressed by cells of different animals can vary significantly, thus determining the binding specificity of the animal noroviruses could

provide insights into zoonotic risk. Studies on the cellular attachment factor of GIII noroviruses have identified the Galα1,3 carbohydrate as the ligand for bovine norovirus (Zakhour *et al.*, 2009). The enzyme that synthesizes the Galα1,3 motif is present in all mammalian species, with the notable exception of humans. This suggests that bovine norovirus cannot bind to human gut cells, and thus cannot cause infection, which is at odds with the serological studies described above.

1.8.2 Animal infection with human noroviruses

Multiple studies have shown that pigs are regularly exposed to HuNoV. Over half of the pigs in a US report were seropositive to both GI and GII HuNoVs (Farkas *et al.*, 2005). This finding was supported by a study that demonstrated human strains can replicate and induce an immune response in gnotobiotic pigs (Cheetham *et al.*, 2006). The hypothesis that pigs can be infected by HuNoV has been further supported by the detection of HuNoV RNA (GII.4) in pigs in Canada and Taiwan (Chao *et al.*, 2012; Mattison *et al.*, 2007) In addition, a Canadian pork retail product was positive for HuNoV, posing a speculative risk to human consumers. A GII.4 HuNoV has also been detected in stool samples from cattle (Mattison *et al.*, 2007).

On a daily basis most humans in the western world do not have close contact with cattle and pigs. However, the domestic dog (*Canis lupus familiaris*) is one of the most popular pets in the UK, with approximately 31% households owning a dog (Murray *et al.*, 2010). Various studies have shown that between 14% and 35% pet dogs sleep on their owner's beds (Chomel, 2011) thus this close relationship between dogs and their owners means that the risk of transmission from dog to human is often considered high. To date, several different enteric viruses have been suggested to spread between humans and dogs, including rotavirus

(Theamboonlers *et al.*, 2013; Wu *et al.*, 2012), hepatitis E virus (HEV) (Borgen *et al.*, 2008; Lewis *et al.*, 2008) and HuNoV.

The first report of dogs in association with HuNoV infection occurred during an outbreak of norovirus gastroenteritis in an elderly care home (Humphrey *et al.*, 1984). Just prior to development of clinical symptoms in humans, the owner's dog was sick on multiple occasions around the home. Serological testing of the dog one month after the incident revealed a moderate titre to HuNoV antigen by immune electron microscopy. This titre to HuNoV was significantly higher than that of the twenty-one control dogs. Further evidence linking dogs with HuNoV infections in man has been provided by an epidemiological study that showed seropositivity to HuNoV in children increased seven fold if there was a dog in the household (Peasey *et al.*, 2004).

More concrete evidence that dogs may be involved in the epidemiology of HuNoV has been the detection of HuNoV in stool samples from pet dogs (Summa *et al.*, 2012). Summa *et al* collected stool samples from dogs if their owners were experiencing acute gastroenteritis lasting 1-3 days. A proportion of these human gastroenteritis cases were predicted to be HuNoV. Canine stool samples were tested for the presence of GI, GII and GIV HuNoV, and four dogs were found to be positive for GII HuNoVs. The quantity of HuNoV detected from the stools in three dogs was low and could be attributed to HuNoV merely passing through the canine gastrointestinal tract and not replicating. Dogs could theoretically act as fomites if personal hygiene is not optimal. However, the fourth positive dog in this study had higher levels of HuNoV in their stools, and the strain identified was identical to that isolated from stools of the owner. This suggested that HuNoV may have replicated within the gastrointestinal tract of this single dog.

Summa et al also noted that 2 of the 4 HuNoV positive dogs showed clinical signs of gastroenteritis. However, it is not possible to infer any causality from these clinical signs, as vomiting and diarrhea can occur for a multitude of reasons in dogs. This will now be discussed in the following section.

1.9 Enteric viruses of dogs

Gastroenteritis is a very common condition in dogs, with 6% of canine veterinary consultations addressing gastroenteritis as a primary complaint (Jones *et al.*, 2014b). Furthermore, an owner questionnaire reported diarrhoea in 14.9% dogs within the preceding two-week period (Hubbard *et al.*, 2007). The causes of canine gastroenteritis can be divided into infectious and non-infectious. A change in diet or scavenging is understood to be a major cause of non-infectious diarrhea (Stavisky *et al.*, 2011), with infectious causes sub-divided into parasitic, bacterial and viral. A number of studies have sought to identify the proportion of diarrhea cases that can be attributed to viral infection, as summarized in table 1.1. Results show that viruses have been identified in 32% (Vieler & Herbst, 1995) to 83% (Mesquita *et al.*, 2010) of diarrhoea cases, with geographical variation evident. The accuracy of these figures is likely to be affected by case selection bias, as well as limitations of screening methodologies, but overall they reveal an important role for viruses in canine gastroenteritis.

Author, year	Case number	Location	Method	Overall virus	Canine parvovirus	Canine enteric coronavirus
(Baumann *et al.*, 2014)	104 d+ 43 d-	Brazil	PCR	44% d+ 7% d-	36% d+ 0% d-	11% d+ 7% d-
(Tupler *et al.*, 2012)	50 d+ 50 d-	Florida, USA	PCR /EM	NR	2% d+ 2% d-	2% d+ 18% d-
(Godsall *et al.*, 2010)	355 d+	UK	PCR	64% d+	58% d+	7.9% d+
(Mesquita *et al.*, 2010)	63 d+ 42 d-	Portugal	PCR	83% d+ 57% d-	57% d+ 35% d-	51% d+ 5% d-
(Decaro *et al.*, 2009)	156 d+	Europe	PCR	62% d+	49%	39% d+
(Martella *et al.*, 2009)	183 d+	Italy	PCR	47% d+	39.3%	19.1%
(Schulz *et al.*, 2008)	936 d+ 200 d-	Germany	EM	44% d+ 18% d-	17% d+ 0.5% d-	12% d+ 18% d-
(Vieler & Herbst, 1995)	4044 d+	Germany	EM	32%	17% d+	12% d+
(Marshall *et al.*, 1985)	157 d- 29d+	Australia	EM	NR	48% d+ 32% d-	1% d+ 4% d-

Table 1.1. Summary of epidemiological surveys identifying viruses in canine stool samples.
The presence of viruses in diarrhoea (d+) and normal stools (d) is compared. The methodology in each survey used was either PCR or electron microscopy (EM). Studies only testing for a single virus are not included, and the absence of reporting (NR) for specific viruses is recorded.

Two canine viruses are responsible for the majority of canine viral gastroenteritis worldwide as highlighted in table 1.1; canine parvovirus (CPV) and canine enteric coronavirus (CECoV). Both viruses have been experimentally proven to cause gastroenteritis in dogs (Keenan *et al.*, 1976; Meunier *et al.*, 1985). Two additional viruses have also been shown to induce gastroenteritis in an experimental setting; canine rotavirus and canine distemper virus (CDV) (Demonbreun, 1937; Kang *et al.*, 2007). CDV is part of a systemic disease syndrome and is not believed to be circulating in UK dogs (Bohm *et al.*, 2004), thus will not be discussed further. A number of other viruses have been associated with diarrhea in dogs which will next be discussed in turn.

1.9.1 Canine parvovirus

Canine parvovirus (CPV) was first identified in 1978, when outbreaks of severe haemorrhagic gastroenteritis in young dogs were reported (Thomson & Gagnon, 1978). In less than a decade the virus had spread worldwide, and this virus has a mortality rate of approximately 20%.

The *Parvoviridae* are a group of small single stranded DNA viruses, divided into two subfamilies; *Desnovirinae* infect invertebrates, and *Parvovirinae* infect vertebrates. There are eight recognised genera in the subfamily *Parvovirinae*, with CPV classified into the genus *Protoparvovirus,* The first strains of CPV to be identified were designated CPV-2 to differentiate them from CPV-1, which is a member of the *Bocavirus* genera in *Parvovirinae*, and now renamed as canine minute virus. The abbreviation CPV thus refers to the historically named CPV-2.

The CPV genome is approximately 5kb in length, and contains two ORFs which code for a total of 4 genes; 2 non-structural (NS1 and NS2) and 2 structural (VP1 and VP2). The virus replicates in rapidly dividing cells, thus targeting cells of the immune system, intestinal epithelia, and in very young dogs, myocardial cells (Meunier *et al.*, 1985). This results in the clinical signs of gastroenteritis, in addition to leukopenia and occasionally myocarditis in young puppies.

1.9.2 Canine enteric coronavirus

In 1971, CECoV was isolated from an outbreak of gastroenteritis amongst military dogs (Binn *et al.*, 1974). Infections with CECoV typically cause mild gastroenteritis only, although young dogs can suffer from more severe disease (Greene, 2012). CECoV

prevalence is generally lower than that of CPV (see table 1.1) and CECoV can be found in stools of healthy dogs.

CECoV is a member of *Coronaviridae*, of the genus *Alphacoronavirus.* Other members of this genus include transmissible gastroenteritis of swine and feline coronavirus. Coronaviruses are enveloped viruses with a single stranded positive sense RNA genome of approximately 30Kb, divided into 7 open reading frames (Pratelli, 2011). Similar to other coronaviruses, CECoV is able to undergo genetic mutations which has led to the emergence of more virulent strains, some with pantropic pathogenicity (Zicola *et al.*, 2012).

1.9.3 Canine rotavirus

Rotaviruses are members of the *Reoviridae,* a family of viruses with double stranded RNA genomes. Rotaviruses can cause gastroenteritis in a wide range of species, and are a very common cause of gastroenteritis in man with an estimated 750,000 cases in 2009 in the UK (Tam *et al.*, 2012). The first reports of rotaviruses in dogs appeared in the late 1970s (Eugster & Sidwa, 1979), and molecular characterization has shown these to be distinct from the major human strains. Rotavirus is a double stranded RNA virus, with 2 outer capsid proteins VP7 and VP4, which are used for classification into G and P genotypes respectively. There are currently 15 VP7 genotypes, and 27 VP4 genotypes. Whereas genotypes G1, G3, G4 and G9, with P[8] or P[4] predominate in human populations, canine strains have been grouped into types G3 and P[3] (Kang *et al.*, 2007; Martella *et al.*, 2001). Rotaviruses have been reported on numerous occasions in dogs with gastroenteritis (Marshall *et al.*, 1985; Tupler *et al.*, 2012), but similar to CECoV infections, clinical signs are typically mild.

1.9.4 Canine astrovirus

Astroviruses are small positive sense RNA viruses of the viral family *Astroviridae*. Canine astrovirus (CaAstV) was first reported over 30 years ago, following analysis of diarrheic canine stools by electron microscopy (Williams, 1980). However, it wasn't until 2009 that the first detailed description and molecular characterization of CaAstV infection in dogs was published (Toffan *et al.*, 2009). Since this report, CaAstV has been identified in Italy and France, Asia (China and Korea), and South America (Castro *et al.*, 2013; Choi *et al.*, 2014; Grellet *et al.*, 2012; Martella *et al.*, 2011; Zhu *et al.*, 2011). This demonstrates global distribution of this virus, with prevalence rates between 2% and 27% reported (Choi *et al.*, 2014; Grellet *et al.*, 2012). However, there are no prior reports of CaAstV from the UK and there are no commercial tests available for this virus.

The pathology caused by CaAstV infection in dogs is uncertain. A number of recent studies have identified CaAstV in the stool of healthy dogs, as well as in dogs with diarrhea. A positive association between CaAstV infection and disease has been shown in China and Italy; 12% of 183 diarrhoeic stool samples were CaAstV positive, and no CaAstV was identified in 138 healthy controls in a Chinese dog population (Zhu *et al.*, 2011). The Italian study identified CaAstV in 24.5% of 110 diarrheic samples, and 9% of the 75 healthy controls dogs (Martella *et al.*, 2011). In contrast, a study of French breeding kennels found CaAstV in 27% diarrhoeic puppies and in 19% puppies with normal faeces, demonstrating no significant association with gastroenteritis (Grellet *et al.*, 2012).

1.9.5 Canine vesivirus

Canine vesivirus (CVV), also known as canine calicivirus, was first reported and characterised almost thirty years ago (Schaffer *et al.*, 1985). Virus from a dog with diarrhoea

and neurological signs was propagated in cell culture and shown to have morphology typical of caliciviruses. This virus was later classified into the *Vesivirus* genera based on sequence analysis (Roerink *et al.*, 1999). Experimental infection of dogs with virus replicated in tissue culture did not induce any clinical signs, and hence the disease association is uncertain. The initial study to characterize CVV also conducted preliminary serological assays. It was shown that CVV did not cross-react with feline calicivirus, but a high seroprevalence was detected in dogs within the same US state as the first case identified. In contrast, a low seroprevalence to CVV was reported in a limited number of UK samples (2/25). Later studies have investigated the epidemiology of CVV in Asia; 1.7% stool samples tested were positive for CVV RNA (2 puppies with diarrhea), 57% (139/244) dogs surveyed were seropositive in Japan (Mochizuki *et al.*, 2002), and 36.5% (116/318) dogs were seropositive in Korea (Tohya & Mochizuki, 2003).

1.9.6 Novel canine viruses associated with gastroenteritis

Reports of the first canine picornavirus, canine kobuvirus, were published in 2011, following identification of the virus in canine stool samples using high throughput sequencing (Kapoor *et al.*, 2011; Li *et al.*, 2011). Picornaviruses are a large family of small icosahedral viruses with a positive sense RNA genome. Canine kobuvirus is genetically similar to the Aichi virus, a gastroenteritis-associated human picornavirus. A follow-up study of 400 dogs in the USA found 20 kobuvirus positive samples, although there was not a significant association between virus identification and the presence of gastroenteritis (Li *et al.*, 2011). Canine kobuvirus has since been reported in Korean and Italian dogs (Di Martino *et al.*, 2013; Oem *et al.*, 2014), in addition to a single case identified in the UK; this case had severe diarrhea, and no virus was found in the 147 control cases. A serological survey was also reported in conjunction with the UK case; 37.4% (74/198) canine serum samples were

positive for antibodies specific for the closely related Aichi virus (Carmona-Vicente *et al.*, 2013).

To date there are two reports of canine circovirus in association with gastroenteritis in dogs (Decaro *et al.*, 2014; Li *et al.*, 2013). *Circoviridae* have a very small single stranded circular DNA genome, and the only other circoviruses identified in mammals are the porcine circoviruses. Canine circovirus was first discovered in 2012 (Kapoor *et al.*, 2012), and has subsequently been identified in diseased and healthy dogs, so the role in disease is unknown. This virus has not been reported in the UK.

Canine sapovirus was identified by high-throughput sequencing of canine diarrhea samples in 2011 (Li *et al.*, 2011). Sapoviruses are *Caliciviridae*, and are a relatively common cause of diarrhea in man (Tam *et al.*, 2012). To date, canine sapovirus has only been identified in one subsequent report from Japan (Soma *et al.*, 2014).

The final virus to be discussed in association with diarrhoea in dogs is canine bocavirus. Bocaviruses are members of *Parvoviridae* (see section 1.9.1), and were found in a litter of dogs with severe gastroenteritis in 2014 (Bodewes *et al.*, 2014).

1.10 Project Aims

A. To determine if CNV is present in the UK dog population

There are no prior reports of CNV circulating in the UK, hence this project aimed to investigate whether CNV is a newly emerging pathogen of dogs in this country. This was achieved by surveying pet dogs for current CNV infection, as well as for evidence of previous exposure by serology.

B. To characterize any novel viruses identified in association with canine gastroenteritis

In addition to screening canine stool samples for CNV, a number of other viruses associated with gastroenteritis were surveyed. Any viruses not previously reported in the UK were characterized further through sequencing and phylogenetic analysis.

C. To identify the cellular attachment factor for CNV

It was predicted that CNV would use carbohydrate structures to attach to cells prior to infection, in a similar manner to many other caliciviruses investigated to date. A key goal was to identify and characterize the interaction of the virus capsid with host cells. Expression of the target carbohydrate was then assessed in a population of dogs.

D. To determine the zoonotic potential of enteric viruses in dogs

It has been proposed that dogs may be susceptible to enteric viruses of humans, which could pose a significant public health risk given the close relationship between dogs and man. To investigate this, the susceptibility of dogs to HuNoV and hepatitis E virus (HEV) was evaluated by a series of assays using canine clinical samples. The presence of HuNoV or HEV in canine stool samples and the presence of HuNoV or HEV specific antibodies in canine serum samples was assessed. In addition, immunohistochemistry was performed to evaluate the ability for HuNoV to bind to the canine gastrointestinal tract.

Chapter 2

Materials and Methods

2.1 Samples and Reagents

2.1.1 Canine stool samples

Stool samples were collected from dogs admitted to six participating veterinary clinics in Cambridgeshire, Kent, Lincolnshire, Middlesex and Suffolk. With owner consent, dogs were recruited to the study if they passed stools whilst hospitalized. This provided samples from dogs with a range of clinical conditions, but the sampling protocol was not designed to reflect the frequency with which gastrointestinal (GI) and non-GI disease were present in the study population. Stools were collected by veterinary personnel, then stored at -20°C until and during transportation to the laboratory, whereafter they were stored at -80°C prior to nucleic acid extraction. Stool samples were also collected from healthy dogs owned by veterinary staff at each clinic, as well as from dogs at participating boarding kennels. A limited number of diarrhoeic samples from dogs at an animal rescue centre in Cambridgeshire were also collected. Basic case data was recorded for each dog from which a stool sample was collected, including age, breed, sex, reason for admission to the veterinary clinic if relevant, and any recent history of enteric disease.

2.1.2 Canine serum samples

Serum samples were obtained from four separate dog populations. Samples from 1999-2001 were collected by the Royal Veterinary College (RVC) from a rehoming kennel as part of an existing study (Erles *et al.*, 2003). The second and third group of samples were collected in 2012-2013, obtained from either the diagnostic service of the RVC, or from the UK Pet Blood Bank. These sera were collected from pet dogs that were either veterinary patients from which blood was collected for biochemical analysis for various reasons, or they were healthy blood donor dogs. The fourth group of dogs from which sera was collected all had a

histological diagnosis of hepatitis and had been recruited to a previous study between 2006 and 2010 (Bexfield *et al.*, 2011).

2.1.3 Canine gastrointestinal tissue samples

Canine tissue samples were donated by a pharmacological research company from six healthy 18-month old female dogs, (labeled A-F) humanely euthanased as surplus to industry research requirements. Sections of the gastrointestinal tract (1 cm^2) were dissected from the duodenum, jejunum, ileum, caecum and colon and placed into 90% ethanol fixative to best preserve the carbohydrate structures. Samples were then incubated at 4°C for 24 h prior to embedding in paraffin and sectioning by the Department of Pathology Histology Service, University of Cambridge. Additional 1 cm long sections from the duodenum, jejunum, ileum, caecum and colon were dissected, rinsed in PBS, opened and scraped into lysis buffer (GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Aldrich)) containing β-mercaptoethanol and guanadinium. The tissue scrapings were homogenized and boiled for 10 minutes prior to storage at -20°C. The mannose-binding lectin Concavalin A was used to confirm that comparable levels of carbohydrates were present in each scraping sample by ELISA (see section 2.6).

2.1.4 Canine and human saliva samples

Canine saliva samples were collected from twenty-three dogs at Wood Green Animal Shelter, Huntingdon UK (numbered 1-23), and a further three samples were collected from three of the dogs at a pharmacological research company in the UK (labeled D, E and F). The dogs at the animal shelter were typically mixed breeds, whereas the research dogs were beagles. Sample collection was achieved using a children's swab (Salimetrics, Newmarket, UK), from which saliva and buccal cells (for DNA samples) were extracted. Collection of canine saliva samples was a non-regulated procedure, hence ethical approval was not

required. Human saliva samples were collected as part of a previous study (Airaud *et al.*, 2005), approved by the Nantes University Hospital Review Board (study no. BRD02/2-P), with informed written consent obtained from all saliva donors.

2.1.5 Antibodies

Anti-CNV polyclonal antibody was generated by serial inoculation of two rats with CNV VLPs (University of Nantes, animal experimentation core facility). This was approved by the national ethic review board from the French Ministry of 'Enseignement Supérieur et de la Recherche' (project license number 01322.01). The animal care and use protocol adhered to the European Directive number 2010/063 and to the national French regulation (Décret n°2013-118 du 1er février 2013 relatif à la protection des animaux utilisés à des fins scientifiques). Recognition of target VLPs using the antibody generated was confirmed using ELISA.

Antibodies utilized to detect HuNoV VLPs included anti-GI.1 HuNoV (rabbit 130) and anti-GII.4 HuNoV (rabbit 132), generously donated by Jacques Le Pendu (University of Nantes, France). For HEV VLP detection, anti-HEV ORF2 (pig), a gift from Sue Emerson (NIH, Bethesda, USA), and HEV serum from an HEV infected human patient (Hamid Jalal, Public Health England) were available. Antibodies used for phenotyping canine gastrointestinal samples were also donated by Jacques Le Pendu and included mouse monoclonal anti-A antibody 2A21, mouse monoclonal anti-B antibody B49, and mouse monoclonal antibodies 7-Le, 2.24LE, 3E1 and 12-4 for Lewis antigen expression.

2.2 Nucleic acid extraction from clinical samples

2.2.1 Stool samples

Stools were diluted 10% w/v in phosphate-buffered saline, pH 7.2, and solids were removed by centrifugation at 8000 x g for 5 min. Viral nucleic acid was extracted from 140µl of each clarified stool suspension by the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Aldrich) according to the manufacturers' instructions.

An internal extraction control was added to each sample during nucleic acid extraction to verify removal of PCR inhibitors and enable precise quantification of viral nucleic acid. A fixed amount of Equine Arteritis Virus (EAV) RNA was added with the lysis buffer to each sample to obtain an EAV concentration of approximately $1x10^8$ copies per ml of faecal suspension.

2.2.2 Buccal swabs

Canine DNA was extracted from the buccal epithelial cells collected by the Salimetric children's swab. The swab was incubated with 500µl lysis buffer plus 5µl β-mercaptoethanol, then 10µl proteinase K in 590µl RNAse free water was added and the sample vortexed. Samples were then incubated at 55°C for 10 min, then the swab plus liquid was placed in a filter column and centrifuged at 15,000 x g for 15 s. The supernatant was added to a binding column and the remainder of the extraction protocol followed as per the manufacturers instructions of a commercial kit (GenElute™, Sigma Aldrich).

2.2.3 Liver samples

Canine liver samples were homogenized in 500µl of lysis buffer and β-mercaptoethanol with 1mm silica beads (BioSpec products) using a reciprocating homogeniser (FastPrep-24, MP

Biomedicals). RNA was extracted using the commercial kit as previously described, then 5µl was treated with 5 units of DNase (Roche).

2.3 In-vitro transcription of viral RNA

In-vitro transcription of GI and GII HuNoV, and hepatitis E virus (HEV) RNA was required to generate positive controls for the RT-qPCR assays. The plasmids encoding the full-length genome of each virus, and the respective restriction enzymes required to linearize these are listed in table 2.1.

Virus	Plasmid	Restriction digest enzyme
GI.1 HuNoV	pNV101	MluI
GII.4 HuNoV	pUC57: GII.4-FLC#3	NgoMI(V)
HEV	p6 Kernow	MluI

Table 2.1 Plasmids used for in-vitro transcription of viral RNA

All restriction enzyme reactions were carried out at 37°C for 90 min and the DNA purified with the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). DNA (250-1000ng) was then added to 0.1M HEPES (pH 7.5), 32mM MgAcetate, 40mM dithiothreitol (DTT), 2mM spermidine, 7.5mM ATP, CTP, GTP, and UTP (each), 2.5µg T7 polymerase, and 80 units of RNaseOUT (Life Technologies) in a 50µl volume. The *in vitro* transcription reactions were carried out at 37°C for 2 h. Afterwards, the reaction mixtures were incubated with 20 units of DNase I (Roche) at 37°C for 30 min. The RNA was then purified by ethanol precipitation and resuspended in RNA storage solution (Ambion). The RNA was quantified using spectrophotometry and stored at -20°C until further use.

2.4 Polymerase Chain Reactions

2.4.1 One-step RT-qPCR screen

A TaqMan based RT-qPCR assay was designed to detect the presence of viral nucleic acid in material extracted from canine stool samples. All six CNV sequences listed in Genbank in August 2012 (table 2.2) were used to design a CNV specific primer-probe (PrimerDesign Ltd, table 2.3).

CNV strain	GenBank Accession Number
GIV.2/Bari/170/07-4/ITA	EU224456.1
GVI.1/Bari/91/2007/ITA	FJ875027.1
FD210/2007/Ita	JF939046
FD53/2007/Ita	JF930689
C33/Viseu/2007/PRT	GQ443611.1
Thessaloniki/30/2008/GRC	GU354246.1

Table 2.2 CNV strains used to design the CNV primer-probe

Samples were also screened for HuNoV (GI and GII), and two other canine enteric viruses known to be circulating in the UK (CPV and CECoV). Primer-probes used are listed in table 2.3, as well as the primer-probe sequence used to detect the internal extraction control, equine arteritis virus (EAV).

A 1-step RT-qPCR protocol was used to improve ease and efficiency of sample handling. 2µl of extracted RNA was added to 2x Precision OneStep RT-qPCR MasterMix (PrimerDesign Ltd), 6pmol/µl primers, and 3pmol/µl probe. The thermal cycle protocol used with a ViiA7 qPCR machine (AB Applied Biosystems), was as follows: 55°C for 30 min, inactivation of reverse transcriptase at 95°C for 5 min, and then 40 cycles consisting of denaturation at 95°C for 15 s, then annealing and elongation at 60°C for 1 min.

Virus	Primer/Probe	Ref
Canine norovirus (CNV)	F: GCTGGATGCGGTTCTCTGAC R: TCATTAGACGCCATCTTCATTCAC Probe: FAM-AGCGAGATTGCGATCTCCCTCCCACAT-BHQ	This thesis / (Caddy *et al.*, 2013)
Canine parvovirus (CPV)	F: AAACAGGAATTAACTATACTAATATATTTA R: AAATTTGACCATTTGGATAAACT Probe:VIC-TGGTCCTTTAACTGCATTAAATAATGTACC-BHQ	(Decaro *et al.*, 2005)
Canine enteric coronavirus (CECoV)	F: TTGATCGTTTTTATAACGGTTCTACAA R: AATGGGCCATAATAGCCACATAAT Probe: Cy5-ACCTCAATTTAGCTGGTTCGTGTATGGCATT-BHQ	(Decaro *et al.*, 2005)
Human GI norovirus (HuNoV GI)	F: CGYTGGATGCGNTTYCATGA R: CTTAGACGCCATCATCATTYAC Probe: FAM-AGATYGCGATCYCCTGTCCA-TAMRA	(Kageyama *et al.*, 2003)
Human GII norovirus (HuNoV GII)	F: CARGARBCNATGTTYAGRTGGATGAG R: TCGACGCCATCTTCATTCACA Probe: FAM-TGGGAGGGCGATCGCAATCT-TAMRA	(Kageyama *et al.*, 2003)
Hepatitis E virus (HEV)	F: GGTGGTTTCTGGGGTGAC R: AGGGGTTGGTTGGATGAA Probe: FAM-TGATTCTCAGCCCTTCGC-TAMRA	(Jothikumar *et al.*, 2006)
Equine arteritis virus (EAV, internal control)	F: CATCTCTTGCTTTGCTCCTTAG R: AGCCGCACCTTCACATTG Probe:Cy5.5-CGCTGTCAGAACAACATTATTGCCCAC-BHQ2	(Scheltinga *et al.*, 2005)

Table 2.3 Primers and probe sequences used in the one-step RT-qPCR screen of canine stool samples for enteric viruses.

Standard curves were generated using positive control amplicons for all viruses in the RT-qPCR screen. Amplicons were serially diluted 10-fold (10^6-10 copies/reaction) to determine the detection limit of each assay.

2.4.2 Two – step qPCR screen

cDNA was generated by reverse transcription using MMLV reverse transcriptase enzyme (Life Technologies) and random hexamers (Life Technologies) with the reaction performed at 42°C for 1 h, followed by an inactivation step at 70°C for 10 min. qPCR was performed using primers listed in table 2.4.

Primer target	Name	IGUC	Sequence (5'-3')	Reference
CaAstV RdRp	625F-1	607	GTACTATACCRTCTGATTTAATT	(Martella *et*
	626R-1	608	AGACCAARGTGTCATAGTTCAG	*al.*, 2011)
Calicivirus RdRp	P289	574	TGACAATGTAATCATCACCATA	(Jiang *et al.*,
	P290	575	GATTACTCCAAGTGGGACTCCAC	1999)
CNV strain HK VP1	-	432	RHYATTGACCCCTGGATW	This thesis
		433	TMYTKGCDGGGAATGCGTT	
CVV VP1	-	501	AAGATGTACACTGGDTGGTCT	This thesis
		502	GTGTCRTGCATRTCRTGCCAM	

Table 2.4 Primers used in the two-step qPCR screen of canine stool samples for enteric viruses. Previously published identities are listed in the 'name' column. IGUC column refers to the number of the primer as listed in the Goodfellow lab database.

qPCR reactions were prepared using the MESA Blue qPCR MasterMix Plus for SYBR Assay (Eurogentech). Briefly, 2µl cDNA was mixed with 2X MasterMix and 0.5µM primers, then incubated at 95°C for 10 min. The thermal cycle protocol used with a ViiA7 qPCR machine (AB Applied Biosystems) was as follows: 40 cycles of 94°C, 15 s; 56°C, 30 s; 72°C, 30 s, followed by generation of a melt curve. Positive controls were available for all four primer sets used; sample 8 for CaAstV, HuNoV DNA transcribed from the full length genome for calicivirus, and the CNV strain HK and CVV primers designed for this survey were designed to target conserved regions of VP1 to enable to use of pTriex1.1 plasmids expressing the relevant VP1 to be used as positive controls.

2.4.3 Conventional PCR

The capsid of all positive CaAstV samples was amplified from cDNA synthesised using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol with 0.5µM AV12 primer TTTTTTTTTTTTTTTTTTGC. The PCR reaction was performed using KOD hot start polymerase (EMD Millipore), with reverse primer s2m-rev CCCTCGATCCTACTCGG, and the forward primer 625F-1 from the original qPCR assay. The amplification programme consisted of an initial 5 min step at 95°C, followed by 35

cycles with 95°C for 20 s, 58°C for 30 s and 72°C for 90 s. A final elongation step at 72°C for 5 min was performed, followed by chilling to 4°C.

To obtain sufficient DNA for sequencing, PCR products were subsequently cloned into pCR-Blunt™ using the Zero Blunt PCR Cloning Kit (Life Technologies) according to the manufacturers protocol. Sequencing of the 5' and 3' regions of the plasmid insert was performed using pCR-Blunt™ specific primers by the University of Cambridge Biochemistry DNA Sequencing Facility. Sequencing primers for the central region of insert were then designed based on the primary sequence data to give a 200nt overlap with each predecessor, and a second round of sequencing reactions was performed (sequencing primer details available in appendix). The complete capsid nucleotide sequence generated using this method was then confirmed by sequencing of PCR products generated from cDNA directly. The same PCR and cloning methodology was used to generate the full length sequence of two CaAstV isolates.

2.4.4 Rapid Amplification of cDNA ends (RACE)

Sequences at the extremity of the CaAstV viral genome were determined using 5' and 3' RACE, utilizing a kit (Life technologies) according to the manufacturers instructions, and gene specific primers designed for this purpose: 5' RACE ATGCAGCGACAAACACAACA and 3' RACE CACAGCCCATTGAAGATG.

2.4.5 Canine α1,2-fucosyltransferase gene amplification

Nucleotide sequence alignment of human α1,2-fucosyltransferase (*FUT2*) gene and the predicted canine *Fut2* gene (GenBank accession number XM_005616863.1) enabled design of primers to target the canine *Fut2* gene and allow amplification of the entire gene for sequencing. The predicted canine *Fut2* gene has an 87.8% identity with human *FUT2*. The

primers IGUC0496 TCCATCCYCCGAGCTAAC and IGUC0497 TCTGTTACTTGCCGCCCAAAGCAT were used to amplify a region of DNA 1020bp in length. Sequencing of PCR products was performed using primers IGUC0496, IGUC0497 and an additional primer GGTACTCCTCCTCCATCCAGTCGT by the University of Cambridge Biochemistry DNA sequencing facility.

2.5 Virus-like particle (VLP) production

2.5.1 Generation of recombinant baculoviruses

Three different CNV strains with maximum sequence difference were selected for VLP production. The sequences of the three CNV-VP1 genes were obtained from GenBank; GIV.2/Bari/170/07-4/ITA, C33/Viseu/2007/PRT, and GVI.1/HKU_Ca035F/2007/HKG, referred to as strains 170, C33 and HK respectively. Restriction enzymes sites were designed (5' NotI for all strains, 3' BbsI for CNV strains 170, C33 and 3' BsaI for CNV HK) to enable later ligation into the baculovirus transfer vector pTriex1.1. Sequences were synthesized by BioBasic Inc. in the vector pUC57. CNV-VP1 sequences were digested from pUC57 and re-ligated into pTriex1.1 that had been digested with NcoI and NotI (NEB). The correct sequence for all three CNV-VP1 inserts was confirmed by sequencing. Recombinant baculoviruses were synthesised using the *flash*BAC baculovirus expression system as per the manufacturers instructions (Oxford Expression Technologies). Stock viruses were generated and titrated in Sf9-ET cells (Hopkins & Esposito, 2009) and stored in the dark at 4°C.

2.5.2 VLP production and purification

VP1 protein expression was performed in Hi5 insect cells (Invitrogen). Briefly, $1x10^7$ Hi5 insect cells were seeded into 10 x T150 flasks then infected with recombinant baculovirus at

a multiplicity of infection of 5 PFU/cell. Infections were allowed to proceed for 6 days prior to protein harvest and VLP purification. VLP purification was performed following a previously published protocol (Belliot *et al.*, 2001). VLP was released from infected Hi5 cells by freeze-thaw, followed by clarification to remove cellular debris (6000 x g, 30 min) then baculovirus removal (14,000 x g for 30 min). VLPs were partially purified through a 30% w/v sucrose cushion in TNC buffer (50mM Tris HCl pH7.4, 150mM NaCl, 10mM $CaCl_2$) containing the protease inhibitor leupeptin for 150,000 x g for 2 h. The pelleted VLP was resuspended in TNC and further purified by isopynic centrifugation in caesium chloride (150,000 x g, 18 h). The resultant VLP bands were collected by puncture and the solution containing VLPs was dialysed against PBS prior to quantification by BCA protein assay (Thermo Scientific) and storage at -80°C.

2.6 ELISA procedure

2.6.1 Detection of serum antibodies

Ninety-six-well polystyrene microtiter plates (Nunc maxisorb, Fisher Scientific) were coated overnight at 4°C with 25ng VLP in 0.05 M carbonate/bicarbonate buffer (pH 9.6). If different VLP samples were pooled together, 25ng of each VLP was coated, giving a maximum of 100ng VLP per well. Plates were washed three times with 0.05% Tween 20 in phosphate buffered saline (PBS-T) before blocking in 5% skimmed milk-PBS-T for 1 h at 37°C and then three PBS-T washes. Plates were then incubated for 3 h at 37°C with 1:50 dilution of each serum sample in duplicate in 5% skimmed milk-PBS-T. Pooled human sera (Sigma Aldrich), diluted 1:400, and 100ng pooled GII HuNoV VLPs were used as a positive control for the norovirus ELISAs, and HEV-positive pig sera diluted 1:1000 and 25ng HEV VLPs were used for the HEV ELISAs. After three washes with PBS-T, 50µl of horseradish peroxidase (HRP)-conjugated anti-canine, porcine or human IgG antibody (Sigma Aldrich)

diluted 1:5000 in 5% milk PBS–T, was added to each well and incubated at 37°C for 1 h. The plates were washed four times with PBS-T and bound antibody detected with 50µl tetramethylbenzidine (TMB, Sigma Aldrich) followed by incubation at room temperature for 10 min. The reaction was stopped with 1N H_2SO_4 and the optical density (OD) was read at 450nm (Spectromax M2 plate reader, Molecular Devices).

To eliminate the possibility that non-specific components of the VLP preparation were identified by the canine sera, an antigenically distinct vesivirus 2117 VLP was included in the assay. The OD450 of a selection of serum samples incubated on either carbonate/bicarbonate buffer coated wells or vesivirus 2117 coated wells was highly comparable. This confirmed that no non-specific reactivity relating to the VLP preparation was occurring. The background signal for each sample was hence determined by measuring the OD450 of serum samples incubated with carbonate/bicarbonate buffer alone. Background signal was then subtracted from the OD450 of VLP coated wells to generate the corrected OD450 value. A threshold value was established as the mean of the OD450 of all buffer coated cells plus 3 standard deviations. A serum sample was considered positive when the corrected OD450 was higher than the threshold. Any serum samples showing a positive response to pooled CNV VLPs were subjected to further testing with individual CNV VLPs. Plates were coated with 25ng of individual VLPs in carbonate/bicarbonate buffer and the protocol then repeated as above.

2.6.2 Competition ELISAs

Evaluation of serological cross reactivity between different norovirus strains was achieved using VLP competition assays. Plates were coated with 25ng/well of VLP overnight at 4°C. Positive test sera was incubated with a range of concentrations of either HuNoV VLPs, or

CNV VLPs (0.5, 1, 2 and 4μg/ml) for 1 h at 37°C. Vesivirus 2117 VLP was incubated with the test sera as a negative control. After the incubation period, 50μl of each serum-VLP combination was added to the previously VLP coated plates. The remainder of the ELISA protocol was followed as detailed above.

2.6.3 Synthetic oligosaccharide assays

Synthetic oligosaccharides as polyacrylamide (PAA) and human serum albumin (HSA) conjugates were coated at 10μg per well onto Nunc Maxisorp immunoplates in 100mM carbonate buffer pH 9.6 by overnight incubation at 37°C in a humidified atmosphere. A full list of the synthetic oligosaccharides used, their conjugate molecule and their graphical structures is presented alongside the relevant data in section 5, table 5.1. For assays using saliva and duodenal samples, these were coated onto immunoplates at a 1:1000 dilution using the same reagents and conditions. Blocking of VLP binding with synthetic oligosaccharides was achieved by pre-incubating 10μg/ml VLPs with 400μg/ml oligosaccharides for 1 h at 37°C. VLPs were then added to wells coated with duodenal samples and the ELISA procedure followed as before.

2.6.4 Saliva phenotyping assays

The saliva phenotyping assay used the ELISA protocol as detailed above, but with the following variations. Following the coating of saliva samples onto immunoplates overnight, A antigen was detected using a mouse monoclonal anti-A antibody, 2A21, and B antigen was detected using a specific mouse monoclonal B49, a B-specific broadly reacting antibody as previously described (Nyström *et al.*, 2011). Lewis antigen expression was investigated using mouse monoclonal antibodies 7-Le, 2.24LE, 3E1 and 12-4. H antigen expression was determined using HRP conjugated Ulex europaeus-I (Sigma-Aldrich, St. Louis, MO).

Secondary HRP conjugated anti-mouse (Uptima/Interchim, Montlucon Fr) was used for A, B and Lewis antigen detection.

1,2α-L-fucosidase (*Bifidobacterium bifidum*) treatment of duodenal samples was performed by incubation at 37°C with 10µg fucosidase in 100 mmol/L, pH 6.5, sodium phosphate buffer for 1 h. Blocking the wells with 5% milk in PBS-T followed. The enzyme shows exquisite specificity for α1,2-linked fucose residues (Katayama *et al.*, 2004; Nagae *et al.*, 2007)

2.7 SDS-PAGE and western blot analysis

VLPs were heated to approximately 100°C for 5 min in the presence of SDS loading buffer and electrophoresed on 12.5% SDS-polyacrylamide gels. For Coomassie blue staining, the gels were incubated with Coomassie Blue for 1 h at room temperature prior to de-staining.

For western blotting, proteins were transferred from SDS-polyacrylamide gels to polyvinylidene difluoride membranes. The membranes were blocked for 1 h at room temperature with 5% milk in PBS–T and then incubated overnight at 4°C with serum samples diluted 1:1000. The excess antibody was washed three times in PBS–T and incubated for 1 h with anti-canine, human or porcine IgG secondary antibody conjugated to horseradish peroxidase (Sigma Aldrich). After washing away excess secondary antibody, the bands were detected using enhanced chemiluminescence reagent (GE Healthcare).

2.8 Tissue samples and immunohistochemical analysis

Tissue sections from the gastrointestinal tract of six dogs were de-paraffinated through baths of LMR-SOL (1-Bromopropane, 2-Methylpropane-2-ol and Acetonitrile) followed by re-

hydration with successive baths of 100, 90, 70 and 50% ethanol. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in PBS. Non-specific binding was blocked with 3% BSA in PBS. HRP conjugated Ulex europaeus-I (Sigma-Aldrich, St. Louis, MO) at 0.8μg/mL, anti A monoclonal antibody 2A21 and anti B monoclonal antibody B49 were used for binding to H antigen, A antigen and B antigen respectively. Lectins and antibodies were incubated with the tissue sections in 1% BSA in PBS at 4°C (lectin) or 37°C (antibodies) overnight. After three washes in PBS, a biotinylated anti-mouse antibody (Vector laboratories, Burlingame, CA) diluted in 1% BSA in PBS was added to the assays with primary mouse antibodies. Sections were washed three times in PBS prior to addition of HRP-conjugated avidin D (Vector laboratories, Burlingame, CA) also diluted in 1% BSA in PBS. Substrate was added to the slides (AEC kit, Vector laboratories, Burlingame, CA) followed by Mayer's haematoxylin solution (Merck, Whitehouse Station, NJ) for contrast staining.

To assess the ability for VLPs to bind to tissue sections, the above protocol was adapted as follows. After sections were blocked with 3% BSA in PBS, 1μg/ml VLPs were incubated with the sections overnight at room temperature. Anti-CNV primary antibody was then incubated with the tissue sections for 1 h at 37°C. After three washes in PBS, sections were incubated with secondary anti-rat biotinylated antibody (Vector labs) for 1 h and the remainder of the protocol completed as previously described. Fucosidase treatment was performed on some sections after the initial blocking step in 3% BSA by incubation at 37°C with 10μg fucosidase in 100 mmol/L, pH 6.5, sodium phosphate buffer for a total of 18 h with a renewal after 6 h.

2.9 Flow Cytometry Analysis

The binding of norovirus VLPs to cells *in vitro* was assessed using HT-29 (human colorectal adenocarcinoma), a cell line with well characterized H antigen and A antigen expression. A total of 2.5 x 10^5 viable cells were incubated with 10µg/mL VLPs in PBS-0.1% BSA for 1 h at 4°C. After 3 washes with the same buffer, a 30 min incubation was performed with anti-VLP antibody. After washings, a third incubation was performed with biotinylated anti-rat secondary antibody under the same conditions. The final incubation step used streptavidin phycoerythrin (BD Pharmingen). After final washings in the PBS alone, fluorescence analysis was performed on a FACSCalibur (Becton-Dickinson, Rungis, France) by using the CELLQuest program. Blocking of VLP binding with synthetic oligosaccharides was achieved by pre-incubating 10µg/ml VLPs with 400µg/ml oligosaccharides for 1 h at 37°C. Fucosidase treatment of cells was achieved by incubation at 37°C with 10µg fucosidase in 100mmol/L, pH 6.5, sodium phosphate buffer for 1 h.

2.10 Tissue culture protocols

2.10.1 Primary cell establishment

Fresh 3 x 2cm sections of canine duodenum were placed into ice cold Opti-MEM media (Gibco®), then cut open and washed five times in ice cold fortified medium (Opti-MEM supplemented with murine epidermal growth factor (20ng/ml), insulin from bovine pancreas (10µg/ml), hydrocortisone 21 hemisuccinate sodium salt (150nM)). Tissue sections were then disrupted with trypsin-EDTA for 5 min at room temperature, before the mucus and villi on the luminal surface were removed by scraping. A second scraping was next performed to collect the tissue layer required for the study, which was transferred to fortified medium in a 50ml tube. Samples were centrifuged at 235 x g for 2 min, then the pellet washed with fortified media. The pellet was next digested with a 1:1 solution of collagenase type I and

dispase (Sigma) for 30 mins at 37°C. The remaining pellet was centrifuged on a 2% sorbitol gradient in fortified media supplemented to 2.5% fetal calf serum (FCS) twice at 235 x g. The pellet was resuspended in fortified media supplemented with 10% FCS to a density of approximately 5 x 10^5 cells/ml. Finally, cells were seeded into a 24-well culture plate and incubated at 37°C with 5% CO2 and a humidified atmosphere, changing the medium after 24hrs and reducing FCS to 2.5%. This protocol was adapted from (Golaz *et al.*, 2007)

2.10.2 Virus isolation

Virus isolation was attempted in MDCK and A72 cells using the first two CaAstV positive samples identified. Samples were diluted 1:10 in PBS solution then clarified by centrifugation and filtered (0.22 mm disposable filter). Samples were inoculated into confluent cell monolayers with and without trypsin (10µg/ml). The inoculum was adsorbed for 1 h at 37°C, then removed and Dulbecco's Minimum Essential Medium was added. Cell cultures were observed daily for cytopathic effect (CPE). Three serial passages were performed for each sample and negative controls. A sample of cells were collected at each passage and nucleic acid extraction performed as described above. qPCR was performed to confirm or refute the presence of CaAstV nucleic acid in cell lysates.

Chapter 3

Prevalence of canine norovirus in the UK

3.1 Chapter introduction

Canine norovirus (CNV) has recently been identified across Europe, the Far East and the US (Azevedo *et al.*, 2012; Martella *et al.*, 2008; Mesquita & Nascimento, 2012b; Soma *et al.*, 2014; Tse *et al.*, 2012). The ability of this virus to cause disease in dogs is uncertain, but there is concern that CNV may be an under-recognised cause of canine gastroenteritis. Improved understanding of the prevalence levels and clinical relevance of CNV is important from a veterinary perspective. This would enable evaluation of the potential need to develop commercial CNV diagnostic tests, as well as CNV vaccines and therapeutics for dogs. In addition, CNV is genetically very similar to HuNoV, a significant human health and economic burden. Studying the epidemiology and viral-host interactions of CNV would therefore provide a more accurate assessment of zoonotic potential of this virus, as well as providing further knowledge of this whole viral genera.

The presence or prevalence of CNV in the UK dog population had not been investigated prior to this project, but based on previous reports of CNV in Europe it was hypothesized that CNV would be circulating in the UK. Determining the prevalence levels of CNV was set as the first aim of this thesis. An epidemiological survey was designed and conducted using two different approaches. The initial part of the study aimed to evaluate the presence of viral RNA in the dog population. This involved collection of stool samples from dogs with and without gastroenteritis, then extracting RNA and performing RT-qPCR to detect viral RNA. The second part of the survey focused on identifying CNV-specific antibodies in dogs, indicative of previous exposure. The generation of CNV VLPs enabled serum screening by ELISAs. Canine serum samples from 1999 onwards were available, thus this strategy also provided an understanding of how CNV prevalence may have changed over time.

3.2 Samples collected

Stool samples and clinical data were collected from 67 dogs with severe gastroenteritis admitted to veterinary clinics or an animal shelter distributed across the UK between August 2012 and June 2014. Control samples were collected from 181 dogs without signs of gastroenteric disease, from either veterinary inpatients with non-gastrointestinal illness, or dogs at boarding kennels or belonging to veterinary staff. A total of 56 breeds of dog were represented, and the mean age of dogs with gastroenteritis was 4.3 years (standard deviation 4.1 years), whereas the mean age of control animals was 6.1 years (standard deviation 3.9 years).

A total of 396 canine serum samples were available for this project. Of these, 223 had been collected from a population of dogs at a large rehoming kennel in 1999-2001. An additional 173 serum samples were obtained specifically for this study, with sample collection taking place in 2012/2013. Of these samples, 102 were collected from patients at the Royal Veterinary College (RVC), London, and 71 were obtained from healthy blood donor dogs through Pet Blood Bank UK. All serum samples were stored at -20°C until use.

3.3 Canine enteric virus nucleic acid survey

RT-qPCR was selected as the screening method for detection of viral nucleic acid in stool samples as its high sensitivity allows for the detection of lower viral RNA levels than conventional PCR. An RT-qPCR assay was designed to screen for three canine enteric viruses (CNV, canine parvovirus (CPV) and canine enteric coronavirus (CECoV)) plus the internal extraction control (equine arteritis virus, EAV). CPV and CECoV have previously been reported to be circulating at moderate levels in the UK dog population (Godsall *et al.*, 2010), hence were included as sampling controls. After initial optimization of the RT-qPCR protocol, it was determined that screening was possible in two duplex reactions; CNV and EAV, then CPV and CECoV. Validation of the assay was achieved by generating standard curves using amplicons of the target nucleotide sequences (synthesised by Primer Design Ltd) as shown in figure 3.1.

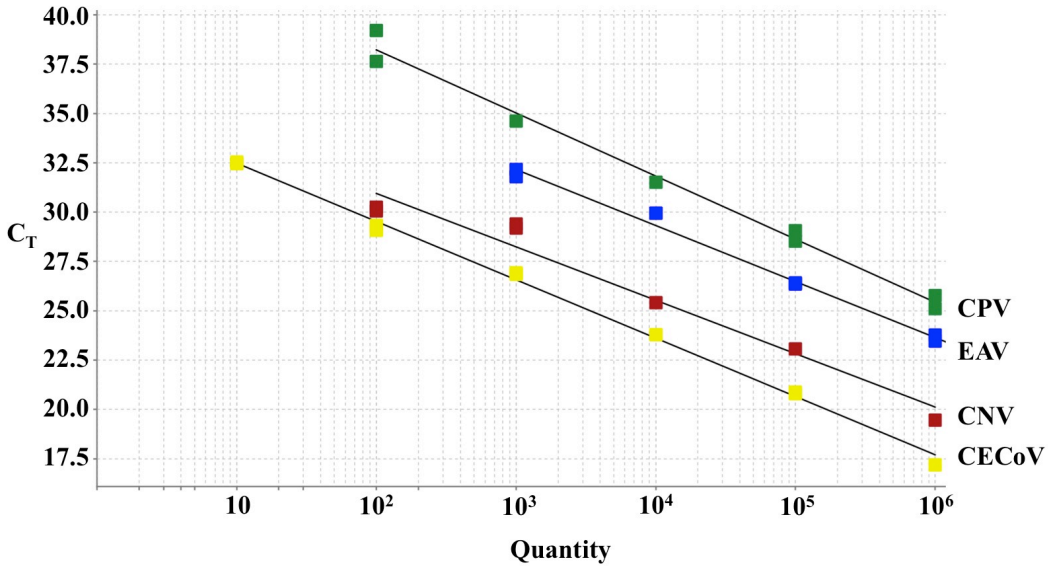


Figure 3.1 Standard curves determining sensitivity of RT-qPCR virus screening assay.
Amplicons of the target sequence for the primer-probe set of the four viruses were serially diluted 10-fold from a known starting concentration of 10^6 copies/µl, and entered into the duplexed RT-qPCR assay. A linear increase in C_T value was identified for each dilution of each virus, and mean R^2 was 0.987. The sensitivity of each primer-probe set was determined to be 100 copies/reaction.

As few as 100 copies of the CNV, CECoV and CPV amplicons were reliably detected in a reaction volume of 20µl. Any samples that generated a C_T value lower than the threshold value of 100 copies of a positive control were deemed negative.

Nucleic acid extraction and RT-qPCR were successfully performed on 248 stool samples as determined by constant C_T values from the internal extraction control RNA. A positive control (10^5 copies) for each virus included in the screening assay was added to every plate and sample results were only accepted if each positive control was within 1.5 C_T of expected values. The overall results of the RT-qPCR screen are presented in table 3.1. CPV or CECoV were detected at high titre (>10^6 copies/ml stool) in 17.0% (12/67) of dogs admitted with primary gastroenteritis. In patients without gastroenteritis or in the healthy control dogs, no viral nucleic acid for CPV or CECoV was detected above the positive threshold level of 100 genome copies/reaction. No samples were positive for CNV viral RNA in any of the canine cohorts. This indicated that the overall prevalence of CNV in the 248 dogs sampled was <1.7% (Wilson binomial approximation, confidence interval 95%).

Sample group (size)	Canine norovirus	Canine parvovirus	Canine enteric coronavirus
Patients with gastroenteritis (67)	0	10 (14.9%)	2 (3.0%)
Patients without gastroenteritis (64)	0	0	0
Healthy controls (117)	0	0	0

Table 3.1 Results of RT-qPCR screen for canine enteric viruses

3.4 Production and purification of VLPs from VP1 of three CNV strains

Recombinant baculoviruses expressing the VP1 proteins from three distinct CNV isolates were generated and used in an insect cell expression system to produce CNV VLPs. A timecourse of VP1 expression was performed and found that day six was the optimum day to harvest the VLPs from the Hi5 cells (figure 3.2). VLP production for all CNV strains was visualized by this method.

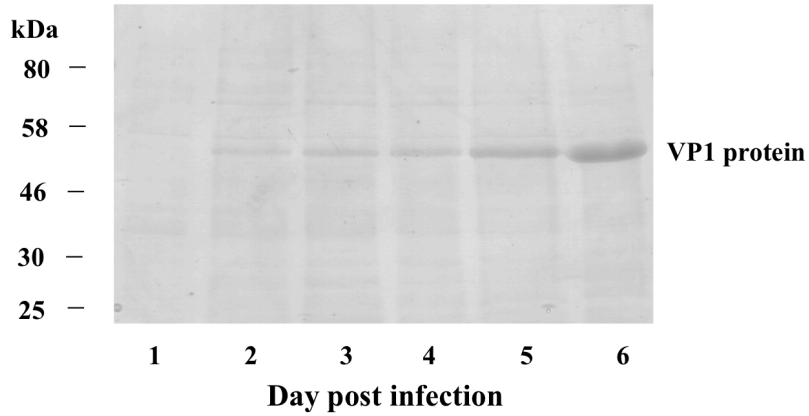


Figure 3.2 Timecourse of CNV strain 170 VLP production by Hi5 insect cells.
An aliquot of cells and media were harvested daily for six days following infection of Hi5 insect cells in shaker culture with recombinant baculovirus at MOI 5 PFU/cell. Protein was extracted in RIPA buffer then separated by SDS-PAGE and stained with Coomassie Blue. A dominant protein band is apparent from day 2, corresponding to the VP1 protein.

Following purification by isopycnic centrifugation on a caesium chloride gradient, VLPs were readily visualized and sedimented with a density of 1.32g/ml. Purified VLPs were quantified by bicinchoninic acid (BCA) assay (Thermo Scientific Pierce), and examined by SDS-PAGE and staining with Coomassie blue (figure 3.3). This showed a single major protein with apparent molecular weight of 63kDa for CNV strains 170 and C33, and single major protein with apparent approximate molecular weight of 57kDa for strain HK. This difference in molecular weight was expected based on VP1 sequence length. VLPs of an unrelated calicivirus, vesivirus 2117, were included as a control. The apparent discrepancy

between molecular weights of CNV strain 170 VLP in figure 3.2 and 3.3 is believed to be due to the different protein markers used (Pre-stained protein marker, NEB and Precision-plus protein standard, Bio-Rad).

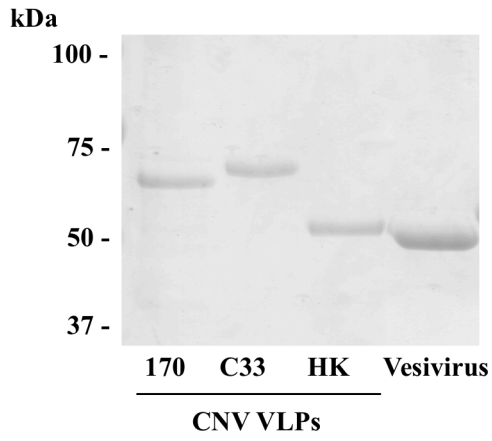


Figure 3.3 SDS-PAGE analysis of purified calicivirus VLPs.
VLPs from three CNV strains and an unrelated calicivirus, vesivirus 2117, were analysed by SDS-PAGE. The molecular weights of VP1 of CNV isolates C33 and 170 are larger than that of the third isolate HK. This is attributed to the length of C33 and 170 VP1 sequences being 52 and 50 amino acids longer than HK VP1 respectively.

The final means by which VLP generation was confirmed was by electron microscopy (EM). This was achieved in collaboration with Mike Hollinshead (Division of Virology, University of Cambridge). Samples were mounted on copper grid, fixed with gluteraldehyde then negatively stained with 0.5% uranyl acetate. EM was performed using an FEI Technai^{TM} transmission scanning electron microscope. Figure 3.4 shows correctly assembled particles were visualized, which were all approximately 30nm in diameter in agreement with previous observations of both norovirus VLPs and infectious particles (Jiang *et al.*, 1992; Kapikian *et al.*, 1972).

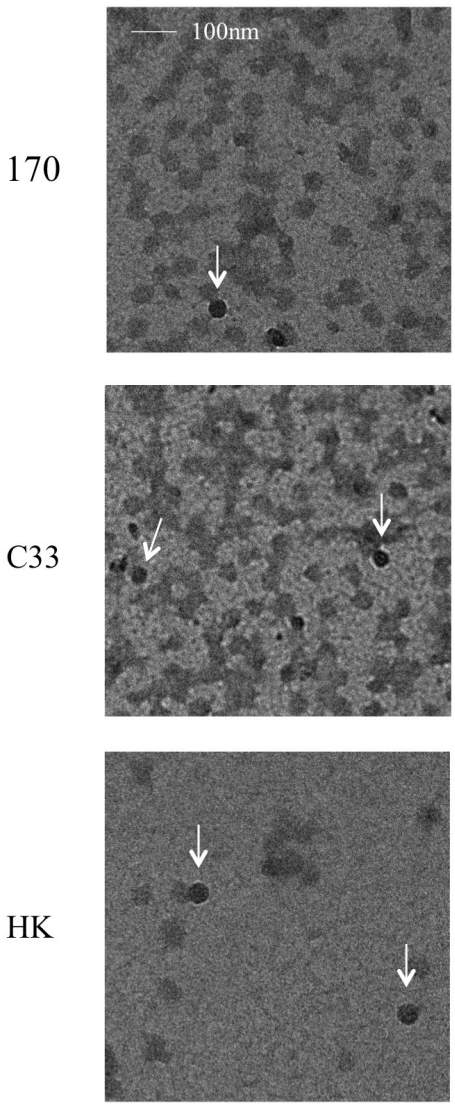


Figure 3.4 Electron micrograph of CsCl-purified CNV VLPs after negative staining. Complete VLPs of all three CNV strains were identified, as indicated by white arrows.

3.5 Serological survey

A total of 396 canine sera were tested by ELISA for antibodies against CNV; 223 from a 1999-2001 cohort, and 173 from a 2012-2013 cohort. For the initial screen, CNV VLPs to three strains (177, C33 and HK) were combined in a 1:1:1 ratio and a 1:50 dilution of canine sera examined for their reactivity to CNV. Overall, 189 samples were found to be seropositive to CNV, using a positive threshold value of the standard deviation of the OD450 of all blank wells multiplied by three, added to the mean OD450 of the blank wells. In the 1999-2001 cohort, 85 dogs (38.1%) were seropositive and in the 2013/2013 cohort, 104 dogs (60.1%) were seropositive. The increase in seroprevalence between the two cohorts was statistically significant (Z-test, $p<0.001$).

To determine the serological titres of seropositive dogs, seropositive dogs were randomly selected from both the 1999-2001 cohort and the 2012-2013 cohort, giving a total of 10 seropositive samples. Sera was serially diluted two-fold from a starting dilution of 1:50, and added to pooled CNV VLPs (25ng each strain per well) coated onto 96-well plates. A range of titres were identified (figure 3.5), varying from 1:1600 (one dog) to 1:100. The most prevalent titre in this preliminary screen was 1:400.

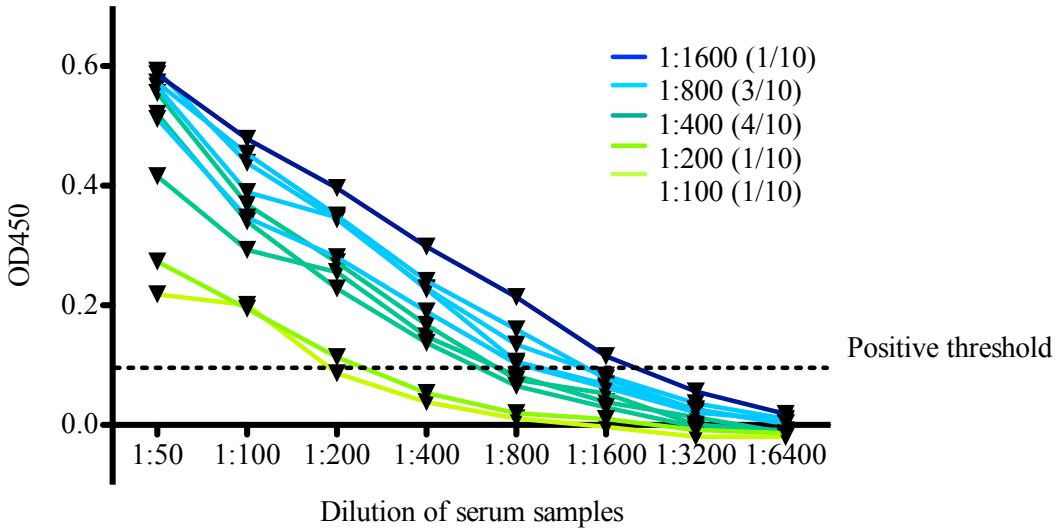


Figure 3.5 Serological titres to CNV.
Ten dogs seropositive to CNV from both the 1999-2001 and the 2012-2013 cohorts were randomly selected to determine anti-CNV antibody titre. Corrected OD450 values are plotted, calculated by subtracting the OD450 value of buffer-only coated wells. The positive threshold was calculated from the mean plus three times the standard deviation of the OD450 reading of buffer-only.

All 189 serum samples identified as positive by the initial ELISA screen against pooled CNV VLPs, were then tested against individual CNV VLPs. The results show significant variation between the seroprevalence of different strains (figure 3.6). CNV strain HK predominated in both cohorts of dogs, whereas strain C33 showed the lowest seroprevalence in both groups. These data also indicated that a proportion of dogs have seroconverted to more than one of the CNV strains used in this study. In the 1999-2001 cohort 30.6% dogs were seropositive to two or three strains, a percentage that increased to 40.4% in the 2012-2013 cohort.

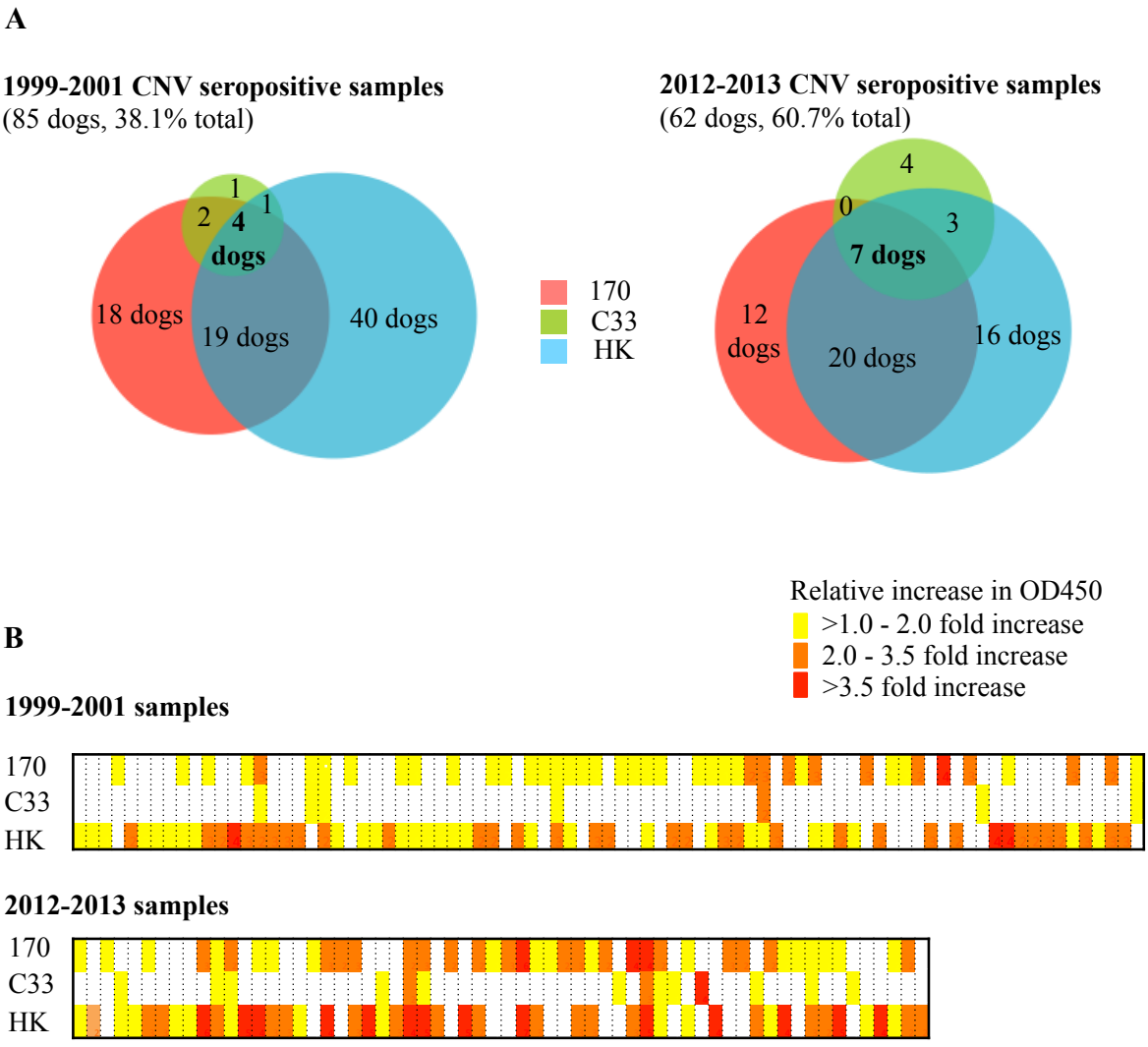


Figure 3.6 Seropositive samples by ELISA to three different strains of CNV.
Canine serum samples that were seropositive to pooled CNV VLPs were screened against individual CNV VLP strains; 170, C33 and HK. (A) Venn diagrams represent seroprevalence of each CNV strain in the 1999-2001 and the 2012-2013 cohorts. The number of dogs seropositive to one strain alone or combinations of strains are represented as percentages. (B) Heatmap representation of the relative OD450 value to each CNV strain of each seropositive. Every column represents a single dog. Positive threshold value was established from the mean OD450 of coating buffer alone plus three standard deviations. Relative increase in OD450 values above the positive threshold were calculated to enable fair comparison between experiments. A relative increase of <1 indicates a seronegative sample, represented by a white box. The degree of relative increase for samples is represented by increasing darkness of the corresponding box.

3.6 Evaluation of CNV antibody cross reactivity

To determine the strain specificity of the anti-CNV reactivity identified in positive samples and to determine if CNV exists as a series of distinct serotypes, a series of blocking assays were performed on two selected samples. Firstly it was shown that pre-incubating a serum sample positive to pooled CNV VLPs with HuNoV VLPs from genogroup I and II (methodology depicted in figure 3.7A), did not diminish antibody binding to CNV (figure 3.7B). This demonstrates that apparent reactivity to CNV is not a consequence of cross-reactivity to antibodies to HuNoV strains. To investigate the antigenic relationship between different CNV strains, blocking assays were next performed using individual CNV strains. When a CNV seropositive sample was pre-incubated with CNV strain 170 VLP, subsequent detection in ELISA of strain 170 VLP coated on a plate was reduced. Pre-incubation with VLPs of a different CNV strain did not diminish signal. A similar result was shown when serum was pre-incubated with CNV strain HK VLPs; detection of HK VLPs by ELISA was reduced (figure 3.7C and 3.7D). No serum that contained anti-CNV strain C33 antibodies was available in sufficient quantities to undertake the blocking assay, though the results from pre-incubation with C33 VLPs were sufficient to indicate that cross reactivity to strain 170 or HK was not occurring. This data overall demonstrates that anti-CNV antibodies against the three strains used in this study are strain specific, suggesting that CNV exists as distinct serotypes.

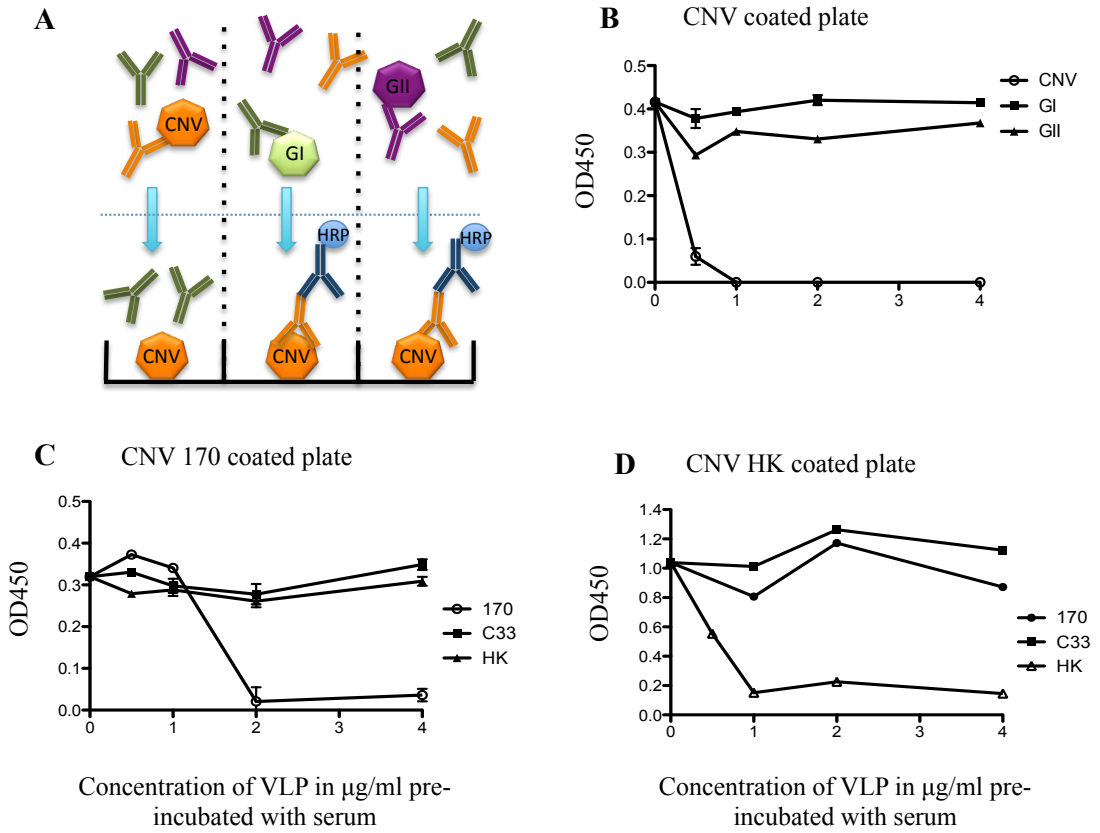


Figure 3.7 Evaluation of cross-reactivity between antibodies against human and canine noroviruses, and between different CNV strains.
The methodology used is presented in panel A; canine serum was pre-incubated with serial dilutions of either pooled human norovirus VLPs from genogroups I and II (GI/GII) or pooled CNV VLPs. The ability of the serum to subsequently detect pooled CNV coated onto ELISA plates was analysed (B). To determine cross reactivity between the three CNV strains, the blocking assay was repeated with canine serum being pre-incubated with serial dilutions of each of the three CNV strains VLPs separately. ELISAs were used to analyse the ability to detect CNV strain 170 (C) and strain HK (D). No C33 seropositive sample of adequate titre was available for the blocking assay.

Western blotting analysis of CNV VLPs using a representative positive canine serum sample was used as an additional method to confirm the specificity of anti-CNV antibodies (figure 3.8). By ELISA, the selected serum was positive to CNV strains 170 and HK, and this was proven to be the same by western blotting.

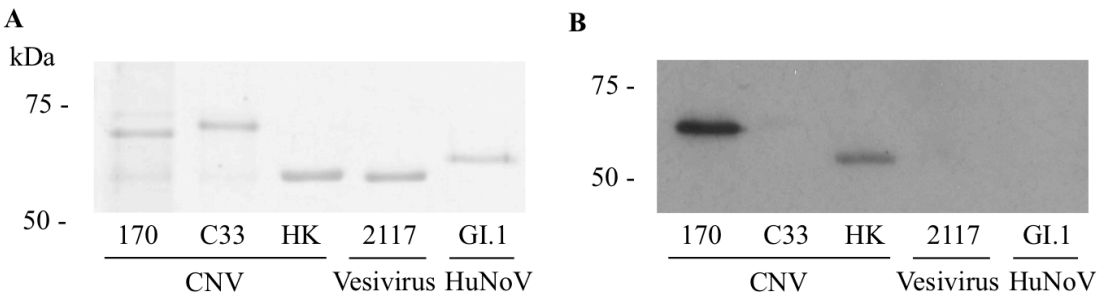


Figure 3.8 Western blotting of purified VLPs using seropositive canine serum.
Five different VLPs were separated by SDS-PAGE (2µg each). Gel A was stained with Coomassie blue to identify purified VLP protein at the expected molecular weight. Gel B was used for western blotting with a canine serum sample which was seropositive to CNV strain 170 and HK by ELISA.

3.7 Serological prevalence by age

The age at the time of blood sampling was known for 93 out of the 173 dogs in the 2012-2013 cohort. The mean age of dogs seropositive to CNV was 8.1 years (SD 3.6), whereas the mean age of seronegative dogs was 5.8 years (SD 3.8). The difference between the age distribution of the two groups was statistically significant ($p = 0.0076$, Mann-Whitney test). Division of the dogs into age-groups and calculation of the proportion of each group seropositive to CNV showed that seroprevalence increases from 14% if less than 2 years of age, to almost 80% in the 6-8 year age group (figure 3.9).

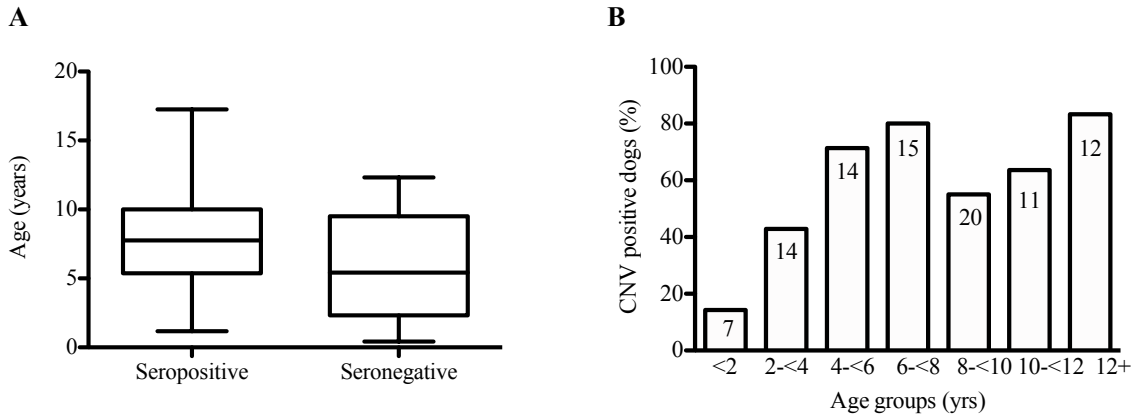


Figure 3.9 Relationship of CNV antibody status to age.
(A) Box plot of age distribution of dogs relative to seroconversion to CNV. The box represents the interquartile range, with the band inside the box representing the median age. The whiskers are the minimum and maximum of all data. (B) Histogram representing the percentage of dogs that have seroconverted to CNV in each age group. Numbers inside bars indicate the quantity of samples associated with each data point.

3.8 Canine vesivirus seroprevalence

An unexpected finding during the seroprevalence study was that a small proportion of dogs were seropositive for vesivirus 2117 VLPs. These VLPs were generated by Ed Emmott (Goodfellow lab, University of Cambridge) using an identical protocol to the CNV VLP production and were initially selected for use as a negative control in the ELISAs performed with canine serum samples. However, 8% dogs tested were seropositive for vesivirus 2117, making this an inappropriate negative control. There was no relationship identified between seropostivity to vesivirus 2117 and seropositivity to CNV as shown by figure 3.10.

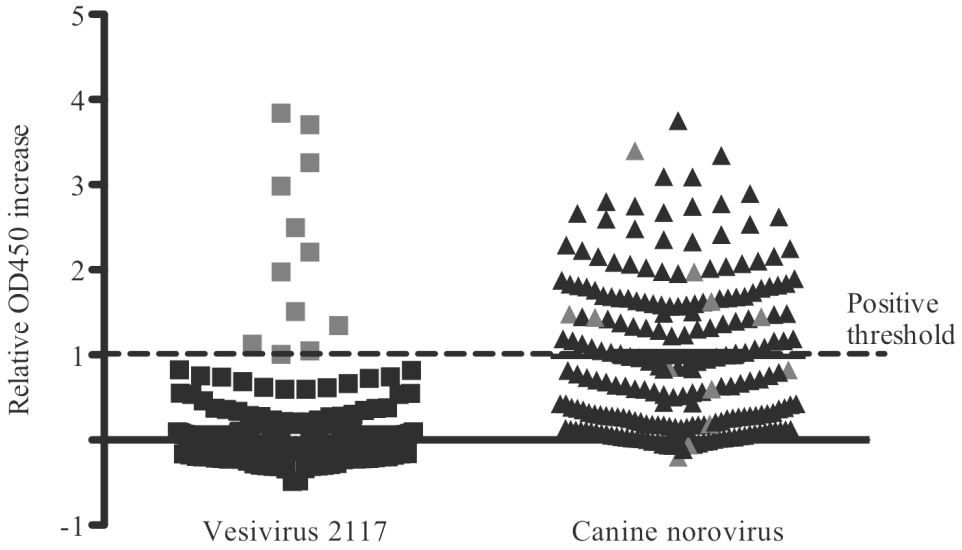


Figure 3.10 Scatterplots of seropositivity to vesivirus 2117 and CNV.
Relative OD450 increase above the positive threshold for each plate was plotted to enable comparison between different ELISA plates. Dogs seropositive to vesivirus 2117 were identified if the relative increase in OD450 was greater than 1. The data points for these dogs are highlighted in grey. The relative OD450 increase to CNV VLPs for every dog is plotted in the second scatter column, with dogs seropositive to vesivirus 2117 again highlighted in grey.

The species of origin of vesivirus 2117 is unknown, with the virus first being identified in Chinese hamster ovary (CHO) cells. However based on sequence analysis, the closest related virus is canine vesivirus (CVV) (Oehmig *et al.*, 2003). It is hypothesized that dogs

seropositive to vesivirus 2117 have previously been exposed to CVV, and cross-reactivity between the two vesiviruses accounts for the positive results. Only a handful of reports have identified CVV in canine populations, and the disease association is uncertain. There is a single reference to CVV in dogs in the UK, which remarkably identifies a seroprevalence of 8% (2/25 dogs) (Schaffer *et al.*, 1985). Comparison of seroprevalence to vesivirus 2117 amongst the canine cohort from 1999-2001 and the cohort from 2012-2013 shows an increase between the two time periods; 4.9% from the earlier group, and 14.7% in the later group. This mirrors the increase in seroprevalence in CNV between the two cohorts, and suggests CVV may be circulating at higher levels in recent years.

To follow on from these unexpected serology results, it was hypothesized that CVV RNA may be detectable in a proportion of the canine stool samples collected. To investigate this, degenerate primers were designed (table 2.4) that would be able to detect the capsid region of both vesivirus 2117 (GenBank AY343325.2), and the three canine vesivirus sequences available (GenBank NC004542.1, AF053720.1, JN204722.1). A SYBR-based two-step qPCR screen was performed with nucleic acid extracted from the 248 stool samples, but no CVV positive cases were identified.

3.9 Chapter summary

In this chapter the first evidence that CNV is present in the UK dog population is presented. Whilst previous reports have confirmed the existence of CNV throughout Europe and the US, CNV-specific antibodies in dogs in the UK have been identified by this work, confirming that CNV is circulating.

Seroprevalence to CNV in the canine serum samples collected from 2012-2013 was 60.1%. The number of positive samples amongst 102 veterinary in-patients at the RVC (60.8%) was highly comparable to the 71 samples from healthy blood donor dogs (59.2%). For comparison to other enteric viruses known to be circulating in the UK, table 3.2 summarises previous seroprevalence studies in UK dog populations.

Virus	Study	Seroprevalence	Population
Canine enteric coronavirus	(Tennant *et al.*, 1991)	54%	Veterinary Hospital
	(Tennant *et al.*, 1993)	76%	Rescue Centre
Canine parvovirus	(Tennant *et al.*, 1991)	70%	Veterinary Hospital
Canine rotavirus	(Tennant *et al.*, 1991)	86%	Veterinary Hospital
Canine kobuvirus	(Carmona-Vicente *et al.*, 2013)	37%	Veterinary Hospital

Table 3.2 Previous seroprevalence studies for canine enteric viruses in the UK

The seroprevalence to CNV shown by this study is not dissimilar from the percentage of dogs seropositive to CECoV. However, CECoV is included in a proportion of canine vaccines, which would increase seropositivity in the absence of natural infection. Similarly, the 70% seropositivity to CPV is likely enhanced by the widespread use of CPV vaccines in the UK. A fair comparison between CNV and canine kobuvirus seroprevalence is possible based on the absence of vaccines and the similar time periods of sample collection; this project does suggest that CNV is a more prevalent virus in the UK.

Following on from this study of CNV seroprevalence, a second report has recently been published on CNV seroprevalence across Europe (Mesquita *et al.*, 2014a). This comprehensive study included 510 serum samples collected from dogs visiting veterinary clinics in 2009-2010. It was found that 36% of all samples were seropositive, with a range of 0-70% when samples were grouped according to the fourteen different countries in which they were collected (0% in Iceland and Hungary, 70% in Finland). The lower overall level of seropositivity in comparison with data from this UK study could be attributed to the use of only a single type of CNV VLP in the ELISAs (strain C33). Results from the UK survey indicate the existence of multiple CNV serotypes, and as only a single serotype is screened for in the European study, the overall CNV seroprevalence in these countries is likely to be an underestimate.

CNV seroprevalence was unexpectedly high in the UK given that CNV RNA was not detectable in 248 canine stool samples analysed in this study. The high seroprevalence suggests that many dogs are exposed to the virus, with the inability to detect actively secreted viral RNA attributable to two possible factors: firstly, it is likely that, as with the majority of HuNoV infections in man, CNV infection is an acute infection with virus only shed during a short period of time. Acute gastroenteritis caused by HuNoV in man results in peak viral shedding at 2-4 days after infection. By 3 weeks after infection only 25% cases are still positive for HuNoV RNA (Rockx *et al.*, 2002). This is in agreement with epidemiological data from the first case identified (22 days viral shedding), and from a study involving a kennel outbreak of CNV in seven dogs (less than 7 days shedding) (Mesquita & Nascimento, 2012b). A second explanation for the low prevalence of CNV RNA detection could be due to the genetic heterogeneity of CNV. Significant sequence variation has been shown between the CNV strains characterized so far, and there is evidence of recombination

between CNV strains and noroviruses of different genogroups (Martella *et al.*, 2009). Design of the primer-probe used in this study was based on a highly conserved region of the CNV genome (RdRp), but small sequence differences will affect the ability of the viral sequence to be amplified. Although strains circulating in the UK are antigenically similar to previously identified strains, minor sequence differences could reduce the chance of detection in a PCR screen. To address this, a primer set designed to be broadly reactive to a wide range of noroviruses and sapoviruses (p289/290, table 2.4 (Jiang *et al.*, 1999)) was used to reanalyze the samples. In addition, it must be noted that the primer-probe used in the RT-qPCR survey for CNV RNA was designed prior to the publication of the HK genome sequence, hence the RT-qPCR survey would not detect strain HK. In light of the results of the serosurvey, this issue was addressed retrospectively to the first viral RNA survey. A new primer set was designed to detect the most conserved region of the HK VP1 protein (table 2.4). A SYBR-based qPCR assay was used to screen all 248 stool samples for the presence of any norovirus strain using primer pair p289/290 and also specifically for CNV strain HK using the newly designed primer pair. No positive samples were identified using either set of primers (data not shown).

Seroprevalence to the CNV strains surveyed in this study has been shown to have significantly increased over the past decade in the populations studied. In the 1999-2001 cohort of dogs 38.1% were seropositive, whereas in the 2012-2013 cohort this proportion has almost doubled. Not only does this data provide the first proof that CNV has been present in dogs for at least 8 years prior to its initial discovery (Martella *et al.*, 2008), it also implies that the number of dogs exposed to these strains of CNV has significantly increased during this period. This conclusion does come with certain caveats however, as the study populations of dogs used are not directly comparable. The 1999-2001 samples were

collected from dogs that had been at a rehoming kennels for 3 weeks whereas the 2012-2013 dogs were all privately owned pets. Seroprevalence and overall viral prevalence to many viruses is known to be higher in facilities where dogs are kept in close contact (Stavisky *et al.*, 2012; Tennant *et al.*, 1993). This does suggest that the apparent increase in seroprevalence to the CNV strains studied is real, as typically owned pets will be exposed less frequently to viruses than dogs in kennels. It would be valuable to assess the current CNV seroprevalence in kenneled dogs, which is predicted to be greater than 60%.

VLPs of three different CNV strains were pooled together to establish the overall seroprevalence to the virus. CNV strains included were identified in 2007 from Italy (170), Portugal (C33) and Hong Kong (HK). Following the initial serosurvey with the pooled VLPs, positive samples were entered into ELISAs with VLPs from each of the individual strains separately. This data would indicate reactivity to all three strains in the UK dog population. Blocking assays demonstrated no significant cross-reactivity between the three CNV strains in the samples tested, proving that antibodies generated in response to infection were likely to be strain specific. This was not unexpected based on the low amino acid identity (58.4% - 60.6%) between the capsid region of the three strains. The human immune response against human norovirus is of short duration (up to 14 weeks) and is homotypic, i.e. the immunity acquired for a genogroup or a particular genotype does not provide effective protection against another genogroup or genotype (Wyatt *et al.*, 1974). This study therefore reveals that at least three antigenically distinct CNV strains have been circulating in the UK dog population. This is similar to the co-existence of human norovirus strains in the human population, with both genogroup I and genogroup II strains circulating (Phan *et al.*, 2007; Tran *et al.*, 2013; Wang *et al.*, 2012).

Despite co-existence of multiple human norovirus strains, genogroup II (GII) noroviruses are the most prevalent genogroup worldwide with 96% of human outbreaks attributed to these strains (Tran *et al.*, 2013). This data also identified variation between the prevalence of the different strains, with the highest seroprevalence in both cohorts demonstrated for CNV strain HK. This strain has been classified as a GVI norovirus, along with the Portuguese strain C33. However, strain HK has the highest sequence identity (51.6%) to an intergenotype GII recombinant human norovirus strain and it is suggested that strain HK may be classified into a novel genogroup (Tse *et al.*, 2012). Aside from this phylogenetic information, it is not possible to determine if the higher seroprevalence to strain HK in this study is due to a viral fitness advantage, and further molecular characterization is required.

The age of seroconversion to CNV shows that exposure to the virus typically occurs in the first few years of life. Seroprevalence increases significantly in older dogs. This is comparable to the seroconversion rates to human norovirus in man; seroprevalence in children less than 2 years old is 20-30%, but this rapidly increases to 70-80% in older children (Kobayashi *et al.*, 2009; Koho *et al.*, 2011). It is speculated that CNV isolation from stool samples using qPCR will be more likely in a younger cohort of dogs.

To conclude, this chapter has not only demonstrated that CNV is present within the UK dog population, but has also shown that multiple strains of CNV have elicited antibody production in dogs. Evidence of exposure to CNV prior to its first discovery in 2007 has also been presented, and the rise in seroprevalence over time suggests this virus is becoming increasingly common in the UK.

Chapter 4

Serendipitous discovery of canine astroviruses

4.1 Chapter introduction

Astroviruses are small non-enveloped, positive sense RNA viruses that have been reported to infect a wide range of mammalian and avian species, including dogs. Astroviruses were first identified in 1975 in the stools of children with diarrhea (Appleton & Higgins, 1975; Madeley & Cosgrove, 1975), and are now estimated to cause 2% - 8% cases of gastroenteritis in children worldwide (King *et al.*, 2011).

Canine astrovirus (CaAstV) is associated with gastroenteritis in dogs, and although it has been detected in a range of countries worldwide, (Castro *et al.*, 2013; Choi *et al.*, 2014; Grellet *et al.*, 2012; Martella *et al.*, 2011; Zhu *et al.*, 2011) it has not previously been reported in dogs in the UK. As 248 canine stools samples were collected for screening against human and canine noroviruses, it was deemed valuable to extend testing of these samples for additional enteric viruses, including CaAstV. The first CaAstV positive sample was identified unexpectedly, which drove the design and completion of a more focused screen for CaAstV in the canine stool samples collected. This enabled estimation of the prevalence of CaAstV in the UK dog population.

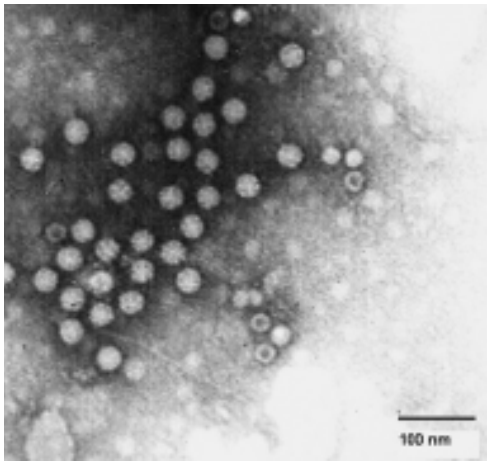


Figure 4.1 Electron microscopy image of canine astrovirus.
Canine astrovirus particles were observed in the faeces of a dog with gastroenteritis (Toffan *et al.*, 2009). Astrovirus particles have a characteristic star-shaped appearance (astron is Greek for star).

Previous studies have only conducted limited genetic analysis of CaAstV isolates, with the full genome of CaAstV not reported. The genome of astroviruses is typically 6.4-7.3kb and divided into three open reading frames, ORF1a, ORF1b and ORF2 with a 5'untranslated region (UTR) and a 3' poly-A tail (King *et al.*, 2011). ORF1 is divided into two coding regions by a ribosomal frameshift sequence, and codes for the non-structural proteins involved in viral genome replication; ORF1a codes for a polyprotein predicted to include a serine protease and VPg (Fuentes *et al.*, 2012) and ORF1b encodes the RNA dependent RNA polymerase (RdRp). ORF2 encodes the capsid precursor protein which is cleaved both intracellularly and extracellularly to generate the mature infectious virion (Bass & Qiu, 2000). It was expected that CaAstV would have the same genome organisation, and to confirm this detailed molecular characterization was performed with full genome sequencing of the CaAstV isolates identified.

4.2 Fortuitous identification of the first canine astrovirus isolate in the UK

A total of 248 canine stool samples were screened using a two-step qPCR protocol for the presence of a number of different enteric viruses, as described in chapter 3. Canine vesivirus (CVV) screening was included as vesivirus-specific antibodies were identified in 8% dogs (chapter 3.8). Primers designed to detect CVV (table 2.4) were shown to amplify a 300bp fragment from RNA extracted from a single stool sample (sample 8/248). This fragment was then purified and the sequence determined using conventional sequencing by the DNA sequencing facility at the Department of Biochemistry, University of Cambridge. The similarity with other sequences was then examined using the nucleotide alignment programme BLAST®. The closest alignment of PCR product was with 3' end of ORF1 of a Californian sea lion astrovirus. This therefore identified the cDNA amplified in the sample as a member of *Astroviridae*. The reverse CVV primer had been designed with degeneracy

at four nucleotide positions in order to detect the four CVV sequences deposited in GenBank, as well as the related virus, vesivirus 2217. It is believed that this degeneracy permitted mis-priming with an astrovirus. As no ORF1 CaAstV sequences were published at this point in time, it was not possible to determine if the sequence amplified from the stool sample had a canine origin.

4.3 Identification of multiple canine astrovirus isolates

It was hypothesized that the astrovirus identified in the single stool sample was a CaAstV, thus to confirm this, published primers designed to specifically target the CaAstV RdRp (table 2.4, (Martella *et al.*, 2011)) were used to screen sample 8 for CaAstV. This resulted in generation of an amplicon of the correct size as determined by gel electrophoresis, and sequencing confirmed this to be a CaAstV. These primers were then used to develop a two-step qPCR protocol, with a positive control generated by cloning the 300bp fragment of CaAstV RdRp amplified from sample 8 into pCR-Blunt™. This qPCR protocol was then used to screen all remaining samples for CaAstV. Of the 248 stool samples, CaAstV was detected in a total of four samples, including the initial sample. All four positive dogs were showing signs of gastroenteritis, whereas CaAstV was not detected in any dogs without gastroenteric signs. The difference between the prevalence of CaAstV in dogs with gastroenteritis (6.0%) and prevalence in dogs without gastroenteritis was statistically significant ($p = <0.001$). Two of the four CaAstV positive dogs were co-infected with CPV, but no co-infections with CECoV and CaAstV were identified. The age range of CaAstV positive dogs was from 7 weeks to 7 years, with a mean age 2.1 years (SD 3.3 years). Table 4.1 summarises the clinical information and viral screening results of the four CaAstV positive dogs.

Case	Age	Breed	Collection date	Collection location	Additional viruses identified
1	7 weeks	Crossbreed	August 2012	Gillingham, Kent	CPV
2	7 years	Border Collie	August 2012	Lincoln, Lincolnshire	None
3	1 year	Staffordshire Bull Terrier	February 2014	Huntingdon, Cambridgeshire	None
4	10 weeks	Crossbreed	May 2014	Braintree, Essex	CPV

Table 4.1 Clinical and co-infection data for CaAstV positive cases

4.4 CaAstV shedding is acute

CaAstV was confirmed in case 4 whilst the dog was still hospitalized at Queen's Veterinary School Hospital (QVSH). This dog had presented collapsed, with severe dehydration and weakness at QVSH, following a six day history of diarrhea and vomiting. The dog received intensive supportive care, including intravenous fluid therapy, a fresh frozen plasma transfusion and intravenous glucose and antibiotics. After a week of therapy, a full recovery was made and the puppy was discharged.

Figure 4.2 10 week old crossbreed dog with CaAstV (case 4).
This image was taken whilst the puppy was hospitalized at Queen's veterinary School Hospital, University of Cambridge (courtesy of James Warland, used with owners permission).

In order to determine the duration of CaAstV shedding, serial stool samples were collected from the puppy at 2-5 day intervals post diagnosis with CaAstV. Veterinary staff and the owners facilitated with sample collection, with the final sample being collected 24 days after the onset of clinical signs. Samples were stored at 4°C until transportation on ice to the Division of Virology, University of Cambridge. qPCR for CaAstV and CPV nucleic acid was performed on all serial samples, and the results are presented in figure 4.3. CaAstV was only identified in the first sample (6 days post start of clinical samples), with all samples collected 9 days or later from the start of clinical signs being below the threshold of detection and thus deemed negative. In contrast, CPV was detected in every sample collected, up to 24 days post presentation, although there was a 1000 fold decrease in viral titre over the sampling period. Overall this indicates that CaAstV was only shed briefly in this case, with cessation of viral shedding correlating with improvement in clinical signs.

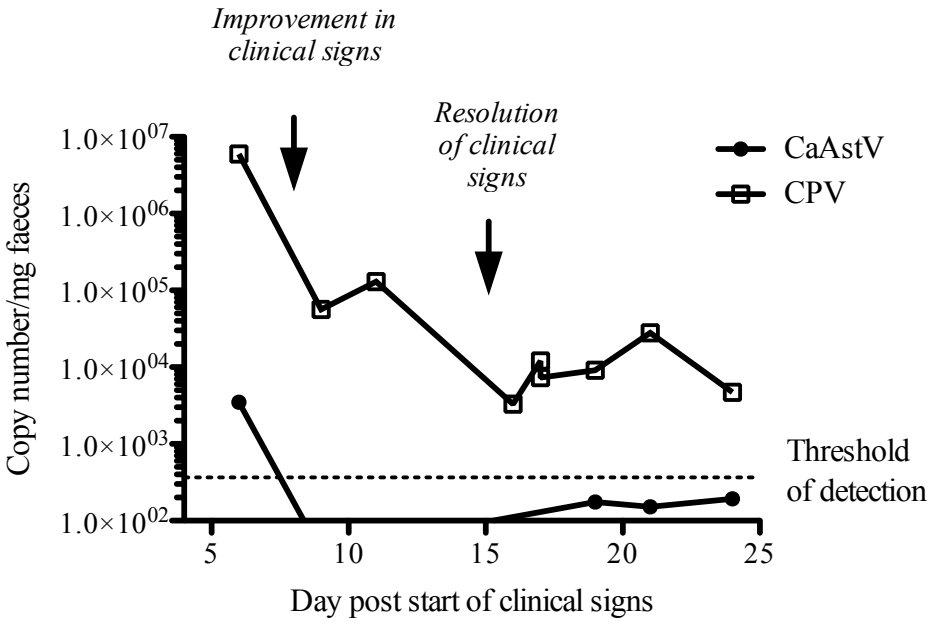


Figure 4.3 Viral shedding in serial canine stool samples as detected by qPCR.
Stool samples were collected up to 24 days post onset of clinical signs and tested for the presence and quantity of CaAstV and CPV nucleic acid using SYBR-based and TaqMan-based qPCR respectively.

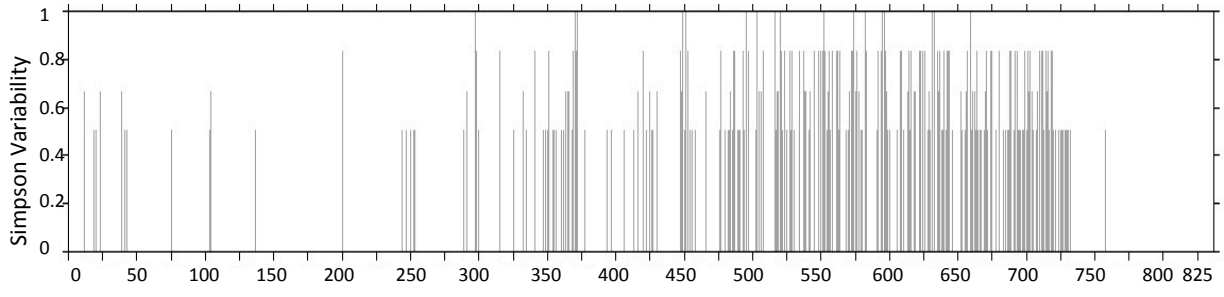
4.4 Comparison of CaAstV capsid sequences

The complete coding sequence of the four CaAstV strains identified was elucidated using conventional PCR and cloned amplicons. This required optimization of the PCR protocol for the fourth positive sample, of which sample quantity was low. A touchdown hemi-nested PCR using primer pairs IGUC 965F and 626R, then 887F and 626R (listed in appendix A.4) was performed (adapted from Debing *et al.*, 2014), with the amplification programme consisting of an initial 5 min step at 95°C, followed by 18 cycles with 95°C for 30 s, 62°C for 45 s and 70°C for 60 s, with a reduction of 0.5°C per cycle for the annealing temperature, then 14 cycles with 95°C for 30 s, 53°C for 45 s and 70°C for 60 s. A final elongation step at 70°C for 10 min was performed, followed by chilling to 4°C.

The ORF2 nucleotide and amino acid sequences of these strains were aligned using ClustalW2. The overall nucleotide identity between strains was 77.1-81.1%, whereas the amino acid identity was 79.3-86.3%. The four sequences are listed in the appendix and have been deposited in the GenBank database and assigned accession numbers KP404149 – KP404152 (cases 1-4 respectively).

A number of studies have reported the N-terminal and C-terminal regions of astrovirus capsids to be relatively conserved, whereas the central region is hypervariable. The human astrovirus (HAstV) capsid protein has previously been divided into three regions; the N terminus (amino acids 1-415), a variable central region (416-707), which includes a hypervariable section from 649-707, and a conserved C terminus (708-786) (Willcocks *et al.*, 1995). An analogous approach for the CaAstV capsid was taken by Zhu et al, who divided the capsid into three regions for analysis: amino acids 1-446, 447-730, and 731-end (Zhu *et al.*, 2011). The four capsid sequences derived from this study have been analysed

according to the latter scheme, and sequence identity compared (figure 4.4). Sequence analysis of the three regions clearly shows the majority of sequence variation is concentrated in region II.



Strain	Gillingham/ 2012/UK	Lincoln/ 2012/UK	Huntingdon/ 2014/UK	Braintree/ 2014/UK	Gillingham/ 2012/UK	Lincoln/ 2012/UK	Huntingdon/ 2014/UK	Braintree/ 2014/UK	Gillingham/ 2012/UK	Lincoln/ 2012/UK	Huntingdon/ 2014/UK	Braintree/ 2014/UK
Gillingham/ 2012/UK	100.0	92.2	91.5	92.4	100.0	59.1	55.1	53.7	100.0	99.0	98.1	99.0
Lincoln/ 2012/UK	92.2	100.0	91.5	92.6	59.9	100.0	52.0	70.8	99.0	100.0	99.0	100.0
Huntingdon/ 2014/UK	91.5	91.5	100.0	94.0	55.1	52.0	100.0	49.1	98.1	99.0	100.0	99.0
Braintree/ 2014/UK	92.4	92.6	94.0	100.0	53.7	70.8	49.1	100.0	99.0	100.0	99.0	100.0

Figure 4.4 Alignment and variability analysis of capsid amino acid sequences of the four CaAstV isolates identified in the UK.
(A) CaAstV capsid amino acid sequences were aligned using ClustalW2, then a variability scan was constructed using the Simpson diversity index and Protein Variability Server software (Garcia-Boronat *et al.*, 2008). (B) Amino acid percentage identity between CaAstV strains in three different regions of the capsid protein

A 24nt deletion was identified in samples 2, 3 and 4 in the 5' end of region II, which has previously been reported in Chinese CaAstV strains (Zhu *et al.*, 2011). It is not possible to predict the location of this deletion on the capsid structure as it is beyond the region of the astrovirus capsid spike for which the crystal structure has been solved (Dong *et al.*, 2011b). However, sequence alignment with the HAstV type 8 capsid (GenBank AAF85964.1) shows this deletion to be located downstream of the caspase cleavage site required for virion

maturation, which truncates the full length capsid protein (VP90) into the mature VP70 form (Banos-Lara & Méndez, 2010). Therefore it is predicted that the 24nt deletion will not alter the mature virion.

Evolutionary analysis of the four CaAstV sequences from the study, alongside the seven previously reported full-length CaAstV capsid sequences are presented in figure 4.5. This analysis indicated that the UK strains do not cluster, contrary to previous studies which have analysed CaAstV strains from a single city (Zhu *et al.*, 2011). Each UK strain is distinct from each other, and whereas one strain clusters most closely with the Chinese strains, the remainder group with strains identified in Italy over a number of years.

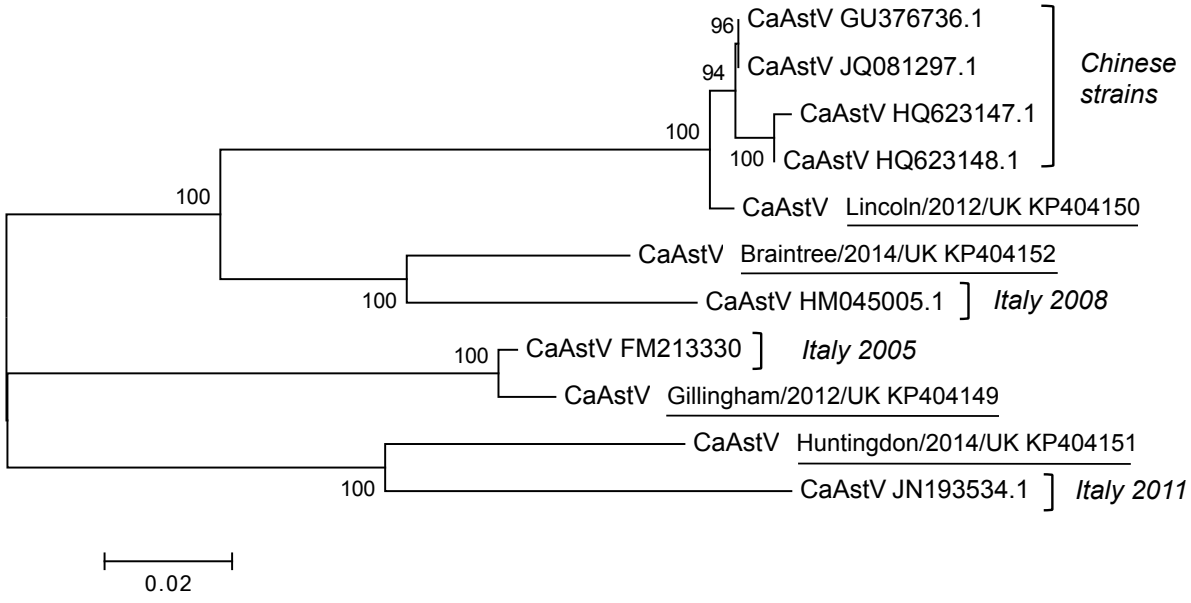


Figure 4.5 Phylogenetic tree based on the full-length amino acid sequence of the capsid protein of CaAstVs.
This includes the four UK strains identified in this study (underlined) and the seven strains previously sequenced and listed in GenBank. The tree was determined using the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA6.

4.5 Full-length CaAstV genome sequenced

The complete CaAstV genome was determined from two samples using conventional PCR and cloned amplicons, coupled with 3' and 5' RACE. The first CaAstV genome was isolated from a 7 week old crossbreed dog (strain Gillingham/2012/UK, GenBank accession number KP404149) and the second isolated from a 7 year old Border Collie (strain Lincoln/2012/UK, KP404150). The total length of CaAstV Gillingham/2012/UK is 6600nt and CaAst/Lincoln/2012/UK is 6572nt, as determined by the use of 5' and 3' RACE. Each genome encodes three open reading frames (ORFs); ORF1a, ORF1b, and ORF2 flanked by a 5' untranslated region (UTR), and a 3' UTR and a poly-A tail (figure 4.6A). In HAstVs, the 5' UTR is 85nt in length, whereas data from both CaAstV strains would indicate that the CaAstV 5'UTR is 45nt. The 83nt 3'UTR of HastV is identical to 3'UTR in CaAstV Lincoln/2012/UK, whereas the 3'UTR of CaAstV Gillingham/2012/UK is 2nt shorter. The nucleotide composition of both CaAstV strains is 29% A, 22% G, 26% T and 23% C. The G/C composition is 45%.

The ORF1a of non-canine astroviruses encodes a serine protease (Willcocks *et al.*, 1994). Sequence alignment of CaAstV with astroviruses of other species shows a high degree of conservation in the predicted serine protease region (figure 4.6B). This is especially pronounced in the regions around the proposed catalytic triad of the serine protease (Speroni *et al.*, 2009).

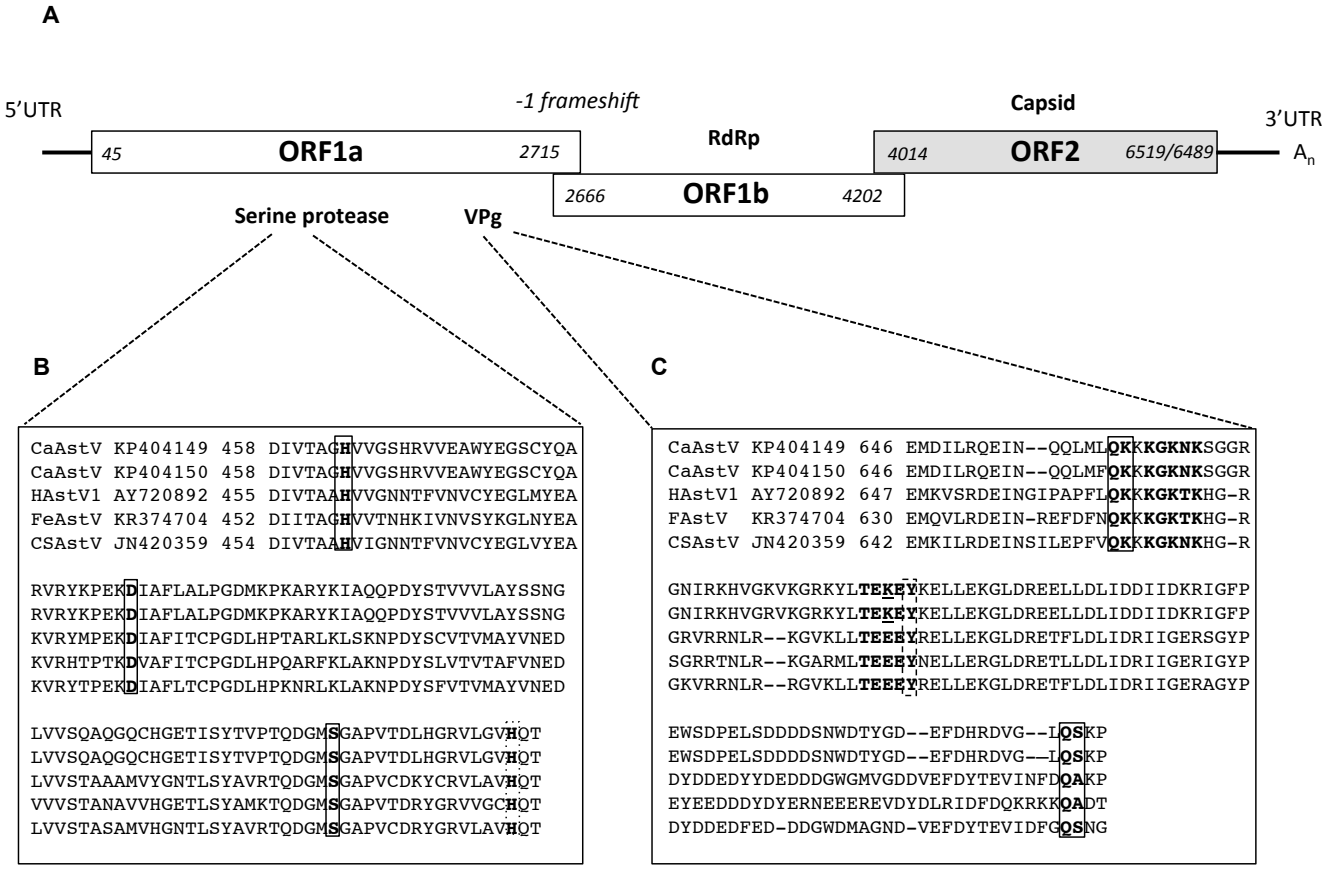


Figure 4.6 Genome organisation of CaAstV
The genome is divided into three ORFs, with ORF1 divided by a frameshift site (A). ORF1a is predicted to encode a serine protease (B) and a VPg protein (C). This is supported by alignment of the two CaAstV sequences with HAstV (human), FeAstV (feline) and CSAstV (Californian sea lion) sequences. The suspected catalytic triad of the serine protease (B) is highlighted by the boxed residues, with the dotted box H residue (histidine) representing the substrate binding region. The boxed residues in (C) represent the C and N termini cleavage sites of VPg. The conserved KGK(N/T)K and TEXEY motifs of VPg proteins are highlighted in bold, with the TEKEY variation identified in CaAstV underlined. The tyrosine residues identified by a dashed box represent the site of covalent linkage of VPg to the RNA genome.

ORF1a also encodes the viral genome-linked protein, VPg (Fuentes *et al.*, 2012) (figure 4.6C). CaAstV VPg is predicted to start at aa 656, at a conserved QK cleavage site and is 90 aa in length. In other astroviruses, it has been proposed that the C-terminal VPg cleavage site is coded by Q(P/A/S/L) (Al-Mutairy *et al.*, 2005), and the presence of a QS dipeptide at the same site in CaAstV is consistent with this prediction. The amino acid motif

KGK(N/T)K is conserved at the N-terminal end of VPg sequences from both astroviruses and caliciviruses, and this is also identifiable in the CaAstV genome. Another conserved VPg motif is TEXEY, with mutagenesis studies indicating that the Y (tyrosine) residue is important for linkage of VPg to viral RNA (Fuentes *et al.*, 2012). Analysis of the CaAstV ORF1a sequence also identifies the conserved TEXEY motif at 684-688, thus this tyrosine is predicted to covalently link to the RNA genome. However, the CaAstV sequence diverges slightly from the other mamastroviruses studied, in that X of the motif corresponds to K, whereas this is E/Q in all other mamastrovirses.

A -1 ribosomal frameshift site between ORF1a and ORF1b, present in HAstVs (Marczinke *et al.*, 1994), is also conserved in CaAstV. This translational frameshift is directed by the slippery heptamer sequence AAAAAAC at position 2666 in the CaAstV genome. A stem loop structure is predicted downstream of the slippery sequence, as shown in figure 4.7. The slippery sequence and downstream stem loop are highly conserved amongst mamastroviruses. The 3'end of ORF1a overlaps with ORF1b by 49 nucleotides. This is shorter than the 71nt overlap reported for HAstV.

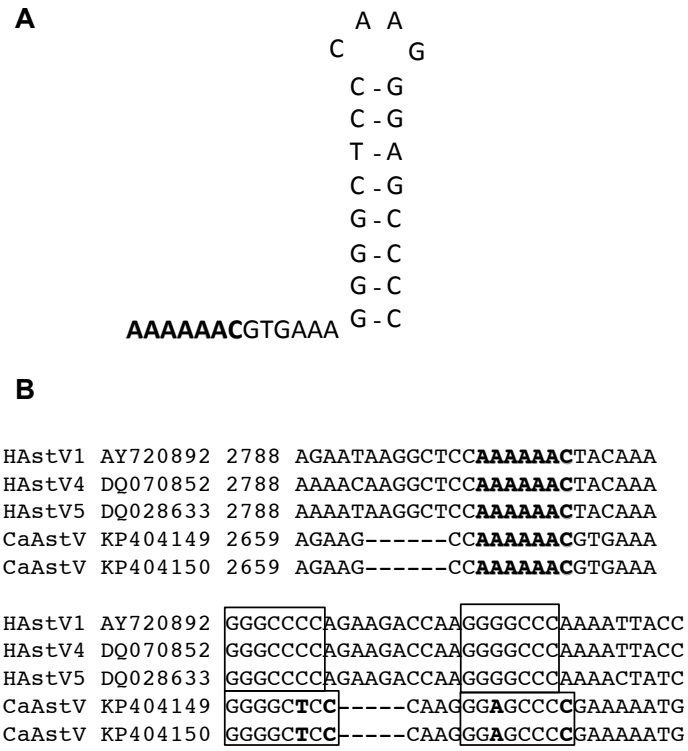


Figure 4.7 Analysis of ORF1a/1b -1 frameshift site.
(A) Predicted structure of the –1 frameshift site, directed by a slippery heptamer sequence and a downstream stem loop structure. (B) Sequence alignment of CaAstV and a range of human astrovirus (HAstV) isolates shows this region to be highly conserved. The slippery heptamer sequence is highlighted in bold, and the stem loop structure is boxed, with nucleotides in bold representing differences

ORF1b is predicted to code for an RNA dependent RNA polymerase (RdRp). The CaAstV sequence contains a YGDD motif at aa 1252, common to RdRps of a variety of RNA viruses, supporting this conclusion. ORF1b of CaAstV aligns with the RdRp of HAstV with 58-60% aa identity (HAstV-1, 4, 5 and 8). There is a similar identity to feline astrovirus (56%) and porcine astrovirus (55%). CaAstV aligns most closely with the Californian sea lion astroviruses (67-71%), though only incomplete Californian sea lion astrovirus sequences were available.

As for all other astroviruses studied to date, an overlapping reading frame exists at the ORF1b-ORF2 junction of CaAstV. The CaAstV ORF1b-ORF2 overlap sequence is 8nt, as reported for other mamastroviruses, hence ORF2 is in the same frame as ORF1a. As previously reported, the capsid sequence was found to have an in-frame start codon 180 nt upstream of the start codon homologous to other mamastrovirus genomes (Toffan *et al.*, 2009).

The 6 aa C terminus of the VP1 (SRGHAE) is highly conserved in mamastrovirses. This motif is within a highly conserved nucleotide stretch, s2m, overlapping the termination codon of ORF2, and has been identified in both CaAstV strains sequenced.

The overall nucleotide identity between the two CaAstV strains sequenced was 88.5%. Sequence comparison of the individual ORFs is presented in table 4.2. This clearly shows that ORF1b (RdRp) is most closely conserved, making this an ideal target for qPCR screens. Conversely, the capsid sequence is most diverse.

	Nucleotide	**Amino Acid**
ORF1a	93.8%	98.4%
ORF1b	97.6%	99.8%
ORF2	77.5%	80.8%

Table 4.2 Comparison of nucleotide and amino acid identity of the two full-length CaAstV sequences

A phylogenetic tree was constructed by multiple alignment of the full-length genome of the two CaAstV strains isolated in this study, and a number of astrovirus reference strains isolated from different mammalian species. This was achieved using MEGA6 software (figure 4.8).

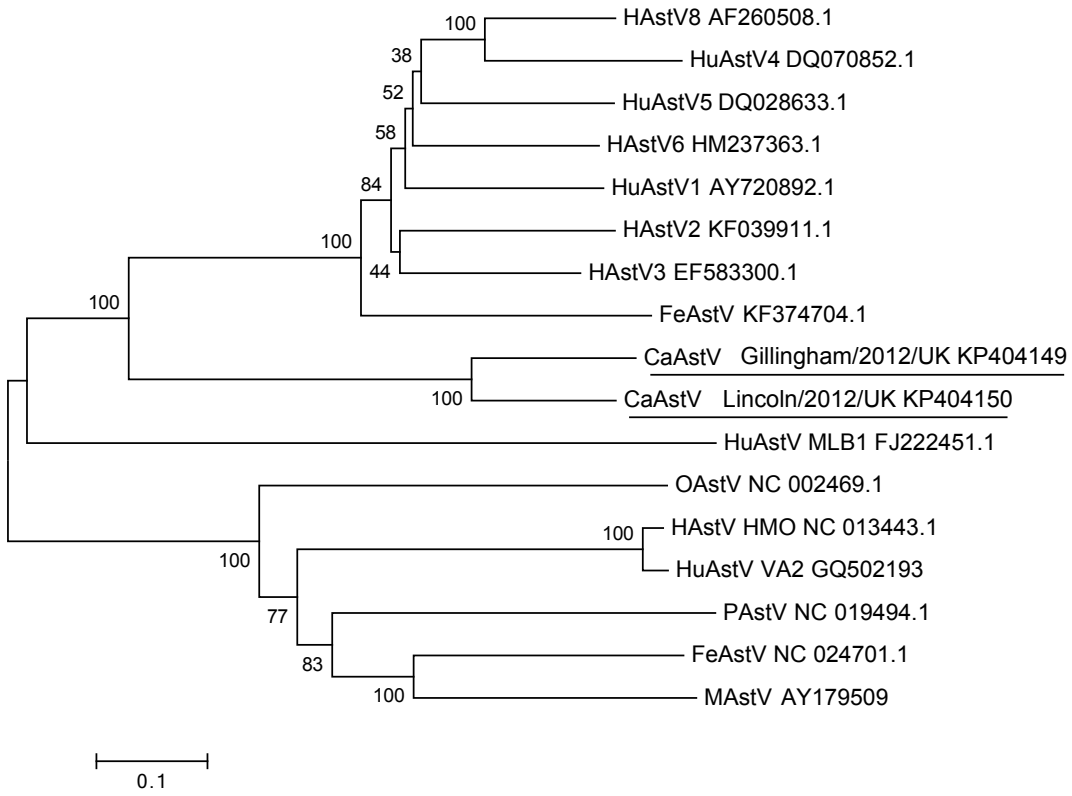


Figure 4.8 Phylogenetic tree based complete genome nucleotide sequences of astroviruses from a range of mammalian species.
GenBank accession numbers are listed, with the strain details included for the CaAstV isolates sequenced in this study (isolates underlined). The tree was determined using the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA6.

4.6 Chapter Summary

CaAstV has previously been detected sporadically in dogs across the world, but the association with disease, prevalence levels and genetic diversity is largely unknown. This chapter presents the first identification and molecular characterization of CaAstV cases in dogs in the UK. Sequencing of the viral capsid for all four strains revealed extensive genetic

diversity, and sequencing of the full genome of two strains has provided the first full-length sequences for CaAstV.

The prevalence of CaAstV in gastroenteritis cases in this study was shown to be 6.0%. This prevalence was unexpectedly high given that CaAstV has not previously been reported in the UK. It may be predicted that this is an underestimation of prevalence based on the population of dogs surveyed. The majority of previous CaAstV epidemiological studies have focused on dogs less than 6 months old, whereas this study included dogs of any age. Serological studies have shown that exposure to CaAstV typically occurs in young animals, with dogs older than 3 months significantly more likely to be seropositive that younger dogs (Martella *et al.*, 2011). This suggests that studies focusing only on young dogs will identify more positive CaAstV cases. However, the decision to survey dogs of any age in this study enabled detection of a CaAstV case in a 7-year old dog. This is oldest case of CaAstV reported to date and highlights the need to have an index of suspicion for infectious causes of gastroenteritis in dogs of any age. Indeed although HAstV is more common in paediatric populations, infections in the elderly are reported (Fernández *et al.*, 2011).

The pathology caused by CaAstV in dogs is uncertain; CaAstV has previously been detected in the stools of both healthy and diseased dogs (Grellet *et al.*, 2012; Martella *et al.*, 2011). However, this study shows a relationship between the presence of CaAstV RNA in stool samples, and the presence of clinical signs of gastroenteritis ($p < 0.001$). This finding is in agreement with two previous studies from Italy and China (Martella *et al.*, 2011; Zhu *et al.*, 2011), but is at odds with a French study which found no significant difference in CaAstV identification between diarrhoeic (27%) or healthy puppies (19%) (Grellet *et al.*, 2012). Determination of the specific pathology induced by CaAstV infection in dogs is often

confounded in a clinical setting by co-infections with other gastroenteric pathogens (e.g. CPV in cases 1 and 4 in this study). Experimental studies will be required to confirm or refute the association of CaAstV with gastroenteritis, but this study does suggest CaAstV that may cause disease.

Sequencing of the capsid region of each CaAstV strain identified in this study revealed significant sequence variation. This mirrors the variation previously identified within HAstV isolates (De Benedictis *et al.*, 2011). At present, astroviruses are named according to the species in which they are isolated, and subsequent classification is based upon serotypes; these are defined if there is a 20-fold or greater two-way cross neutralization titre (King *et al.*, 2011). Sequence analysis has verified this classification, with HAstV 1-8 having 86-100% nucleotide identity within a serotype, based on capsid sequences (Noel *et al.*, 1995). The nucleotide variation within the capsid region of the four CaAstV strains was shown to be 77.1-81.1%, which strongly suggests these strains are also different serotypes. Confirmation of this requires serological analysis, but unfortunately repeated attempts to grow the CaAstV isolates identified in this study in cell culture failed (data not shown).

Identification of four possible CaAstV serotypes circulating in the UK alone raises questions regarding the possible origins of these strains. Phylogenetic analysis of the UK capsid strains alongside the limited number of CaAstV sequences previously listed in GenBank was unexpected. There was no clustering of the UK strains, unlike the grouping of all Chinese strains. Instead UK strains each grouped with a different CaAstV isolate from either China or Italy. With such limited sequences available it is not possible to determine whether CaAstV strains have spread globally, or independent evolution has occurred. Clearly a high rate of evolution does occur within all astroviruses however, with their RNA genome

facilitating introduction of point mutations and recombination events (Wolfaardt *et al.*, 2011).

Given the strain diversity identified, it has been suggested that some CaAstV strains may be more pathogenic than others (Martella *et al.*, 2012). This has previously been reported for astroviruses of mink which show variation in their ability to invade the central nervous system in a strain related manner (Blomström *et al.*, 2010). Assessment of this risk will require wider epidemiological and clinical studies. Another concern raised by the existence of multiple circulating CaAstV strains, is regarding future disease control. Management of viral causes of gastroenteritis in dogs is best achieved by widespread vaccination, exemplified by the widely used canine parvovirus vaccine. However the presence of multiple strains will make CaAstV vaccine design challenging.

Full genome sequencing of two CaAstV isolates revealed them to be closely related and possess a typical astrovirus organization. The first full length sequence of an astrovirus was for HAstV in 1994 (Willcocks *et al.*, 1994), and relatively few full length sequences have since been determined. Sequence analysis of the CaAstV strains identifies the presence of a serine protease and VPg within ORF1a as for other astroviruses, which is separated from the conserved RdRp of ORF1b by a -1 frameshift.

In summary, this chapter has not only identified CaAstV circulating in the UK dog population, but also found significant genetic diversity within the CaAstV strains. Furthermore, full genome sequencing of two CaAstV isolates has enabled detailed molecular characterisation of this astrovirus species, and provides the astrovirus field with further examples of genome variation.

Chapter 5

Identification of CNV carbohydrate attachment factor

5.1 Chapter introduction

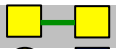
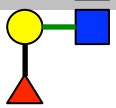
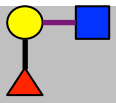
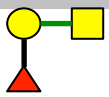
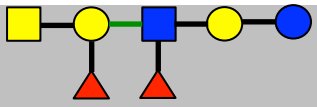
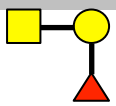
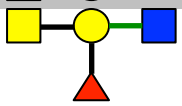
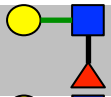
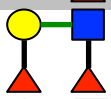
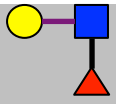
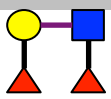
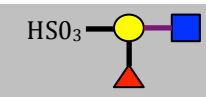
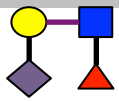
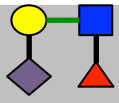
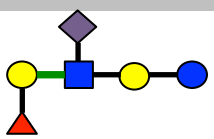
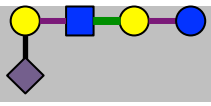
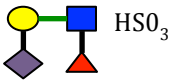
Understanding of CNV interactions with host cells is negligible, with previous studies on CNV, including chapter 3 of this thesis, restricted to epidemiological and genetic analyses. As this virus has been shown to be emerging in the UK and worldwide, there is now greater necessity to study the detail of the virus-host relationship at the cellular level. Traditionally this would require considerable quantities of purified virus, but no studies have described successful culture of CNV *in vitro*, and infectious clinical samples often lack sufficient material. However, the production of CNV VLPs for the epidemiological aspect of this thesis provided a valuable opportunity to examine virus-cell interactions in the absence of purified infectious CNV particles. Therefore, the third aim of this thesis was to use CNV VLPs to develop a greater understanding of how CNV interacts with canine cells. This would allow comparison with other genogroups and genotypes of noroviruses, and it was hoped this would provide knowledge of both norovirus evolution and the risk of transmission of CNV between species.

The first step in viral invasion of a host cell is the attachment of viral capsid proteins to specific host cell receptors. Many caliciviruses studied to date use carbohydrate structures to attach to cells prior to invasion (Marionneau *et al.*, 2002; Ruvoën-Clouet *et al.*, 2000; Taube *et al.*, 2009; Zakhour *et al.*, 2009) and it was consequently predicted that CNV would also recognize a carbohydrate attachment factor on canine cells. Therefore the goal was set to identify and characterize the interaction of the capsid of CNV with cell surface carbohydrates. Confirmation that this carbohydrate receptor was important *in vivo* was achieved by studying expression of the target carbohydrate in dogs and performing immunohistochemical and ELISA-based assays to study binding *in vitro*. This also enabled evaluation of the proportion of the canine population that may be susceptible to CNV.

5.2 CNV VLPs bind to synthetic neoglycoconjugates related to HBGAs

To commence investigations, a panel of immobilized neoglycoconjugates (table 5.1) were used to assess the ability of CNV to attach *in vitro*. In the absence of purified CNV particles, CNV VLPs were used to represent the capsid protein; the binding properties of purified norovirus virions and norovirus VLPs have been shown to be highly comparable (Harrington *et al.*, 2004). VLPs of the three different strains of CNV generated as described in chapter 3, were incubated with neoglycoconjugates immobilized on a 96-well immunoplate in an ELISA-based assay at 37°C. VLPs of CNV strain C33 were first tested against an extensive panel of neoglycoconjugates attached to either polyacrylamide (PAA) or human serum albumin (HSA) (Figure 5.1A). Four different carbohydrate structures were identified to which VLPs could bind. These were H type 1, A heptasaccharide, Lewis b and lacto-N-fucopentose. Each of these neoglycoconjugates incorporates the H type 1 motif Fucα1-2Galβ1-3GlcNAcβ1-R1 (structures shown in figure 5.1A and table 5.1), suggesting that these three carbohydrate moieties in the specific H type 1 configuration are important for CNV binding. This is in agreement with the finding that although A heptasaccharide could bind to CNV VLPs, the closely related A di and A tri neoglycoconjugates could not. A di and A tri neoglycoconjugates incorporate a GalNAc moiety which differentiates A antigen from H antigen, but they lack GlcNAc which is one of the three moieties that constitute the H antigen.

Table 5.1. Neoglycoconjugates used to determine the carbohydrate binding specificity of CNV VLPs

Name	Conjugate	Structure	Graphic representation of structure
αN-acetylgalactose	PAA	GalNAcα1	
LacNAc	PAA	Galβ1-4GlcNAcβ1	
Forsmann disaccharide	PAA	GalNAcα1-3GalNAcβ1	
H type 1	HSA	Fucα1-2Galβ1-3GlcNAcβ1	
H type 2	Both	Fucα1-2Galβ1-4GlcNAcβ1	
H type 3	PAA	Fucα1-2Galβ1-3GalNAcα1	
A heptasaccharide	HSA	GalNAcα1-3(Fucα1-2)Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc β1	
A trisaccharide	Both	GalNAcα1-3(Fucα1-2)Galβ1	
A disaccharide	PAA	GalNAcα1-3Galβ1	
A type 2b	PAA	GalNAcα1-3(Fucα1-2)Galβ1-4GlcNacβ1	
Le[a] (Lewis a)	PAA	Galβ1-3(Fucα1-4)GlcNAcβ1	
Le[b] (Lewis b)	Both	Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1	
Le[x] (Lewis x)	PAA	Galβ1-4(Fucα1-3)GlcNAcβ1	
Le[y] (Lewis y)	Both	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1	
3-sulfo Le[x]	PAA	HSO_3-3Galβ1-4(Fucα1-3)GlcNAcβ1	
Sial Le[x] (sialyl-Lewis x)	Both	NeuAcα2-3Galβ1-4 (Fucα1-3)GlcNAcβ1	
Sial Le[a] (sialyl-Lewis a)	Both	NeuAcα2-3Galβ1-3 (Fucα1-3)GlcNAcβ1	
Sialyl LNF V (lacto N-fucopentose V)	HSA	Fucα1-2Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc β1	
Sialyl LNnT (lacto-N-neotetraose)	HSA	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1	
6-sulfo Sialyl Le[x]	PAA	NeuAcα2-3Galβ1-3(Fucα1-4)(HSO_3-6)GlcNAcβ1	

All four neoglycoconjugates shown to bind VLPs were attached to HSA, although there were six additional HSA-conjugated oligosaccharides that did not bind to the VLPs. The conjugate molecule is suspected to have some effect on accessibility of the oligosaccharide, as Lewis b conjugated to PAA was not able to bind to the VLPs. This is evidently a limitation of the synthetic neoglycoconjugate binding assay, and subsequent experiments using clinical samples were deemed essential to investigate these binding preferences further.

Temperature has previously been shown to affect the ability of norovirus VLP binding to H type carbohydrates; red blood cells only express H type 2 carbohydrates (Fucα1-2Galβ1-4GlcNAcβ1-R1), and HuNoV is able to induce haemagglutination at 4°C but not 37°C (Hutson *et al.*, 2003). To investigate the possible effect of temperature on CNV binding, synthetic oligosaccharides were next incubated with VLPs at 4°C. H type 1, H type 2 and H type 3 were coated onto immunoplates and C33 CNV VLP was added for a 1 h incubation at 4°C. The results presented in figure 5.1B show that H type 2 and H type 3 oligosaccharides were able to weakly bind CNV VLPs at 4°C but showed no binding ability to CNV VLPs at 37°C.

Given the apparent specificity of CNV strain C33 VLP binding to neoglycoconjugates, it was predicted that the two other CNV strains available for this study would also behave in a similar manner. To investigate this, strains 170 and HK were incubated with the four neoglycoconjugates shown to bind strain C33, plus a negative control (A tri). The results as shown in figure 5.1C confirmed that at 37°C all 3 strains of CNV were able to recognize H type 1, A heptasaccharide, Lewis b and lacto-N-fucopentose, but could not bind to A tri.

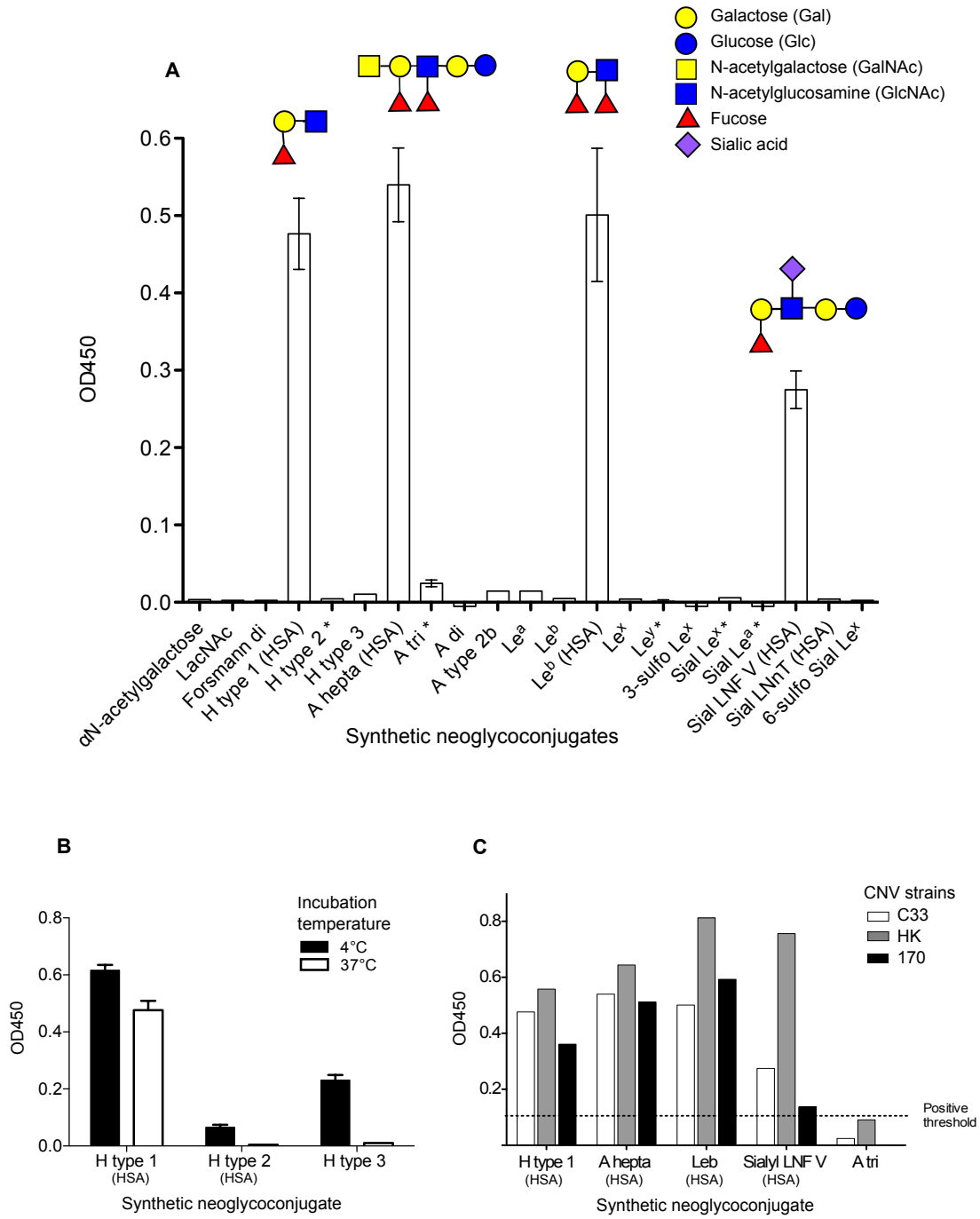


Figure 5.1 Binding of CNV VLPs binding to synthetic neoglycoconjugates.
CNV strain C33 VLPs were incubated with a panel of 26 neoglycoconjugates at 37°C (A) and a panel of 5 neoglycoconjugates at 4°C immobilized on immunoplates (B) to determine binding ability. Bound VLPs were detected using an anti-CNV antibody. Synthetic oligosaccharides were either conjugated to human serum albumin (HSA), or polyacrylamide (PAA, unlabeled). An asterisk indicates that the oligosaccharide was available for testing attached to both types of conjugate, and the mean OD450 for both is presented. VLP binding to Lewis b was significantly different for each conjugate, hence each is shown independently. Schematic structures of the neoglycoconjugates recognized by CNV VLPs are presented above the associated bars on the chart. Neoglycoconjugates shown to bind CNV strain C33 were also incubated with two additional CNV strain VLPs (170 and HK) at 37°C (C). All error bars on bar charts are demonstrative of the standard error.

5.3 Major capsid protein (VP1) sequence analysis of different noroviruses

Genogroup I and genogroup II HuNoV can bind to HBGAs (Huang *et al.*, 2003; Marionneau *et al.*, 2002), and following the identification that CNV VLPs were able to bind to synthetic HBGA carbohydrates *in vitro*, comparison of the major capsid protein amino acid sequences was performed. The major capsid protein of a representative GI.1 Norwalk strain (Genbank AAA59229.1) and a GII.4 strain (Norovirus Hu/II.4/2201480/HK/2010, Genbank ADR78268.1) were aligned with the three CNV strains using ClustalW2. Figure 5.2 illustrates only the region of capsid sequence alignments that incorporate the amino acid residues shown to interact with HBGAs in previous crystallography studies for GI.1 and GII.4 noroviruses (Bu *et al.*, 2008; Cao *et al.*, 2007; Choi *et al.*, 2008). Only very limited similarities were identified between the GI.1 or GII.4 norovirus HBGA-binding amino acids and the capsid sequences of the three CNV strains. This suggests that different mechanisms of carbohydrate binding exist between CNV and the GI and GII HuNoVs.

Figure 5.2 Alignment of GI.1, GII.4 and CNV major capsid protein sequences.
Major capsid protein sequences of a representative GI.1 (GenBank AAA59229.1) and GII.4 (GenBank ADR78268.1) HuNoV and the three CNV strains used in this study were aligned using ClustalW2. The 8 (GI.1) and 7 (GII.4) residues implicated in HBGA binding from crystallographic studies are highlighted by grey boxes in the figure. Amino acids residues in the three different CNV strain major capsid proteins that are identical to the key HBGA binding residues of GI.1 or GII.4 are highlighted by white boxes.

5.4 Dogs express A, H and Lewis antigens in their gastrointestinal tract

To determine if dogs express the carbohydrates identified as potential CNV attachment factors by the neoglycoconjugate assay, saliva samples from 26 dogs (1-23, D - F) and intestinal scrapings from 6 dogs (A-F) were phenotyped using ELISA-based assays. H antigen carbohydrate was present in the saliva and intestinal tissue of all dogs tested (figure 5.3A). This data was verified by sequencing the complete *Fut2* gene of 14 of these dogs (identified by * in figure 5.3A). The canine *Fut2* gene showed high conservation of the nucleotide sequence between samples, with no evidence of polymorphisms that would result in an inactive transcript. A single non-coding nucleotide polymorphism (C777G) was identified in one dog.

Expression of H antigen along the length of gastrointestinal tract was examined in samples collected from the duodenum, jejunum, ileum, caecum and colon, using Ulex europaeus agglutinin (an H antigen binding lectin). Concavalin A was used to confirm that the amount of carbohydrate present in each duodenal scraping was comparable (data not shown). Figure 5.3B shows H antigen expression in the intestines varies between dogs, but it is apparent that this inversely correlates with A antigen expression (see figure 5.3C). Within the gastrointestinal tract of each dog, H antigen expression is relatively constant from the duodenum to the colon. This in contrast to the H antigen expression patterns reported in humans and in cattle, whereby expression of the H antigen diminishes in the distal parts of the gastrointestinal tract (Yuan *et al.*, 1985; Zakhour *et al.*, 2009).

A antigen was shown to be present in 12/26 (46.2%) dogs, whereas no dogs were B antigen positive. A antigen expression in saliva correlated with A antigen expression in gastrointestinal secretions, demonstrated by phenotyping of saliva samples (D, E and F)

obtained from three dogs from which gastrointestinal tissues were also available. Of the dogs from which tissues were obtained, two were identified as being A antigen positive (C and E), and A antigen expression throughout the length of the gastrointestinal tract in these two animals was relatively constant (figure 5.3C). The A antigen positive dogs had comparatively reduced expression of H antigen as detected by Ulex binding, apparent in figure 5.3B. This is understood to be due to the ability of the A antigen to mask the H antigen, therefore preventing detection by Ulex (Nyström *et al.*, 2011).

Lewis a, Lewis b and Lewis x were not detectable in canine saliva or canine gastrointestinal scrapings (data not shown). A lack of expression of Lewis a and Lewis x antigen was expected based on previous data which had already confirmed H antigen expression and therefore secretor status in every dog studied (figure 5.3A). Lewis y was detectable in 12/26 (46.2%) dogs, although Lewis y antigen expression was not linked to A antigen expression; dogs could express both, either or neither antigen (figure 5.3A). Lewis a and b are derived from α1,3-fucosyltransferase activity on the type 1 precursor and H type 1 respectively, whereas Lewis x and y are derived from α1,3-fucosyltransferase activity on the type 2 precursor and H type 2. Amongst the Lewis y positive dogs, Lewis y expression varied significantly, unlike the all-or-nothing expression of A antigen.

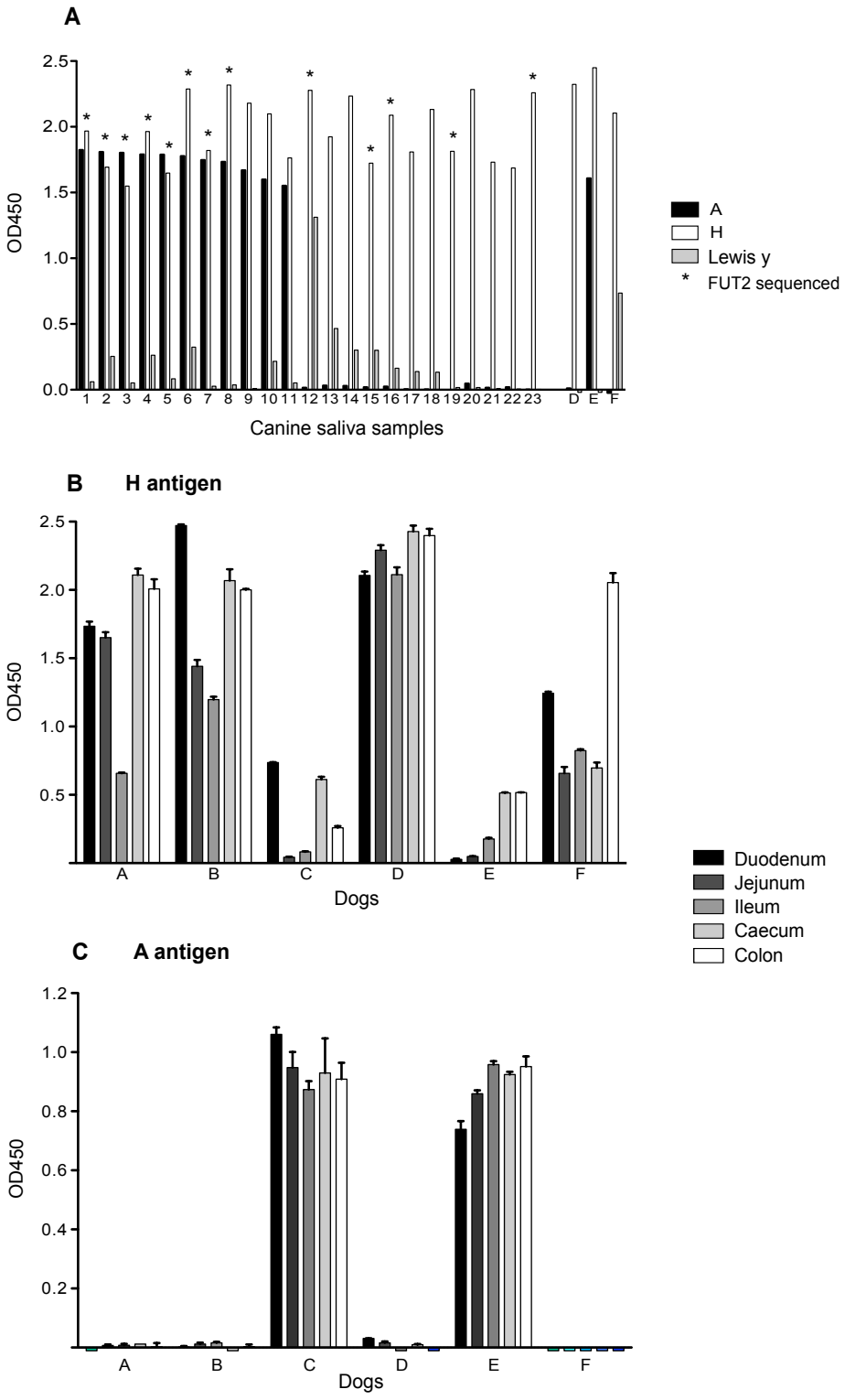


Figure 5.3 Phenotyping canine saliva and gastrointestinal samples using ELISA-based assays. Twenty-six canine saliva samples were anaysed to determine the expression of H antigen, A antigen and Lewis y antigen (A). Saliva samples 1-23 were collected from kenneled dogs, whereas samples D, E and F were collected from research dogs from which tissues were also collected. The *Fut2* gene was sequenced for 14 dogs, identified by an asterisk, in addition to dog B from which saliva was not available. Phenotyping for H antigen (B) and A antigen (C) was also performed for tissue scrapings from the duodenum, jejunum, ileum, caecum and colon of the six dogs A-F.

5.5 CNV VLPs bind to intestinal tissues of A antigen positive and negative dogs

Following identification of putative attachment factors for CNV and the confirmation that expression of most of these factors is present in the canine intestinal tract, the ability of CNV VLPs to bind to canine samples was examined. Immunohistochemistry (IHC) was used to confirm CNV VLPs could bind to canine intestinal sections, and also to investigate the pattern of VLP binding and compare this to carbohydrate expression. Based on the data obtained using the panel of synthetic neoglycoconjugates, it was hypothesized that CNV binding would follow H antigen and A antigen expression in the tissues. Tissue sections from the length of the canine intestinal tract were incubated with CNV VLPs and their binding detected using primary and secondary antibodies. VLPs from the three representative strains of CNV (C33, 170 and HK) were incubated with tissue sections separately and it was shown that all strains were able to bind to the intestinal tissue sections used in this study (Figure 5.4 and data not shown). Figure 5.4 shows only data obtained using the CNV strain C33 VLPs binding to tissue sections, as this is representative of the two additional strains.

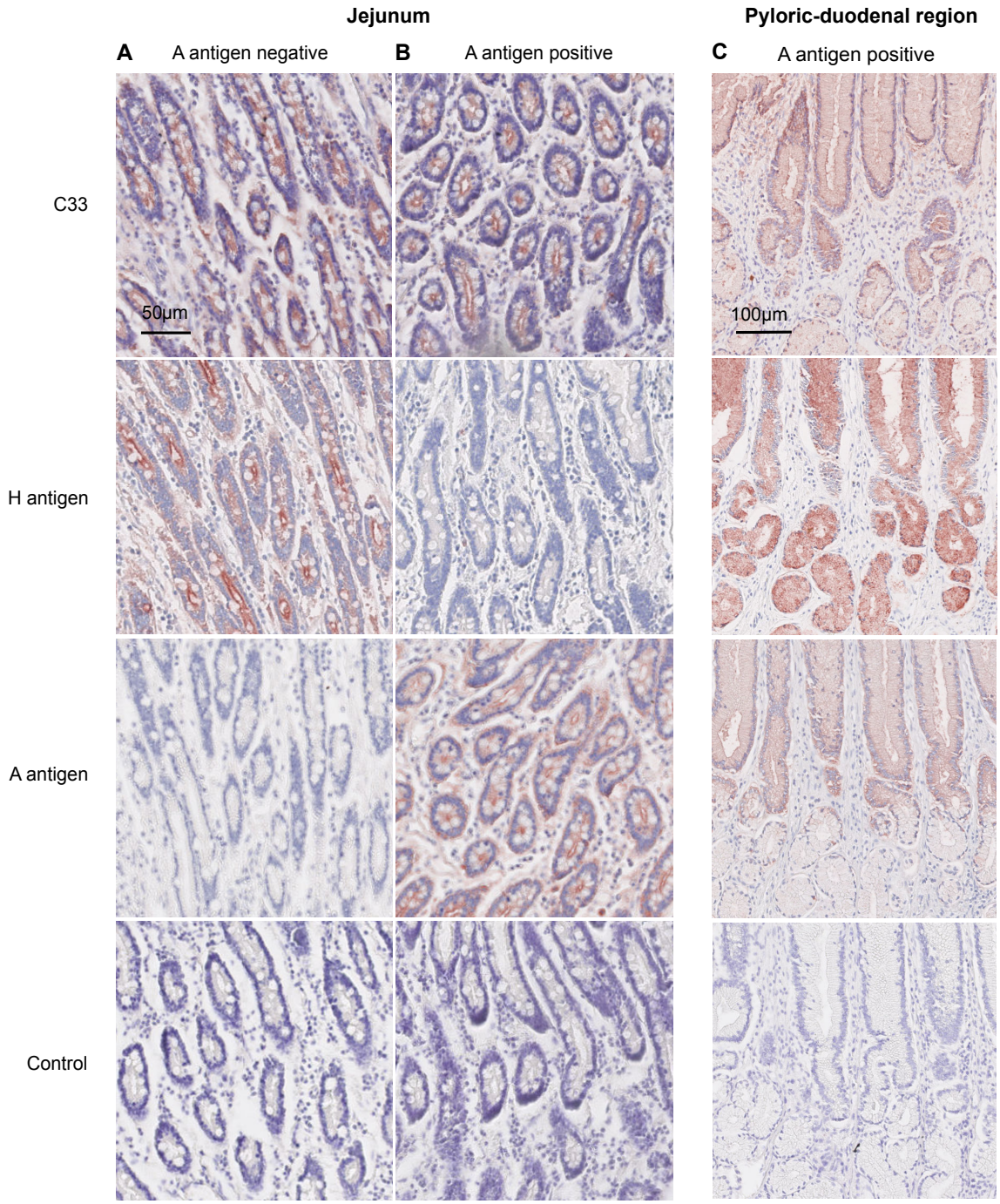


Figure 5.4 Immunohistochemical analysis of CNV VLPs binding to canine intestinal tissues. VLPs were incubated with tissue sections overnight and binding was detected using anti-CNV antibody and biotinylated secondary antibody. HBGA expression was determined using anti-A antigen antibody and Ulex lectin. Binding of either VLPs or antibodies/lectin is indicated by the presence of red signal. Panel A presents the binding of CNV strain C33 to jejunal tissue from an A antigen negative dog, and panel B presents C33 binding to an A positive dog. Panel C shows binding of C33 to tissue from the pyloric duodenal region of intestine from an A positive dog.

To determine the presence or absence of H antigen and A antigen in the tissue sections used for the VLP binding, sections were incubated with Ulex and anti-A antigen antibody respectively and immunohistochemistry performed. Both A positive and A negative dogs were compared, as shown in figure 5.4 panels A and B. H antigen expression was not detectable in the A positive dogs in agreement with the ELISA phenotyping data. Figure 5.4 demonstrates that CNV VLPs can bind to both A antigen positive and A antigen negative dogs, and that expression of both of these carbohydrates follows a comparable pattern to CNV VLP binding. In particular, VLP binding closely follows the pattern of A expression when examining deeper tissues of the pyloric duodenal region (figure 5.4 panel C). VLP binding was concentrated at the mucosal surface of intestinal villi, with no binding observed in deeper tissues. Whilst this does not prove a direct association between CNV VLP and HBGA binding, it does add support to the initial data.

5.6 CNV VLP binding ability shows variation related to carbohydrate expression pattern

The phenotyping results demonstrated that different dogs exhibit variation in carbohydrate expression, comparable to most species studied. To investigate whether this affected the ability for CNV VLPs to bind to tissues, further assays were required to extend the findings provided by the initial immunohistochemical studies.

CNV VLPs were incubated with 26 canine saliva samples in an ELISA-based assay, and shown to bind to all of these samples (figure 5.5A). CNV strain C33 was selected as a representative strain for these experiments as binding was more consistent. The OD450 value for the binding of VLPs to each saliva sample was variable, with a range from 0.076 to

0.784. This variation was compared to the HBGA phenotype identified in each dog to see if any correlation between binding and expression of A antigen or Lewis antigen could be established. Although no statistically significant patterns were identified, a general trend was seen with regards to samples that most weakly and most strongly bound the CNV VLPs. Of the 3 saliva samples that bound the VLPs most weakly (OD <0.2), none were A antigen positive. In contrast, of the 4 saliva samples that bound to VLPs with OD450 >0.7, all samples were A antigen positive. A significant relationship between Lewis antigen expression and VLP binding was not identified (data not shown).

As the carbohydrate repertoire of canine saliva samples has, as far as we are aware, not been characterized, it is likely that carbohydrates are present that were not detected during the phenotyping studies (Figure 5.3), that contribute to the variation in CNV VLP binding. In contrast to the paucity of data on carbohydrate expression in canine saliva, carbohydrate expression in human saliva is much better characterized. Therefore it was anticipated that use of a comprehensively phenotyped human saliva panel would provide more conclusive results regarding CNV VLP binding ability. To this end, 26 human saliva samples representing the six main groups of HBGA expression patterns known (O Lewis negative, O Lewis positive, A Lewis negative, A Lewis positive, B expression and non-secretor) were used in ELISA-based assays to quantitatively study CNV VLP binding. All three CNV strains available were first incubated with a single sample from each of the six HBGA groups, and identical binding patterns were identified for each strain (figure 5.5B). CNV strain C33 was then selected as a representative strain for the complete 26-sample panel (figure 5.5C).

Figures 5.5B and 5.5C showed that human saliva samples from non-secretor individuals were not recognized by CNV VLPs. Non-secretors do not have a functional *FUT2* gene, hence cannot express HBGAs on the surface of epithelial cells. This confirms that expression of HBGAs is essential for CNV binding. The single B antigen positive saliva sample available in this panel also did not bind CNV VLP. B antigen expression was confirmed using the anti-B antibody B49. The structure of the B antigen (Galα1-3[Fucα1-3]Galβ1-GlcNAcβ1-R1) must therefore preclude binding, showing that the terminal galactose cannot be accommodated by the CNV VLP.

Saliva samples containing the O antigen (H antigen) and A antigen, with or without the presence of Lewis antigen could all bind CNV VLPs to varying degrees. Excluding non-secretor and B antigen positive samples, the difference between the OD450 values for each saliva phenotype was statistically significant (p=0.013, one-way analysis of variance). Figure 5.5C demonstrates that human saliva samples have an increased ability to bind CNV VLPs if they contain A antigen and/or Lewis antigen instead of only O (H) antigen alone.

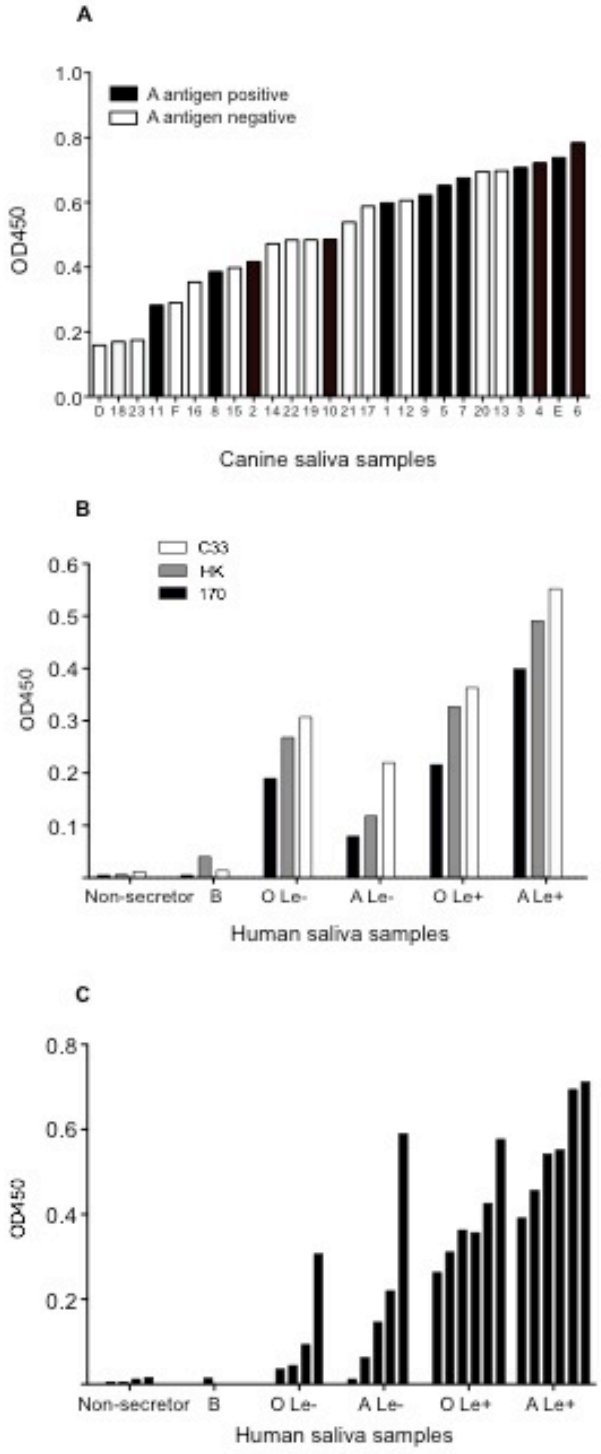


Figure 5.5 CNV VLP binding to canine and human saliva samples.
Twenty six canine saliva samples with characterized phenotypes (A antigen and Lewis) were coated onto ELISA plates and the ability for CNV VLPs (strain C33) to bind was assessed (A). Samples are ordered according to CNV VLP binding ability (low to high). Black bars correspond to dogs expressing the A antigen and white bars are A antigen negative dogs (Lewis antigen expression is not shown). Six human saliva samples, each with a different ABO and Lewis phenotype, were used to assess binding of CNV VLPs from the three CNV strains available (B). CNV strain C33 was next selected as the representative strain to analyse binding to a wider panel of twenty six human saliva samples (C).

To extend these findings further, binding of VLPs to Chinese hamster ovary (CHO) cell lines transfected with glycosyltransferases was studied using flow cytometry (figure 5.6). Using the HuNoV and CNV VLPs generated specifically for this project, this work was performed by A. Breiman at the University of Nantes. CHO cells do not express α1,2-fucosyltransferase activity, hence are devoid of ABH antigens (Marionneau *et al.*, 2002). Transfection of fucosyltransferase and A or B enzymes enabled the precise control of ABH antigen expression. Assessment of HuNoV GI.1 VLPs and three strains of CNV VLPs binding to transfected and untransfected cells was achieved using FACS. This demonstrated that the GI.1 HuNoV and CNV VLPs were able to bind CHO cells expressing H and A antigens, with a preference for A, but binding to cells expressing B antigen, or no HBGAs at all was substantially less. It is interesting to note that HBGA expression of the transfected CHO cells was primarily type 2 structures (personal communication, Jacques Le Pendu). Binding of CNV strain C33 VLPs to type 2 structures at 37°C is contrary to the results obtained with the synthetic oligosaccharide assay (figure 5.1A), although does agree with the data from ELISAs with 4°C incubation steps (figure 5.1B). This indicates that the CNV C33 H antigen specificity is not absolutely dependent on the β1,3 linkage of the Gal-GlcNAc moiety (H type 1), and can accommodate a β1,4 linkage (H type 2).

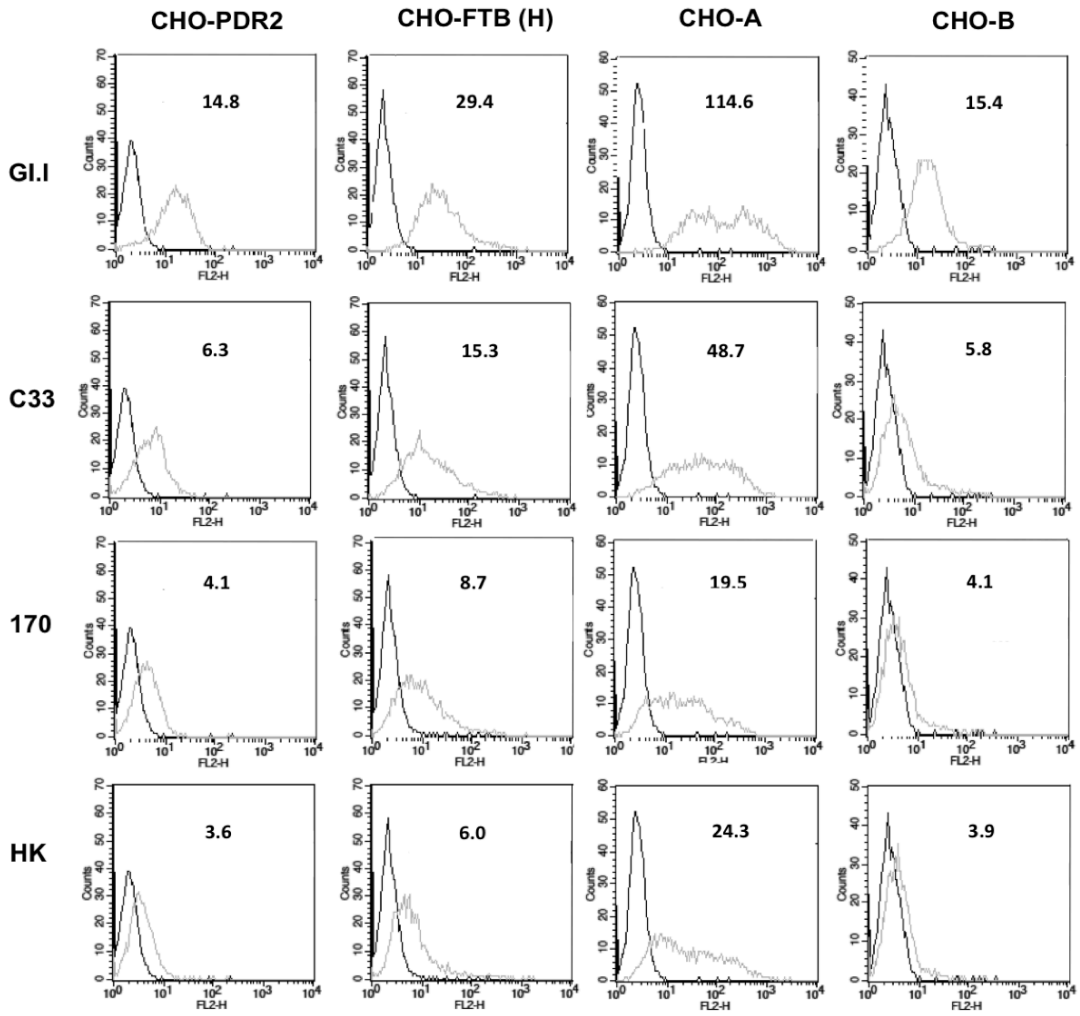


Figure 5.6 Binding of CNV and GI.1 VLPs to CHO cells transfected with glycosyltransferases. CHO cells were transfected with rat α1,2-fucosyltransferase B (FTB) cDNA to induce H antigen expression (CHO-FTB (H)) and were co-transfected with FTB and A enzyme to express A antigen (CHO-A) or transfected with FTB and B enzyme to express B antigen (CHO-B). CHO cells transfected with the empty PDR2 vector were used as control cells not expressing HBGAs. Binding of the three CNV strain VLPs (C33, 170 and HK) and GI.1 VLP to the different cell lines was assessed using flow cytometry. The black line represents signal in the absence of VLPs, but in the presence of the primary and secondary antibodies. The grey line represents VLP binding. The number above each histogram is the mean fluorescent intensity (MFI, geometric mean).

5.7 Synthetic neoglycoconjugates incubated with VLPs can block binding to canine samples

To further characterise the canine carbohydrates involved in recognition of CNV VLPs, a series of blocking studies were performed. The initial ELISA-based synthetic neoglycoconjugate assays had identified H antigen, A antigen and Lewis b antigen as likely binding partners of the CNV VLPs. To investigate the role of these carbohydrates further, CNV strain C33 VLPs were pre-incubated with each synthetic neoglycoconjugate prior to addition of the VLPs to either duodenal scrapings coated onto immunoplates (figure 5.7A), cells in tissue culture (figure 5.7B) or tissue sections (data not shown). Pre-incubation of VLPs with H type 1, A hepta (A Lewis b) and Lewis b neoglycoconjugates significantly reduced VLP binding to canine duodenal scrapings (figure 5.7A) and to HBGA-expressing HT-29 cells in tissue culture (figure 5.7B). In contrast, when VLPs were pre-incubated with H type 2, H type 3 and A tri (truncated form of A antigen lacking GlcNAc) at 37°C, no reduction in binding to canine samples was observed. Human norovirus G1.I VLPs were included in the FACS studies to provide a comparison set of data for a well characterized norovirus-carbohydrate binding pattern. Overall these blockade studies provided further evidence of the importance of H type 1, A antigen and Lewis antigen in CNV binding.

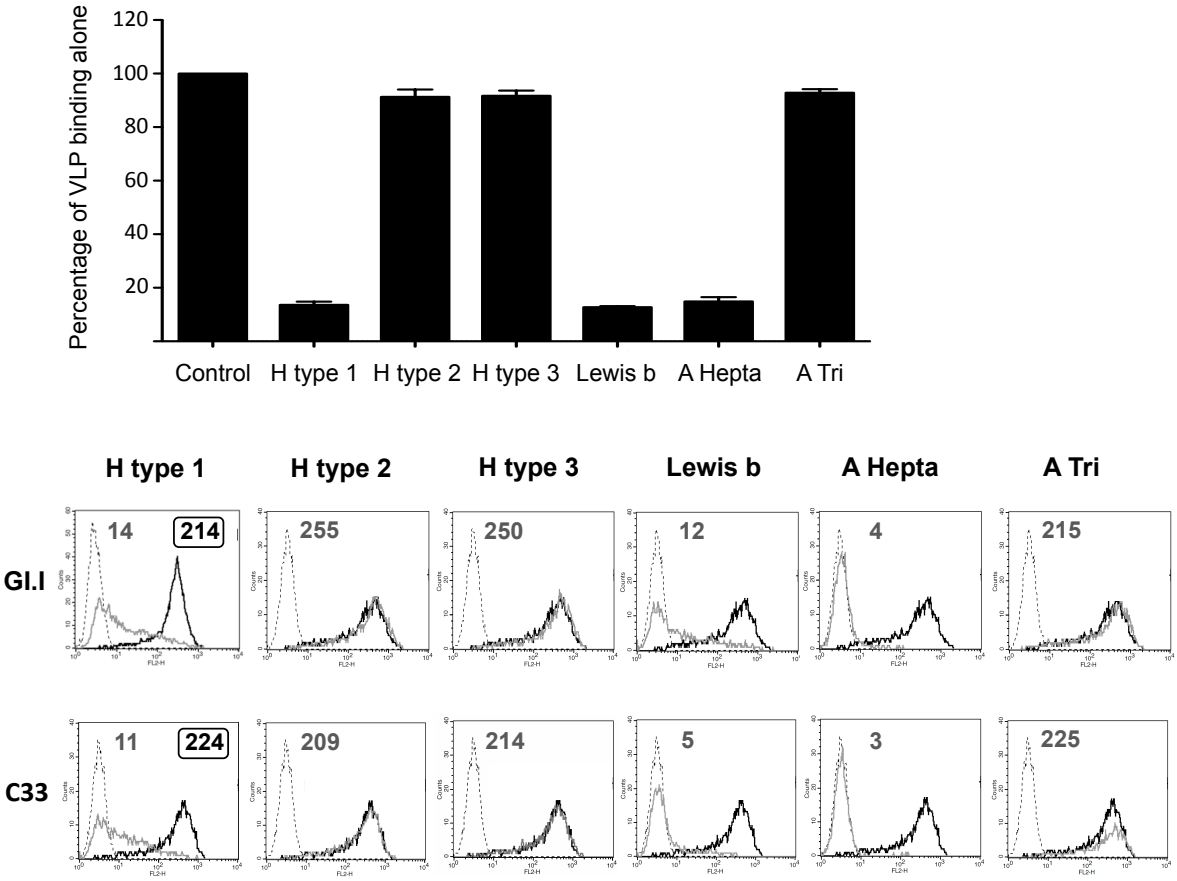


Figure 5.7 Blocking CNV binding to canine samples using synthetic neoglycoconjugates.
Neoglycoconjugates were incubated with CNV VLPs for 1 h at 37°C prior to VLPs being added to either duodenal scrapings in an ELISA-based assay (A), or human HT29 cells in suspension for flow cytometry (B). Synthetic oligosaccharides were either conjugated to human serum albumin (HSA), or polyacrylamide (PAA), as labeled in brackets beneath each oligosaccharide in part A. The same conjugates were used in the flow cytometrey experiments, where the binding of both CNV strain C33 VLPs and HuNoV GI.1 VLPs were studied. The dashed black line represents signal in the absence of VLPs, but in the presence of the primary and secondary antibodies. The black line represents VLP binding when VLPs were pre-incubated with PBS only. The grey line represents the binding of VLPs to cells after they have been pre-incubated with different synthetic oligosaccharides. The number in grey above each histogram is the mean fluorescent intensity (MFI, geometric mean) of all cells when VLPs were pre-incubated with neoglycoconjugate. The boxed number by the H type 1 histograms is the MFI of all cells when VLPs were pre-incubated with PBS only. This number applies to all conditions and hence was not repeated in each histogram.

5.8 Enzymatic removal of specific intestinal carbohydrates reduces CNV binding

Carbohydrate moiety-specific enzymes were used to cleave off HBGAs shown to play a role in CNV attachment to the cell surface. 1,2α-fucosidase was incubated with duodenal scrapings, tissue sections and cells in tissue culture to remove the fucosidase of the H antigen (figure 5.8). Fucosidase treatment was shown to completely abolish CNV VLP binding in A antigen-negative dogs. In dogs expressing A antigen however, fucosidase treatment had no effect on CNV binding to duodenal, and only a moderate effect in binding to tissue sections. Similarly, in A antigen expressing cells used for flow cytometry, no significant change in CNV VLP binding was observed after fucosidase treatment (data not shown). From this is can be concluded that in A negative dogs, the H antigen is essential for CNV binding. Fucosidase treatment of A positive dogs does not result in a change in binding pattern because it is understood that the A antigen masks the H antigen blocking fucosidase action (Nyström *et al.*, 2011), thus A antigen will therefore still be present and sufficient to mediate CNV VLP binding.

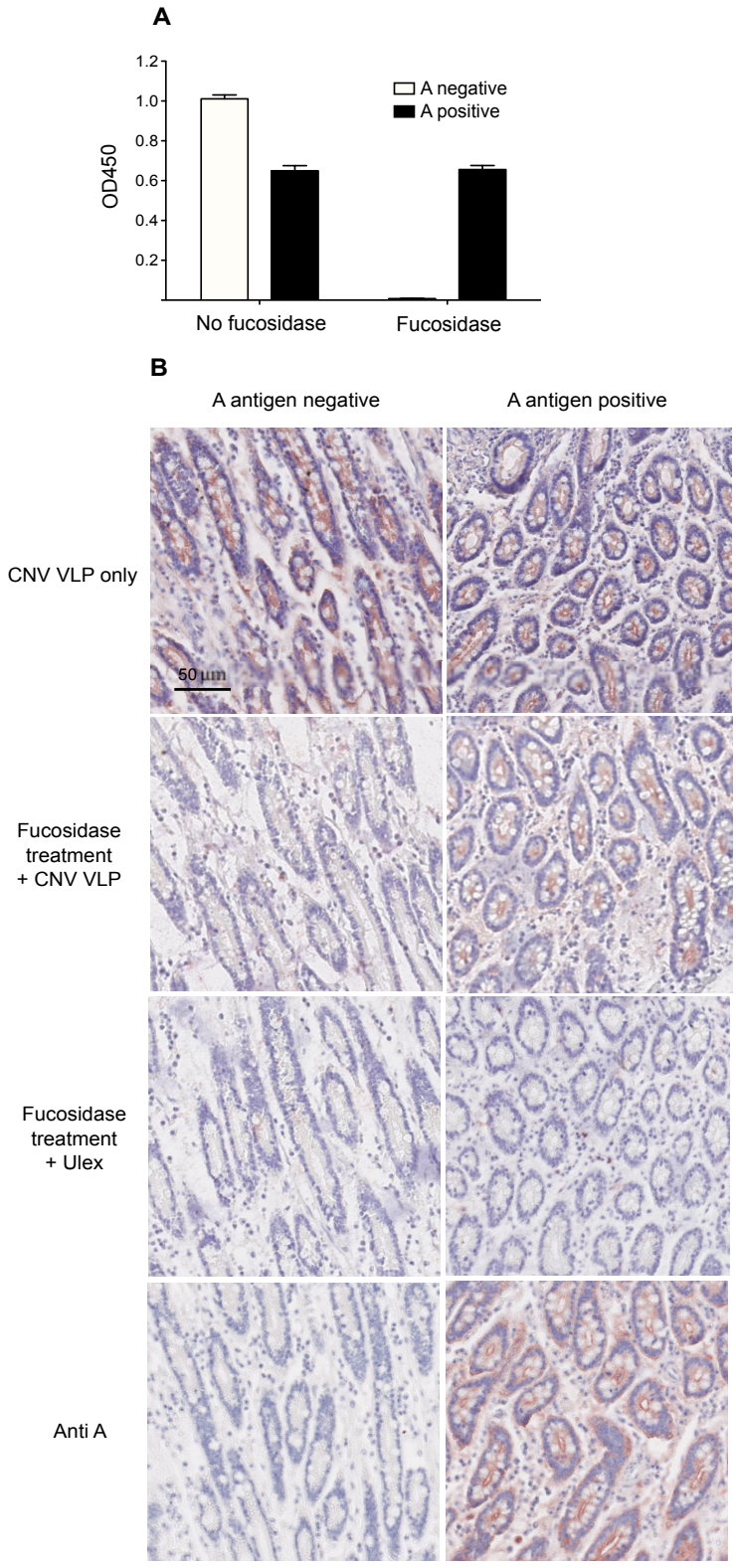


Figure 5.8 Enzymatic treatment of canine samples reduces CNV VLP binding.
Canine duodenal scrapings and canine intestinal tissues sections from an A antigen positive and an A antigen negative dog were treated with 1,2-α-fucosidase or a control for 1 h (scrapings) or 18 h (tissue sections) at 37°C. The ability for CNV VLPs (strain C33) to bind to the scrapings or tissue sections was assessed by an ELISA-based assay (A) and IHC (B) respectively. Confirmation that the 1,2-α-fucosidase removed H antigens was achieved by incubating tissue sections with Ulex.

5.9 Chapter summary

This chapter demonstrates that the CNV capsid can attach to the H antigen of the HBGA carbohydrates expressed on the surface of epithelial cells. The interaction between the CNV capsid and the H antigen can accommodate the A antigen (addition of N-acetylgalactosamine) and the Lewis antigen (addition of a α1-3/1-4 fucose) but not the B antigen (addition of galactose). These conclusions have been drawn from initial assays using synthetic neoglycoconjugates, and CNV VLP binding to intestinal sections, saliva, duodenal scrapings and cells *in vitro*. Blocking assays and enzymatic removal of identified carbohydrates were then used to confirm these findings. Whilst the merits of the individual assays may be limited, for example it is predicted that the conjugate molecules of the neoglycoconjugates can affect VLP binding and may not be an accurate reflection of the situation in vivo, the multiple lines of evidence for HBGA binding to CNV support this overall conclusion.

The discovery that CNV binds to HBGAs was unexpected with regards to the amino acid sequence of the CNV strains. When compared to the GI.1 Norwalk virus capsid amino acid sequence, there was almost no similarity in the amino acids shown to interact with HBGAs (Asp327, His329, Gln342, Asp344, Trp375, Ser377, Pro378 and Ser380) (Bu *et al.*, 2008; Choi *et al.*, 2008). When CNV major capsid protein sequences were compared to a GII.4 norovirus, a comparable lack of amino acid identity was observed in the key HBGA binding residues (Ser343, Thr344, Arg345, Asp374, Ser441, Gly442 and Tyr443 (Cao *et al.*, 2007)). The difference between the amino acid and HBGA interaction profiles for the GI and GII noroviruses indicate convergent evolution of the two genogroups has occurred (Tan & Jiang, 2011). CNVs have been classified into GIV and GVI genogroups, genetically distant from the GI and GII HuNoV strains studied to date. This is the first data to suggest that norovirus

recognition of HBGAs may have evolved in at least three different lineages. An alternative explanation for this finding would be the existence of a distant ancestor to all norovirus strains, that evolved the ability to recognize the HBGA carbohydrates which are conserved amongst many species.

Identification of H, A and Lewis antigens as the key ligands for CNV correlates well with the canine carbohydrate expression phenotypes identified in this study. All dogs tested were positive for the H antigen, and no inactivating mutations were identified in the canine *Fut2* genes sequenced. This is in contrast to *FUT2* polymorphism in humans, whereby approximately 1 in 5 Caucasians have inactivating *FUT2* polymorphisms resulting in the 'non-secretor' phenotype. The polymorphism G428A is responsible for this phenotype in over 95% non-secretors of European and African decent, and the A385T polymorphism is predominant in Asian non-secretors (Kelly *et al.*, 1995; Kudo *et al.*, 1996). In comparison, the single polymorphism identified in canine *Fut2* was a non-coding mutation, predicted to be a random change as opposed to a fixed polymorphism. Overall, the phenotyping and genotyping data obtained indicated that non-secretor individuals were absent from this canine population. This could be a reflection of the lack of evolutionary pressure to induce resistance alleles.

No dogs were positive for the B antigen, in agreement with two previous reports examining carbohydrate expression in canine intestines (Miller-Podraza H, Stenhagen G, Larsson T, Andersson C, 1997; Smith *et al.*, 1973). A total of 12/26 dogs were positive for the A antigen, a proportion comparable to a 1982 study which analysed fucolipid expression in the intestines of 37 dogs and identified 17 as A antigen positive (46%) (McKibbin *et al.*, 1982). This same study identified Lewis b antigen in 12/37 dogs (32.4%), and Lewis a in the

intestines of 8/37 dogs (21.6%). These antigens are derived from activity of α1,3-fucosyltransferase on the type 1 precursor and H type 1 antigen respectively. Unexpectedly, the results regarding Lewis antigen expression did not correlate with this previous data. Lewis a and b expression could not be demonstrated in the canine saliva or intestinal samples used in this study. This discrepancy might be due to the fact that the 1982 study involved analysis of glycolipids, whilst in this immunohistochemical approach, glycolipids were largely removed during the tissue processing steps. The 1982 study did not consider expression of Lewis x and y however, which are generated from activity of α1,3-fucosyltransferase on the type 2 precursor and H type 2 antigen. In this sample population, Lewis y was identified in 12/26 dogs (46.2%), which is comparable to the level of Lewis b expression reported in the primary study. Though the precursor antigen is still questionable, these results confirm that Lewis antigen expression is present and polymorphic in the canine population. More extensive phenotyping studies are required to solidify the results for all types of HBGA expression, but in terms of the ability of CNV to recognise HBGAs expressed in different dogs, all dogs in this study would be potentially susceptible to infection. This conclusion is supported by data in chapter 3, which identified unexpectedly high seroprevalence to CNV in the UK dog population, indicating that the majority of dogs studied would be susceptible to infection.

The confirmation that A antigen and Lewis antigen can be expressed in dogs, and that expression is polymorphic, raises questions regarding the possible biological role of these HBGAs in the canine population. It has previously been suggested that variation in cell surface carbohydrate expression could be an evolutionary strategy to avoid pathogens affecting an entire population equally (Marionneau *et al.*, 2001; Ségurel *et al.*, 2013). The identification of CNV as a pathogen that can bind to A antigen and Lewis antigen starts to

add support to this theory for the dog population. It is hypothesized that dogs expressing these antigens will have an altered susceptibility to CNV infection when compared to dogs negative for these antigens. Though natural CNV in dogs is not believed to have high mortality rates (Martella *et al.*, 2009; Ntafis *et al.*, 2010), it is theorized that a survival advantage may be conferred by specific phenotypes. However, confirmation or refutation of this theory requires a much greater understanding of the pathology induced by CNV, as well as a much wider appreciation of carbohydrate polymorphism in dogs.

A number of pathogens in addition to caliciviruses are known to interact with HBGAs. These include *E.coli*, whose heat labile enterotoxin binds preferentially to A and B type 2 antigens, and *Pseudomonas aeruginosa*, a cause of external ear canal infections, which specifically interacts with GalNAc moieties of the terminal part of the A antigen (Steuer *et al.*, 1995). Rotaviruses have also been shown to interact with HBGAs; human rotavirus strains P[4], P[6], P[8] bind to H type 1, P[4]and P[8] interact with Lewis b, and P[9], P[14], P[25] bind the A antigen (Huang *et al.*, 2012; Liu *et al.*, 2012). Crystallographic studies have shown the A antigen interacts directly with the VP8* terminal portion of the VP4 spike protein (Hu *et al.*, 2012). Rotaviruses can infect dogs, and although these are typically P[3] strains, zoonotic transmission to humans has been reported (Luchs *et al.*, 2012; Wu *et al.*, 2012). In addition, a P[9] rotavirus strain has been identified in a child with a possible canine origin (Theamboonlers *et al.*, 2013). It may be hypothesized that the transmission of a P[9] strain was facilitated by expression of HBGAs in the canine gastrointestinal tract, with this viral attachment moiety playing a key role in zoonotic spread. Though not yet studied, it may also be supposed that P[3] strains interact with HBGAs in a similar manner, and these may be a key factor in enabling transmission of rotavirus infection from dogs to humans.

The range of HBGAs identified as being attachment factors for CNV was found to be most similar to those of GI.1 Norwalk virus. Norwalk virus was shown to attach to H type 1 by Marionneau et al (Marionneau *et al.*, 2002), and subsequent studies identified Lewis b and the A antigen as additional attachment factors (Harrington *et al.*, 2002; Huang *et al.*, 2003; Hutson *et al.*, 2003). In addition, Norwalk has been shown to bind to H type 2 and H type 3 structures (Hutson *et al.*, 2003; Marionneau *et al.*, 2002), although binding is of weaker affinity in comparison with H type 1 (Huang *et al.*, 2005). This binding pattern is remarkably similar to that identified for CNV, and although there appear to be minor variations between the CNV strains and the degree with which HBGAs are recognised, the overall specificity is very similar. The results from the synthetic oligosaccharide experiments at 4°C and the flow cytometry data from the glycosyltransferase-transfected CHO cells studies have demonstrated that CNV can recognise H type 2 and H type 3 structures, albeit with reduced binding ability in comparison with H type 1. Whereas CNV and Norwalk virus HBGA recgonition patterns are highly comparable, HBGA binding patterns of other HuNoVs are quite distinct from that of Norwalk virus and thus CNV. For example GII.5 strains recognise the A and B antigen but not H antigen, GII.9 strains can recognise the Lewis a antigen, and GII.4 strains recognise A/B/H and Lewis antigens (Huang *et al.*, 2003, 2005).

The likely ability for CNV to infect all dogs is in contrast to the proportion of the human population that are susceptible to GI.1 Norwalk virus. As with CNV, Norwalk virus cannot bind to human tissues or saliva that do not express HBGAs on epithelial cells ('non-secretors') and binding to B positive saliva or red blood cells is significantly less than A or O positive samples (Hutson *et al.*, 2003; Lindesmith *et al.*, 2003). Consequently, non-secretors are not susceptible to Norwalk virus and B positive humans have a reduced risk of

infection (Hutson *et al.*, 2002; Lindesmith *et al.*, 2003). Between 10% and 40% of the human population are B positive, although this varies geographically (Agarwal *et al.*, 2013) and 20% Caucasians are non-secretors (Marionneau *et al.*, 2002). It has previously been suggested that noroviruses were initially introduced to humans from non-human hosts using HBGAs as a common niche (Tan & Jiang, 2011). The similarities identified between HBGA recognition of GI.1 Norwalk virus, and CNV are marked. We have established that CNV appears well adapted to a secretor positive, B antigen negative population. Animal species other than dogs also exhibit a similar spectrum of carbohydrate expression; both cattle and pigs do not express B antigen (Rydberg *et al.*, 2001; Zakhour *et al.*, 2009), and no non-secretor pigs were conclusively identified in a recent study (Cheetham *et al.*, 2007). It is interesting to speculate that GI.1 may have arisen from a norovirus adapted to a B negative-animal species such as dogs, hence explaining why it lacks this HBGA specificity. GI.1 infections are now fairly uncommon in the human population, with GII.4 human norovirus strains dominant globally. This is believed to be due in part to the ability of GII.4 to bind to a wider range of HBGAs than any other human strain (Shirato *et al.*, 2008). GII.4 noroviruses are thus able to recognise a greater proportion of the human population, out-competing the norovirus strains with a narrower host range such as Norwalk (Ruvoën-clouet *et al.*, 2013).

The canine saliva binding studies demonstrated that CNV VLPs do not bind to all canine samples with equal abilities. This is despite all dogs expressing H antigen, a primary determinant of CNV binding, at similar levels. Therefore this data suggests that additional factors contribute the ability of CNV to bind to canine tissues. This in turn suggests that variability in susceptibility to infection will exist in the population. It is suspected that this might be comparable to the binding and susceptibility patterns identified for RHDV in

rabbits. Like CNV, RHDV is able to bind to HBGAs, and *in vitro* has been shown to be able to recognise tissue samples from any rabbit regardless of phenotype. In clinical studies however, some rabbit phenotypes are significantly more likely to become infected that others. It has been demonstrated that this is viral dose-dependant. Certain rabbit phenotypes are susceptible to very low titres of RHDV, whereas other rabbit phenotypes will only become infected if exposed to very high (and thus clinically rare) viral loads (Nyström *et al.*, 2011). It is predicted that a similar situation might occur during CNV infection outbreaks.

In summary, this chapter has identified HBGAs as the carbohydrate attachment factor for CNV strains in both GIV and GVI. It is hypothesized that HBGAs will be attachment factors for other non-canine strains in these genogroups, and identification of a third and fourth genogroup of noroviruses to recognize HBGAs raises key questions regarding the evolutionary ancestors of these viruses.

Chapter 6

Evidence for human norovirus infection of dogs

6.1 Chapter introduction

The studies on carbohydrate expression in the canine gastrointestinal tract (chapter 5) suggest that dogs express the correct carbohydrate attachment factor (HBGAs) to enable both GI and GII HuNoV to bind and possibly enter canine cells. This indicates that there is a theoretical risk that dogs may be susceptible to infection by HuNoV. Three previous studies lend support to this theory; two of these have identified HuNoV specific antibodies in dogs (Humphrey *et al.*, 1984; Mesquita *et al.*, 2014a), and the third identified HuNoV RNA in canine stools (Summa *et al.*, 2012). With approximately 10 million dogs in the UK, divided amongst 31% of the households (Murray *et al.*, 2010), the suggestion that HuNoV may be transmissible between these species is of considerable public health concern.

The aim of this chapter was to investigate the ability for HuNoV to infect dogs, and the frequency with which this might be occurring in the UK. This has been achieved by firstly exploring the relationship between canine HBGA expression and HuNoV binding to canine tissues, and secondly by determining the occurrence of current and past HuNoV infections in dogs by a survey of canine stool samples for HuNoV RNA, and a survey of canine serum for HuNoV-specific antibodies.

6.2 HuNoV VLPs bind to canine gastrointestinal samples in ELISA-based assays

A panel of six HuNoV VLPs were generously donated to this study by Alexis de Rougemont, (University Hospital of Dijon), and an additional VLP (GI.1 Norwalk virus) was generated specifically for this project, using a recombinant baculovirus synthesized by Ed Emmott using a plasmid containing the full length genome of Norwalk virus (pNV101). The sequences of HuNoV VP1 proteins used are recorded in GenBank, with their associated accession numbers listed in table 6.1.

HuNoV genotype	GenBank Accession number
GI.1	NC001959.2
GI.2	KP064095
GI.3	KP064096
GII.3	KP064097
GII.4	AF472623
GII.6	KP064098
GII.12	KP064099

Table 6.1 GenBank Accession numbers of HuNoV strains used to generate VLPs

Characterisation of these VLPs is illustrated in figure 6.1, with a Coomassie blue stained SDS-PAGE gel demonstrating their purity and confirming quantification (A), as well as an EM image confirming correct particle assembly (B).

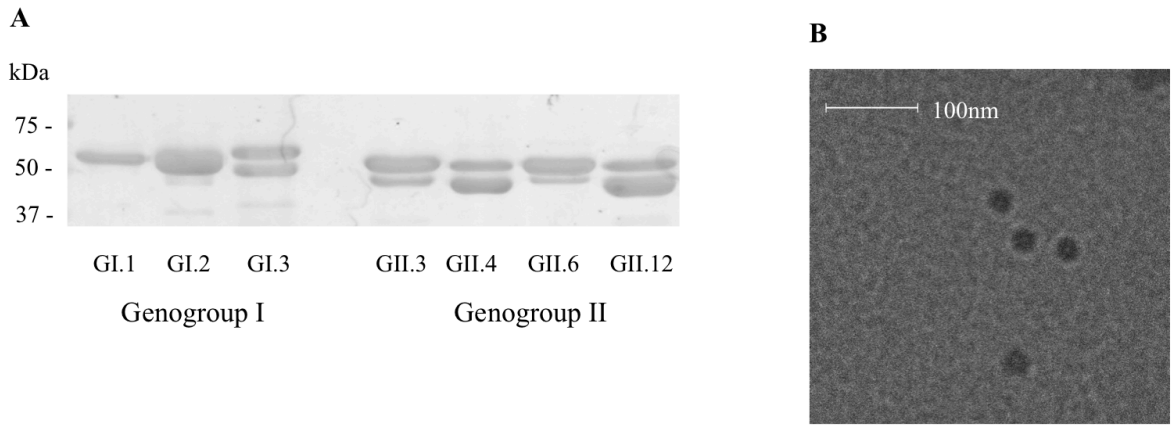


Figure 6.1 Characterisation of HuNoV VLPs.
A) SDS-PAGE analysis of all seven purified HuNoV VLPs with Coomassie Blue staining. The 64kDa band is present in all samples, and the presence of a second band of 58kDa in certain samples is expected based on previous reports (Belliot *et al.*, 2001)**.** B) Electron micrograph of an example HuNoV VLP (GII.4) with negative staining.

Saliva samples from 26 dogs (1-23, D-F), and duodenal scrapings from 6 dogs (A-F) were analysed in ELISA-based assays for their ability to bind to HuNoV VLPs (figure 6.2). As all canine samples had been phenotyped for HBGA expression (chapter 5), it was therefore known that H antigen expression was present in every canine sample, and A antigen and Lewis antigen expression was polymorphic. Human saliva samples representing the major HBGA phenotypes present in man were used as controls. These human samples included saliva from a non-secretor individual (no HBGA expression), and saliva from humans expressing either A antigen, B antigen or H antigen alone (O phenotype). Saliva samples with variation in Lewis antigen expression (+/-) were also included.

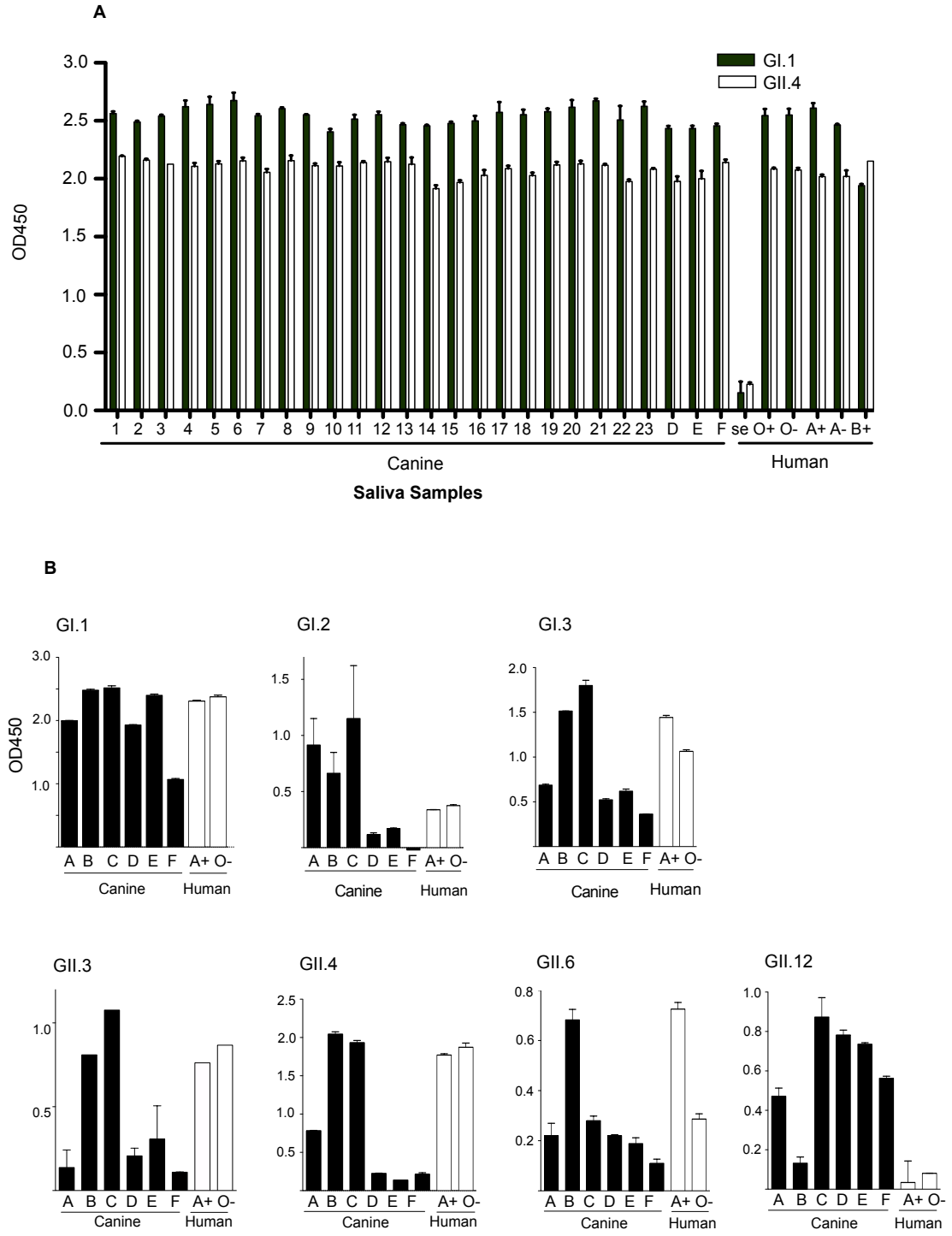


Figure 6.2 HuNoV binding to canine samples in ELISA-based assays.
Saliva from twenty six dogs (A) and duodenal scrapings from six dogs (B) were analysed to assess their ability to bind to HuNoV VLPs. GI.1 and GII.4 HuNoV VLPs were used to assess binding to both saliva and duodenal samples, and an additional five genotypes of HuNoV VLPs were used in the duodenal sample binding assays. Human saliva samples representing a range of HBGA phenotypes were used as positive and negative controls; secretor negative (se) or O/A/B antigen positive, with Lewis expression represented by +/- . All experiments were performed in duplicate, with error bars representing the standard error for each sample.

In the saliva binding assay (figure 6.2A), the non-secretor human sample was unable to bind to HuNoV VLPs as expected based on previous reports (Marionneau *et al.*, 2002). In contrast, all canine saliva samples and all secretor human samples were able to bind to HuNoV GI.1 and GII.4 VLPs. There were comparable OD450 values between the canine and human saliva samples, indicative of similar levels of binding.

VLPs of seven different HuNoV genotypes were used to assess their ability to bind duodenal scrapings from six dogs (A-F) (figure 6.2B). Human saliva samples from an A antigen, Lewis positive individual (A+) and an A antigen negative, Lewis antigen negative (O-) individual were used as positive controls; both samples were shown in figure 6.2A to bind to GI.1 and GII.4 HuNoV VLPs. Figure 6.2B demonstrates that canine duodenal scrapings could bind to every HuNoV genotype tested. Individual variation between the samples was identified, for example canine samples D, E and F showed decreased binding to GI.2 and GII.4 HuNoV VLPs. Other dogs however, most notably dogs B and C, were able to bind to all HuNoV VLPs tested. This was not apparently related to HBGA phenotype; all dogs were H antigen positive, and dogs C and E were A antigen positive whereas dogs A, B, D and F were A antigen negative (as described in chapter 5.4). In addition, dogs were phenotyped for Lewis antigen, with dogs A and B being Lewis positive and the remainder Lewis negative (data not shown). Variation in OD450 scales between genotypes was due to the variation in the reactivity of the primary antibody used; for detection of the GI VLPs the primary antibody used had been raised against GI.1, whereas for the GII VLPs the primary antibody was raised against GII.4.

6.3 HuNoV VLPs bind to canine gastrointestinal tissue sections

To determine whether HuNoV VLPs are able to bind to canine gastrointestinal tissues, fixed sections of duodenum from two dogs (B and C) were incubated with HuNoV VLPs for 1 h, then IHC used to detect binding of HuNoV VLPs to the tissue surface. As polymorphism for the A antigen is present in dogs (approximately 50% are A antigen positive (chapter 5.4)), and due to the known interaction between A antigen and HuNoV (Huang *et al.*, 2003), HBGA phenotyping was also required. Confirmation of the presence or absence of H antigen and A antigen in the tissue sections used for the VLP binding was achieved by incubating the tissue sections with Ulex binding lectin or an anti-A antigen antibody, and IHC was performed. This demonstrated that dog C was A antigen positive, and dog B was A antigen negative, hence enabling comparison of HuNoV VLP binding to canine samples representing the two major HBGA phenotypes. H antigen expression was not detectable in the A positive dog, which is understood to be due to the ability of the A antigen to mask the H antigen, therefore preventing detection by Ulex binding (Nyström *et al.*, 2011).

Figure 6.3 demonstrates that GI.1 and GII.4 HuNoV VLPs can bind to both A antigen positive and A antigen negative dogs. In addition it was shown that HuNoV VLP binding has a similar pattern of expression to H and A antigen expression. Given the known interaction between HBGAs and HuNoVs, these similar binding patterns were expected (Marionneau *et al.*, 2002).

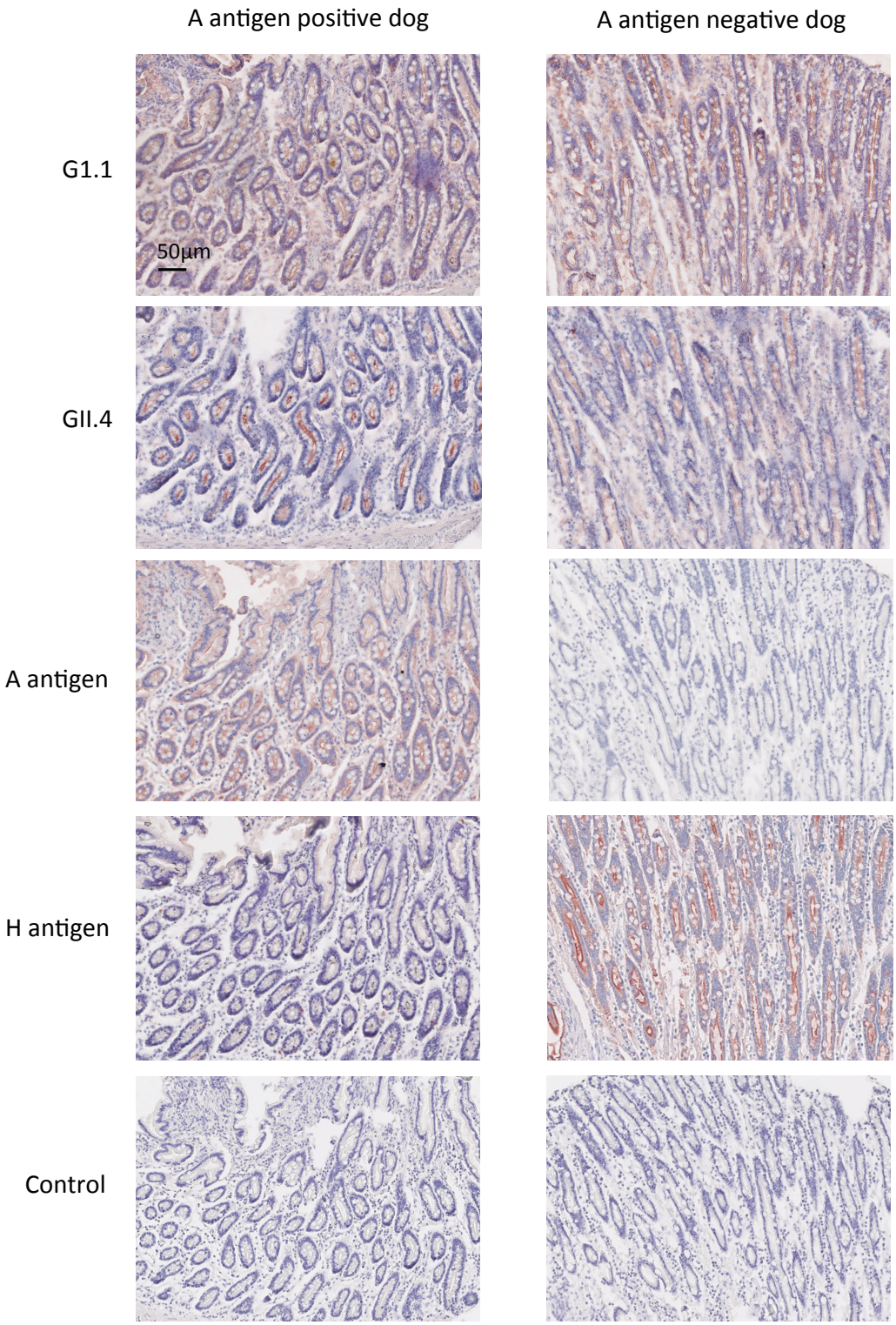


Figure 6.3 Binding of HuNoV VLPs to canine gastrointestinal tissue sections.
HuNoV VLPs (GI.1 and GII.4) were incubated with tissue sections prior to staining for IHC analysis. Two different canine phenotypes were compared; a dog expressing A antigen (A positive), and a dog negative for A antigen expression. A positive signal, either VLP binding or HBGA expression, is represented by red-brown staining.

6.4 HuNoV RNA was not detected in canine stool samples

The bank of 248 canine stool samples collected as described in chapter 3.2 were analysed for the presence of GI or GII HuNoV RNA by an RT-qPCR screen using the primer-probes listed in table 2.3 (Kageyama *et al.*, 2003). No samples were identified as being positive for HuNoV, indicating that the overall prevalence of HuNoV in this population at the time of sample collection was <1.5% (Wilson binomial approximation, confidence interval 95%).

6.5 HuNoV-specific antibodies are present in dogs

Seven genotypes of HuNoV VLPs were used in ELISAs to screen for anti-HuNoV antibodies in a total of 325 dogs. Serum samples were available from two groups of dogs; 223 samples collected in 1999-2001 (cohort A) and 102 samples collected in 2012/2013 (cohort B). Three GI HuNoV VLPs (GI.1, GI.2 and GI.3) were pooled together for preliminary assays, as were four GII VLPs (GII.3, GII.4, GII.6, GII.12). Each pool of VLPs included HuNoV strains isolated prior to 1999 (GI.1 and GII.4).

The primary anti-HuNoV antibody screen identified anti-HuNoV antibodies at detectable levels in serum from 43 dogs; 24 from cohort A (10.7%), and 19 from cohort B (18.6%). Of these 43 dogs, 32.5% were seropositive for both GI and GII HuNoV, whereas the remainder were seropositive for either GI or GII HuNoV. This is summarised in figure 6.4. Seropositivity to CNV and vesivirus 2117 in the same canine serum samples has previously been described (chapter 3), and this data has been added to the heatmap in figure 6.4B for comparison.

The age of dog at time of samples was known for 93/102 dogs in cohort B. No relationship between seropositivity to HuNoV and age was identified (data not shown).

A

Year of sera collection	Canine samples screened	HuNoV positive sera			
		GI only	GII only	GI and GII	Total
1999-2001	223	5 (2.2%)	11 (4.9%)	8 (3.6%)	**24 (10.7%)**
2012-2013	102	3 (2.9%)	9 (8.8%)	7 (6.9%)	**19 (18.6%)**
All	325	8 (2.5%)	20 (6.2%)	15 (4.6%)	**43 (13.2%)**

B

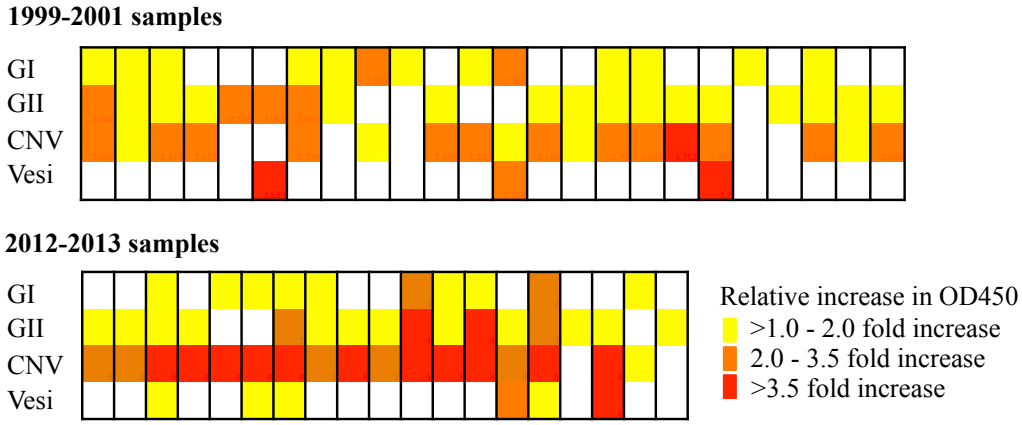


Figure 6.4 Seroprevalence of canine and human noroviruses in two canine cohorts.
Serum samples were screened in ELISAs against pooled HuNoV or CNV VLPs. The HuNoV GI pool consisted of genotypes GI.1, GI.2 and GI.3. The HuNoV GII pool consisted of GII.3, GII.4, GII.6 and GII.12. CNV pool consisted of strains 170, C33 and HK. The results of the ELISAs are presented in table format (A) and in heatmaps (B). The heatmaps include all dogs which were seropositive to either GI and or GII HuNoV, with each column representing a single dog. The serology results for CNV and vesivirus 2117 (vesi) for these dogs is included. Positive threshold value was established from the mean OD450 of coating buffer alone plus three standard deviations. Relative increase in OD450 values above the positive threshold were calculated to enable fair comparison between experiments. A relative increase of <1 indicates a seronegative sample, represented by a white box. The degree of relative increase for samples is represented by increasing darkness of the corresponding box.

To estimate the magnitude of the canine anti-HuNoV antibody response, anti-HuNoV titres were determined for 21/23 samples seropositive to GI HuNoV, and 33/35 samples seropositive to GII HuNoV. As presented in figure 6.4, the antibody titres to GI in the 21 dogs seropositive in the primary ELISA screen are relatively low, but the OD450 values obtained in the titre ELISA showed strong consistency in comparison with the original ELISA screen. For the majority of the anti-GII HuNoV positive serum samples, a similarly low antibody titre (mode 1:100) was determined, but in contrast to GI, three samples (9% of GII seropositive samples tested) had antibody titres 1:800 or higher.

Anti-HuNoV antibody titre	GI positive samples	GII positive samples
1:50	8 (38.1%)	5 (15.2%)
1:100	8 (38.1%)	12 (36.4%)
1:200	5 (23.8%)	7 (21.2%)
1:400	0	6 (18.2%
1:800	0	2 (6.1%)
1:1600	0	1 (3%)

Table 6.2 Anti-HuNoV antibody titres in canine serum

To extend the findings of the preliminary ELISAs, all canine serum samples positive for HuNoV were entered into a second round of ELISAs with individual genotypes of HuNoV. This was to investigate whether it was possible to identify the HuNoV genotype that may be eliciting the anti-HuNoV immune response. It is acknowledged that immunological cross reactivity does exist between norovirus genotypes (Hansman *et al.*, 2006), thus conclusive identification of the primary genotype inducing antibody production was not the aim of these experiments. However, the genotype to which the highest OD450 value was induced in ELISAs was tentatively suggested to be the major HuNoV genotype involved. For example, a serum sample for which the OD450 was highest against GII.4 HuNoV VLPs, was designated GII.4-specific for the purposes of this study. Figure 6.5 presents genotype

distribution of HuNoV GII positive samples, comparing cohort A (1999-2001) with cohort B (2012/2013). The results showed that GII.4 specific antibodies are most common in both cohorts, although whereas 42.1% samples showed the highest OD450 for GII.4 in cohort A, this figure increases to 87.5% in cohort B.

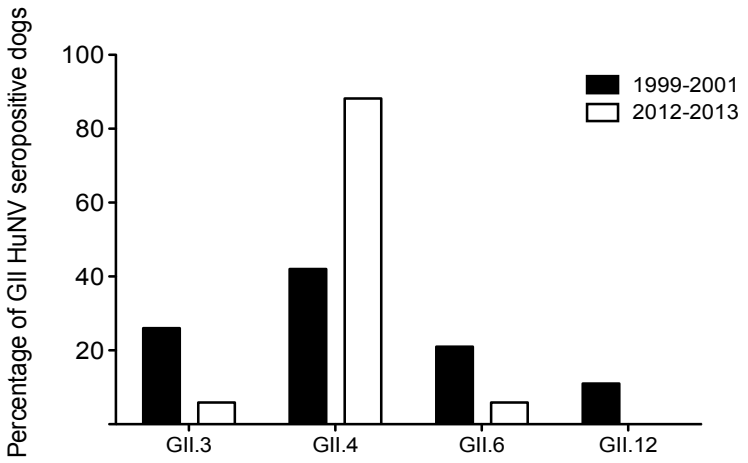


Figure 6.5 Genotype specificity of GII HuNoV seropositive canine samples.
Serum samples positive to pooled GII HuNoV were screened against GII.3, GII.4, GII.6 and GII.12 HuNoV VLPs individually. The genotype to which the highest OD450 reading was obtained was designated the primary genotype to which the antibody response was elicited. The proportion of GII HuNoV positive samples from 1999-2001 (cohort A) and 2012-2013 (cohort B) reactive to each GII genotype tested were compared.

6.6 HuNoV antibodies do no cross react with CNV

To confirm that the anti-HuNoV antibodies identified in dogs were not merely the result of cross reactivity to canine specific noroviruses, a series of blocking assays were performed (figure 6.6). In this thesis it has previously been shown that seroprevalence to CNV was high in the same population of dogs analysed for HuNoV reactivity (chapter 3 and figure 6.4), so firstly it was necessary to establish that the CNV-specific antibodies were not cross reactive

with HuNoV. This was achieved by pre-incubating varying concentrations of HuNoV and CNV VLPs with a representative anti-CNV antibody positive canine serum (serum S), then analysing the ability of serum S to detect CNV VLPs (figure 6.6A, methodology previously pictorialized in 3.7A). Pre-incubation with CNV VLPs was clearly able to block recognition of CNV VLPs by canine serum, whereas pre-incubation with GI or GII VLPs had no effect on CNV VLP recognition. This confirmed that the epitopes recognized by the anti-CNV antibodies were distinct from epitopes present on HuNoV VLPs.

Next, the specificity of the anti-GII antibodies identified in canine sera was examined using a similar VLP competition assay with GII VLPs coated onto a microtitre plate instead of CNV VLPs (figure 6.6B). The concentration of HuNoV or CNV VLPs required to block 50% binding to GII VLPs was calculated by fitting a sigmoidal curve to the OD450 values for the serial dilution of VLPs. Seven different canine serum samples (i-vii) identified as being positive for anti-GII antibodies were analysed, and serum S (negative for GII binding) was added as a negative control. For samples i-vii, the type of VLP inducing the lowest EC50 for blocking GII VLP recognition by canine serum was GII HuNoV VLPs. CNV VLPs did induce a decrease in GII recognition below the upper threshold of detection in 4/7 cases, but a greater concentration of CNV VLPs than GII VLPs were required. This suggests a degree of cross reactivity is likely between GII HuNoV and CNV, but that differentiation is possible.

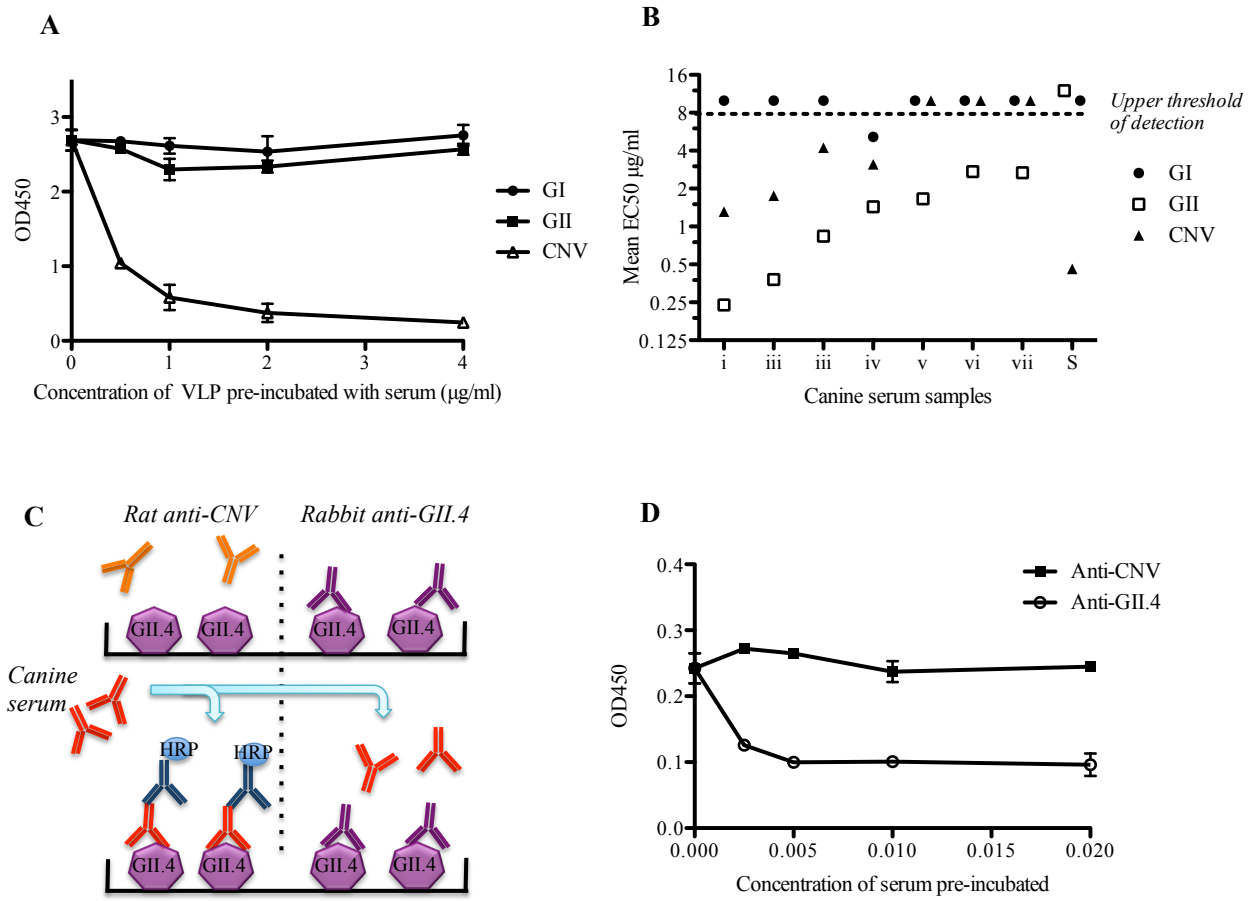


Figure 6.6 Evaluation of cross-reactivity between antibodies against human and canine noroviruses in canine sera.

VLP competition assays assessing the ability for canine sera to detect CNV (A) or GII.4 HuNoV (B) in the presence of alternative norovirus VLPs were conducted. A representative CNV positive canine serum sample (A) and seven different GII.4 HuNoV positive canine serum samples (B) were pre-incubated with serial dilutions of either pooled GI or GII HuNoV VLPs or pooled CNV VLPs. The concentration of VLP required to block 50% binding (EC50) was calculated by fitting sigmoidal curves to the serial dilution data, to allow comparison between serum samples (B). The dashed line represents the upper limit of detection. An antibody competition assay was performed using antibodies specifically raised against CNV (rat) and GII,4 HuNoV (rabbit). The experimental design is presented in panel (C); anti-CNV and anti-GII.4 antibodies were pre-incubated with GII.4 VLPs on a microtitre plate, then after three plate washes GII.4 positive canine serum was added and the OD450 of this interaction determined (D).

The final blocking assay conducted to investigate the specificity of antibodies detected in canine serum, used antibodies generated in animals immunized solely with either CNV or GII HuNoV VLPs. These animals, rat and rabbit respectively, would not have been exposed to natural infection, hence antibodies in their serum were deemed specific for their VLP immunogen. As pictorialized in figure 6.6C, anti-CNV or anti-GII HuNoV sera (rat and rabbit respectively) was serially diluted and incubated directly with GII VLPs coated onto microtitre plates, then after plate washing, GII positive canine serum (serum vi) incubation followed. The results showed that rat CNV specific antisera was unable to block recognition of GII HuNoV by canine sera, whereas rabbit GII specific antisera induced blocking of GII VLP recognition by canine sera (figure 6.6D).

Western blotting was used as alternative method to demonstrate the presence of anti-HuNoV antibodies in canine sera. Five serum samples identified as being positive for anti-HuNoV antibodies by ELISA were selected for use in western blots (figure 6.7). A single serum sample (sample S2) shown to be negative for both human and canine noroviruses was selected as a negative control. Western blotting confirmed that canine sera from five representative samples could detect GII.4 VLPs, and that this expression was independent of recognition of genogroup IV or VI CNV.

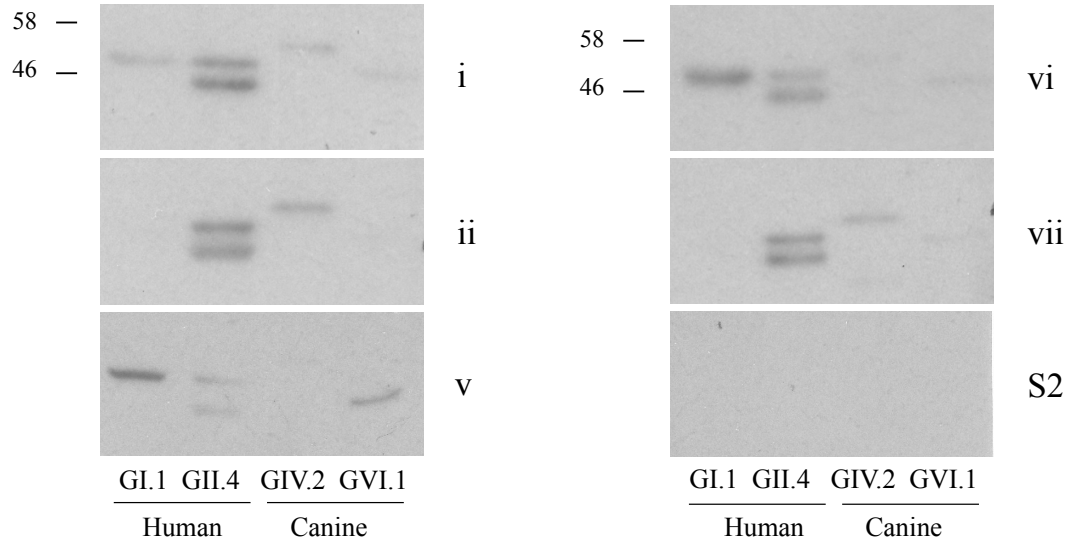


Figure 6.7 Western blotting of purified VLPs using seropositive serum.
Norovirus VLPs from 4 genogroups; GI and GII (HuNoV) and GIV and GVI (CNV) were separated by SDS-PAGE. The polyacrylamide gel was then used for western blotting with five different canine serum samples positive to GII.4 by ELISA, and a single canine serum sample negative to all norovirus VLPs tested.

6.7 HuNoV cannot be grown in canine primary duodenal cells

Given the cumulative evidence that HuNoV can infect dogs, it was hypothesized that HuNoV may be able to infect canine cells in tissue culture. If so, this would be of significant scientific importance as no cell line has yet been shown to enable HuNoV replication with efficient and robust results in all labs (Duizer *et al.*, 2004; Jones *et al.*, 2014a; Lay *et al.*, 2010). Duodenal cells were isolated (as described in methods 2.10.1) from fresh canine duodenal samples from two beagles and seeded into tissue culture flasks. After overnight incubation at 37°C, a proportion of cells had adhered to flasks and there was evidence of cell division. Cells were inoculated with purified HuNoV isolated from human stool samples (purification performed by Jia Lu) at a multiplicity of infection (MOI) of 500 genome copies

per cell. After 1 h, cells were washed with media to remove the inoculum. RNA was extracted from cells and extracellular medium harvested at 0 h and 96 h post infection and RT-qPCR using a GII HuNoV specific primer-probe (table 2.3) was performed. At 96 h, although HuNoV was detected at low levels in the cellular and extracellular fractions, this was over 100 fold (7C_T values) lower than the starting inoculum, indicating that replication had not occurred.

6.8 Chapter Summary

The work in this chapter sought to investigate the likelihood that dogs can be infected with HuNoV. The results of this serological survey and VLP binding studies strongly suggest that dogs are theoretically able to be infected with HuNoV. However, the frequency with which this occurs is deemed low based on the epidemiological results from this report. Furthermore, the clinical implications for both dogs and people in contact with dogs still remain to be confirmed.

In human cells, it has been shown that HuNoVs bind to cell surface carbohydrates of the HBGA family prior to internalization. HBGAs are expressed on epithelial cells of many species, and in chapter 5 it was confirmed this includes dogs. This finding led to the hypothesis that HuNoV would be able to bind to the gastrointestinal tract of dogs, and the ELISA and IHC data presented in this section were able to confirm this. This demonstrates that the initial step required for HuNoV entry into canine cells is present. However, it should be noted that RHDV, a related but distinct member of *Caliciviridae*, can bind to HBGAs (H type 2, A antigen and B antigen) (L'Homme *et al.*, 2009) and infect wild and domestic rabbits of the *Oryctolagus cuniculus* species, yet there is no evidence RHDV can infect

humans (Carman *et al.*, 1998; Greenslade *et al.*, 2001). HBGA binding may be an initial step in calicivirus-host interaction, but a subsequent host-restrictive step(s) must be necessary for RHDV infection, and potentially HuNoV infection in dogs.

The viral RNA survey conducted as part of this project did not reveal any canine stool samples containing HuNoV RNA. This implies that the incidence of HuNoV shedding by this population of dogs is negligible, despite samples being collected from both healthy dogs (117 animals), dogs with non-gastroenteric disease (64 animals), and dogs with severe gastroenteritis (67 animals). Inclusion of samples from the latter two groups was essential as it has been suggested that HuNoV may be more likely to infect dogs with underlying disease or immunodeficiency (Summa *et al.*, 2012). In addition, canine-specific noroviruses are associated with gastroenteritis in dogs (Mesquita & Nascimento, 2012b; Mesquita *et al.*, 2010; Ntafis *et al.*, 2010) and it was hypothesized that HuNoV infection of dogs may cause signs of gastroenteric disease. Of the 67 dogs with gastroenteritis in this survey, CPV (10 dogs) and CECoV (2 dogs) were detected in 17.9%. This proves that whilst viral gastroenteritis is relatively common in dogs, noroviruses are not a major cause of viral disease in the population of dogs sampled. The likelihood of HuNoV infection in a dog resulting in clinical signs of gastroenteritis is clearly much lower than that of CPV and CECoV, and as such, there is no immediate cause for concern by owners and veterinarians.

The absence of HuNoV positive stool samples from dogs in this study is in contrast to the results of a study from Finland which identified HuNoV RNA in 4/92 canine stool samples (Summa et al., 2012). However their sampling strategy was significantly different from the approach used in this study; canine stool samples were only collected if the owner had shown symptoms of gastroenteritis within the past week, whereas stool samples in this study

were collected with no reference to recent owner illness. HuNoV in man is typically an acute infection, with peak viral shedding occurring 2–4 days after infection. By 3 weeks after infection only 25% cases are still positive for viral RNA (Rockx *et al.*, 2002). In addition, although HuNoV is responsible for millions of infections worldwide each year, the virus is only identified in approximately 18% of human diarrhoeic samples submitted (Ahmed *et al.*, 2014). Detection of HuNoV RNA in faeces can be limited by factors such as low virus concentrations, improper storage of samples, inefficient viral RNA extraction, and the presence of fecal reverse transcriptase inhibitors (Patel *et al.*, 2008). Overall this indicates that positive identification of HuNoV shedding in dogs will only be possible within a very narrow timeframe, and that a proportion of cases will be false negatives.

Serological analysis of 325 canine serum samples in this study strongly suggests that dogs mount immune responses against HuNoV. It has been demonstrated that almost 20% of dogs sampled in 2012/2013 had antibodies that could recognise HuNoV VLPs. This suggests that 1 in 5 dogs have been exposed to HuNoV in the UK. This proportion was lower than the proportion of dogs (43%) reported to be seropositive to HuNoV by a recent survey across Europe (Mesquita *et al.*, 2014a), and may be a reflection of population differences. An important conclusion from both studies is that the HuNoV seroprevalence rate identified in dogs is substantially lower than HuNoV seropositivity amongst human populations. In the UK nearly 100% people are seropositive for GII.4 (Menon *et al.*, 2013a). This indicates that either dogs are exposed much more rarely to HuNoV, or they are much less susceptible to infection than humans. Given that in one questionnaire-base study, 96% dogs sleep in their owners houses and that when owners are at home almost 60% dogs were allowed anywhere in the house (Westgarth et al., 2008), it seems unlikely that dogs would not be exposed to

HuNoV in a household with infected humans. Therefore, it is proposed that dogs are susceptible to HuNoV but at a much lower level than man.

It could be argued that the anti-HuNoV antibodies identified in canine sera may have been generated in response to infection with related non-human noroviruses, and are merely cross-reactive with HuNoV. For example, anti-CNV antibodies were detected in 45.2% of serum samples used in this thesis. To investigate this further, a series of blocking assays were performed using canine serum samples and serum samples from rats inoculated with CNV VLPs. These were able to show that the anti-GII HuNoV antibodies were specific for GII HuNoV VLPs and not three different strains of CNV. It is acknowledged that there are other non-human and non-canine noroviruses to which dogs may have been exposed, for example swine and bovine noroviruses, to which cross-reactivity was not assessed. However, due to UK farming practices, the frequency with which dogs in the study population would come into contact with either pigs or cattle was deemed to be significantly lower than the frequency of contact with humans. In addition, although the feeding of raw pork/beef to dogs does infrequently occur, animal noroviruses are extremely unlikely to be found in commercial pet food due to UK manufacturing processes and regulations (DEFRA & APHA, 2014).

Comparison of canine serum samples from two time periods (1999-2001 and 2012-2013) allowed analysis of the change in the presence of anti-HuNoV antibodies over time. Although the two study populations are not directly comparable as the earlier group was from a rehoming kennels and the later from a veterinary referral hospital, it was shown that the proportion of dogs seropositive for HuNoV increased over time. The prevalence of HuNoV in man in UK has increased over a similar time period from 6% of acute

gastroenteritis cases in 1999 to approximately 16% in 2009 (Tam et al., 2012). It is possible to speculate that the rise in HuNoV seroprevalence in dogs from 1999 onwards is a reflection of the increased levels of infection in the human population.

The initial serosurvey demonstrated that dogs were more likely to be seropositive to GII HuNoV strains than GI strains. This was in line with the findings from a recent European study (Mesquita *et al.*, 2014a). To explore this further, any HuNoV positive samples were entered into a second round of ELISAs with VLPs from seven individual genotypes. This showed that the highest seroprevalance was to GII.4 strains. This is remarkable as this is the most common genotype infecting man worldwide. This also correlates with the report which identified HuNoV in the stools of four dogs (Summa et al., 2012). GII.4 HuNoV was detected by qPCR in the stools of 3 dogs, and GII.12 in the stools of 1 dog.

In summary, whereas HuNoV infection of dogs has been shown to be theoretically possible, the risk of this causing significant clinical disease in dogs is believed to be very low. As for the potential for HuNoV infection transmitting between dogs and their owners, this has yet to be established, though it is recommended that sensible hygiene precautions are taken around pets, especially when gastroenteritis in either humans or dogs is present in a household.

Chapter 7

Hepatitis E Virus in dogs

7.1 Chapter introduction

Following the results of chapter 6 which indicate HuNoV can infect dogs, these findings were extended by investigating the possibility that other enteric viruses of man can also be transmitted to dogs. Enteric viruses closely related to HuNoV were considered as likely zoonotic candidates, and based on existing literature, reagents available and virus characteristics, hepatitis E virus (HEV) was selected for further study. The virion properties of HEV are very comparable to HuNoV; HEV is a small icosahedral, single stranded, positive sense RNA virus with a genome of approximately 7.2kb (figure 7.1). HEV was initially classified as a calicivirus (Bradley & Balayan, 1988), but is now classified as the sole member of the viral family *Hepeviridae*, genus *Hepevirus* (King *et al.*, 2011).

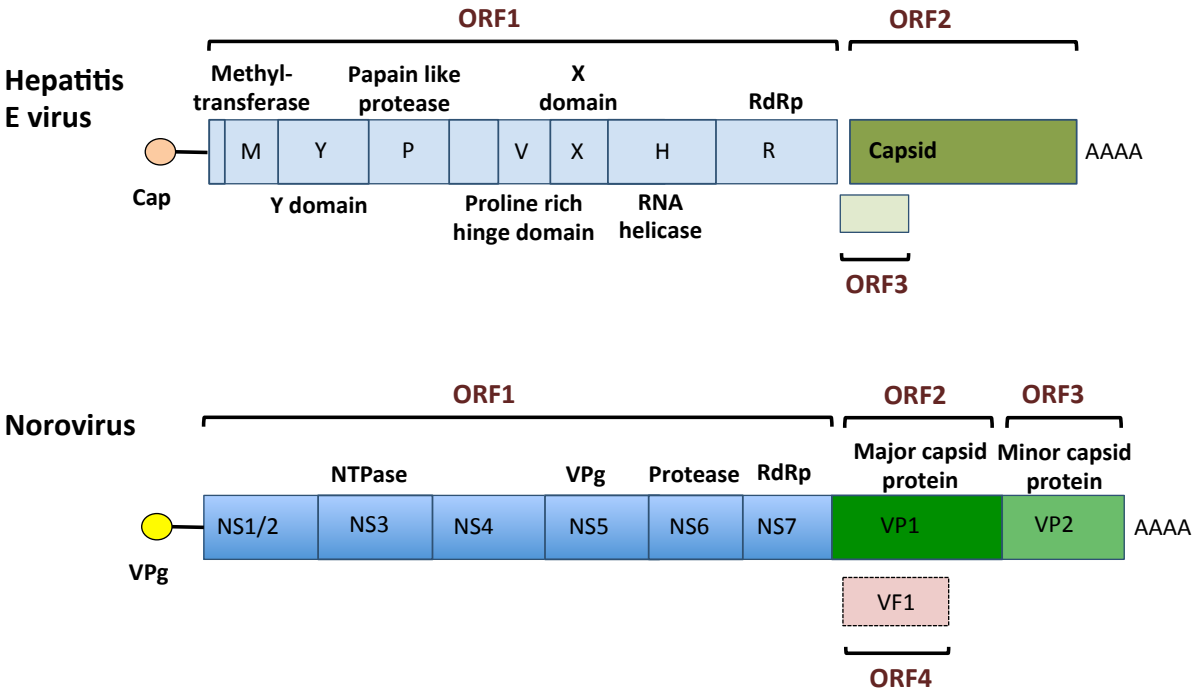


Figure 7.1 Comparison of the genome organization of HEV and noroviruses.
The HEV genome is capped at the 5' end of the RNA, whereas noroviruses have VPg covalently linked at the 5'end. Both genomes have polyA tails at the 3' end. ORF1 of both HEV and noroviruses is encoded by the genomic RNA, which consists of a polyprotein that is subsequently cleaved into separate proteins by a viral protease. The structural proteins of both viruses are encoded by the subgenomic RNA; ORF2 for HEV and ORF2 and ORF3 for noroviruses. The function of the overlapping reading frame ORF3 in HEV is uncertain.

Similarly to HuNoV, HEV is also spread by the faeco-oral route, but following entry via the gastrointestinal tract, HEV infects hepatocytes to cause acute hepatitis. HEV is a major cause of acute epidemic viral hepatitis in developing countries (Arankalle *et al.*, 1999) and has recently emerged as the most common cause of acute hepatitis in the UK (Harvala *et al.*, 2014). There are four HEV genotypes; genotypes 1 and 2 are endemic in humans in developing countries, whilst genotypes 3 and 4 are zoonotic agents mainly associated with sporadic outbreaks of HEV worldwide (figure 7.2) (Meng, 2010).

Figure 7.2 Geographical distribution of HEV disease in man.
Countries where HEV is endemic in man are highlighted in grey, and countries reporting sporadic cases are identified by asterisks (*) (adapted from (Khuroo, 2011).

Disease severity following HEV infection is extremely variable in man, ranging from asymptomatic to fatal infections. In the UK there are an estimated 60,000-100,000 human HEV infections each year (Hewitt *et al.*, 2014; Ijaz *et al.*, 2009), and although the majority are subclinical, this is a developing concern for blood transfusion services; 79/225000 blood donors in southeast England were viraemic with HEV in 2012-13, and 43% recipients followed up had evidence of infection (Hewitt *et al.*, 2014). Severe manifestations of HEV infection include acute liver failure and chronic infection, and despite mortality rates

typically <1%, up to 25% pregnant women die following HEV infection in endemic regions (Aggarwal & Krawczynski, 2000).

The first animal strain of HEV was identified in pigs in the US (Meng *et al.*, 1997). Porcine HEV strains are antigenically and genetically related to human strains of HEV and experimental evidence has shown that cross species infection can occur between humans and pigs (Meng *et al.*, 1998). Furthermore, contact with pigs or ingestion of pork products has been implicated in disease transmission in man (Chaussade *et al.*, 2013; Colson *et al.*, 2010; Meng *et al.*, 2002; Said *et al.*, 2014).

Multiple studies have sought to determine whether other animal species could be additional zoonotic sources of HEV infection. Anti-HEV antibodies have been identified in a number of different farmed animals, including cattle, goats and chickens (Arankalle *et al.*, 2001; Sanford *et al.*, 2013), but their role in transmission of disease to man is unclear.

7.2 Hepatitis E virus in dogs

Pet dogs have been implicated in HEV disease transmission in a number of previous reports. Anti-HEV antibodies have been identified in dogs in developing countries where HEV is endemic in humans, including China, India and Brazil (Arankalle *et al.*, 2001; Geng *et al.*, 2010; Liang *et al.*, 2014; Liu *et al.*, 2009; Vitral *et al.*, 2005; Zhang *et al.*, 2008). Two studies have previously investigated the seroprevalence of HEV in dogs from regions with sporadic HEV cases in humans; no positive samples were identified in Japan, and only 2/212 positive dogs were identified in the US (Dong *et al.*, 2011a; Mochizuki *et al.*, 2006). Despite this very low seroprevalence, an epidemiological link between HEV infection and dogs has still been implied in industralised nations. Periodic contact with dogs was reported in 74%

(14/19) of cases of indigenously acquired infection in a Dutch study (Borgen *et al.*, 2008), and owning pets was reported by 60% (17/28) of patients with indigenous HEV infection from the UK (Lewis *et al.*, 2008).

More robust evidence that HEV can infect dogs, requires identification of HEV RNA within canine samples. Previous studies in Asia have examined canine stool and serum samples for HEV RNA, but no positive cases have been identified (Dong *et al.*, 2011a; Geng *et al.*, 2010; Liu *et al.*, 2009; Mochizuki *et al.*, 2006). Similarily, no HEV RNA was detected in a Dutch study examining canine liver samples collected prior to 2005. Given the rise in human HEV cases with a suspected zoonotic origin in the past decade (Arends *et al.*, 2014), it is possible that previous attempts to identify HEV RNA in canine samples have produced negative results due to very low prevalence levels.

This chapter aimed to investigate the potential for dogs to be infected with HEV using stool and serum samples previously banked and screened for HuNoV, in addition to a set of clinical samples from dogs with histologically confirmed hepatitis, collected as part of previous studies (Bexfield *et al.*, 2011, 2014) and generously donated to this study by Nick Bexfield (University of Nottingham). The clinical samples from canine hepatitis cases were particularly valuable for HEV screening because it was hypothesized that if HEV can infect dogs, it would cause hepatitis. This was based on reports that indicate HEV induces hepatitis in a range of species other than man, including pigs and rabbits (Halbur *et al.*, 2001; Ma *et al.*, 2010).

7.3 Prevalence of HEV antibodies in canine sera

A total of 247 canine serum samples were collected and screened by ELISA for anti-HEV antibodies. HEV VLPs were kindly donated to this study by Rintaro Hiraide (Goodfellow Lab, University of Cambridge). VLPs were synthesised following generation of recombinant baculoviruses with DNA encoding amino acids 112-608 of the HEV genotype 3 ORF2 gene. These recombinant baculoviruses were propagated in Sf9 cells, and VLP expression was achieved in Hi5 insect cells. VLPs were then purified using the same methodology as for the CNV and HuNoV VLPs.

Of the 247 canine serum samples, 92 were from healthy dogs, 34 were from patients with a range of clinical diseases at a veterinary hospital, and 121 were from dogs with confirmed hepatitis. Two dogs were identified as seropositive, both from the group of healthy animals (2/92, 2.2%). The antibody titre of the two positive samples (designated samples A and B) was determined to be 1:400 by serial dilution of sera as shown in figure 7.3. No seropositive cases were identified amongst the dogs with clinical disease, hepatitis or otherwise.

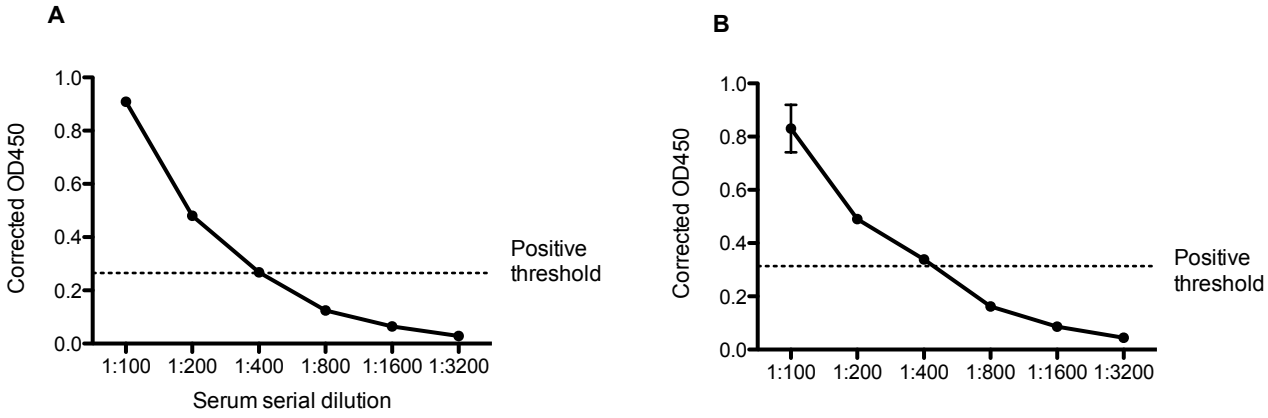


Figure 7.3 Anti-HEV antibody titres in positive canine serum samples.
Positive canine serum samples were prepared in dilutions of 1:100,1:200, 1:400, 1:800; 1:1600 and 1:3200 and entered into an ELISA. The corrected OD450 was obtained by subtracting the background signal from the VLP coated well OD450 value. The positive threshold was determined by calculating the mean OD450 of buffer coated wells with the highest serum dilution, plus 3 standard deviations.

Western blotting was used an additional means of confirming samples deemed positive by ELISA. HEV VLPs as well as GI.1 HuNoV and vesivirus 2117 VLPs included as controls, were separated on a SDS-polyacrylamide gel, then samples were either transferred to polyvinylidene difluoride membranes for western blotting, or stained with Coomassie Blue. Figure 7.4 confirms that antibodies present in samples A and B could detect HEV VLPs as predicted. These two samples were also seropositive for vesivirus 2117 although this is not believed to be due to cross-reactivity as demonstrated by inclusion of sample C (positive for vesivirus 2117 only). Canine sample D represents a dog seronegative by ELISA to all VLPs analysed, with the same result demonstrated in the western blot, adding additional support to the conclusion that samples A and B are specific for HEV. Sera from an HEV-seropositive pig and a seropositive human were included as positive controls. Human reactivity to GI.1 HuNoV was not unexpected given that approximately 70% human population in the UK are seropositive to this virus (Gray *et al.*, 1993).

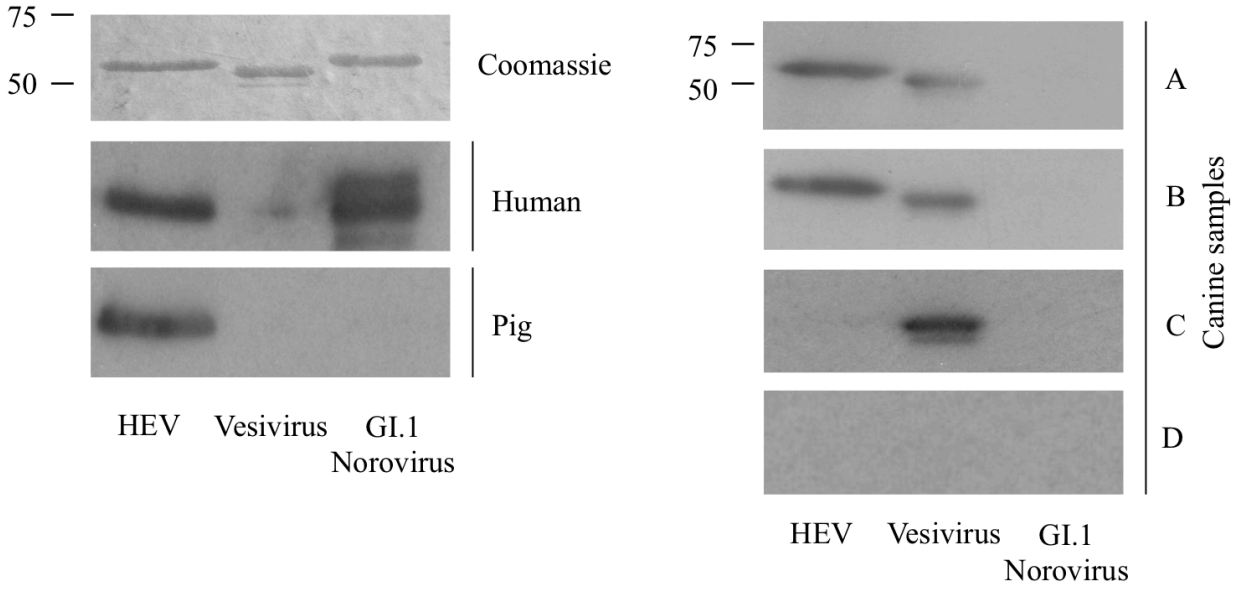


Figure 7.4 Western blot analysis of serum sample reactivity with HEV, vesivirus 2117 and HuNoV G1.1 VLPs.
Three types of VLP were separated by SDS-PAGE. One gel was stained with Coomassie Blue to identify VLP protein at the expected molecular weight. Additional gels were used for western blotting with canine serum samples positive by ELISA for HEV (samples A and B). A pig serum sample and a human serum sample known to be positive for anti-HEV antibody were used as a positive control for the HEV VLPs. Canine sample C, previously confirmed positive for anti-vesivirus antibody by ELISA, was used as a positive control for the vesivirus VLPs. Canine sample D was used as a negative control for all VLPs.

Both samples positive for HEV were collected in August 2013 at separate canine blood collection events in the UK. The average age of these two dogs was 7.5 years (SD 3.55). The age of seronegative dogs was known for 97/124 samples, with the average age being 6.7 years (SD 2.19). The two seropositive animals were both of greyhound type breeds. A variety of dog breeds were included in this study, although larger dogs were over represented in the healthy cohort due to the population studied (blood donation requires dogs to be heavier than 25kg).

7.4 Prevalence of HEV RNA in canine samples

One step RT-qPCR was performed using primers designed to detect all four genotypes of HEV, targeting a conserved region of HEV ORF3 (table 2.4 (Jothikumar *et al.*, 2006)). RT-qPCR reactions were prepared using 2µl of extracted RNA added to 2x Precision OneStep RT-qPCR MasterMix (PrimerDesign Ltd), with primers and probe at concentrations of 250nM and 100nM, respectively. The thermal cycle protocol used was as follows: RT reaction 50C for 30 min, 95C for 15 min, then 42 cycles of 95°C, 10 s; 55°C, 25 s; 72°C, 25 s.

Optimization of the previously published RT-qPCR protocol for HEV RNA detection (Jothikumar *et al*., 2006) was first performed using *in vitro* transcribed HEV RNA from an infectious clone. This demonstrated that 100 genome copies of HEV per reaction were reliably detected (figure 7.5A).

Following on from this, a total of 2.5 x 10^7 genome copies of HEV RNA were spiked into a single canine liver sample, serum sample and a stool sample, to determine the effect that additional material in clinical samples had on assay sensitivity. RNA extraction was conducted according to the protocol described, then 2µl of the extracted RNA was entered into the RT-qPCR reaction, corresponding to 10^6 copies of HEV RNA per reaction. HEV RNA added directly to lysis buffer in the absence of any canine samples was included as a control. It was demonstrated that HEV RNA could be detected in spiked canine liver and stool samples at levels comparable to the lysis buffer-only control. However, detection of HEV RNA from the spiked serum sample was significantly impaired, with almost a 1000 fold decrease in the number of copies detected by RT-qPCR (figure 7.5B). Therefore serum samples would have to contain at least 10^5 genome copies/µl to be detectable by this assay,

which was deemed highly unlikely based an average HEV RNA concentration of 10^5 – 10^8 genomes copies/ml in infected human serum (Takahashi *et al*., 2010). Therefore, screening for HEV RNA was conducted for RNA extracted from liver and stool samples only.

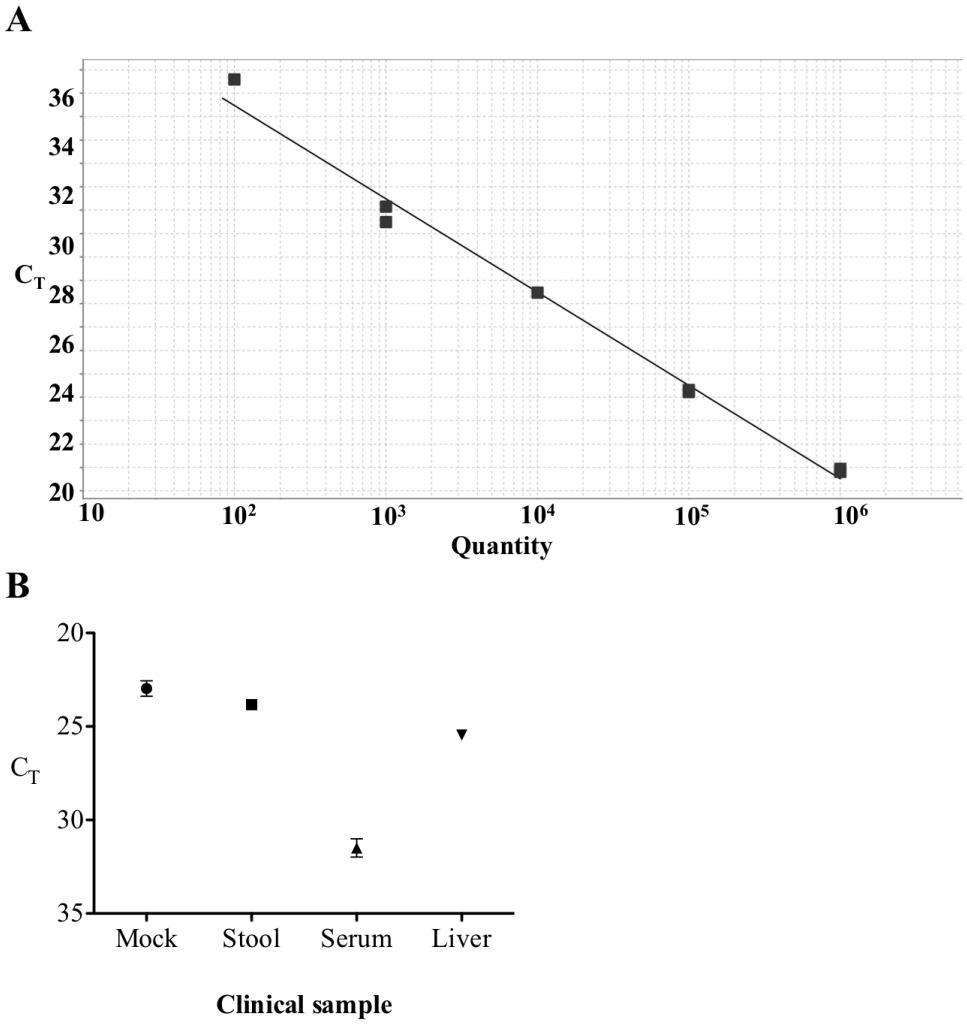


Figure 7.5 Optimization of RT-qPCR protocol for HEV RNA
(A) In-vitro transcribed HEV RNA was serially diluted 10-fold from a known starting concentration of 10^6 copies/µl, and entered into the RT-qPCR assay. A linear increase in C_T value was identified for each dilution. The sensitivity of the primer-probe set was determined to be 100 copies/reaction. (B) In-vitro transcribed RNA (10^6 copies/reaction) was spiked into canine clinical samples, then RNA extraction performed using a commercial kit. RNA samples were then entered into the RT-qPCR assay and the resulting C_T values compared on a scatterplot.

A total of 248 canine stool samples were screened for the presence of HEV RNA using the RT-qPCR protocol with primers designed to amplify a highly conserved 71bp fragment of ORF3. Products of any samples showing uncertain results were analysed by gel electrophoresis. A positive control was included on each plate to ensure RT-qPCR had been performed correctly. No HEV positive cases were identified.

Eighty-four canine liver samples were analysed for the presence of HEV RNA using the same RT-qPCR protocol. In all other species studied, HEV induces varying degrees of hepatitis (Billam *et al*., 2005; Halbur *et al*., 2001; Ma *et al*., 2010), hence to optimise the chances of identifying HEV RNA, liver samples from dogs with histologically diagnosed hepatitis were screened in this study. No HEV RNA was detected in any canine liver samples.

7.5 Chapter summary

In this chapter it has been shown that of 247 dogs tested from the UK, 0.8% were positive for antibodies specific for HEV. This strongly suggests that HEV had replicated within the two positive animals, inducing an immune response. This is the first study investigating the seroprevalence of anti-HEV antibodies in dogs in Europe, and the low canine HEV seroprevalence reflects the low human seroprevalence in this region. Seroprevalence of HEV in humans in the UK is 13% (Ijaz *et al.*, 2009), whereas up to 25% people are seropositive in China (Zhuang *et al.*, 2014) where canine HEV seroprevalence can be up to 29% (Liang *et al.*, 2014). This also correlates with the negative HEV canine serological study from Japan (Mochizuki *et al.*, 2006), where human seroprevalence is only 3.4% (Takeda *et al.*, 2010).

An alternative explanation for the two seropositive cases could be the existence of a unique canine-specific HEV strain. By the use of serology alone it is not possible to determine the relatedness of HEV strains, as all HEV genotypes exist as a single serotype (Emerson & Purcell, 2003). This is exemplified by the recently identified rabbit-specific strains of HEV; these are genetically distinct from human strains, but they share the same serotype as the human genotypes 1-4 (Wang *et al.*, 2013). To ascertain whether human or a putative canine HEV strain is inducing an HEV-specific immune response in dogs, identification and characterisation of HEV RNA from canine samples is required.

Multiple studies have previously attempted to identify HEV RNA in canine samples. The majority of these have analysed canine serum (Geng *et al.*, 2010; Liu *et al.*, 2009; Zhang *et al.*, 2008), with no positive cases reported. Experimental infection of dogs with swine HEV has been shown to induce an antibody response, but despite this no HEV RNA was detectable in serum post inoculation (Liu *et al.*, 2009). This suggests viraemia is not readily identified in infected dogs, and this coupled with our optimization experiments that failed to reliably detect HEV RNA spiked into serum samples, indicated that analysis of serum samples is not the optimum method for RNA detection in dogs.

In order to increase the chances of identifying HEV RNA in canine samples, alternative clinical samples were deemed necessary. Detection of HEV RNA in the liver is possible for up to 1 month post inoculation in experimentally infected pigs, which is twice the duration of viral detection in serum samples (Halbur *et al.*, 2001; Williams *et al.*, 2001). It was therefore hypothesised that analysis of canine liver samples would be the most likely strategy to detect virus. Hepatitis is frequently reported in dog populations (Poldervaart *et al.*, 2009) and yet the aetiology of most cases of canine hepatitis remains unknown despite

numerous studies to identify a viral cause (Bexfield *et al.*, 2014; Boomkens *et al.*, 2005). HEV was included in a PCR screen for a viral cause of canine liver disease in a study of dogs with hepatitis over a decade ago, but no HEV positive cases were identified (Boomkens *et al.*, 2005). The incidence of HEV in man in the UK has increased dramatically over the intervening ten years, and it is predicted that HEV might be more frequent in canine hepatitis samples collected more recently. However, despite this reasoning HEV RNA was not detectable in the canine liver samples screened from the UK. This is in contrast to recent surveys of livers from pigs and wild boar in Europe, where almost 15% samples were HEV positive (Baechlein *et al.*, 2013; Schielke *et al.*, 2009).

It is important to note that the primer-probe set used for our RT-qPCR screen targeted a highly conserved region of HEV ORF3. Although originally designed to detect HEV genotypes 1-4 (Jothikumar *et al.*, 2006), sequence analysis showed that this primer-probe set should also detect the more distantly related HEV strains identified in rabbits and camels. A rabbit HEV strain (GenBank Accession number KJ013414) and a camel HEV strain (KJ496143) had 94-100% sequence identity to the RT-qPCR primer-probe sequence. It was therefore theorised that any novel canine HEV strains should be detectable using this assay.

A common method of detecting HEV RNA in pigs is from stool samples (Banks *et al.*, 2004; McCreary *et al.*, 2008), and hence to extend our study we also tested 248 canine stool samples for HEV RNA using qPCR. Again, no positive canine samples were identified which differs from the detection levels in pigs; HEV RNA has been reported in stools of 5-35% pigs across the UK (Banks *et al.*, 2004; McCreary *et al.*, 2008). Clearly viral shedding by dogs is substantially lower than in pigs.

Altogether, samples from over 500 dogs have been screened by qPCR or ELISA in this study. However, in spite of being a sizeable cohort in comparison to many canine studies, this number of cases is still significantly fewer than several of the large-scale human studies where over 10,000 human samples were analysed (Gallian *et al.*, 2014; Hewitt *et al.*, 2014; Sauleda *et al.*, 2014). Surveys this large have been required to detect HEV in healthy humans, where prevelance is as low as 1 in 3000 individuals (Hewitt *et al.*, 2014). Studies of this magnitude would be extremely challenging to achieve in canine populations, which means determining accurate prevalence levels of rare viruses is seldom possible.

The zoonotic potential of HEV in dogs was recently assessed by an alternative method in a serosurvey of veterinarians. It was hypothesised that if dogs were a common source of HEV, then humans with high exposure to dogs would be more likely to be seropositive. However it was found that HEV seroprevalence did not increase with occupational exposure to dogs (Mesquita *et al.*, 2014b). This is at odds with a serosurvey of swine veterinarians, which found significantly higher levels of seropositivity to HEV in comparison with people not in regular contact with swine (Meng *et al.*, 2002). These human prevalence studies indicate that even if it is definitively proven that dogs can become infected with human strains of HEV, they play a much lesser role in disease transmission than pigs.

To summarise, this chapter has provided evidence that dogs in the UK can become infected with HEV, although strains involved are unknown. The low seroprevalence coupled with the absence of detection of HEV RNA suggests HEV infection is very rare in dogs. Nonetheless, these preliminary findings warrant further investigations to determine if contact with HEV infected dogs could be a transmission route for HEV to man.

Chapter 8

Discussion

8.1 Overview

This thesis has provided the first detailed analysis of noroviruses, both human and canine, in dogs. This thesis also includes examination of two additional enteric RNA viruses in dogs, specifically astroviruses and HEV.

Studies commenced with the most comprehensive epidemiological survey of CNV to date, in which it was demonstrated that multiple strains of CNV are circulating the UK dog population. Canine astroviruses were also identified for the first time in the UK, and genetic characterization of four isolates was conducted.

Studies of CNV were extended with the first analysis of CNV interactions with host cells. This led to the identification that HBGAs are the carbohydrate attachment factor for CNV strains in both genogroup IV and genogroup VI. HBGA recognition has thus been shown to be a feature of virus-host interactions in five out of the six norovirus genogroups, and sequence analysis suggests co-evolution of at least three mechanisms of HBGA binding in the norovirus genera.

Concern about the zoonotic potential of enteric viruses in dogs has been raised by the results in this thesis. HBGA expression in the canine gastrointestinal tract has been characterized and shown to be very similar to human HBGA expression, thus it is postulated that noroviruses may be able to cross the species barrier. The close relationship between dogs and humans means disease transmission via direct contact is very possible. HEV is an additional enteric virus hypothesised to infect dogs, and serological analyses in this thesis demonstrated the presence of HEV-specific antibodies in dogs. Further evaluation of the

potential risk of both HuNoV and HEV infection in dogs is recommended to satisfactorily address this public health concern.

8.2 Pathogenesis of CNV and CaAstV in dogs

This thesis initially hoped to determine the clinical impact of CNV or CaAstV infection in dogs following identification of positive cases. Due to the limited study size, and the absence of positive results for CNV, this has not proven definitively possible for either virus. The evidence that CNV causes gastroenteritis is currently based on the premise that CNV is genetically similar to HuNoVs experimentally proven to cause gastroenteritis in man, and CNV has been identified in dogs with gastroenteritis in the absence of other known pathogens (Martella *et al.*, 2009). However, the high seroprevalence to CNV in conjunction with a lack of identified cases in the RNA screen, suggests that viral shedding is only brief and veterinary attention is rarely required.

CaAstV was identified in 6% dogs with severe gastroenteritis, in comparison with 0% in the control group. This strongly suggests that CaAstV can cause significant disease in dogs, which fits with the knowledge that HAstVs can cause gastroenteritis in children. However, in 2/4 CaAstV positive dogs, co-infections with viruses known to induce gastroenteritis were identified (CPV). Overall, the evidence for CNV or CaAstV causing severe disease in dogs is insufficient to be conclusive at present.

The optimal way to answer the question of the pathogenic effect of CNV and CaAstV is with experimental canine studies. This is important for deciding appropriate management strategies for suspect cases. This would help to answer the following concerns:

1. Would commercial tests for CNV and CaAstV be valuable for adequate management of cases, e.g. isolation?
2. Would vaccination against CNV or CaAstV be important for animal health?
3. Would the use of anti-virals be beneficial in CNV or CaAstV infected dogs?

Experimental infection studies would require inoculation of specific-pathogen-free dogs with purified virus, then monitoring for signs of clinical disease. Viral shedding could be analysed by RT-qPCR analysis of stool samples, and sites of viral replication in the body could be assessed from tissue samples collected post-mortem. Tissue sections could be screened for CNV particles by immunofluorescence utilizing the anti-CNV antibodies that have been generated for this work. RT-qPCR on RNA samples extracted from tissue samples would be an additional method of confirming any immunofluorescence results.

Ongoing surveillance for CNV in the UK and worldwide would not only drive greater understanding of the clinical relevance of this virus, but it would also facilitate identification of novel strains of CNV. The evolutionary rate of noroviruses is high, and new variants of HuNoVs commonly emerge through antigenic drift and shift. It is reasonable to predict that CNV variants will emerge at a similar rate. Of CNV strains characterized to date, strain diversity is very high and hence strains can be divided into two or possibly three genogroups (Vinjé, 2014). Evolution of new strains generates two main concerns. Firstly, there is potential that novel strains may have a greater pathogenic effect in the host. Secondly, novel strains may be more likely to jump between hosts. It is possible that point mutations in key genes, or recombination events could increase the likelihood of inter-species transmission, and recombination events have already been reported for CNV (Martella *et al.*, 2009).

8.3 Role of HBGAs in CNV infection

Following identification that CNV interacts with a subset of HBGAs (H, A and Lewis antigen), it is hypothesized that dogs expressing these HBGAs will have an enhanced susceptibility to CNV infection when compared to dogs negative for these antigens. Although all dogs in this study expressed H antigen, only 50% expressed the A or Lewis antigens. Though natural CNV infection in dogs is not believed to have high mortality rates (Martella *et al.*, 2009; Ntafis *et al.*, 2010), it is theorized that a survival advantage may be conferred by specific phenotypes. This work has shown that approximately 40% dogs in the UK group have not seroconverted to CNV, and it is hypothesised that this may in part be due to an A antigen and Lewis antigen negative phenotype. Confirmation or refutation of this theory requires a much greater understanding of the pathology induced by CNV, as well as a much wider appreciation of carbohydrate polymorphism in dogs. Testing of this hypothesis could be achieved by phenotyping of dogs identified with natural CNV infection. This has not proven possible in this study due to the lack of CNV positive cases identified.

So far in this thesis only HBGA-CNV interactions with HBGAs expressed by the host have been considered. However, it has long been known that certain species of bacteria can express HBGA like-substances on their surface (Springer *et al.*, 1961). Recently it has been demonstrated that HuNoV VLPs can bind to cells of *Enterobacter cloacae,* a commensal bacteria isolated from human faeces (Miura *et al.*, 2013). Furthermore, HuNoV infection of B cells was enhanced by the addition of this bacteria to filtered stool samples (Jones *et al.*, 2014a). This suggests commensal bacteria in the gastrointestinal tract could play a role in norovirus infections. This theory is supported by follow up experiments using antibiotics to deplete commensal bacteria in mice, which resulted in a significant reduction in MNV titres (Jones *et al.*, 2014a). Broad spectrum antibiotic therapy has also been shown to prevent

development of persistent MNV infections in mice (Baldridge *et al.*, 2015). The exact mechanism by which commensal bacteria enhance norovirus infections is yet to be elucidated, however bacteria have been shown to enhance stability of the viral capsid in poliovirus infections (Robinson *et al.*, 2014), and it is possible that similar effects are induced by HBGA-norovirus interactions. A role for bacteria-induced alteration of the innate immune response has also been indicated in MNV studies; bacteria are believed to limit IFNλ-dependant innate immunity, enabling establishment of persistent infection (Baldridge *et al.*, 2015). In summary, it is suspected that carbohydrate expression of both the host and commensal bacteria is important in norovirus infections. This is supported by observations that susceptibility to HuNoV is reduced in the absence of HBGA expressing bacteria *in vitro* (Jones *et al.*, 2014a), and also the absence of secreted HBGAs *in vivo* (human non-secretor phenotype) (Hutson *et al.*, 2005; Lindesmith *et al.*, 2003).

Relating this to CNV, it is possible to speculate that canine commensal bacteria could be enhancing viral infection in dogs. CNV could be binding to HBGAs expressed on the surface of gastrointestinal bacteria, which may have either direct (e.g. altering virion stability or localisation) or indirect (altering immune response) effects on viral infection. It is therefore theorized that depletion of the canine microbiota could diminish CNV titres. This brings into question the use of antibiotics for CNV therapy; could treating CNV positive dogs with antibiotics help resolve infection? Previous studies focusing on commensal-viral interactions have only reported decreased viral titres if animals are pre-treated with antibiotics. No studies have analysed the effect of antibiotic therapy after infection. This is because adequate decreases (i.e. 10^4 fold) in the gastrointestinal microbiota using antibiotics takes several days, by which time acute enteric viruses have already reached their target cells and replicated to high titres (personal communication, Julie

Pfeiffer, University of Texas, and Christiane Wobus, University of Michigan). Thus it is unlikely that antibiotic therapy would be beneficial for managing CNV infections after clinical signs are apparent as virus replication would already have occurred before commensal depletion is achieved. However, there is the possibility that antibiotic treatment of dogs prior to CNV infection could limit infection. Whereas this would not be relevant for individual cases, this could be extremely beneficial for managing outbreaks of CNV where many dogs are housed together, e.g. rescue centres or boarding kennels. Investigation of this theory will require experimental studies as described above, but given that several different enteric viruses are now known to be enhanced by microbiota (Jones *et al.*, 2014a; Kuss *et al.*, 2011), it is possible that antibiotic therapy may be of benefit to control of a number of different canine enteric viruses. However, it is imperative to consider the negative impact that indiscriminate use of antibiotics can have on small animal populations. This includes development of antibiotic resistance, a risk of adverse reactions and increased susceptibility to certain pathogens (BSAVA, 2014; Willing *et al.*, 2011). A decision to use of antibiotics to reduce viral infections would therefore have to be based on rigorous clinical trials and an extensive cost-benefit analysis.

8.4 CNV as a potential zoonotic pathogen

Given the close genetic relatedness of CNV to the GIV HuNoV strains, it has been proposed that CNV could be a zoonotic agent (Martella *et al.*, 2008; Mesquita *et al.*, 2010). The finding that CNV recognizes HBGAs common to both dogs and humans provides support for this theory. This work has shown that CNV VLPs bind to human saliva samples and human cells in tissue culture (HT-29 colorectal adenocarcinoma cells), confirming that CNV can attach to carbohydrates present on human cells and in secretions. This demonstrates that the initial step required for CNV entry into human cells is present. However, it should be

noted that RHDV can bind to HBGAs (H type 2, A antigen and B antigen) (Ruvoën-Clouet *et al.*, 2000) and yet there is no evidence RDHV can infect humans. HBGA binding may be an initial step in calicivirus-host interaction, but a subsequent host-restrictive step(s) must be necessary for RHDV infection, and potentially CNV infection.

Two epidemiological studies have investigated the possibility that CNV can infect humans (Martino *et al.*, 2014; Mesquita *et al.*, 2013). Both have provided serological evidence of GIV.2 infection in man, although it is possible that there are GIV.2 HuNoVs circulating in the human population that have yet to be identified. However, compelling evidence is provided by the reported higher GIV.2 seroprevalence in individuals (veterinarians) who have regular contact with dogs in comparison with people who are not often exposed. More conclusive evidence of CNV infection in man would be identification of viral RNA in human stool samples. As far as it is possible to tell, no such studies have been performed. It would be interesting to screen dog owners with clinical symptoms of gastroenteritis for the presence of CNV RNA in clinical samples.

8.5 Carbohydrate binding specificity of GIV human noroviruses

The discovery that group IV CNV strains use HBGAs as attachment factors makes it likely that GIV HuNoV strains also recognize this class of carbohydrates. A limited number of HuNoV strains have been classified into genogroup IV (Fankhauser *et al.*, 2002; Zintz *et al.*, 2005), alongside several of the CNV strains. The prototype CNV strain was shown to have 69% amino acid identity to human strains (Martella *et al.*, 2008), hence CNV has been designated a GIV.2 norovirus, and the humans strains are GIV.1. The carbohydrate binding specificity of the human GIV strains has not been studied, and to confirm a role for HBGAs, GIV HuNoV VLPs will be required. The first GIV HuNoV VLPs to be generated were

recently reported in an Italian study of seroprevalence to GIV HuNoV in humans (Martino *et al.*, 2014). Screening these VLPs against a panel of synthetic oligosaccharides would be a simple way to confirm this hypothesis.

8.6 Evolution of the norovirus genera

The results in this thesis identifying two additional norovirus genogroups able to bind to HBGAs, suggests that norovirus recognition of HBGAs may have evolved in at least three different lineages. This is based on sequence comparison with GI and GII HuNoVs, for which the crystal structures of GI and GII HuNoV complexed to HBGAs have been solved (Bu *et al.*, 2008; Cao *et al.*, 2007; Choi *et al.*, 2008). An alternative explanation for co-evolution of HBGA binding abilities would be the existence of a distant ancestor to all norovirus strains, which evolved the ability to recognize the HBGA carbohydrates conserved amongst many species. To begin establishing which proposal is correct, crystallographic studies of the CNV major capsid protein will be essential to enable identification of amino acids involved in HBGA recognition.

A recent study has reported solving the x-ray crystal structure of a feline norovirus (FNV) P domain (Singh *et al.*, 2015). FNV is a GIV.2 norovirus, with 90% amino acid identity to GIV.2 CNV. Similar to CNV, the HBGA-binding residues identified on GI and GII noroviruses are not conserved in FNV. It did not prove possible to crystalize FNV in complex with HBGAs, and analysis of the FNV VP1 structure at the equivalent GI.1 HBGA binding pocket showed a blocking P2 subdomain loop. At the equivalent GII.10 HBGA binding pocket, an extended P1 subdomain interface loop blocked the predicted HBGA binding site. This indicates that there is likely to be an alternative HBGA binding site for the GIV noroviruses, although this has yet to be elucidated.

8.7 HuNoV infection of dogs

This thesis strongly suggests dogs can become infected with HuNoV. However, definitive proof that HuNoV can infect dogs requires experimental studies. This could be achieved by inoculating dogs with HuNoV purified from human stool samples, and then analyzing canine faecal samples for viral shedding by qPCR. Experimental studies would also be valuable for a investigating a number of other unanswered questions regarding both HuNoV infection dogs and also general pathogenesis of HuNoV infections.

1. Do dogs seroconvert following experimental exposure to HuNoV? This would validate the serology data presented in chapter 6. This could be answered by collecting serum samples pre and 2-4 weeks post infection.

2. What is the duration of HuNoV shedding in dogs? Humans typically shed HuNoV for four weeks (Atmar *et al.*, 2008), and it would be valuable to compare shedding duration in dogs. This would be important for epidemiological understanding and possible quarantine of dogs during HuNoV outbreaks.

3. Do dogs show clinical signs following HuNoV infection? There is only very limited data addressing this question; one dog with suspected HuNoV infection had a severe vomiting episode (Humphrey *et al.*, 1984), whereas two other dogs showed very minor clinical signs (nausea and inappetance) (Summa *et al.*, 2012). Daily clinical exams and assessment of appetite/demeanor following experimental infection of dogs could start to address this question.

4. Do dogs become viraemic following HuNoV inoculation? It has been reported that 15% humans become viraemic during HuNoV infection (Takanashi *et al.*, 2009), but this has not been consistently reported by other groups.

5. What is the tissue or cellular tropism of HuNoV infection in dogs? qPCR of RNA extracted from different organs post-mortem could identify the main sites of replication in the body. Analysis of tissue sections from these regions could then be valuable for two reasons. Firstly, histological analysis could determine the presence any specific pathology; intestinal pathology in man caused by HuNoV infections is typically mild despite the severity of clinical signs (Karst *et al.*, 2014). Secondly, immunofluorescence could be used to identify the sites of viral replication. Cellular tropism for HuNoV is still uncertain, and as it may be hypothesized that HuNoV will replicate within the same cell types for humans and dogs, this could lead to a breakthrough in understanding HuNoV pathogenesis.

Confirming HuNoV replication can occur in the canine gastrointestinal tract alone will not be sufficient to determine a significant role for dogs in the epidemiology of HuNoV infection. The majority of HuNoV outbreaks do not occur in places where dogs are commonly found, e.g. outbreaks on cruise-ships or in hospitals, but a role for dogs perpetuating outbreaks in communities is possible. To investigate transmission of HuNoV between humans and dogs, a focused sampling approach will be required. Targeting dog owners with confirmed HuNoV infection, and testing both canine and human stools for HuNoV could provide insights into spread of infection. Full genome sequencing of any isolates identified would provide confirmation of any epidemiological relationship.

8.8 HEV infection of dogs

The preliminary HEV epidemiological surveys in this thesis have demonstrated that dogs mount an immune response to HEV. However it is not possible to determine whether this is to a human strain or a novel canine strain as all HEV strains studied to date belong to a single serotype (Wang *et al.*, 2013). The existence of a canine-only HEV strain would only be proven by the identification of HEV RNA representing a new genotype from a dog, and although clinical samples from almost 250 dogs were screened by RT-qPCR, no positive cases were found.

The host cell receptor for HEV is unknown, thus it is not possible to determine whether dogs express the correct receptor to enable HEV to invade canine cells. Similar to the canine HuNoV experiments, immunohistochemical studies with HEV VLPs and canine tissues could be a way to investigate the ability of HEV to bind to canine cells. This alone would be insufficient to confirm dogs are susceptible to the virus however. As discussed above for HuNoV, experimentally infecting dogs with HEV strains isolated from man would be required to provide definitive evidence that HEV can replicate in dogs.

8.9 Closing remarks

Although this thesis provides the first detailed study of how CNV interacts with host cells, there are still many questions yet to be answered about this virus. Continued research into the pathogenesis and viral-host relationship of CNV would be valuable for expanding our understanding of the whole norovirus genera. The possibility that CNV can be transmitted to humans makes this line of study even more important.

From a veterinary viewpoint, this thesis has highlighted the issue that our awareness of emerging viral causes of gastroenteritis in dogs is minimal. This is best exemplified by identification in this study of a novel virus in 6% canine gastroenteritis cases (CaAstV) in the UK. Canine viral gastroenteritis is understudied due to the lack of resources for veterinary research, but the emotional and economic impact of canine disease is ever increasing as owner expectations and veterinary medicine progresses. Given the potential zoonotic transmission of gastroenteric viruses between dogs and humans as shown by this thesis and other reports, it is now apparent that infectious disease research should not overlook viruses identified in dogs.

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Appendix

A.1 Full genome sequence of CaAstV Gillingham/2012/UK KP404149.

Primers used for amplification and sequencing are highlighted. Primers previously published are underlined, whereas all other primers were designed specifically for this analysis, and are numbered according to the Goodfellow Lab IGUC system. The start codon (ATG) of ORF1 and ORF2, and the stop codon (TAG) of ORF1a, ORF1b and ORF2 are highlighted in bold.

```
CCAAGAGTTGGTTTGGGTGATTGATTCGCCCATAAGATCTCTAGT**ATG**TGTTCAGTGCCAC 853 F
CTTACTTCCGCAACCAAGCGGACCAAGATTTCTCCTTCGGGAGCACTATGGCGCGGAGGAT
GATGACCATTAATATGATCAACACCCTCCCACCATTTGAAGAGCACACACCACTTCACTAT
GACTGGGTTGTTAAACACCTTATATTTCCAGGACCAAATACCACTGAGCGTTGTGTAGTCA
TAACCGGTGGTCTTGAGAACGGTAACTATGTTACTGTTGTTCATGATGGTGAAACCTGGAT
AGAAATAAACCCGGCATACAAATTTGAAGGACTGCTCAGAGTGCTCCGCATGACAGCCCGC 683 F
AACAACTCCTTGCGCGAGCGTCTGCGTCTTAGTCAGGAGGAAAAATCTAAACTAATTCTTG
ACCATCAACTCTTGAGGCATGACTATGAACGCATCAAATCTCAAACTAGCACCAGCACTAA
GACTAAAATCCCTATGTTTGTTGTGTTTGTCGCTGCATTGATCATTTTCCTGAACTTTGTA 682 R
CCAACCACAGAGGCAGCACAGGCTTACACAAGCAAGTATACTCAAACTGGCACTGAGGGAT 5'RACE
TTAGTCTCTTGGACAAATGTGCACAAAATGTGGCCAGGCTTAACAACGAGATAAGTCTAAG
ACTGAAACTGGCCCTAGGTAATGTGACCTGGTCTGACCGCTATGGTGCAATTAAAGAGATC
TTCTATGCCAACCTCTTGCCGAGATCGCACTGGATTGTGCGTCTCTTTTCTTTCCTTTCAT 878 F
ATTTCCATATTTGGACCATCTTTTCAACATGCATCGCTGTGGCAACACTAACTCGGTCTAC
AAACCCTATTGTTGACATAGTATTTCTGTTCTTTGCCCACATATCAAAATGGCAGTTGGGT
ATTGTTCCAGCTCTTCCATATTTCACCACTACTATTGTCTGGATTGCCATAACTTGTATGG 941 R
GGGCCTATGTTCTGGACCCATATCTTGCCATCACACTCACATGGCTCCAGCTTCCCTTTTG
TGTTGTATGCCTTTCTTTCCTAAGTGATGATAAGTTCATAGAGCACGTGAGAGGGTCATTC
ATTCTCACTGTGACAGCAACATCTATTCATACTTGCCTTGTTCTAACTGGCAGCACCACCT
ACTGCTTTATACTCCTGATGTTTTTTAGAAGCCTTCGGCTTCTGATGTCCTCTGTCGGTAA
CAAGATAGAGTTTAAGGACTTCCAGGGTAAGGTCGTTGGGTCGATTTCTTCAGGAACACGT
AACCGTGTCTGGAATTTCATCCAGCGAATGAAGCAGGTTAGAACTGGTTCCAATCCATTTG 952 R
CTATCATAAAACCTGAAGCACTTGTCAAAATCGTAACCGATGAAGGATGTGGAACTGGTTT 896 F
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TGACTACTCCACTGTGGTTGTGCTAGCATACAGCTCTAATGGACTTGTTGTCTCACAAGCA
CAAGGCCAATGTCACGGGGAGACCATCTCCTATACCGTCCCAACACAGGATGGTATGTCAG
GGGCACCAGTCACTGACTTACATGGTCGTGTGCTTGGTGTACATCAAACTAACACTGGCTT
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AAGAAGAAAGGTAAAAACAAGAGTGGTGGTCGTGGTAACATCAGAAAACATGTTGGTAAGG
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AGAAATTTGATTATGCACCACCTTCAAATTTCTTTGAGCAGTATCCAGAATTTTGTAAATT
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TATGTCCGGAGCAAAACCTAAGGTCCTCTGGTACTTGTTCCTCAAGAAAGAAGTTATTAAA
GTTGACAAAATTGCTGACTCAGACATAAGACAAATTCTATGCAGTGACCCTATTTATGTTA
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CCATGGACAATGTGGTTGGACACCAATGGAAGGTGGCTTCACTTCTCGTATGAAACGCCTT
ATCTCCAAAGGCAATGCACATTTCATCGAGTTTGATTGGACCAGATTTGATGGTACTATAC 607 F
CGTCTGATTTAATTCGACATATCAAGAAACTCCGCTGGAGTTTGGTTAATGCCGAACAGAG (625F1)
GAGGAAATACCAAAAGCTTCATGATTGGTATGTTGAAAACCTGGTCAATCGTACTGTGCTA
CTTCCATCTGGTGAGGTCACAGAGCAACATCGTGGTAATCCATCTGGCCAATTCTCCACTA 854 R
CCATGGATAACAATATGATCAACACATGGCTTCAGGCTTTTGAATTTGCCTACTTCCATGG
ACCCAACAAACAGCTTTGGCTGAACTATGACACCTTGGTCTATGGTGATGATAGGCTGTCA 608 R
ACAACACCACTAATTCCTGATAACTATGTTGAGAGAGTTGTACTAATGTACAAAGATGTCT (626R1)
TTGGAATGTGGGTTAAGCCTGAAAAAGTCAAGATTTCAAACACTATTGTTGGTCTCAGTTT 625 F
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GCCTCACTGCTTAAACCAGCATCCAAACTTCCGGATCTTGAATCACTCCATGGGAAACTCC
TGTGCTATCAGCTCCTCTCGGCCTTCCTACCTGAGGAACACCCTTTTAAGGTGTACGTCGA
GAGTTGTCTGGCTGCCACTAGCAGGCAGCTTCGTGATTCTGGCTTACCTACCAGATTCACA
GAAGAGCAAATGCATCGCATATGGAGGGGAGGACCAAAAAATTGCG**ATG**GC**TAG**CAAGCCA
GGCAAAGATGTCACCGTTGAGGTTAAAACCTCCGGAACAAAATCAACATCCTCTAGGAGCA
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GAGGAGACGAAACAGACCTAACAATCGTGGTCGCAAGAGAGTTGAGGCTGTCGTTAAACGA 931 F
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CTCTGGGAACTATTGGCCCGAATACTTCGGGTGCAGTAGAGCTGGAACTTGCAACATTCAT
GAATCCATGCTTAGTCAAAGAATCAACAGCTTCCAACTCTTTTGGACCCATTCAAGCATCA
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TTCCACAGGCTGGTCAGGCTTAGGGGCTAGAAAGCACAAAGATGTCAAAGTTGGCTCCATG
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GACCCAGTCAACCTACCAAAACCAGAGGTACACTGGGCCTGTTTTCCTTGTCGAATTGCAT
TGTACATGGCAATTTTCAAACTACAGTGCAAATCCAGCTCTAGCCCAGCTAGAGAAAGGAG 953 R
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TTTTTTTCCCTGCTTCTGATCAGCAGGTTTTTTCCAGTCGCCAGGTCCTCTTCAGAGCAAT
GGTCAATGAGGGTTGGCCAGAGGACCAGGCTGAGCGCCTTGCCAAACGCGCTCTCCCTACA
CTAAGTGAGAAAGAACTTAGGGATGAATTTTTAGTCGGACTCGCTGACGGCTTTTCGCCAC
GTCAAGCAGCCGCAAATGCTCGTGAAAAGTGTTCCCGAGGCCACGCCGAG**TAGG**ATCGAGG 626 R
GTACAGGTTCACTTTCACTCTTTTCTTTTACAGGTTCACTTTCACTCTTTTCTTTTCTGTC
TTTAACAATCACTTATTTTTAAGTTAGATTAGTTTAGGCAAAAAAAAAAAAAAAAA

A.2 Full genome sequence of CaAstV Lincoln/2012/UK KP404150.

Primers used for amplification and sequencing are highlighted. Primers previously published are underlined, whereas all other primers were designed specifically for this analysis, and are numbered according to the Goodfellow Lab IGUC system. The start codon (ATG) of ORF1 and ORF2, and the stop codon (TAG) of ORF1a, ORF1b and ORF2 are highlighted in bold.

```
CCAAGAGTTGGTTTGGGTGATTAAATCGCCCATAAGATCTCTAGTATGTGTTCAGTGCCAC
CTTACTTCCGCAACCAAGCGGATCAAGATTTCTCTTTCGGGAGCACTATGGCGCGGAGGAT 853 F
GATGACTATTAATATGGTCAACACCCTCCCACCATTTGAAGAGCATACACCACTTCACTAT
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TAACCGGTGGTCTTGAAAACGGTAACTATGTTACTGTTGTTCATGATGGCGATACCTGGAT
TGAAATAAACCCGGCATACAAATTTGAAGGACTGCTCAGAGTGCTTCGCATGACGGCTCGC
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GACCAGAATCCCTATGTTTGTCGTGTTTGTCGCTGCATTGGTCATCTTCCTGAATTTTGTA
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TCAGTCTTCTGGATAAATGCGCCCAAAATGTAGCCAGGCTTAACAACGAGATTAGTCTAAG 5'RACE
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ATTCTCACTGTGACAGCAACATCCATTCATACCTGCCTTGTTCTAACTGGCAGTACCACCT
ACTGCTTCATACTCCTGATGTTCTTTAGGAGTCTTCGACTCCTGATGTCCTCTGTCGGTAA
CAAGATAGAGTTTAAAGATTTCCAAGGTAAGGTTGTTGGATCAATTTCTTCAGGGACACGT
AATCGTGTCTGGAATTTCATCCAGCGAATGAAGCAAGTTAGAACTGGTTCCAATCCATTTG
CTATCATAAAACCTGAAGCACTTGTCAAAATCGTAACCGATGAAGGATGTGGAACCGGCTT
CTTTTGTGGTAATGACATTGTCACAGCAGGCCACGTGGTCGGTAGTCATCGCGTTGTTGAA 896 F
GCCTGGTATGAGGGCTCGTGCTACCAAGCAAGAGTTCGCTACAAGCCAGAAAAAGACATCG
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TGACTACTCCACTGTGGTTGTGTTAGCATACAGCTCTAATGGACTTGTTGTCTCACAAGCG
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TACAGGTGGTGCCGTAGTTATTAAAACAACTGATGTAACACCTCCAACACGCCCAACTGAG
GATGATTTGCGCAAACAAATTGAGGATCTACGTAAGCAACTTGAGGAAATCAACAAACCTC
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TTAAGGGCAGGAAATACCTAACTGAGAAAGAATACAAAGAACTGTTGGAGAAGGGTCTTGA
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ATGTGTCATTCAGGTTCAGGAAGTTGTCCCAATTGATGAGGTTACAATTTCAAAAGCAACT
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GGTCAAGACGATGTGTACCGGAAAGCTACCCATTATTAGGCCATCTAAAATTAGACAGACC 940 F
AATTTATGATGATAAAATACCCAAAGATGATCTGCTCAAATCCCTACCAGTTCCTGATTGG 893 R
CACGAATTTGAGAAATTTGGACCAACTGTGTGGGGACCACAAGCTTTCACCAAGTCTTTTG
AGAAATTTGATTATGCACCACCTTCAAATTTCTTTGAGCAGTATCCAGAATTTTGTAAATT
TGCTGATTGGGCATTTTATAAACAATATGGGTTTCTTGAAGATTCTCGTGTGATACATGTC
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TCAGCCACTGAAAAGAATCAAGATTCCACGCCTGCCTATCCCAAGATGCTCGACTATGACA
CCGAAGCCGACTTCTTGGAAGCGAACGGTTGGTCACCTTATGTTTCTGAGATTTCAAGGAT
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GTTGACAAAATTGCTGACTCAGACATAAGACAAATCCTATGCAGTGACCCTATTTATGTTA
GGATAGGTGCGGTACTTGAAGGACATCAGAACCAGCTCATGAAGAACAATACTGAAAAAAC
CCATGGACAATGTGGTTGGACACCAATGGAAGGTGGCTTCACTTCTCGCATGAAACGCCTT
ATCTCCAAAGGCAATGCTCATTTCATCGAGTTTGATTGGACCAGATTTGATGGTACTATAC 607 F
CGTCTGATTTAATTCGACATATCAAGAAACTCCGCTGGAGTTTGGTTAATGCCGAACAGAG
GAGGAAATACCAAAAACTTCATGATTGGTATGTTGAAAACCTGGTCAATCGTACTGTGCTA
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CCATGGATAACAATATGATCAACACGTGGCTTCAGGCTTTTGAATTTGCCTACTTCCATGG
ACCTAACAAACAGCTTTGGCTGAACTATGACACCTTGGTCTATGGTGATGATAGGCTATCA 608 R
ACAACACCACTAATTCCTGACAACTATGTTGAGAGAGTTGTACTAATGTACAAAGATGTCT 625 F
TTGGAATGTGGGTTAAGCCTGAAAAAGTCAAGATTTCAAACACCATTGTTGGTCTCAGTTT
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GAGTTGTCTGGCTGCCACTAGCAGGCAGCTTCGTGATTCTGGCTTACCTACCAGATTCACA
GAAGAGCAAATGCATCGCATATGGAGGGGAGGACCAAAAAATTGCG**ATG**GC**TAG**CAAGCCA 887 F
GGCAAAGATGTTACCGTTGAGGTTAAATCCTCCGGGACAAAATCAACATCCTCCAGGAGCA
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GCAGCCCAATATAATCTCTGGAGAGTAACTAAAGCAGATGTACGGCTTACACCTATGGTAG
GCCCGTCTGCAATCTCTGGTACCGCTTACCGAGTATCCCTCAATACAGCGGGGACCCCTTC
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TCAACTTTCAGAGTTACTGCGCGAGATATGGCGGGCCCACGCGAGGGTTGGTTTGTCACCA
ACACAAATGAATCAGGAGGTGAATCAGTAGGACCTACAATAGAGCTACATTCACTTGGTCA
AACACAATCCACCTACCAGAATGCTCCCTATTCTGGCCCTGTATTTCTTGTTGAGCTTCAC
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TTCACCACATTCCAACCTGGCTAGGGCTCTTGAAACAAAATCAGCAATGCCCTATGCCTCA
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CCTATTTGTACCACTCCTACAACAAAGTGGTTGTTAAAATAGGTGAGCACTATGATGAAAT
CAACTTTGTGTTCAGGGCAACAACACCCCACATCTGGCTCTCAGGTACTCAACCCAATTTT
CAGGTCTCCAGCAAACCCGGTGCGCTGGATTTCATGGAAGTTGGACAGAAGAGTAACAATC 1060 R
AATACCTAACCCGGGGAGAAATTTGGGGCTATTCACAACATAAGATCTCTAGCACCAATGG
CCAAAGTGTGTTAACATTCTATGTTGGTCAGCTTAGCCGCCAGATACGCTACTCCAACTAC
AAACAGGTTAGGTATGCCTTCTCTGGTAATGGGTCAACAACTTCACTCAATCCAATTGCCA
CTGAGTATGATCTGCAGTTCCTTGAACTCAACCCAGGCCCCTGGTTCATTTTTGTCTCCAT
GCACTTTGTCAACAGTTCAATCCAAGGCTTTGTTGCTACTGAAAATCCATCAAGACCAACA
ATTGTGTGTCCTTCTGCTGCACAAAACCTGCTTGTGTGTACAGGAAACAATGTCGCCAGCA
CAATGGTGATTGATTTTCTCAGGACACCACCAACTTTCACACAACAGGCAGAACAAGCCTT
GTTTGATTTGCCACAGCCCATTGAAGATGGTTTTCTTCCAGATTCTGAAGATGAAGATTTT 894 F
TCTGATGATGATTCTCTTCTTGATGATGATGATTTTTTCCCTGCTTCTGATCAGCAGGTTT 3′ RACE
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TGAGCGCCTTGCCAAACGCGCTCTCCCTACACTAAGTGAGAAAGAACTTAGGGATGAATTT
TTAGTCGGACTCGCTGACGGCTTCTCGCCACGTCAAGCAGCCGCAAATGCTCGTGAAAAGT 888 R
GTTCCCGAGGCCACGCCGAG**TAG**GATCGAGGGTACAGGTTCACTTTCACTCTTTTCTTTTC 626 R
TGTCTTTAACAATCACTTATTTAGTTAAGTTAGATTAGTTTAGGCAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAA

A.3 Complete capsid sequence of CaAstV Huntingdon/ 2014/UK KP404151

Primers used for amplification and sequencing are highlighted. All primers except 626R were designed specifically for this analysis, and are numbered according to the Goodfellow Lab IGUC system. The start codon (ATG) of ORF2, and the stop codon (TAG) of ORF2 are highlighted in bold.

ATGGGAAACTCCTGTGCTATCAGCTCCTCTCGGCTTTCCTACCTGAGGAACACCCCTTTAA 965 F
GGTGTATGTCGAGAGTTGTCTGGCTGCCACTAGCAGGCAGCTTCGTGATTCTGGTTTACCA
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GCTAGCAAGCCAGGCAAAGATGTTACCGTTGAGGTTAAATCCTCCGGAACAAAATCAACAT
CCTCCAGGAGTAAATCCCGGGGCCGAAACCGGAATGTCAAAATCACTGTCAACTCACAACC
AAGGGCAAACAGAAGGAGACGAAACAGACCTAACAATCGTGGTCGCAAAAGAGTTGAGGCT
GTCGTTAAACGACAACTCGATAAGGCTGGAGTCACAGGACCAAGACCAGCGATCACCCAGA
CTGCTACATCTACTCTTGGGACTATTGGCCCGAATACTTCGGGTGCAGTAGAGCTGGAACT
TGCAACTTTCATGAACCCATGCTTGGTCAAAGAATCAACAGCTTCCAACTCTTTTGGGCCC
ATCCAAGCATCTGCAGCCCAATACAACCTCTGGAGGGTAACTAAAGCAGAAGTCCGACTCA
CACCTATGGTAGGTCCCTCTGCCATCTCTGGTACTGCTTATCGAGTCTCACTCAATACAGC
AGGAACACCATCCTCAACAGGATGGTCTGGACTAGGTGCCAGAAAACACAAAGACGTGAGA 959 F
GTGGGCTCCATGTCCACTTTCAGGGTCACAGCCAGAGATATGGCCGGACCACGTGAGGGCT
GGTTTGTTACTAACACTAATGAGTCAGGAGGTGAGTCAGTAGGTCCCACCATCGAACTGCA
CTCGCTTGGTGAAACTAAGTCCACCTATCAAAATAGTTCATACACAGGGCCTGTCTTCCTC
GTTGAGCTACATTGTACTTGGCAATTTACCAACTACAGCGCCAATCCAGCTTTAGCTCAAC
TTGAGAAAGGAGAGGACAGGGATGCCCAAATCAAATTTGAAGGGGATGCTGGACAACCCCT
CAAGATGGTCATCCAACCACATACGGCTTTTGCGCGGGCGCTTGAAGCAAGATCTACAGTC
CCATATTCTAATGGGTCCCGTGCTGCCGGTGAGTCCACCTCTGACACAATTTGGCAAATAG
CGTCGACTGCAGTTGACGCAACCTCTGTTGTTGTGCCACCACCTTTTAACTGGCTCATAAA
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CAAGTCTATGCAAGTTATGAGGATGCACAAAACAATAGGCCAGCAATATGTACTGGCCCGG
TGTCTGGTGAAAACACACCGCGACAACACACTGTCAAGTTTGTGCAAATGAACTCTCCATC
CACAGGTATGCCACCCGAGTCCTCTCTCGGAGCGTACTCATTGAACATGCCAGAAACACCA
ACAATTACTAATGAATTTATGCTTGTCACTGGGTTTAACCAGCCTTACCAAGAAAACCCGC
CCTGTCCTACGTACATCTACAAGGACGATGTTAAAGTAGTTGTCAAGTGTGATACCAACTA
TGATGAAGTTAATTTCTTCTTCAAGGCAGCGCATCCACATGCGTGGCTTGTTGGTTCCGCA
GGAAACTGGACGGGTTCTACCAAGCCCGACTTCTTAACATTCATGGAAGTTGGCATAAGAA
ACAACGCAGGCAACTACAACAAGGCTGGAAACCTTTGGGGCTACTCTCAGCATAAGATCCA
GAGTGGAGACACAACCAACATCTTGACTTTCTATGTTGGGCAATTGACAAGCACCATCAGA
ATACAAAACCCTAAGAAACAGAGGTACCAATACAGAGGTTCAGGCTCTACAACTCTCCTTG
AGCCAACAGGACCTGAAACTACACCTAGGACTTTTGAACTTAACCCAGGCCCGTGGATTGT
CTTCGTTGCCATACATTCTATCAGTGGCAACCCACAAGGTTTTGTTATGAGGACTAACCCA
TCAAGGCCAACTATCACTTGCCCAGCTGCAGCACAAAACATAGCAGTCTGTGTCAACACAA
ACACTGACAGCATCATGTCGGTGTTGTATCAAAGGGTGCAACCAACATCAATTGTGAATGA
AGATGTAGTAAATTATGATCTGCCACATCCCATTGAAGATGGTTTCCTTCCAGATTCTGAA
GATGAAGACTTTTCTGATGATGACTCTCTTCTTGATGATGATGATTTTTTCCCTGCTTCTG
ACCAGCAGGTTTTTTCCAGTCGCCAGGTCCTCTTTAGAGCAATGGTCAATGAGGGTTGGCC 960 R
AGAGGACCAGGCTGAGCGCCTTGCCAAACGCGCTCTCCCTACACTAAGTGAGAAAGAACTT
AGGGATGAATTTTTAGTCGGACTTGCTGACGGCTTTTCGCCACGTCAAGCAGCCGCAAATG 626 R
CTCGTGAAAAGTGTTCCCGAGGCCACGCCGAG**TAG**

A.4 Complete capsid sequence of CaAstV Braintree/2014/UK KP404152

Primers used for amplification and sequencing are highlighted. All primers except 626R were designed specifically for this analysis, and are numbered according to the Goodfellow Lab IGUC system. The start codon (ATG) of ORF2, and the stop codon (TAG) of ORF2 are highlighted in bold.

ATGGGAAACTCCTGTGCTATCAGCTCCTCTCGGCTTTCCTACCTGAGGAACACCCTTTCAA
GGTGTATGTCGAGAGTTGTCTGGCTGCCACTAGCAGGCAGCTTCGTGATTCTGGTTTACCT
ACCAGGTTCACAGAAGAGCAAATGCATCGCATATGGAGGGGAGGACCAAAAAATTGCGATG 965 F
GCTAGCAAGCCGGGCAAAGATGTCACCGTTGAGGTTAAATCCTCCGGCACAAAATCAACAT
CCTCCAGGAGCAAATCCCGGGGGCGAAACCGGAATGTCAAAATCACTGTCAACTCGCAACC 887 F
AAGGACAAACAGGAGGAGACGAAACAGACCTAACAATCGTGGTCGCAAGAGAGTTGAGGCT
GTCGTTAAACGGCAGCTCGATAAGGCTGGAGTCACAGGACCAAAACCAGCGATCACCCAGA
CTGCTACATCTACTCTGGGAACTATTGGCCCGAATACTTCGGGTGCAGTAGAGCTGGAACT
TGCAACCTTCATGAATCCATGTCTTGTCAAAGAGTCAACAGCTTCCAACTCTTTTGGGCCC
ATTCAAGCCTCTGCGGCCCAGTACAATCTCTGGAGGGTGACCAAAGCAGAAGTTCGACTCA 1060 F
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AGGAACACCATCTTCAACAGGATGGTCTGGACTAGGTGCCAGGAAACACAAAGACGTGAGA
GTGGGCTCTATGTCTACTTTCAGAGTCACAGCCAGGGATATGGCTGGCCCACGTGAGGGCT
GGTTCGTCACCAACACTAACGAGTCAGGAGGCGAATCTGTGGGTCCCACCATTGAGTTGCA
CTCGCTAGGTGAAACCAAATCCACCTATCAAAACAATTCTTATACAGGACCTGTTTTTCTT
GTTGAGCTACATTGTACTTGGCAGTTTACCAACTATAGTGCTAATCCAGCTTTAGCTCAGC
TCGAGAAAGGAGAAGACAGGGATGCACAGATCAAATTTGAAGGCACCGCTGGGCAGCCATT
AACAATGACTATTGCACCACATTCAGCATTTGCTCGTGCTCTGGAGACAAAATCAACAATG
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CTTCAACCGCAGTAGATGCTGCCTCAGTTGTGGTGCCACCACCCTTCAATTGGCTCATTAA
GGGTGGCTGGTGGTTTGTAAAGAAAATCGCTGGTCGCACAAGGGCTGGAGAACTCCAGGTC
CAAGTCTATGCCAGCTATGAGGATGCTCAAAACAACAGGCCAGCTATCTGCACCGGCTCAG 1408 F
CAGAAGGTAGTAATGTTCCTAGACTCCACAATGTCAAATTTGTTCAAATGAATGCGCCTTC
GACTGGGATGCCACCTGAGTCTGCATTAGGTGCTTTCAGTCTGCCACTCCCAGACACGCCA
GCTACTATTGGGGATAGCTTTAGGCTTGTAACAGGGGTTTACCAACAATTCCAACAAAACC
CTCCTTGCCCTACATATGTCTANCATAGTACCAACAAAATGGTTATCAAAATTGGCACTCA
CTATGATGAGTTGAATTTTGTATATAGGGCAACTACACCTCATATCTGGCTTGCTGGAGGA
CCTCAAACCTGGCAGGTTGCTGATAAACCACCTGAGTTGGAGTTTATGGAGATTGGTATTA
AATCAGGTTCCACCTACCTTAAACGTGGTATTGTGTGGGGCTACTCACAACATAAGATAGT
CAGTGGAAGCGAACTAAGCGTTCTTACCTTCTATGTGGGTAAATTGGACAACCAAATACGC
TACACCAACTACAAGAAAGTTCGCTATCTCTTTACAGGAACAGGCCCTAGCCTCAACCTAA
CACCAGATGCAACAACCACTGAATTACAGGTGCTCGAGCTCAACCCTGGGCCATGGTTTGT
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ACAGACAGACCTACAATAGTCTGCCCTGCTGCAGCTCACCAATTGTTTGTTTGCACCAACA
ACAATGTTGCCAGCACAATTGTCATTGATTTCTTGAGAACACCACCAAAAATTGCACAACA
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GAAGATGAAGATTTTTCTGATGATGATTCTCTTCTTGATGATGATGATTTTTTCCCTGCTT
CTGATCAGCAGGTTTTTTCCAGTCGCCAGGTCCTTTTCAGAGCAATGGTCAATGAGGGTTG
GCCAGAGGACCAGGCTGAGCGCCTTGCCAAACGCGCTCTCCCTACACTAAGTGAGAAAGAA
CTTAGGGATGAATTTTTAGTCGGACTTGCTGACGGCTTCTCACCACGTCAAGCAGCCGCAA
ATGCTCGTGAAAAGTGTTCCCGAGGCCACGCCGAG**TAG**
626 R