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Genetic characterisation and phylogenetic analysis of bovine astroviruses and kobuviruses

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Declaration

I declare that this thesis is my own work, and that its contents have not previously been submitted for the award of any other degree. The work described in this thesis was carried out by myself, except where indicated throughout the thesis.

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Abstract

Calf diarrhoea remains the most important cause of economic loss to both dairy and beef cattle industries worldwide, with approximately 50% of deaths among weaning calves resulting from diarrhoeal disease. Complex etiopathogenesis involving the infection of one or multiple pathogens, as well as other non-infectious factors such as the environment and nutrition, contributes to its devastating effects. Astroviruses (AstVs) and Kobuviruses (KoVs) are two single-stranded, positive-sense RNA viruses previously detected in healthy and diarrhoeic calves. AstV was identified in healthy and diarrhoeic calves in similar proportions, while KoV was predominantly associated with diarrhoeic individuals.

In order to investigate the KoV strains found in diarrhoeic calves, the full genome of KoV from a diarrhoeic calf was sequenced. This KoV was then compared with the bovine U-1, porcine KoV and Aichi virus strains. Specifically-designed PCRs were used to target the full KoV genome of positive samples, and amplicons were cloned to allow the internal sequencing of one single KoV detected among possible mixed infections. Upon assembly of the genome sequences, some animals were found to be co-infected with multiple KoVs. The main region of diversity, the VP1 (capsid) region, was amplified from multiple samples to determine the diversity of KoV in Scotland. The genome sequenced in this study will be used to produce an infectious clone for future challenge studies to establish the potential role of bovine KoV in calf diarrhoea.

Another aspect of the study was to explore the diversity and epidemiology of AstV in diarrhoeic and healthy calves by capsid gene analysis. As the AstV capsid protein is the major target of host antibody production, a serological test for AstV infection could then be developed. Following amplification of AstV capsid genes by PCR, phylogenetic analysis identified 4 lineages from which capsids from 2 of these lineages were successfully cloned ready for use in the baculovirus expression system. This information and the expression plasmids containing representative AstV capsid genes can then be used to develop serological tests for AstV, enabling estimation of the prevalence of AstV in the British cattle population.

Lay Summary

Calf diarrhoea remains the most important cause of economic loss to both dairy and beef cattle industries worldwide, with approximately 50% of deaths among weaning calves resulting from diarrhoeal disease. The complex interactions between multiple pathogens (including viruses, bacteria and parasites) contribute to the fatalities associated with calf diarrhoea. AstV and KoV have both been previously detected in calves suffering with diarrhoea. AstV have been identified in healthy and diarrhoeic animals, while KoV have been predominantly found in diarrhoeic animals. Further characterisation of both of these viruses is required to determine the role they each play in diarrhoeal disease, either as a primary pathogen or a co-factor for infection.

The aims of this project were to investigate the diversity of bovine AstV and bovine KoV within the samples and others taken from the GenBank database. In terms of bovine KoV, a full genome was sequenced from an infected calf, which is the first bovine KoV to be sequenced from an animal rather than cell culture. For the AstV, genes known to be involved with pathogenicity of the virus and interact with the animal's immune system, were also sequenced. This collection of AstV sequences were then both compared with each other, and other known sequences in the database, to locate them into groups according to diversity.

Acknowledgements

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1. Introduction

1.1 Calf diarrhoea

Diarrhoea in young calves remains the most important cause of economic loss to both dairy and beef cattle industries worldwide, despite technological improvements in animal facilities and herd management (Cho and Yoon, 2014). In 2007, the U.S. dairy industry reported 57% of unweaned heifer deaths were due to diarrhoeal disease, with the majority of calves under 1 month old (USDA, 2007). A similar rate of 53.4% deaths for dairy calves due to calf diarrhoea was also recently reported in South Korea (Hur *et al.* 2013).

Calf diarrhoea has a complex etiopathogenesis encompassing infectious and non-infectious pathogens, including infection by one or multiple pathogens, and non-infectious factors such as environment, management practices and nutrition (Blanchard, 2012). Causative pathogens include a range of viruses, bacteria and parasites with the most important in the UK being coccidia, rotavirus, coronavirus, enterotoxigenic strains of *Escherichia coli* and *Cryptosporidium parvum* (Takiuchi *et al.* 2006; Foster and Smith 2009; Cho and Yoon, 2014). Other viral pathogens such as bovine torovirus, bovine AstV and bovine KoV have been associated with calf diarrhoea, although the extent of their contributions to pathogenesis are still to be determined (Khamrin *et al.* 2008; Gülaçti *et al.* 2014; Candido *et al.* 2015).

There are a limited number of longitudinal studies of enteric virus infections in calves performed, and most of these studies only investigated one enteropathogen. This makes defining the relationships between pathogens in co-infections challenging (Takiuchi *et al.* 2006; Tse *et al.* 2011; Gülaçti *et al.* 2014; Ribeiro *et al.* 2014). The Coura *et al.* (2015) study is one of the few studies to investigate the epidemiology of enteric disease associated with Salmonella spp., enterotoxigenic strains of *Escherichia coli*, rotavirus and coronavirus infections in calves up to 70 days old in one dairy herd. It identified a series of complex interactions taking place between pathogens and multiple internal and external factors throughout infection. In order to establish the roles of individual pathogens within calf diarrhoeal disease, the identification of new or previously undetected pathogens is crucial. More studies on

novel pathogens are required to understand the extent of pathogen interactions and environmental factors, with the aim of providing preventative and control strategies (Coura *et al.* 2015).

1.2 Kobuvirus

1.2.1 Background to kobuviruses

1.2.1.1 Kobuvirus

The first KoV to be discovered was Aichi virus in Japan in 1989. It was isolated from faecal samples of human patients suffering from non-bacterial shellfish-associated gastroenteritis (Yamashita *et al.* 1991). In 1997, complete genome sequencing and phylogenetic analysis of this virus confirmed it to be a new member of the *Picornaviridae* family, with just 15-36% amino acid homology to those of other viruses in the family (Yamashita *et al.* 1991; Yamashita *et al.* 1998). It was classified into a new genus of *Picornaviridae* called Kobuvirus, the name kobu (meaning 'bump' or 'knob' in Japanese) coming from the morphological appearance of the virions observed under an electron microscope (King *et al.* 1999).

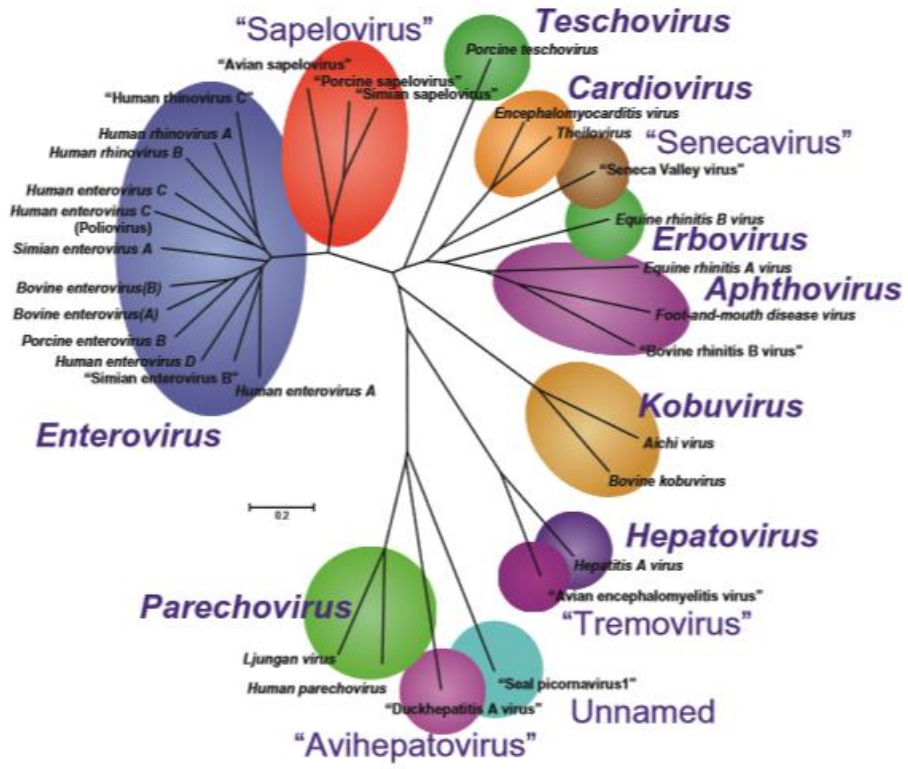

Figure 1: Unrooted neighbour-joining tree of the *Picornaviridae* family, based on a P1 capsid region comparison (Dubovi and MacLachlan, 2010).

The *Picornaviridae* family includes several important animal and human pathogens with a range of host species, such as foot-and-mouth disease virus and poliovirus, making picornaviruses an interesting family to study (see Figure 1) (Yamashita *et al.* 2000; Hughes, 2004). The kobuvirus genus currently comprises of six accepted or proposed species with canine KoV, murine KoV and ovine KoV being added most recently (Sweeney *et al.* 2012). KoVs are divided into three Aichivirus species - A, B and C. The distinction criteria for Aichivirus species are the following: viruses must share a greater than 70% amino acid identity in the polyprotein and P1 region (see Figure 3); share a greater than 80% amino acid identity in 2C and 3CD; and share a common genome organisation. Aichivirus A includes Aichi virus, murine KoV, canine KoV and feline KoV (Cho *et al.* 2015); Aichivirus B includes bovine KoV, ovine KoV and ferret KoV (Cho *et al.* 2015); Aichivirus C includes porcine KoV (Ribeiro *et al*, 2013). These species groups can be seen below in Figure 2.

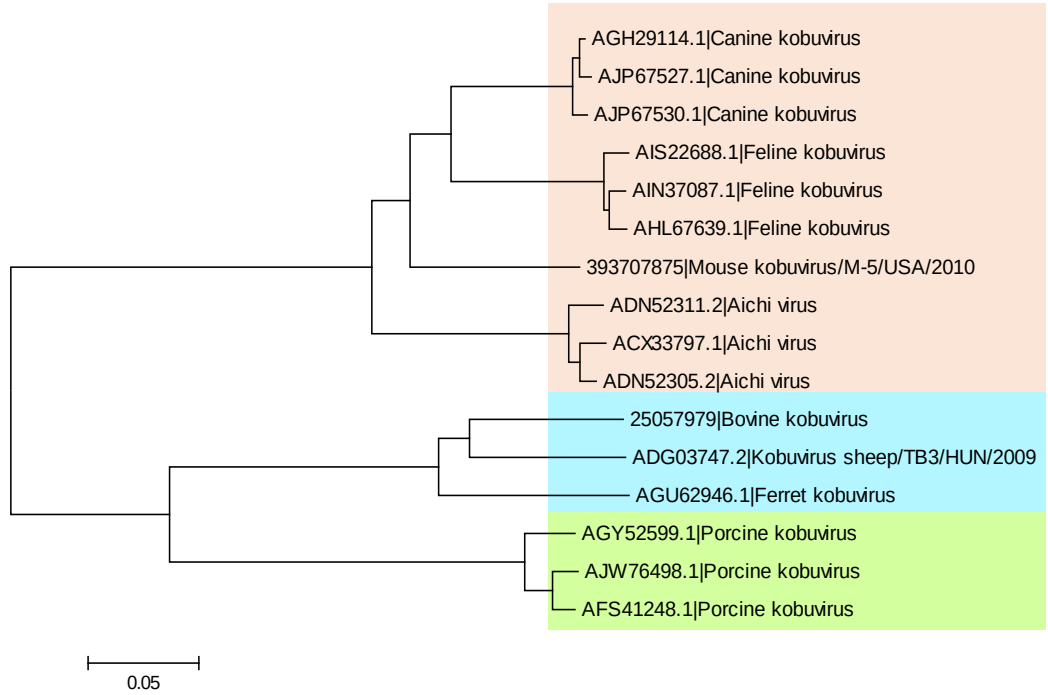

Figure 2. Phylogenetic grouping of the three species in the Kobuvirus family. The red box represents species Aichivirus A, the blue box represents Aichivirus B and the green box is Aichivirus C.

Recently, numerous other KoVs have been detected in a range of mammalian and avian, wild and domestic animals. KoV has been present or associated with enteric disease in all the hosts indicated below in Table 1.

Table 1: The first detection information of mammalian KoVs.

Species	Detection	Reference
Human (Aichi virus)	1989	Yamashita *et al.* 1991
Cow	2003	Yamashita *et al.* 2003
Pig	2008	Reuter *et al.* 2008
Sheep	2010	Reuter *et al.* 2010
Insectivorous bat	2010	Li *et al.* 2010
Mouse	2011	Phan *et al.* 2011
Dog	2011	Kapoor *et al.* 2011
Goat	2012	Lee *et al.* 2012
Cat	2013	Chung *et al.* 2013
Ferret	2013	Smits *et al.* 2013
Fox	2014	Bodewes *et al.* 2014
Norway rat	2014	Firth *et al.* 2014
Roe deer	2015	Di Martino *et al.* 2015
European roller (bird)	2015	Pankovics *et al.* 2015
Golden jackal	2015	Olarte-Castilloa *et al.* 2015
Side-striped jackal	2015	Olarte-Castilloa *et al.* 2015
Spotted hyena	2015	Olarte-Castilloa *et al.* 2015

1.2.1.2 Bovine kobuvirus

Following the discovery of Aichi virus, bovine KoV was detected for the first time in 2003 as a cytopathic agent in a Vero cell culture medium. Yamashita and colleagues (2003) explained the calf sera used in the medium had been contaminated with virus from the faeces of an infected animal. The virus, termed U-1 strain, was found to be different from Aichi virus since it reacted with antibody raised to Aichi virus by ELISA, but could not be neutralised by Aichi virus antisera. Antibodies against U-1

were not detected in human, monkey, pig, horse, dog or cat sera samples, compared to the bovine sera in which 60% of individuals were positive for neutralising antibodies (Yamashita *et al.* 2003).

1.2.2 Genome structure and genetic diversity

1.2.2.1 Genome structure

KoVs are small, spherical, non-enveloped viruses with a single-stranded, positive sense RNA genome between 8.2 and 8.4Kb in size (Yamashita *et al.* 2003; Reuter *et al.* 2009). The genome encodes a polyprotein (approximately 2400 aa) which undergoes a cleavage cascade to obtain the active viral proteins (Yamashita *et al.* 1998). These predicted cleavage sites occur between a glutamine residue and another amino acid, commonly glycine, alanine or threonine, and are similar to those found in other picornaviruses. Viral proteins include a non-structural protein (L), three structural proteins (VP0, VP3 ad VP1), and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D), of which the VP1 capsid protein has been found to be the most immunogenic (Chang *et al.* 2014). All KoVs share the same genome organisation (see Figure 3), although genes encoding 3D and VP1 show the highest and lowest sequence homology, respectively, between bovine KoVs, porcine KoVs and Aichi viruses (Cho *et al.* 2014). Viral protein functions vary within the *Picornaviridae* family, with 2A working as a protease in most picornaviruses; 2B alters host cell membrane permeability; 2C is important for the formation of viral replication vesicles; 3C is a proteinase; 3D is the RNA-dependent RNA polymerase (RdRp); and 3A is involved in modulating the host immune response by inhibiting expression of host class 1 major histocompatibility complex (MHC) molecules (Dietz *et al.* 2000). These functions in *Picornaviridae* are largely consistent among KoVs, with noted differences in 2A, VP0 and L protein functions (Reuter *et al.* 2011). The L protein is known to have no autocatalytic activity and isn't involved in polyprotein cleavage, although it is expected to play a role in viral RNA replication and encapsidation (Sasaki *et al.* 2003). In other picornaviruses, the VP0 protein is also cleaved into VP4 and VP2 in the later stage of capsid assembly, whereas in KoVs VP0 is found uncleaved in mature particles (Yamashita *et al.* 1998).

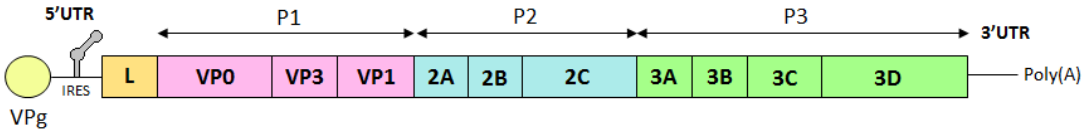

Figure 3: Genomic organisation of three recognised KoV species: Aichi virus, bovine KoV and porcine KoV (adapted from Reuter *et al.* 2011).

The polyprotein is preceded by a 576-808 nucleotide 5' untranslated region (UTR) which has been predicted to contain complex secondary/ tertiary stem-loop and cloverleaf structures that are crucial for genome replication (Sasaki *et al.* 2001; Yamashita *et al.* 2003). The GC-rich nature of the first 59 nucleotides of the genome has made it difficult to sequence and therefore produce an infectious clone (Sasaki *et al.* 2001; Martínez-Salas *et al.* 2015). The first 108 nucleotides and the secondary structure of this stem-loop domain are thought to be distinctive to KoVs, and also play an essential role in the production of virions (Sasaki *et al.* 2001; Oem *et al.* 2014). The Sasaki *et al.* study (2001) on Aichi virus indicates the folding of the stem-loop at the 5' end, but not the 3' end, is vital for viral RNA replication. Also, nucleotide changes to this stem-loop structure were not found to reduce the efficiency of virus replication in the same study.

In addition to the 59-terminal element found at the 5' end, a longer element termed the internal ribosome entry site (IRES) is present which is involved in cap-independent translation (Sweeney *et al.* 2012; Asnani *et al.* 2015). The secondary structures of IRESs (Type IV and V) of human and bovine KoVs are unknown, although there are genetic differences between the three defined KoV species: human, bovine and porcine (Sasaki *et al.* 2001; Asnani *et al.* 2015). The covalent link between the 5' end and the viral protein g (VPg) enables the initiation of translation via the IRES element (Martínez-Salas *et al.* 2001). Correct function of the IRES results in optimum replication, and determines viral pathogenesis and virulence, making it a target for inactivation by antiviral drugs (Martínez-Salas, 2008).

1.2.2.2 Genetic diversity

Genetic variability among KoVs is difficult to determine, with relatively few sequences available that span a whole gene and even fewer complete genomes available. There are partial sequences entered in GenBank cover the 3C/3D junction, due to the suggested benefit of using this section for classification of other picornaviruses. High nucleotide and amino acid sequence homology has been observed within VP1 (capsid) regions of bovine (>86% and 96%, respectively) and porcine (>85% and 92%, respectively) KoVs (Reuter *et al.* 2011). While these data suggest KoV infection is host-specific, cross-species infections have been detected in some farm animals. Another study by Chen *et al.* (2013) investigating VP1 regions of porcine KoV isolates, found two diarrheic pigs were co-infected with multiple KoV strains. Following phylogenetic analysis, sequence diversity of the isolates was found within the same host and between different hosts, possibly as a result of recombination. Naturally, co-infection of multiples KoV strains in the same host could be favourable to the occurrence of these recombination events (Lau *et al.* 2011; Chen *et al.* 2013; Fan *et al.* 2013). In the studies by Khamrin *et al.* (2010) and Okitsu *et al.* (2012), phylogenetic analysis of the VP1 and 3D regions of a bovine KoV-like strain detected in pigs suggested it may be a natural recombinant from porcine and bovine KoVs, as there was a higher sequence identity to the bovine U-1 strain than any of the porcine KoVs (Yang *et al.* 2014).

The pathogenic and zoonotic potential of these viruses remains uncertain. Antibodies to Aichi virus have not been detected in pigs or cattle and bovine U-1 strain antibodies have not been detected in human or pig sera either (Yamashita *et al.* 2003). However, this does not rule out the possibility of other animals acting as carriers for the viral transmission and as a site of recombination events. Full genome sequencing of KoVs detected in different species would enable more detailed phylogenetic studies to be carried out, with incidences and sites of recombination to be estimated more effectively.

1.2.2.3 Replication

Currently, there are few studies exploring KoV replication or pathogenesis, with even less known about bovine KoV specifically. For many picornaviruses the cell receptors are known, with low-density lipoproteins, extracellular matrix-binding proteins and integrins being just some of the cell surface molecules targeted by the viruses. As with other virus families, picornaviruses follow a basic replication cycle (detailed in Figure 4) which provides valuable information about the role of the VPg and IRES structures characteristic of this family. Cell entry mechanisms vary among picornaviruses. Poliovirus, for example, interacts with its CD155 receptor resulting in a structural change that in turn takes part in the generation of a membrane pore. Through this pore it is able to enter the cell cytoplasm (Dubovi and MacLachlan, 2010). Following the virus uncoating, VPg at the 5' end of the genome is removed by cellular enzymes allowing the ribosome to bind to viral RNA via the IRES. The IRES has a cloverleaf-like structure that can bind specifically to host-cell proteins, stimulating the synthesis of viral protein and RNA. Synthesis of the complementary strand of the replication complex (comprising of RNA templates, the virus-encoded RNA polymerase and several other viral and cellular proteins) is initiated at the 3' terminus of the viral RNA, using the VPg as a primer. The strand generated from this step then acts as a template for further synthesis of viral RNA (Whitton *et al.* 2005; Dubovi and MacLachlan, 2010). This series of events is typical of picornaviruses and the presence of the VPg and IRES in Figure 4, suggests their roles in replication will be similar, if not the same.

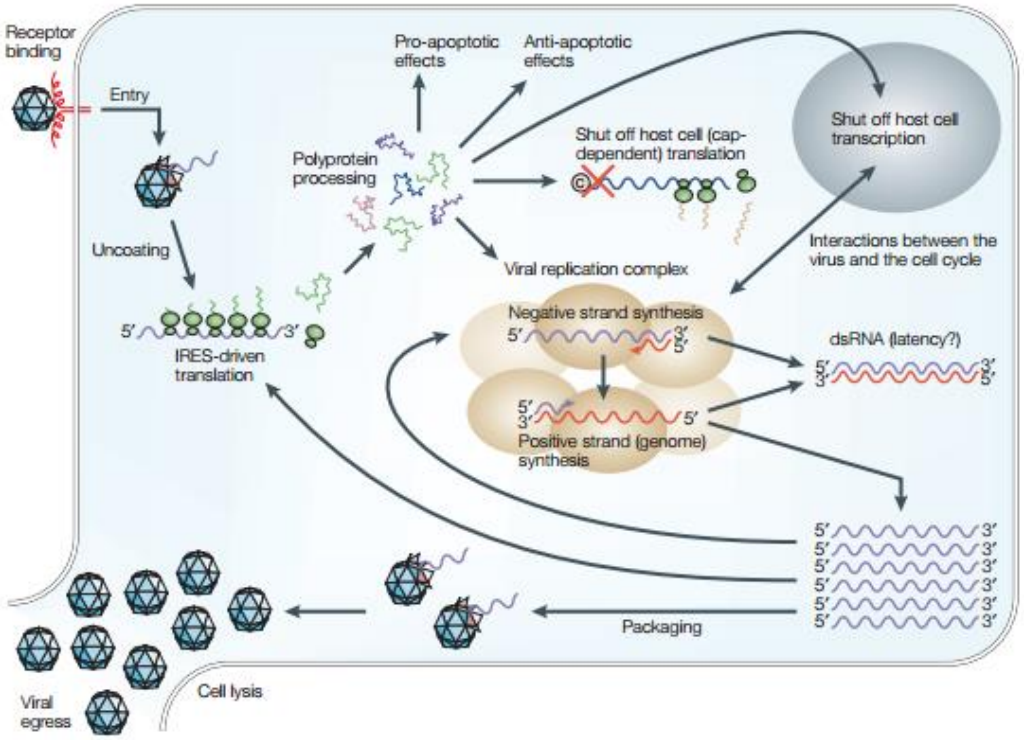

Figure 4: Summary of the picornavirus replication cycle illustrating the cell entry and involvement of VPg and IRES detailed in the text (from Whitton *et al.* 2005).

1.2.3 Disease association

1.2.3.1 Kobuviruses

A connection between KoV and diarrhoeal disease has been strongly suggested in a range of species, although limited pathogenesis studies have been carried out. They have been associated with diarrhoeal disease in humans, pigs, goats, cats and dogs among others; although a definitive relationship is yet to be established (Carmona-Vicente *et al.* 2013; Chung *et al.* 2013; Oem *et al.* 2014; Di Bartolo *et al.* 2015). Transmission is expected to be via the faecal-oral route, including the contamination of food, water and environmental surfaces, as was the case when consumption of raw shellfish was found to be responsible for Aichi virus-related gastroenteritis in humans (Le Guyader *et al.* 2008; Sdiri-Loulizi *et al.* 2010). Mammalian KoVs have been detected in Europe, Asia, South America, Africa and North America (Reuter and Egyed, 2009; Khamrin *et al.* 2010; Barry *et al.* 2011; Sisay *et al.* 2013; Olarte-Castillo *et al.* 2015). KoV-associated diarrhoea in young animals appears to be more severe than in adults, which is perhaps unsurprising as immunity plays a major role in protection against enteric viruses.

A study by Reuter *et al.* (2010) reported porcine KoV viraemia, suggesting this KoV (and potentially others) are able to escape the gastrointestinal tract and move into the circulatory system of immunocompromised and young individuals. If this is also true for bovine KoV it could provide an explanation for the contamination of cell culture described in Yamashita *et al.*'s (2003) first bovine KoV detection study, as it was believed the fetal bovine serum was the contaminated component. This area of KoV research requires further investigation.

1.2.3.2 Bovine kobuvirus

One study has found bovine KoV to be endemic within the Korean diarrhoeic cattle population, further epidemiological studies are required to define whether it is endemic globally (Park *et al.* 2011). The small collection of studies which used a sample set of both calves and adult cattle (Table 2) makes age at which immunity is acquired difficult to predict. Diarrhoea in young calves (under the age of a 1 month,

especially) can be fatal often as a result of co-infections and highly pathogenic strains.

Studies detecting bovine KoV in diarrhoeic and asymptomatic cattle are limited (Table 2), meaning a fair assessment of the overall impact of bovine KoV on calf diarrhoea is not possible at present. Most research groups will look at only one or two of the groups (between age and diarrhoea status), which reduces the impact of the conclusions in these studies. The Jeoung *et al.* study had errors in the number of samples used for each group, with the summary table representing the samples differently to that described in the text. Another observation from these studies is the higher prevalence in calves compared to adult cattle. While this may be expected with younger animals being more susceptible to enteric viruses, a study by Di Martino *et al.* (2012) found only a small difference between prevalence in asymptomatic and diarrhoeic calves (4.8% and 5.3%, respectively). Co-infections with other enteric viruses, including bovine rotavirus, bovine coronavirus and bovine viral diarrhoea virus, in both calf and adult cattle populations have been reported (Jeoung *et al.* 2011).

Table 2: The sample sizes and distributions between symptomatic and asymptomatic, calves and adult cattle.

No. of Calves (<1 year)		No. of Adult cattle (>1 year)		Reference
Symptomatic	Asymptomatic	Symptomatic	Asymptomatic	
300	0	0	0	Mauroy *et al.* 2009
61		46		Jeoung *et al.* 2011
13	0	49	0	Park *et al.* 2011
38	104	0	0	Di Martino *et al.* 2012
0	0	166	0	Chang *et al.* 2014
182	0	40	0	Ribeiro *et al.* 2014

1.2.3.3 Kobuvirus pathogenesis

The site of infection for bovine KoV is unknown; however, other picornaviruses studied in more detail (such as poliovirus and hepatitis A virus) target the M cells on the Peyer's patches of surface epithelial cells in the small intestine (Takahashi *et al.* 2008). A study by Ouzilou *et al.* (2002) suggested a method by which poliovirus crosses the epithelial barrier using M cells. The CD155-covered basolateral face of the enterocytes may allow poliovirus to infect the cells most efficiently, which then releases virions into the intestinal lumen where further amplification of poliovirus can occur in the gut. Hepatitis A virus gains entry to enterocytes in a similar mechanism but targets the apical surface more efficiently than the bilateral surface of the cells (Blank *et al.* 2000).

As mentioned in the previous section (1.2.3.1), porcine KoV has been detected in pig serum although it is not known whether this viraemia was acquired actively or passively (Reuter *et al.* 2010). Porcine KoV has also been found to undergo nucleotide changes consistent with other picornaviruses, indicating good adaptation to virus-host relationship (Reuter *et al* 2010).

1.3 Astrovirus

1.3.1 Background to Astroviruses

The first detection and identification of AstV was by electron microscopy in 1975 from infant diarrhoea cases on a maternity ward, and then later at a Glasgow hospital in the same year (Appleton and Higgins, 1975; Madeley and Cosgrove, 1975a, b). AstVs are small, icosahedral, non-enveloped positive sense RNA viruses, initially identified by the negative staining of their characteristic star-like projections on their virions, and thus deriving their name from the Greek word "astron" meaning star (Caul and Appleton, 1982; Tse *et al.* 2011). The family *Astroviridae* is comprised of two genera: Mamastrovirus and Avastrovirus, which infect mammalian and avian hosts, respectively (Smits *et al.* 2010). AstVs are generally associated with enteric disease in humans and has been reported as the second most common cause of viral gastroenteritis in young children, with rotavirus being the first (Liu *et al.* 2007). Symptoms in humans include diarrhoea and vomiting, although infections in other species are presumed to be subclinical in immunocompetent individuals (Moser and Schultz-Cherry, 2005).

AstV-associated disease can be found away from the gastrointestinal tract, for example, nephritis in domestic chickens and wild birds (Todd *et al.* 2011; Honkavuori *et al.* 2014); hepatitis in ducks (Gough *et al.* 1984; Fu *et al.* 2009); shaking syndrome in mink (Blomström *et al.* 2010); and most recently a potential cause of encephalitis in humans and cattle (Quan *et al.* 2010; Li *et al.* 2013; Bouzalas *et al.* 2014; Brown *et al.* 2015). At present, AstVs have been detected in numerous terrestrial and aquatic, domestic and wild animal species. Some examples can be found in Table 3.

Table 3: Chronological order of AstV discovery with their associated disease.

Species	Detection	Associated disease	Reference
Human	1975	Gastroenteritis in infants and young children	Madeley and Cosgrove, 1975a
Ovine	1977	Diarrhoea in lambs	Snodgrass and Gray, 1977

Bovine	1978	Diarrhoea in calves (asymptomatic)	Woode and Bridger, 1978
Chicken	1979	Interstitial nephritis in young chicks, enteritis	Yamaguchi *et al.* 1979
Turkey	1980	Poult enteritis complex, poult enteritis mortality syndrome	McNulty *et al.* 1980
Pig	1980	Diarrhoea in piglets, asymptomatic	Bridger, 1980
Dog	1980	Diarrhoea in pups, asymptomatic	Williams, 1980
Cat	1981	Pyrexia and mild diarrhoea, asymptomatic	Hoshino *et al.* 1981
Red deer	1981	Diarrhoea	Tzipori *et al.* 1981
Duck	1984	Acute hepatitis and mortality in ducklings	Gough *et al.* 1984
Mouse	1985	Diarrhoea, asymptomatic	Kieldsberg and Hem, 1985
Mink	2002	Pre-weaning diarrhoea, shaking mink syndrome	Englund *et al.* 2002
Guinea fowl	2005	Enteritis	Cattoli *et al.* 2005
Insectivorous bat	2008	-	Chu *et al.* 2008
Cheetah	2009	Lethargy and anorexia, watery diarrhoea	Atkins *et al.* 2009
Brown rat	2010	-	Chu *et al.* 2010
California sea lion	2010	Pup with diarrhoea, adults clinically healthy	Rivera *et al.* 2010
Steller sea lion	2010	Pup without signs of diarrhoea	Rivera *et al.* 2010
Bottlenose dolphin	2010	Clinically healthy	Rivera *et al.* 2010
Roe deer	2010	Diarrhoea	Smits *et al.* 2010
Pigeon	2011	-	Zhao *et al.* 2011

Vast improvements have been made to existing techniques and the development of better molecular assays and investigative tools, for example enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), new cell lines for DNA cloning and high throughput sequencing (Willcocks *et al.* 1990; Hale, 1997). These methods have been used successfully to discover new AstVs in different species, by isolating and sequencing the virus (De Benedictis *et al.* 2011). ELISA tests have already been applied to chicken AstV capsids to detect virus-specific antibodies and differentiate between maternally acquired antibodies and antibodies produced following acute infection (Caballero *et al.* 2004; Skibinska *et al.* 2015). In cattle, ELISAs could be used to determine the prevalence and diversity of AstVs occurring in the British population, as well as investigating the effect of acute and persistent infections in calves.

1.3.2 Genome structure and genetic diversity

1.3.2.1 Genome structure

AstVs have a linear, non-segmented, positive-sense RNA genome containing three open reading frames (ORFs) and a poly-A tail at the 3' end. ORF1a encodes non-structural polyprotein 1a and ORF1b encodes the viral RNA dependent RNA polymerase (RdRp), with an overlap of approximately 70nt between them. ORF2 encodes the viral capsid protein which has a small overlap with the RdRp of 40nt providing a highly conserved region compared to the remainder of the AstV genome (Smitts *et al.* 2010; Farkas *et al*. 2012a, b). During the translation of ORF1a a -1 ribosomal frameshift occurs resulting in the translation of ORF1b. Unlike other viruses, this frameshift is mediated by a slippery $(A)_6C$ sequence and downstream structural hairpin (see Figure 5), opposed to an RNA pseudoknot as more commonly found, for example in coronaviruses (Jiang *et al.* 1993; Tse *et al.* 2011; De Benedictis *et al.* 2011). ORF2 encodes a polyprotein that undergoes proteolytic cleavage by caspase protease and processing by trypsin, with the purpose of promoting structural capsid protein maturation in preparation for virus particle release. The role of trypsin in acquiring the mature capsid proteins VP34 and VP27/25, forming surface spikes, have been associated with a considerable increase of virus infectivity (Méndez *et al.* 2004; van Hemert *et al*. 2007).

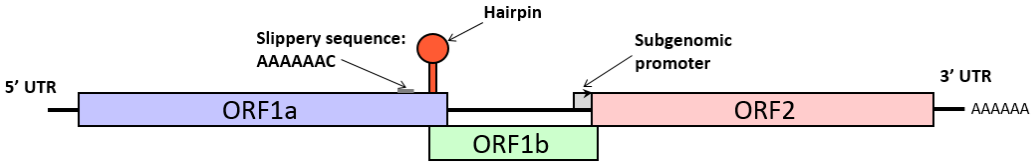

Figure 5: Genome structure for Astroviridae family, showing the key characteristic features for replication (modified from de Benedictis *et al.* 2011)

The proteolytic maturation of the virus particles described causes consequent conformational changes in the AstV capsid, perhaps most importantly in the spike domains. High-resolution crystal structures of mammalian and avian AstV capsid spike domains have shown differences between potential host receptors, requiring

further investigation into species specificity and host entry (Dong *et al.* 2011; DuBois *et al.* 2012, 2013). AstV virion structure and capsid spikes have a strong similarity to those of Hepatitis E virus (HEV) (see Figure 6), indicating a closer evolutionary relationship than previously thought. This information could provide vital clues to unknown areas of AstV research such as pathogenesis and innate immune responses (Dong *et al.* 2011; DuBois *et al.* 2012).

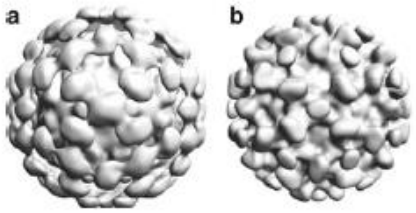

Figure 6: Comparison of human astrovirus (HAstV) and HEV structures. The tertiary structure of immature HAstV (a) is very similar to that of HEV (b) (modified from DuBois *et al.* 2013).

Spike domains of human and avian AstVs have some fundamental differences in their structures. The b-strands being solely responsible for the bowl-shaped dimeric spike of HAstVs contrasts to the additional helices found in spike domains forming the heart-shaped structure of avian AstVs (see Figure 7). Studies into structural homology have found the HEV spike domain to be more closely related to the avian astrovirus spike domain to that of HAstV. The low sequence homologies and structural conservation between human and avian AstVs suggests they may have substantial differences in host entry and immune cell interactions (Dong *et al.* 2011; DuBois *et al.* 2012).

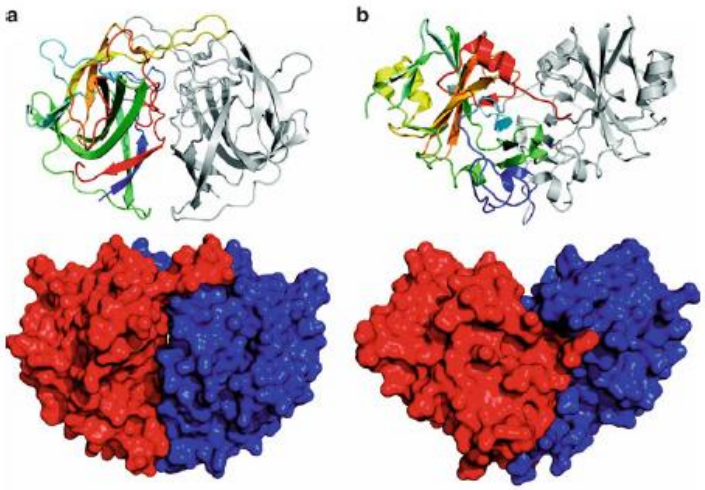

Figure 7: Comparison of the human and avian AstV spike structures. Dimeric structure of the HAstV 8 capsid spike (a) and of the turkey AstV 2 (TAstV-2) spike (b) shown as cartoon (top) and surface (bottom) representation. In the cartoon representations, one protomer has a blue-to-red colouration from N- to C- termini whereas the other protomer is coloured grey. In the surface representations, one protomer is coloured red and the other blue (modified from DuBois *et al.* 2012, 2013).

AstVs have a viral genome-linked protein (VPg) covalently linked to the 5' end of the genome by a phosphodiester bond. In picornaviruses, VPgs have numerous functions including playing vital roles in genome replication, viral protein synthesis and potentially genome encapsidation (Goodfellow, 2011). Several studies have suggested the probability of a VPg on the HAstV genome, which would play a crucial role in viral infectivity as found in other virus families. Proteinase K treatment removes this VPg from the 5' end of the genome, therefore preventing protein synthesis and replication of the virus. This action eliminates the recovery of infectious viruses post transfection, making these viruses safer during infection studies without the risk of infection to researchers (Al-Muitairy *et al.* 2005; Velázquez-Moctezuma *et al.* 2012; Fuentes *et al.* 2012).

1.3.2.2 Host range and capsid variability

Genetic variability, as common with most RNA viruses, has been described for the vast majority of mammalian and avian AstV species including porcine AstVs (Lee *et al.* 2015; Luo *et al.* 2011), bovine AstVs (Tse *et al.* 2011; Candido *et al.* 2015; Sharp *et al.* 2015), bat AstVs (Chu *et al.* 2008; Drexler *et al.* 2011), turkey AstVs (Cattoli *et al.* 2007; Strain *et al.* 2008) and chicken AstVs (Todd *et al.* 2009; Pantin-

Jackwood *et al.* 2011), to name a few (Figure 8). Studies on AstV capsid structure have suggested a greater host immune and environmental pressure resulting in synonymous or non-synonymous nucleotide substitutions, with receptors that interact with immune cells most susceptible to these changes. Recombination events are known to occur across the mam- and avastrovirus species, with at least one expected during the evolution of bovine astrovirus and roe deer AstVs (Tse *et al.* 2011) and evidence of recombinations in two separate studies on turkey AstVs in the USA (Pantin-Jackwood *et al.* 2006; Strain *et al.* 2008). Oem and An's study (2014) provides evidence for probable recombination in ORF2 of bovine faecal samples.

Naturally, the ability of AstVs to undergo recombination in a relatively short period of time has raised the important question of whether these animal AstVs could recombine with HAstV, thus facilitating zoonotic transmission and the emergence of novel viruses into the animal and human populations. Current evidence for this is limited with just one study by Rivera and colleagues (2010) reporting the potential interspecies transmission between a Californian sea lion and its carer, and another by Ulloa and Gutierrez (2010) describing a possible recombination event between a porcine AstV and HAstV3. Lukashov and Goudsmit's study in 2002 suggests that a least two cross-species transmissions involving pigs, cats and humans may have taken place in the virus' history, potentially through the use of intermediate hosts. The recent association of encephalitis with neuro-AstV in humans (Quan *et al.* 2010) and cattle (Li *et al.* in 2013), has also attracted interest to the potential of recombination events leading to zoonotic transmission and even causing inflammation in the brain.

As expected, ORF2 shows the most diversity among different host species as this enables the virus to interact with specific host cell receptors and successfully replicate. This equates to a single host species being susceptible to infection by divergent AstVs, for example the classic human genotypes (1-8), VA and MLB strains of HAstV do not cluster to form a monophyletic group (Strain *et al.* 2008; Tse *et al.* 2011). Conversely, some studies have suggested that some HAstVs have multiple hosts. The Kapoor *et al.* (2009) study reports the presence of human-mink-ovine AstVs or 'HMOAstVs' from human stools, which are phylogenetically distinct

to other AstVs previously found. Another example is from Finkbeiner and associates' (2009) work where they noted the close genetic relationship between HAstVs and circulating rat AstVs in Hong Kong. These phylogenetic studies suggest the ability of these viruses to infect multiple species, although this hasn't yet been explicitly studied. While HAstV genotypes have been found to differ in virulence (Caballero *et al.* 2003; Holtz *et al.* 2011), the pathogenic potential of different phylogenetic groupings have not been collectively studied to the same extent in non-human mammals.

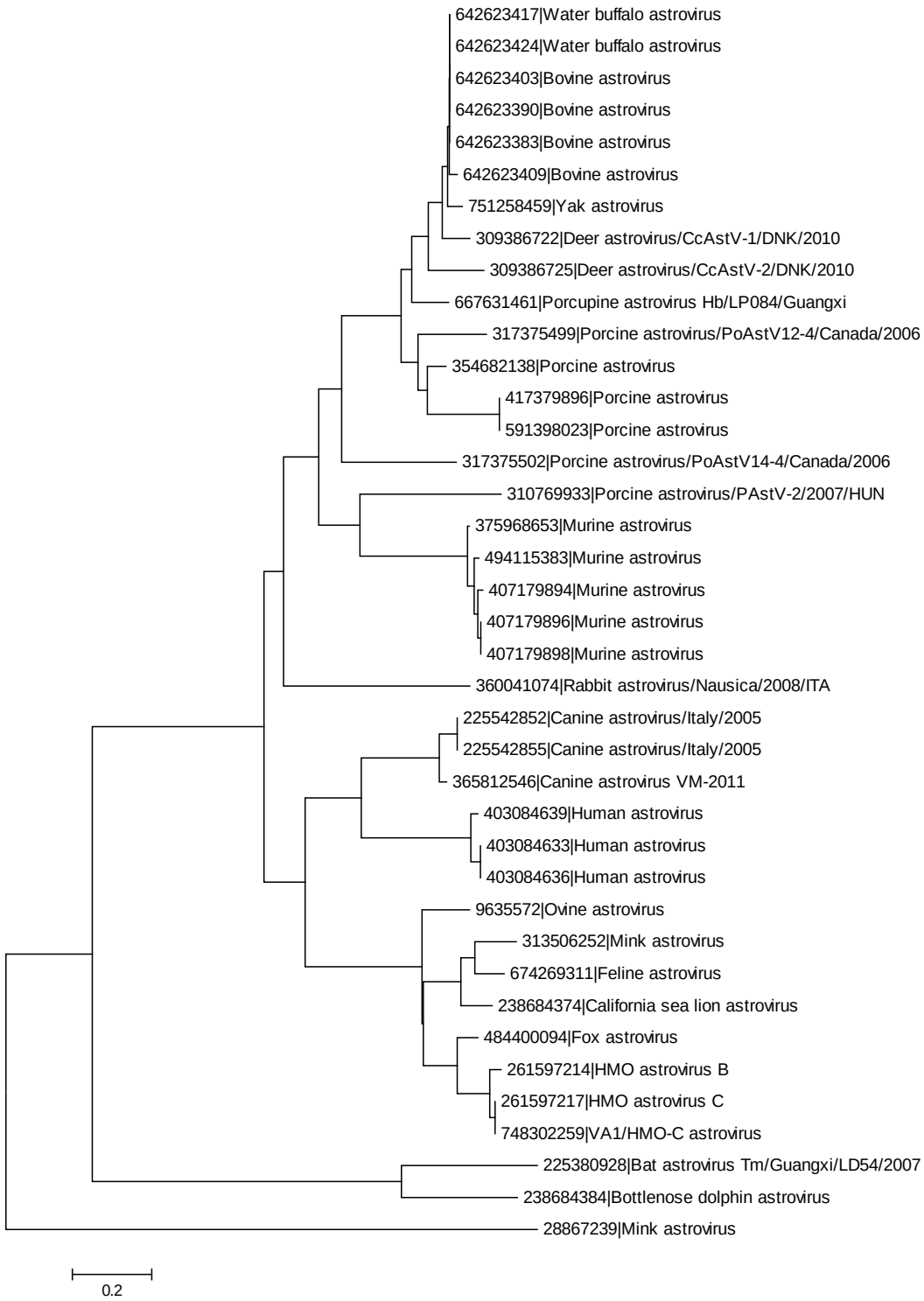

Figure 8: Neighbour-joining tree comparing the diversity of mammalian AstVs, based on full genome sequences (including GenBank numbers).

1.3.2.3 Replication

AstV replication has been most extensively studied for human AstVs (summarised in Figure 9), although the mechanism is expected to be very similar, if not the same, for other mammalian AstVs. The virus entry mechanism adopted by HAstV is clathrin-mediated endocytosis, although the method for releasing the genome from the virus particle into the cytoplasm for translation, and the site where this occurs, remains unknown (Méndez *et al.* 2012). The extra-cellular signal-regulated (ERK1/2) pathway is activated by an interaction between HAstV and the host cell, and is thought to be required to establish productive infection. Following uncoating of genomic RNA, it has been suggested the VPg recruits the cap binding protein eIF4E to initiate translation, as observed in caliciviruses. Non-structural proteins are translated as two polyproteins (nsp1a and nsp1ab), of which nsp1ab requires a translational fusion of ORF1a and ORF1b that produces a -1 frameshift to be synthesised (Lewis and Matsui, 1996). Individual non-structural proteins are consequently produced by the cleavage of nsp1a and nsp1ab by viral serine protease (Gibson *et al.* 1998). The non-structural protein, nsp1a has been found to be essential for virus replication (along with the viral polymerase) and has been suggested to produce the VPg characteristic of AstVs (Al-Mutairy *et al.* 2005; Guix *et al.* 2005; Fuentes *et al.* 2011).

In addition to the VP34 and VP27/25 spike proteins, other proteins such as VP90 and VP70 are also synthesised from the ORF2. The 90-kDa protein VP90 assembles into virus particles before being cleaved by caspases to produce the 70-kDa VP70 protein through several intermediate stages. VP90 and VP70 have been associated with the exit of virus particles from the cell (Méndez *et al.* 2002). In order for the VP70 virions to fully activate their infectivity they require treatment with trypsin, which cleaves the VP70 into three smaller products and improves virus infectivity up to 105-fold. The mechanism for this cleavage is, however, unknown (Lee and Kurtz, 1981; Bass and Qiu, 2000).

The release method of viral particles from the infected cell is uncertain, although it has been found that the capsid assembles in the absence of viral RNA, carried out in association with cellular membranes and requires the action of cellular caspases during the final stages of assembly (Méndez *et al.* 2012).

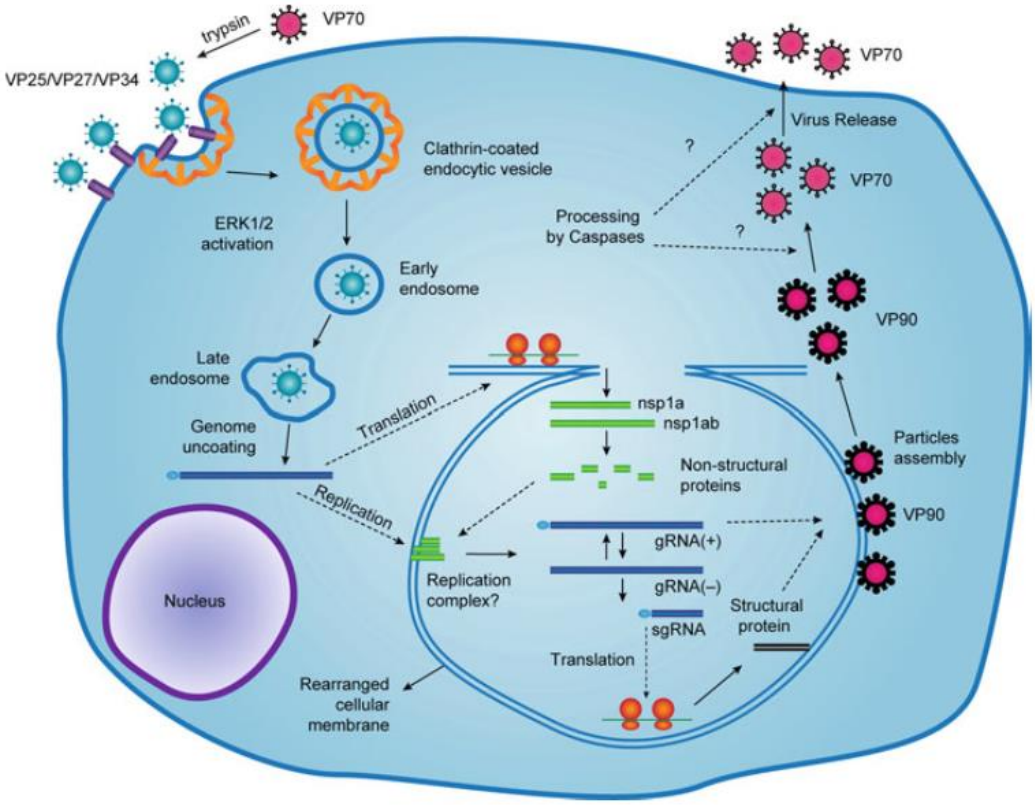

Figure 9: The replication mechanism for HAstVs (Méndez *et al.* 2012).

1.3.3 Disease association

Since the first observations of AstV-associated outbreaks of gastroenteritis in infants in 1975, AstVs have been strongly connected to viral gastrointestinal disease in infants, the elderly and immunocompromised people (Noel and Cubitt, 1994; Maldonado *et al.* 1998; Koci *et al.* 2003; Meyer *et al.* 2015). Furthermore other studies have reported a similar trend with the AstVs detected in the diarrhoea of young animals. Recent studies have found a low prevalence in adult cattle populations compared to calf populations (Tse *et al.* 2011; Sharp *et al.* 2015), which suggests there may be acquired immunity after initial infection (Bosch *et al.* 2010).

1.3.3.1 Bovine astrovirus

Association between bovine AstV and calf diarrhoea has been disputed since its initial discovery in the 1980s, with new technological developments providing evidence for its involvement in calf diarrhoea and its role as a co-factor of mixed infections. The series of studies by Woode and colleagues often found the presence of rotavirus particles in faecal samples or rotavirus antibodies in serum whilst testing for AstV infection (Woode and Bridger, 1978; Woode *et al.* 1984; Woode *et al.* 1985). Initial studies suggested AstV was avirulent in calves following the absence of clinical signs in infected gnotobiotic calves. Later investigation discovered cytopathological changes to the M cells of the dome epithelium covering the Peyer's patches when infected with either AstV and rotavirus or AstV and Breda virus 2 (Woode *et al.* 1984). However, rotavirus and AstV infected calves were found to produce a strong rotavirus serological response similar to that produced by AstV negative, rotavirus positive calves. This suggested rotavirus may be the primary pathogen but the effect of AstV on reducing or enhancing the function of Peyers' patches remained unknown. Calves infected with AstV and rotavirus produced severe diarrhoea and excretion of both viruses, compared with the yellow soft faeces of calves infected with AstV alone (Woode *et al.* 1984). The Woode *et al.* 1985 study found AstV could be shed in faeces in this instance for 25 days post-infection. It was also concluded from these studies that more mixed infection studies with other enteric viruses and cryptospiridia would be beneficial to establish the role of different pathogens in calf diarrhoea.

Later studies of AstV in European roe deer and cattle (in 2010 and 2011, respectively) revisited the diarrhoea association with new data showing the presence of AstV in a deer population with gastroenteritis, which was genetically distinct from the bovine AstVs previously described by (Smits *et al.* 2010; Tse *et al.* 2011). This diversity was believed to be as a result of co-infections with multiple virus strains in one individual potentially causing recombination events, as found in a similar study looking at porcine AstV diversity (Luo *et al.* 2011). A new study by Sharp *et al.* (2015) found no association between the presence of AstV and calf diarrhoea, but did find a strong link between the presence of AstV and rotavirus Group A. Following previous studies on the various connections between enteric viruses and calf diarrhoea (Cho *et al.* 2013; Coura *et al.* 2015), this study helps to clarify the association of AstV with diarrhoea, and suggests the contribution of rotavirus to be more substantial.

1.3.3.2 Neuro astrovirus

Recently, several cases of human AstV infection have been reportedly associated with encephalitis and other neurological symptoms. In a study by Quan *et al.* (2010), AstV was exclusively identified in the CNS of a 15 year old immunocompromised male with X-linked agammaglobulinemia showing signs of encephalitis. Another study by Naccache *et al.* (2015) used next-generation sequencing (NGS) to identify an AstV, of the VA/HMO clade (see Figure 8), as the causative pathogen of a fatal progressive encephalitis in an adult male following a bone marrow transplant. Furthermore, the Brown *et al.* (2015) investigation into the encephalitis after a stem cell transplant in an infant, found a HAstV-VA1/HMO to be responsible, also by NGS. The VA1/HMO clade are human-mink-ovine-like AstVs which are highly divergent compared to the enteric AstVs and have been associated with these encephalitis cases in humans (Finkbeiner *et al.* 2009).

An AstV associated with neurological disease was discovered on Danish mink farms in 2000 and 2003. Upon sequencing of the causative pathogen, its identification as an AstV has provided a whole new area of interest for AstV research (Mittelholzer *et al.* 2003a, b; Blomström *et al.* 2010). The clinical signs included shaking, staggering

gait and lesions characteristic of nonsuppurative encephalomyelitis identified post-mortem (Blomström *et al.* 2010). Congenital tremor, another disease of the central nervous system (CNS) is linked to detection of AstV in the cerebrum, brain stem and cerebellum in newborn piglets. However, these porcine "neuro AstVs" have been detected in healthy piglets as well as those with congenital tremor, casting doubt on their aetiological role (Blomström *et al.* 2014).

1.3.3.3 Bovine neuro astrovirus

Bovine neurotropic AstV (BoAstV-NeuroS1) was first recorded in a study by Li *et al.* in 2013, in which a brain sample from a calf with encephalomyelitis and ganglioneuritis was analysed by viral metagenomics. Another study in 2014 also reported the presence of neurotropic AstV (BoAstV-CH13) in 5/22 brain samples from cattle with nonsuppurative encephalitis, phylogenetically closely related to ovine AstV (enteric) and other AstVs isolated from animal and human encephalitis cases (Bouzalas *et al.* 2014; Oem and An, 2014). While cattle with neurological signs are attentively screened, particularly since the 1996 BSE outbreak in the UK, the causative pathogen often remains unknown (Lloyd *et al.* 2006; Li *et al.* 2013). AstVs are not part of this routine screen, although recent findings and the potential for zoonotic transmission via the faecal-oral route may bring about the use of next-generation sequencing and *in situ* hybridization for diagnosis (Bouzalas *et al.* 2014).

While neurotropic AstVs show a low phylogenetic similarity to enteric AstVs, they are closely related to the lineage of AstVs also found in association with neurological disease in mink and cattle (Brown *et al.* 2015). Transmission of this AstV group is under speculation, with intravenous immunoglobulin, close residence to mink and cattle farms and the faecal-oral route being suggested (Quan *et al.* 2010; Brown *et al.* 2015). Similar to enteric strains, these AstVs have so far been found in immunocompromised adults and children (often following tissue transplants and blood transfusions) therefore establishing transmission routes is vital to ensuring the satisfactory testing of transplanted and transfused human products (Naccache *et al.* 2015).

1.3.3.4 Astrovirus transmission

Enteric AstVs are transmitted via the faecal-oral route, as common with other viruses affecting the gastrointestinal tract (Méndez and Arias, 2007). Replication of these viruses within the host results in large quantities of virus being excreted for up to 5 days post-infection in cattle, and can therefore spread into the communal environment (Woode *et al.* 1984; Prevost *et al.* 2015). The ability of AstVs to remain stable at cold temperatures means AstV outbreaks are more likely during late autumn through to early spring (Bosch *et al.* 2010).

Husbandry can have a considerable impact with segregating diarrhoeic animals, frequently changing bedding and regularly cleaning out pens thus reducing transmission. The calving pattern of cattle means they can calf all year round, therefore choosing a calving period between late spring and early autumn would be beneficial to reducing risk of viral gastroenteritis. Contamination of surfaces and other fomites has been studied for HAstVs and have been found to act as alternative transmission routes (Abad *et al.* 2001; Gallimore *et al.* 2005). Chloro-derivitives, such as chlorine, and high concentration ethanol (≥ 90%) have been found to inactivate AstVs in water and on surfaces and are common components of strong disinfectants (Kurtz *et al.* 1980; Superti *et al.* 1990; Espinosa *et al.* 2008).

1.3.3.5 Astrovirus pathogenesis

Upon the first detection of AstV in calves in the 1970s, pathogenesis studies took place to investigate the infection mechanism in animals inoculated with AstV. These studies infected gnotobiotic and conventionally reared calves with AstV (without the presence of other gut pathogens) and found lesions in the M cells of the Peyers patches on post mortem (Woode *et al.* 1984; Woode *et al.* 1985). The lesions were suggested to have a damaging effect on the gut immune response, thus making them more susceptible to infection from other gut pathogens (Woode *et al.* 1985). Since these initial investigations the mechanism of AstV -induced diarrhoea remains unknown, however, there has been speculation of different mechanisms such as destruction of the intestinal epithelia; modulation of ions channels; or induction of intestinal epithelial barrier permeability (Meliopoulas and Schultz-Cherry, 2011). In

turkeys, where AstV is known to cause enteritis, increased intestinal barrier permeability has been found to be the primary factor responsible for infection (Moser *et al.* 2007). In the Moser *et al.* study, an increase in permeability correlated with the disruption of tight-junction proteins, further increasing when monolayers were treated with UV-inactivated virus or purified recombinant HAstV capsid. This suggests AstV-induced permeability occurs independently of viral replication and is controlled by the capsid protein, a characteristic thought to be unique for AstVs.

1.3.3.6 Site of infection

If AstV infection does occur in cattle the mechanism is unknown, although multiple cellular attachments or receptor molecules are believed to be involved (Méndez *et al.* 2012). For neurological bovine AstVs, viral RNA has been detected throughout the brain and nervous system including the neurons in the spinal cord, brainstem and cerebellum (Li *et al.* 2013). When examined further, lesions were of widespread neuronal necrosis, microgliosis (accumulation of microglial cells) with damage also found to the cerebellum and brainstem (Li *et al.* 2013; Bouzalas *et al.* 2014). This neuropathology is consistent with the AstV-related encephalitis in human cases (Brown *et al.* 2015; Naccache *et al.* 2015).

1.4 Background to study

A family-owned farm in South West Scotland, Farm M, experienced two outbreaks of fatal diarrhoeal disease in newborn calves between spring 2012 and winter 2012/13, causing serious concern for the calves' welfare and distress for the farmer. Following the routine screening for common enteric pathogens with no pathogenic agent consistently detected in the diarrhoeic calves, the samples were screened for novel pathogens at the Roslin Institute. Samples from diarrhoeic and non-diarrhoeic calves from 6 farms in the Dumfries and Ayrshire region were found to be negative for parvoviruses and bocaviruses, while perhaps unsurprisingly; AstV, rotavirus and KoV were detected across all the farms sampled. AstV was not found to be associated with diarrhoea, whereas rotavirus and KoV were (Sharp *et al.* 2015).

1.5 Aims of the project

Following the recent detection of both viruses in samples from diarrhoeic and healthy calves in south west Scotland, I will be using the samples available to explore these objectives:

a) Sequence the full bovine KoV genome from a diarrhoeic calf
b) Assemble the bovine KoV genome for future virus challenge studies.
c) Sequence and prepare bovine AstV capsids for use in baculovirus expression system, to develop an ELISA for detection of AstV in serum.

2. Materials and methods

2.1 Sample selection

The samples were provided by SAC Consulting: Veterinary Services during an investigation of calf diarrhoea across 36 different farms in South West Scotland between November 2012 and January 2013. On collection, the samples were suspended in 1ml RNAlater (Ambion) and stored at -20°C until extraction. For this study, 7 calf samples from Farm M (6 diarrhoeic, 1 healthy) and 8 diarrhoeic calf samples from Dumfries and Ayrshire which had tested positive for AstV and/or KoV were selected (Table 4).

Table 4: Sample collection locations, time points and naming system.

Virus detected	Collection location	Date received (by Roslin Institute)	Health status	Calf number
KoV	Dumfries/ Ayr	Dec 2012 - Jan 2013	Diarrhoeic	1
KoV	Dumfries/ Ayr	Dec 2012 - Jan 2013	Diarrhoeic	2
KoV	Dumfries/ Ayr	Dec 2012 - Jan 2013	Diarrhoeic	3
KoV	Dumfries/ Ayr	Dec 2012 - Jan 2013	Diarrhoeic	4
KoV	Dumfries/ Ayr	Dec 2012 - Jan 2013	Diarrhoeic	5
KoV	Dumfries/ Ayr	Dec 2012 - Jan 2013	Diarrhoeic	6
KoV	Dumfries/ Ayr	Dec 2012 - Jan 2013	Diarrhoeic	7
KoV	Dumfries/ Ayr	Dec 2012 - Jan 2013	Diarrhoeic	8
AstV	Farm M	Nov - Dec 2012	Healthy	HC 8
AstV	Farm M	Nov 2012	Diarrhoeic	Calf 2
AstV	Farm M	Nov 2012	Diarrhoeic	Calf 3
AstV	Farm M	Nov 2012	Diarrhoeic	Calf 4
AstV	Farm M	Nov - Dec 2012	Diarrhoeic	Case 2
AstV	Farm M	Nov - Dec 2012	Diarrhoeic	Case 3
AstV	Farm M	Nov - Dec 2012	Diarrhoeic	Case 5

2.2 RNA Extraction

In preparation for extraction, samples were thawed and pelleted by centrifugation at 13,000xg for 5 minutes allowing the formed pellet of waste debris material to then be removed. The RNeasy Mini Kit (Qiagen) was used to extract nucleic acids from 120µl of faecal supernatants using wash buffers RLT and RPE before elution in 30µl nuclease-free water.

2.3 Reverse Transcription

For the generation of cDNA, 11µl of RNA was added to a tube containing 1µl of random hexamer primers and 1µl nuclease-free water, before incubation at 65°C for 5 minutes. The product is then added to a mixture containing 4µl of 5X First-Strand Buffer; 1µl of 0.1M DTT; 1µl of RNaseOUT™ Recombinant RNase Inhibitor and 1µl of SuperScript™ III reverse transcriptase (Life Technologies), and incubated at 25°C for 5 minutes. Finally, the mixture was incubated for 30 minutes at 50°C and then 70°C for 15 minutes to inactivate the reaction.

2.4 PCR

During this project several DNA polymerases, reagent quantities and conditions were used to optimise PCR for the desired regions of the virus genomes. Details on the final optimal reagent quantities and conditions can be found in the results section. Below are the standard reagents and reaction conditions used before any optimisation.

GoTaq® (Promega)

For a 50µl reaction, it would contain the following: 10µl of 5x GoTaq® buffer, 1 µl of dNTPs, 1µl of 10µM forward primer, 1µl of 10µM reverse primer, 0.25µl of GoTaq® DNA polymerase, 2µl of cDNA template (5µl for 2nd round) and nuclease-free water up to a final quantity of 50µl. The conditions advised are: 94°C for 1 minute, 30 cycles of [94°C for 30 seconds, 42-65°C for 30 seconds, 68°C for 1 minute/Kb], 68°C for 5 minutes and hold at 4°C.

LongAmp® (New England Biolabs)

The LongAmp® 25µl reaction contains 5 µl of 5x LongAmp® *Taq* buffer, 0.75µl of 10mM dNTPs, 1µl of 10µM forward primer, 1µl of 10µM reverse primer, 1µl LongAmp® *Taq* DNA polymerase, 1µl of template cDNA and nuclease-free water to the total of 25µl. The conditions described are: 94°C for 1 minute, 35 cycles of [94°C for 30 seconds, 45-65°C for 1 minute, 65°C for 1 minute/kb], 65°C for 10 minutes and hold at 10°C.

SequalPrep™ Long (Life Technologies)

The SequalPrep™ Long reaction contains: 2µl of SequalPrep™ 10X buffer, 0.9µl of DMSO, 1µl of SequalPrep™ 10x Enhancer A, 0.36µl of SequalPrep™ Long Polymerase, 1µl of 10µM forward primer, 1µl of 10µM reverse primer, 1µl cDNA template and nuclease-free water up to 18µl. The conditions are the following: 94°C for 2 minutes, 9 cycles of [94°C for 10 seconds, primer Tm-5°C for 30 seconds, 68°C for 1 minute/kb], 29 cycles of [94°C for 10 seconds, primer Tm-5°C for 30 seconds, 68°C for 1 minute/Kb (+20 seconds/cycle)] and a final extension at 72°C for 5 minutes.

Herculase II Fusion (Agilent)

For a PCR target of 1-10Kb the following reaction is recommended: 10µl of 5x Herculase II buffer, 5µl of dNTPs, 1µl of 10µM forward primer, 1µl of 10µM reverse primer, 1µl of Herculase II fusion DNA polymerase, 1µl of cDNA template and nuclease-free water up to a total volume of 50µl. The conditions are: 94°C for 2 minutes, 35 cycles of [94°C for 18 seconds, primer Tm-5°C for 21 seconds, 72°C for 60 seconds/Kb] and a final extension at 72°C for 5 minutes.

Phusion® High-Fidelity (New England Biolabs)

For a 50µl reaction the following is recommended: 10µl of 5X Phusion® HF (or GC) Buffer, 1µl of 10mM dNTPs, 2.5µl of 10µM forward primer, 2.5µl of 10µM reverse primer, 0.5µl Phusion® DNA polymerase, 2µl of DNA template and nuclease-free water to the total of 50µl. The conditions are: 98°C for 30 seconds, 30 cycles of

[98°C for 8 seconds, 45-72°C for 25 seconds, 72°C for 1 minute per kb] and a final extension of 10 minutes at 72°C.

AccuPrime™ (Invitrogen)

A 50µl reaction contains the following: 5µl 10X AccuPrime™ PCR buffer I, 1µl of 10µM forward primer, 1µl of 10µM reverse primer, 0.2µl of AccuPrime™ Taq high Fidelity, 2µl of DNA template and nuclease-free water to the total volume of 50µl. The conditions are: 94°C for 2 minutes and 35 cycles of [94°C for 18 seconds, 52-64°C for 21 seconds, 72°C for 1 minute per kb] and a final extension of 5 minutes at 72°C.

2.5 Electrophoresis and gel extraction

PCR products were visualised on 2% agarose gels by electrophoresis. The gels were made using LE agarose powder (Promega) and TAE buffer, adding the DNA stain SYBERSAFE as a safer alternative to ethidium bromide. The products were loaded alongside GeneRuler DNA Ladder Mix (Thermo Scientific) and viewed under UV light using a UV-transillumination advanced imaging system. Where amplicons of appropriate size were seen with other products present, they were extracted and purified using QIAquick Gel Extraction kit (Qiagen) according to manufacturer's instructions. When just one amplicon was produced of the appropriate size, the PCR product was used directly for cloning and sequencing.

2.6 Sequencing

2.6.1 BigDye sequencing

Single amplicons from PCR reactions, on plasmid DNA were sequenced using the BigDye V 3.0 kit (Applied Biosystems™) and the same primers used to initially generate the PCR product. The sequence reaction contained 7.9µl nuclease-free water, 2µl 5x BigDye Buffer, 0.6µl primer (either forward or reverse primer used), 0.5µl BigDye and 1µl PCR product (1 in 10 dilution). The reaction took place under the following conditions: 20 cycles of 30 seconds at 96°C, 20 seconds at 50°C and 4

minutes at 60°C. On completion reactions were sent to the Edinburgh Genomics Sequencing Service.

For amplicons where end sequencing was insufficient for complete coverage, specific primers were designed (see Table 5) by primer walking. These primers were designed to contain a high GC content (between 50 and 65%) and annealing temperatures of <60°C.

Table 5: Primers used for internal sequencing of DNA plasmids (in addition to primers used for PCR).

Primer name	Orientation	Length (bp)	Sequence (5'-3')
BKoV			
1495AS	Antisense	21	RACCGTTAGCACCGAGATCGG
1887s	Sense	20	GGCTCCTGGACACTTTCGAG
2111s	Sense	22	GTGCAGGCGTATCCAGACTGCC
2878s	Sense	21	GCCTGGTCATATGCTATTCAC
2878s	Sense	21	GCCTGGTCATATGCTATTCAC
2908AS	Antisense	26	CAGGGTGTGYCAYATCCAYGGCGCRT
2883s	Sense	26	TRCGCGGYACCTAYACYGTGTGGGAC
4082s	Sense	24	GCCCARTGGRTGTASGTCATGCGC
4199s	Sense	21	GGTCTACCRAGARGTAGAACC
4720s	Sense	20	GGCTTCGCACTACTTCACCG
480s	Sense	22	CCAGTGTGCGCAACAGTATGTT
4898AS	Antisense	20	TCTCTCGGTGAAGTAGTGCG
4983s	Sense	23	ATGGCAGCAGCCTCAGCCTCCTC
4997s	Sense	24	ACACCGTCGTTGAAATCACGCACG
7093-7112s	Sense	20	CCTACCGCACCTGTACTTGG
7154-7175s	Sense	22	GCGAGTAACAAGTAACTTCTCC
7581-7562AS	Antisense	20	GGTAGGCTTGCATGTACTCG
7628-7609AS	Antisense	20	CCAGTGGAGGTCTGGGTTGC
7918s	Sense	23	GGAGTTCGCTACCAAACAAGGCC
8199AS	Antisense	22	CAGCCACATGACGGACTGCTCG
8199as	Antisense	22	CAGCCACATGACGGACTGCTCG
8270s	Sense	22	GCGACCACGCTTGCACAAGCAC
Calf 1 6916s	Sense	19	GCGTGATGCTCGTCTCGAG
Calf 1 6917s	Sense	20	CGAGCAGAGCTCGGACCTTG

Calf 1 6941s	Sense	20	GACCTACTGGTTGAGAAGGA
Calf 1 6978s	Sense	20	GCTTAAGGGCGCCGGCGGTA
Calf 1 7978s	Sense	20	CCACGCTAGGAGGTTAGGTG
Calf 7 1150s	Sense	22	CTGGTGTGACATCCTTGACGAG
Calf 7 1589s	Sense	20	CTTGGACGACGAAGCTTCCG
Calf 7 7509AS	Antisense	20	CTGACTCGTGTGGTTGAGGC
Calf 7 7512AS	Antisense	20	CAGCCTCAACCACACGAGTC
Calf 8 3392s	Sense	20	CCACTGTGGGACGGAGAGGA
Calf 8 4720s	Sense	20	GGCTTCGCACTACTTCACCG
Healthy calf 8 4666s	Sense	20	CGCATGGCACGCGCTGAAAC
BAstV			
4334s	Sense	20	GGCGGGACCATCTAAATCCG
4712s	Sense	20	GGATGGTGACAGACACGGTC
4898s	Sense	24	CATCCACCTCCTTTCAGGAAGTTG
5590AS	Antisense	22	TGAAGCTATGCTCAGTCTCAGC
BovAstCapsid 1-2S	Sense	23	GTGTATTCATCTATAAATGACGC
BovAstCapsid 2-2S	Sense	23	GTGTATTCATCTATAAATGACGC
BovAstCapsid 3-2S	Sense	23	CATCAATAAATGATGCACGTGCC
BovAstCapsid 4-2S	Sense	27	CTTTGATGTGTTCCCCTCCATGTCAGA

2.6.2 Sequence Analysis

Returned sequences were analysed using NCBI BLAST to check nucleotide identity to other sequences of the virus in the database, with high similarity sequences imported into an alignment using the SSE program (Peter Simmonds, 2013). This alignment program enables the alignment, annotation, classification and direct analysis of sequences using a number of built-in bioinformatic programs. For this project the program was mainly used to import sequences from different resources for designing primers; assembling large fragments from single sequences and carrying out analysis between both groups and individual sequences. NEB Cutter V2.0 was used to identify restriction sites in sequences to enable the selection of restriction enzymes for assembly and cloning into different vectors. MEGA6 was

used to align imported sequences from GenBank and those generated from this study to carry out phylogenetic analysis.

2.7 Molecular Cloning

2.7.1 Ligation

For PCR products produced using Go*Taq*® (Promega), LongAmp® (New England BioLabs), SequalPrep™Long (Thermo Scientific) or AccuPrime™ (New England Biolabs) DNA polymerases, the pGEM®-T Easy Vector kit (Promega) was used for ligation and transformation. These ligation reactions contained 1µl nuclease-free water, 5µl 2x ligation buffer, 1µl pGEM®-T Easy Vector, 1µl T4 DNA ligase and 2µl PCR product. They were incubated at 4°C for between 24 and 72 hours. PCR products generated using Herculase II Fusion (Agilent) were ligated and transformed using the Zero Blunt® TOPO® PCR cloning kit. The ligation reaction comprised of 2µl blunt-ended PCR product, 0.5µl salt solution and 0.5µl pCR®Blunt II-TOPO® vector, which was then incubated for 10 minutes at room temperature.

For the ligation of DNA fragments in the FastBac vector, the reagents included were: 7µl of purified product, 1µl of FastBac vector, 1µl of 10x T4 ligase buffer and 1µl T4 ligase. This reaction was then incubated at 16°C overnight, to provide sufficient time for ligation of the fragment into the vector.

2.7.2 Transformation

Transformation of both the pGEM®-T Easy Vector ligation and pCR®Blunt II-TOPO® Vector were carried out by adding 2µl of the ligation reaction to 50µl thawed DH5α™ Competent Cells (Invitrogen) on ice. Following an incubation of 20 minutes, the reaction was heat-shocked in a water bath at 42°C for 30 seconds before being returned to the ice to cool for 2 minutes. SOB medium was then added to the tube to a total volume of 1000µl, before incubation at 37°C for 1 hour with horizontal shaking. The cells were plated out with 100µl or 200µl per plate and incubated at 37°C overnight. The LB plates for the pGEM®-T Easy Vector ligations contained ampicillin (100µg/ml), IPTG (100mM) and X-Gal (50mg/ml), while pCR®Blunt II-TOPO® Vector ligation plates contained kanamycin (30µg/ml).

2.7.3 Colony PCR

A colony PCR is used to check the fragment has been ligated successfully into the plasmid. M13 primer sites are located either side of the insert region in both pGEM®-T Easy pCR®Blunt II-TOPO® vectors. For the ampicillin plates only white (as opposed to blue) colonies were selected for PCR, while all colonies on the kanamycin plates were available for PCR. Up to 8 colonies were picked from each transformation and added to 15µl LB in PCR strip tubes for storage at 4°C. Specific primers for the M13F (5'-GTA AAA CGA CGG CCA G-3') and M13R (5'-CAG GAA ACA GCT ATG AC-3') sites located either side of the cloning site of pGEM®-T Easy or pCR®Blunt II-TOPO® vectors were used for PCR. The PCR master mix included 18.6µl nuclease-free water, 0.5µl dNTPs, 5µl 5x GoTaq® Buffer, 0.15µl M13F, 0.15µl M13R, 0.1µl GoTaq®, and 1µl of colony LB solution. Conditions for the PCR were 95°C for 2 minutes, 30 cycles of [95°C for 30 seconds; 50°C for 30 seconds and 72°C for 3 minutes (1kb= 1 minute)] and a final extension of 72°C for 5 minutes. The PCR products were visualised on a 2% agarose gel, extracted and purified as previously described (2.5).

2.7.4 Plasmid DNA extraction

Extraction of plasmid DNA from the bacterial cells was completed using QIAprep Miniprep kit (Qiagen) according to manufacturer's instructions. Briefly, 1µl of colony LB solution (stored at 4°C) was added to 5ml of LB and incubated at 37°C in a shaking incubator for 16 hours. The culture was pelleted by centrifugation at 4000rpm for 8 minutes and the supernatant removed, before being re-suspended in Buffer P1. This solution was then transferred to a 1.5ml tube where Buffer P2 was added and mixed by inversion, followed by Buffer N3 in the same way. Particulate material was pelleted by centrifugation for 10 minutes and the supernatant carefully extracted and applied to QIAprep spin columns. Following centrifugation and washing with Buffers PB and PB, DNA was eluted using 10µl Buffer EB and collected in a sterile 1.5ml tube.

2.8 Rapid amplification of 5' and 3' ends

The GeneRacer™ Kit (Invitrogen) was used to amplify the 5' and 3' ends of the KoV genome, according to manufacturer's instructions. The method involved the generation of cDNA with an attached GeneRacer™ dT oligo which was consequently used for PCR with the 5' GeneRacer™ primer and a pre-designed sequence-specific antisense primer. To produce the cDNA, total RNA extracted from the sample undergoes stages of dephosphorylation; decapping of mRNA; ligation of the GeneRacer™ oligo; and reverse transcription with phenol extractions and ethanol precipitations at each stage. The kit is designed to produce a single product which can be visualised and extracted from an agarose gel before cloning into pCR® 4-TOPO or pCR® 4Blunt-TOPO.

2.9 Restriction digest and ligation into FastBac vector

Restriction digests contained the following reagents to total a 20µl reaction: 2µl of buffer (3.1 or CutSmart®), 0.5 µl of 5' restriction enzyme, 0.5 µl of 3' restriction enzyme, 2µl plasmid and 15µl of nuclease-free water. For the digestion of FastBac vector, 1µl of Antarctic phosphatase was added to 14µl of nuclease-free water, and other reagents were added as described. The reactions were incubated at varying temperatures (according to the restriction enzyme used) for 2 hours. Products were extracted and purified from a 2% agarose gel as previously stated in 2.5.

2.10 DNA Assembly

The NEBuilder® HiFi DNA Assembly Cloning Kit (NEB) was used to assemble DNA fragments in a ligation reaction. The assembly reaction required the following reagents: 5µl of DNA product; 10µl of master mix and 5µl of nuclease-free water. For the assembly of 4-6 DNA fragments, the reactions were incubated at 50°C for 60 minutes. An appropriate PCR would be used to check the success of each assembly reaction.

3. Kobuvirus

The primary aims for the KoV section of the project was to sequence the full bovine KoV genome from a diarrhoeic calf, in order to assemble the genome for future virus challenge studies.

3.1 Results

3.1.1 Bovine kobuvirus PCR optimisation

3.1.1.1 Sample selection and PanKobu Screening

Previous work identified KoV in the faeces of calves with clinical enteritis and diarrhoea (Gregory *et al.* 2015, manuscript submitted). The Pan-KoV primers that were used in this surveillance study were designed from a selection of GenBank human, bovine, porcine, ovine and murine sequences. In order to better characterise the strain of bovine KoV present, the faecal samples from diarrhoeic calves were used again to amplify the full genome of the virus.

The cDNA reverse transcribed from these samples was carried out with two separate primers: one with random hexamers and the other with 3' GeneRacer (GR) primer. The random hexamers produced N6 cDNA by random binding throughout the genome, while 3' GeneRacer primer produced GR cDNA which includes the polyA and some sequence prior to it on the 3' strand. The N6 cDNA was used to generate the first fragment, and GR cDNA was used to amplify the other three fragments.

3.1.1.2 Bovine KoV PCRs targeting specific genome fragments

A combination of bovine, porcine and/or ovine full genome sequences (GenBank numbers: NC_004421.1; NC_011829.1; KC204684.1; GU292559.1; KJ452348.1; KF539763.1; KC424640.1; KC424639.1; KC424638.1; JX177612.1; NC_016769.1; JQ692069.1; JN630514.1; GQ249161.1; KF695124.1; GU245693.2) were used to design primer sets (sense, S and antisense, AS) for amplification of the full bovine KoV genome (Figures 10 and 11. Four PCRs were designed to cover the genome (excluding the 5' end) comprising of 57-1898 (57S and 2908AS hemi-nested with 57S and 1898AS); 1577-5260 (1577S and 5260AS), 4695-6714 (2883S and GR

nested with 4695S and 6714AS) and 6638-GR nest (6638S and GR hemi-nested with 6638S and GR nest).

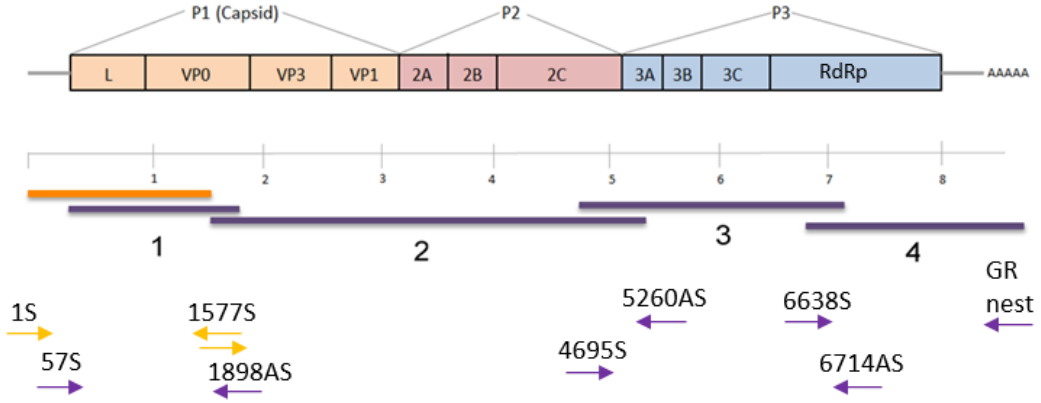

Figure 10: The four regions PCR primers were designed to amplify, spanning the length of the genome (Region 1: 57-1898; region 2: 1577-5260; region 3: 4695-6714 and region 4: 6638-GR nest). The orange band represents the 1-1597 PCR that failed to amplify the appropriate product.

Figure 11: The primers used to amplify the KoV genome, as designed from bovine, ovine and porcine GenBank sequences. The 1597AS primer is the antisense of the 1577S primer, and so was not included.

When designing primers the criteria below were followed, where possible (advised by Life Technologies):

1. The length of between 18-25 nucleotides.
2. Annealing temperature of primer pairs to be 50-55°C.
3. GC content was between 50-65% with 3 GC in the first and last five nucleotides.
4. Avoid repeats of 4 or more bases.

Originally, LongAmp polymerase was used in the reactions targeting the four fragments, however, proof-reading taq polymerases (Herculase II, AccuPrime and Phusion) were used in later PCRs. SequalPrep is a non-proof-reading taq polymerase, and was the second option for the initial PCRs if LongAmp was not working efficiently, for example in the generation of the 6638-GR nest fragment.

3.1.1.3 Fragment 1-1597

This PCR was designed to specifically include the 5' end of the genome in the resulting fragment. The reagent and conditions information can be found in Table 14 (Appendix).

Initially, the PCRs produced three faint products of the appropriate size (1.6kb), as shown in Figure 12B which were extracted, purified and cloned (methods 2.5 and 2.7). The consequent colony screen for these products (Figure 13) produced numerous amplicons for each colony with the faint products at 1.8kb being the appropriate size and more unexpected products at 950bp. This colony screen was repeated (Figure 14) as the appropriately sized amplicons were much fainter than the other product. The amplicons in lanes 11 and 12 of Figure 13 were the expected size, however, following extraction, purification and sequencing of the amplicons they were found to be bacterial contamination and so were not included in the SSE alignment. As this PCR was unsuccessful new primers were designed to target the 57-1898 region.

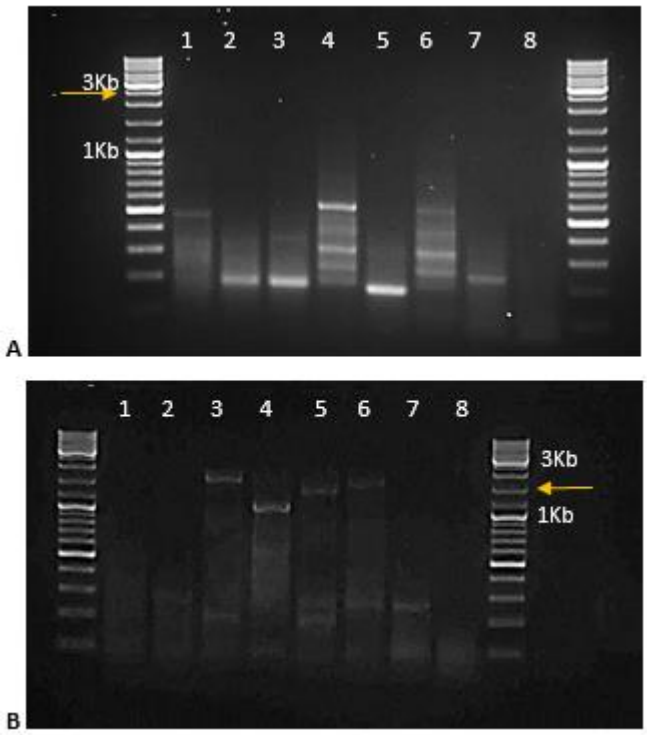

Figure 12: *A* 1-1597 PCR 1st round and *B* nested 2nd round. Lanes 1-8 are calves 1-8, respectively. Products in lanes 3, 5 and 6 in *B* were extracted and purified for cloning.

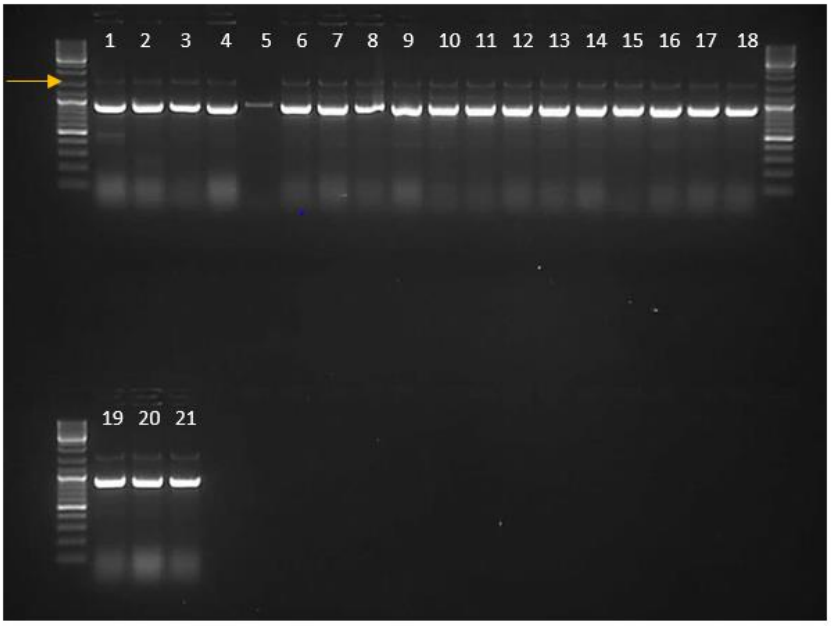

Figure 13: Colony Screen for 3 cloned amplicons described in Figure 12, with lanes 1-7 representing amplicon 3, 8-14 representing amplicon 5 and 15-21 representing amplicon 6.

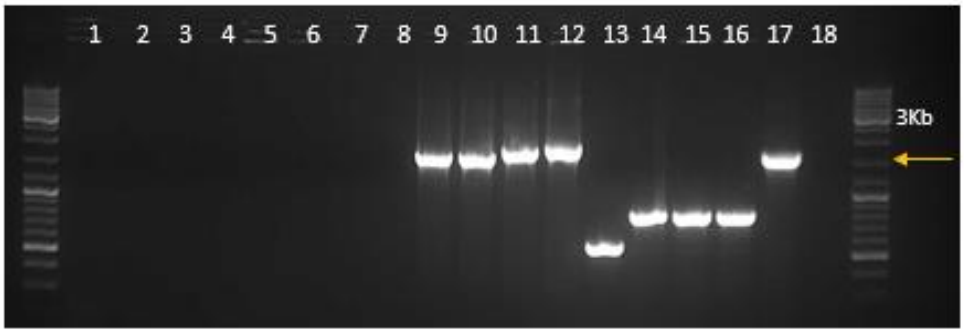

Figure 14: Colony screen repeat of Figure 13. Lanes 1-3 are from product 3, 4-11 are product 5 and 12 and 17 are product 6.

3.1.1.4 Fragments 57-1898, 1577-5260 and 4965-6714

From the primers that were designed, the following PCRs were carried out to obtain several clones for each fragment spanning the genome. Following the unsuccessful 1-1597 PCR the 57-1898 PCR was designed to use in its place. For these three segments of the genome, LongAmp PCR reactions were performed with the reagent quantities and conditions in Table 15 (Appendix).

Reactions with cDNA template from the faeces of calves 1, 7 and 8 produced amplicons of appropriate size (Figures 15 - 17). The calf 1 reaction consistently produced an intense amplicon for each of the three PCRs whereas calf 7 produced weaker amplicons for the 1577-5260 and 4695-6714 PCRs, and calf 8 produced a weaker amplicon for the 57-1898 PCR. The products for the 3 calves were extracted, ligated into pGEM®-T Easy Vector and transformed into DH5-α cells (methods 2.7). Following the colony screen, colonies were selected for plasmid extraction to produce three plasmid clones for each calf (methods 2.7.4).

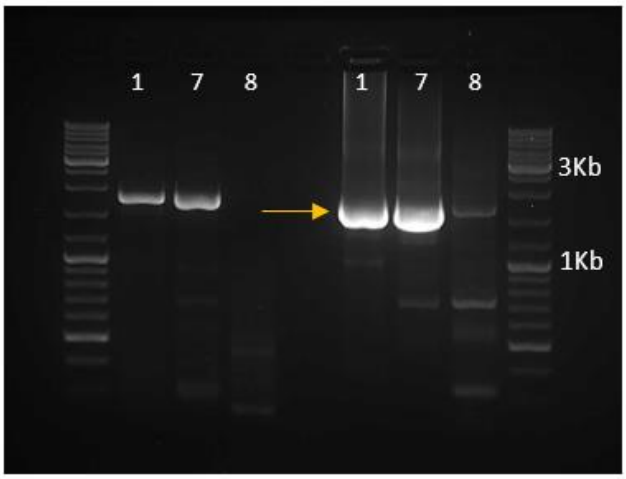

Figure 15: 57-1898 PCR showing the 1st and 2nd rounds for calves 1, 7 and 8. The arrow indicates strong positive amplicons for calves 1 and 7, with a weak product for calf 8. The 2nd round amplicon for calf 8 was not extracted.

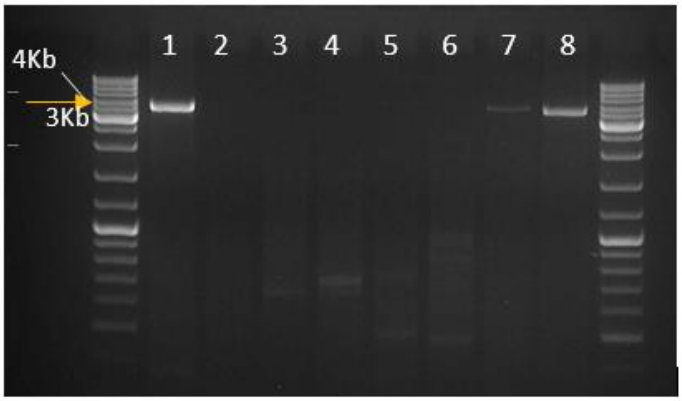

Figure 16: 1577-5260 PCR for calves 1-8 with the arrow indicating the appropriate size product for calves 1, 7 and 8 all of which were extracted, purified and sequenced.

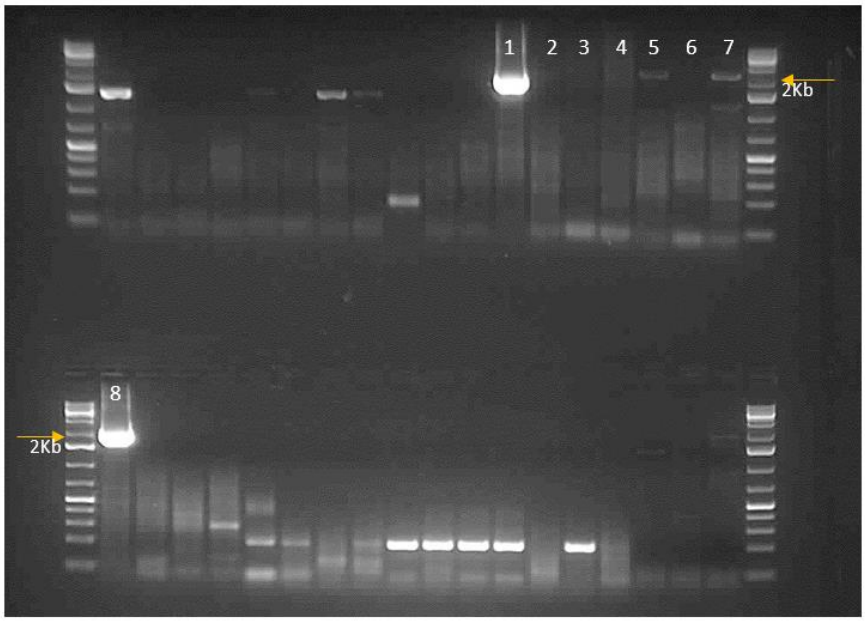

Figure 17: 4695-6714 PCR 2nd round for calves 1-8 (indicated by lanes 1-8). The arrow represents the appropriate size product of 2.3kb. The remaining lanes on the gel are unrelated samples.

3.1.1.5 Fragment 6638-GR nest

The 6638-GR nest fragment was attempted with LongAmp taq polymerase, along with the other three fragments, however, a product of appropriate size could not be detected. The PCR was repeated using SequalPrep taq polymerase with the reagents and conditions found in Table 16 (Appendix), and an amplicon of appropriate size was produced and cloned (Figure 18).

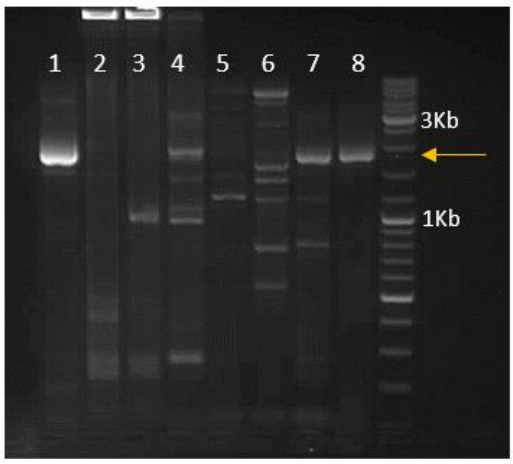

Figure 18: 6638-GR nest PCR with the arrow indicating expected size products for calves 1, 7 and 8. These products were extracted, purified, cloned and sequenced. The product for calf 4 was not investigated further as the other PCRs were unsuccessful with this calf.

A review of the cloned amplicons is presented in Table 6. Given the success of calf 1 cDNA in producing all four fragments, these PCR products were used to create the full genome, and a selection of the PCRs repeated using a proof-reading taq polymerase.

Table 6: The number of clones produced for the calves 1, 7 and 8 for each genome fragment, following the first PCRs.

	Clones			
Calf	57-1898	1577-5260	4695-6714	6638-GR nest
1	1.1, 1.2, 1.3	1.1, 1.2, 1.3	1.1, 1.2, 1.3	1.1, 1.2, 1.3
7	7.1, 7.2, 7.3	cloning unsuccessful	PCR unsuccessful	7.1, 7.2, 7.3
8	PCR unsuccessful	8.1	PCR unsuccessful	8.1, 8.2, 8.3

3.1.1.6 5' end amplification

The final part of the BKoV genome to amplify was the 1-50bp region, following the difficulty optimising the 1-1597 PCR previously mentioned. The GeneRacer™ Kit (Invitrogen) was used to target this region with primers designed from the U-1 BKoV and calf 1 57-1898 clone sequences. The 391AS and 156AS primers were used in a hemi-nested PCR with the 5' end primer included in the kit, the result of which can be seen in Figure 19. From the gel image it is apparent this method was unable to amplify this section.

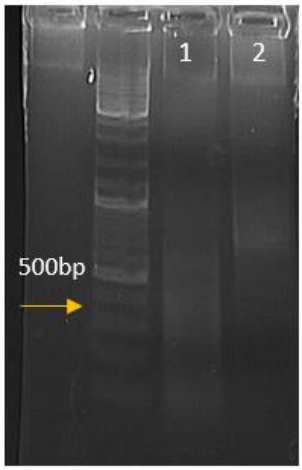

Figure 19: 3' nested PCR with 391AS and 3' (1st round) and 3' nest and 156AS (2nd round). Lane 1 is the 1st round reaction and lane 2 is the 2nd round. Approximate amplicon size was 300bp (1st) and 150bp (2nd).

3.1.1.7 Proof-reading PCRs

In order to confirm the accuracy of amplicons generated using LongAmp and SequalPrep taqs, Herculase II taq polymerase was used to repeat the same four fragment PCRs for calf 1. As shown in Table 7, Herculase II taq polymerase has a 10x higher fidelity than GoTaq, therefore the sequences from these clones would be more representative of the KoV present in the sample.

Table 7: Comparison of the fidelities of taq polymerases used for generating KoV genome fragments. *compared to GoTaq (1x10^{-5} errors/base). **with Enhancer A.

Taq polymerase	Fidelity*
LongAmp	2x
SequalPrep Long	3.6x**
Herculase II	10x

Herculase PCRs were performed with the same primers in the first and second rounds and with the same reagents/quantities as in the LongAmp PCRs previously described (Table 8). The conditions can be found in the Appendix.

Table 8: Details of the Herculase PCRs showing variations in conditions and starting cDNA.

Lane	Fragment	Annealing temperature	Extension time	cDNA template	Produce size
1	57-1898	50°C	2 min	N6	1.9Kb
2	4695-6714	50°C	3 min	GR	2.3Kb
3	6638-GR nest	55°C	2 min	GR	1.9Kb
4	1577-5260	55°C	4 min	N6	3.8Kb
5	4695-6714	55°C	3 min	GR	2.3Kb

From the amplicons shown in Figure 20, the amplicons in lane 1, 2 and 4 indicated by the arrows were extracted, purified, cloned and sequenced (methods 2.5 and 2.7). The 1577-5260 sequencing reaction failed to produce any sequence and therefore was not taken any further. As the calf 1 57-1898 and 4695-6714 fragments were able to be generated using Herculase II taq polymerase, these same fragments for calves 7 and 8 were targeted with the same reagents and conditions including the 50°C annealing temperature (Table 17 in the Appendix). The calf 8 57-1898 and calf 7 4695-6714 fragment PCRs are shown in Figure 21. The amplicons indicated by the arrows were extracted, ligated into pCR®Blunt II-TOPO® vector and transformed into DH5-α cells. After incubation of the plates, the calf 7 4695-6714 transformation failed to produce any colonies. The successful Herculase PCRs were added to clones previously collected (Table 6) to make the final collection of clones from calves 1, 7 and 8 (Table 9).

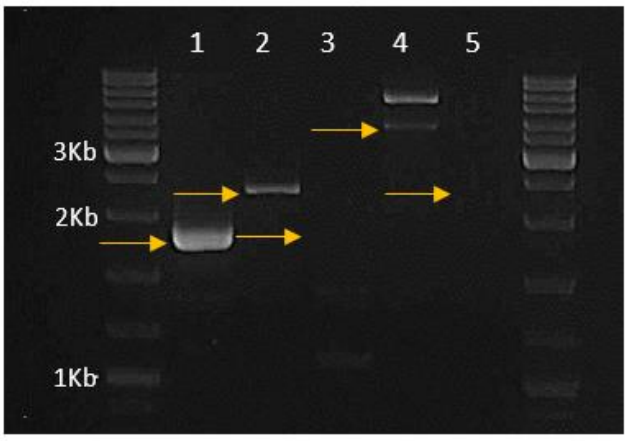

Figure 20: Herculase PCR for each fragment of the KoV genome.

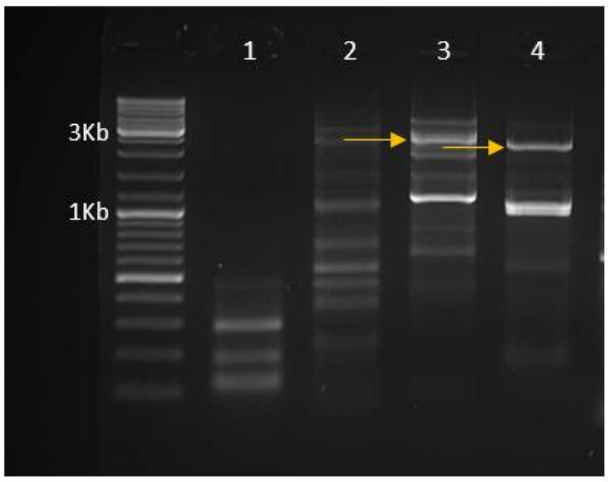

Figure 21: Herculase Calf 7 4695-6714 1st and 2nd PCRs and Calf 8 57-1898 1st and 2nd PCRs. Lanes 1 and 2 are 1st rounds and lanes 3 and 4 are 2nd round reactions. The amplicons indicated by the arrows were extracted, purified and sent for sequencing, although they were both negative for KoVs.

Table 9: Final collection of clones produced from PCRs, with the addition of the Herculase PCRs (indicated in red).

	Clones			
Calf	57-1898	1577-5260	4695-6714	6638-GR nest
1	**H1**, 1.1, 1.2, 1.3	1.1, 1.2, 1.3	**H1**, 1.1, 1.2, 1.3	1.1, 1.2, 1.3
7	7.1, 7.2, 7.3	-	-	7.1, 7.2, 7.3
8	-	8.1	-	8.1, 8.2, 8.3

The calf 1 genome comprised of four fragments spanning from nucleotide 57 on the U-1 KoV genome to the 3' polyA, with overlaps of 300-600bp to ensure one complete genome is assembled without combining separate KoVs from mixed infections. The next steps for these clones was the assembly of an infectious clone and to compare the sequenced genome to the U-1 strain and other mammalian KoVs.

3.1.2 Genome assembly and comparison of VP1 regions

3.1.2.1 Sequence assembly

Following the generation of cloned fragments of calf 1, 7 and 8 genomes, the SSE alignment was used to assemble the sequence reads of each fragment obtained through primer walking. Primer sites were removed and complete fragments were aligned, where possible, observing the main regions of diversity between the same fragment for each calf (e.g. 57-1898). This alignment revealed diversity in the overlapping regions between fragment sequences, for example with the 57-1898 and 1577-5260 overlap in Figure 22. The calf 7 57-1898 fragment had a 100% identity to the calf 1 1577-5260 fragment in the overlapping region and the Herculase 57-1898 fragment. For this reason the Herculase 57-1898 fragment was selected for the KoV genome in place of the more diverse calf 1 57-1898 fragment. The remaining fragments from calf 1 were used, including the Herculase 4695-6714 fragment instead of the LongAmp fragments for this region. The plasmids used for the genome were as follows: Herculase calf 1 57-1898; LongAmp calf 1 1577-5260 1.2; Herculase calf 1 4695-6714; and LongAmp calf 1 6638-GR nest 1.3.

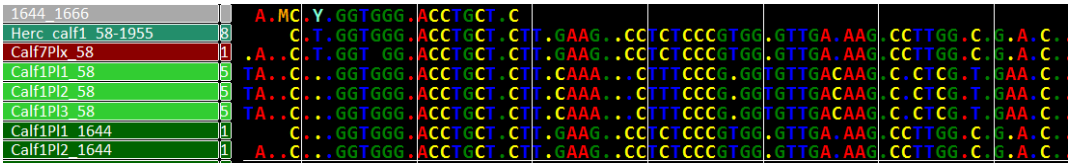

Figure 22: SSE alignment comparing the calf 1 and 7 57-1898 sequences with the calf 1 1577-5260.

The selected fragments for the complete genome were assembled using the same method as for the assembly of the sequence reads to form each fragment. Where sequence errors occurred between the overlapping sequence reads of a fragment, the most common base between the reads was selected. The completed genome was named BKoV_UK_SC1 and submitted to GenBank (GenBank number: KT003671) and will appear in the Gregory *et al.* 2015 paper (manuscript submitted). Completion of the genome enabled its comparison with the U-1 strain and other Aichivirus B KoVs via a maximum likelihood phylogenetic recombination tree calculated using the MEGA6 program (Figure 23).

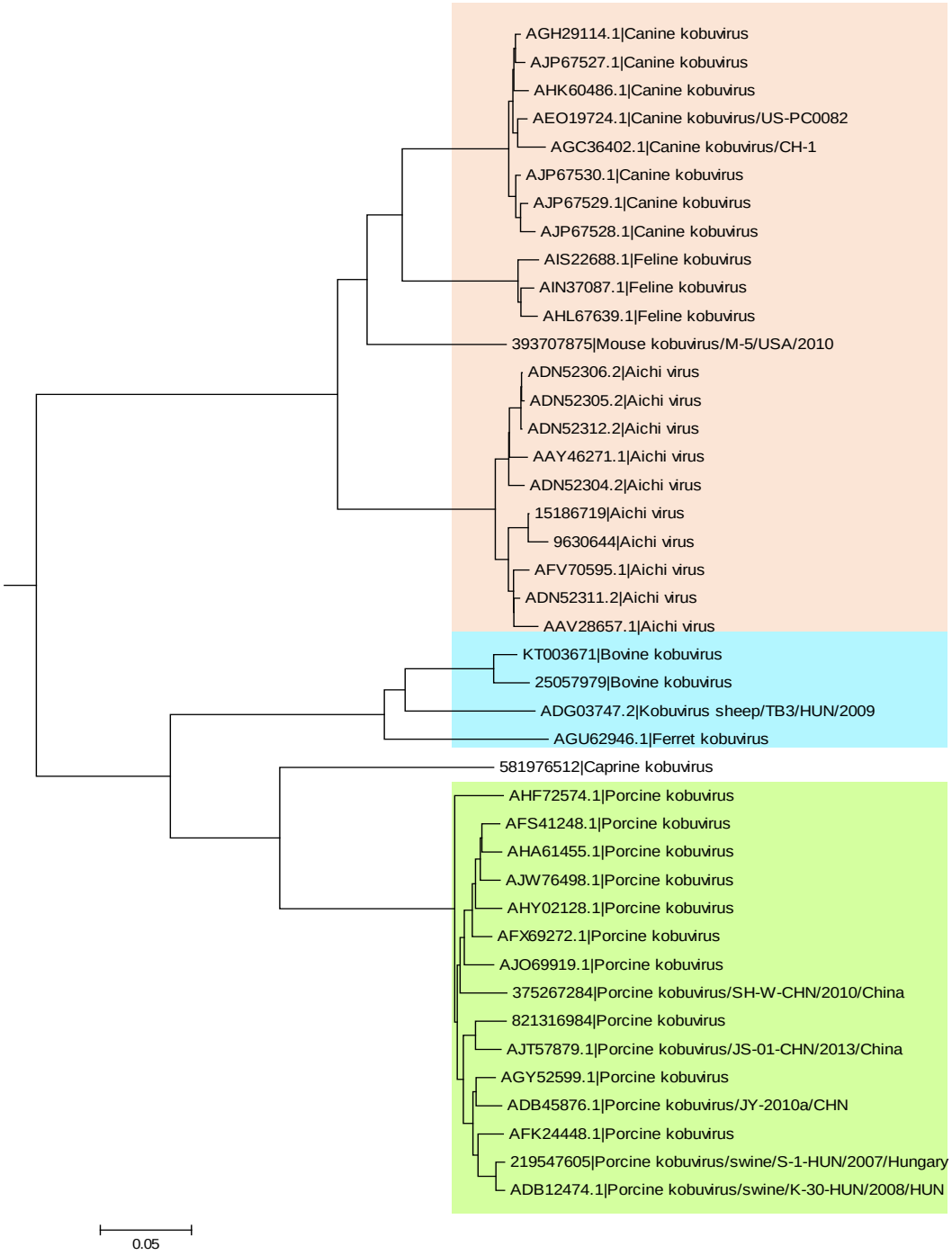

Figure 23: Phylogenetic relationship of BKoV_UK_SC1 (KT003671) compared to other known KoVs. The coloured boxes separate the genomes into their Aichivirus groups: red is Aichivirus A; blue is Aichivirus B; green is Aichivirus C. Caprine KoV has not yet been classified, although it was found to share an amino acid identity of 75.9% with porcine KoV genomes (Oem *et al.* 2014).

3.1.2.2 Pairwise distance analyses

Pairwise distance analyses were performed using the SSE alignment program and numerous sequences from the GenBank database. The comparison graph in Figure 24 represents the amino acid difference between the bovine KoV (NC_004421.1; KT003671) and a selection of porcine KoV (JX401523.1; JX177612.1; NC_011829.1; NC_016769.1; JQ692069.1; JN630514.1) and Aichi virus (NC_001918.1; GQ927711.2; GQ927705.2; GQ927712.2; GQ927706.2; GQ927704.2) genomes. Porcine and bovine KoVs have a higher amino acid similarity to each other than either do to Aichi virus. It is important to note that although the Vero cell culture the U-1 strain was isolated from contained fetal bovine serum, this KoV has been presumed to be of bovine origin.

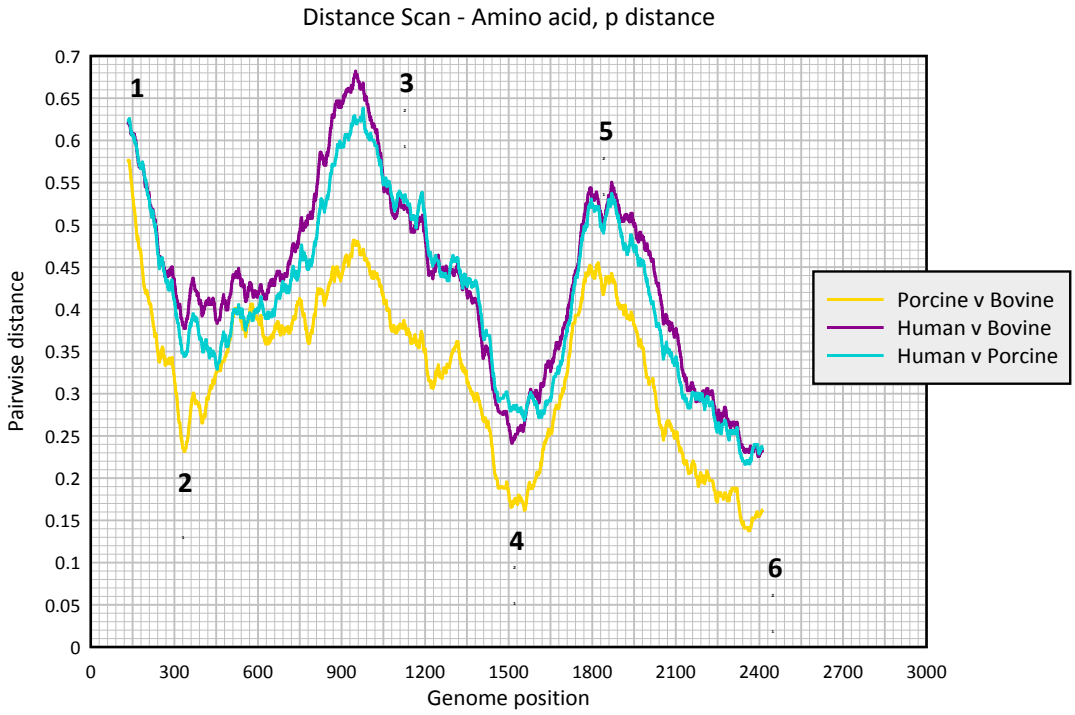

Figure 24: Pairwise distance comparison of bovine KoV, porcine KoV and Aichi virus human genomes according to amino acid sequences.

The same genome sequences used in Figure 24 were also used to produce the pairwise distance graph in Figure 25, directly comparing the BKoV_UK_SC1 strain in this study to the U-1 strain, the porcine KoVs and the Aichi viruses previously listed. It is clear from this group there is a high amino acid similarity between the

BKoV_UK_SC1 and U-1 strain, compared to the other porcine and human sequences.

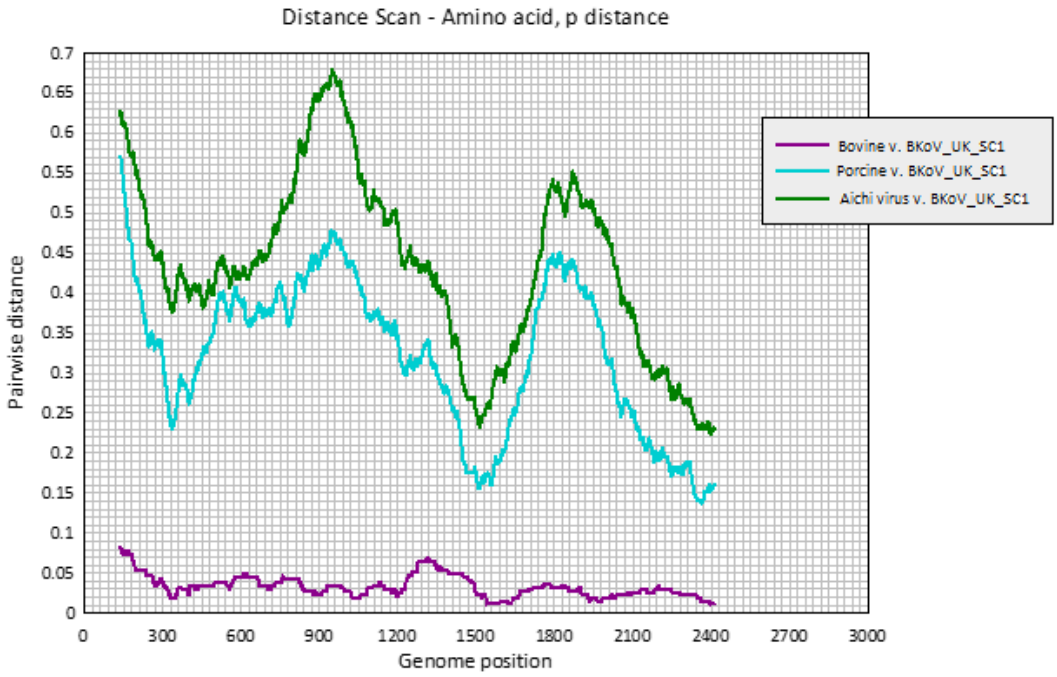

Figure 25: Pairwise distance comparison between U-1 strain and BKoV_UK_SC1; porcine KoVs and BKoV_UK_SC1; BKoV_UK_SC1 and Aichi viruses, according to amino acid sequences.

3.1.2.3 Assembly of the bovine KoV genome

When preparing the plasmids selected to assemble the complete genome, the Herculase calf 1 57-1898 and 4695-6714 were unable to be recovered in sufficient quantity. In their place the LongAmp calf 7 57-1898 plasmid 7.3 and LongAmp calf 1 4695-6714 plasmid 1.1 were used as these clones shared an identical sequence with the Herculase fragments. The NEBuilder® HiFi DNA Assembly Cloning Kit (NEB) suggested small overlaps between 15 and 80bp were most effective with this kit, therefore the NEB cutter tool V2.0 (NEB) tool was used to identify restriction sites on the 5' and 3' ends of each fragment with the aim of reducing the overlap size to between the recommended range. The restriction enzymes selected for each fragment are shown in Table 10.

Table 10: The restriction enzymes selected for each genome fragment with the relevant buffer, incubation temperature and expected product sizes (with the appropriately cut product in bold).

Genome fragment	Restriction enzymes	Buffer	Incubation temperature	Product sizes (Kb)
57-1898	XbaI GQ/ BciVI	CutSmart®	37°C	0.6, 0.9, 1.0, 1.2, **1.7**
1577-5260	SfcI/ AhdI	CutSmart®	37°C	<1, 1.2, **3.2**
4695-6714	BsrDI/ BsmBI	3.1	55-65°C (1 hour at each)	1.2, **1.9**, 2.6 or 1.5, **1.9**, 2.3
6638-GR nest	AleI/ EcoRI (-HF)	CutSmart®	37°C	0.3, **1.5**, 3.0

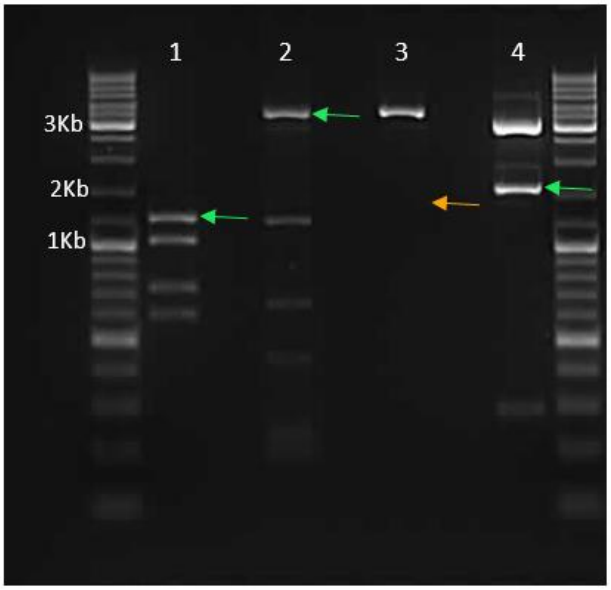

Figure 26: Bovine KoV fragments restriction digest. Lane 1: 57-1898, lane 2: 1577-5260, lane 3: 4695-6714 and lane 4: 6638-GR nest. Amplicons indicated by green arrows were extracted and purified. The orange arrow represents the expected size for the 4695-6714 fragment.

The restriction digest of the four fragments was carried out (methods 2.9), and produced the amplicons visualised in Figure 26. The appropriate amplicons were produced for the 57-1898, 1577-5260 and 6638-GR nest fragments, however, the 4695-6714 fragment didn't appear to have been cut on this gel. This fragment digest was repeated with the 1.2 plasmid, which then produced the correct sized amplicon. The extracted products were purified and taken into the NEBuilder® HiFi DNA Assembly Cloning Kit. (methods 2.10).

The assembly reactions included a mix containing each of the following plasmids: 1 and 2; 2 and 3; 3 and 4; 1, 2, 3 and 4. These assembly reactions were used as the template for the PCRs in Figure 27. The arrows for lanes 1-4 indicate the expected sizes for the amplicons, therefore only the 1-2 and 2-3 assembly mixes were successful. Lanes 5-8 show the same PCR primers and conditions as lanes 1-4 but with the 1, 2, 3, 4 assembly mix. As the same result was gained by both starting templates, this suggests there was something wrong with the overlap between fragments 3 and 4, rather than there being a PCR error or fault with the starting assembly mix. There was a faint product at approximately 500bp in lane 4. As this amplicon should have been 400bp and the product's intensity is poor no further action was taken.

Lane	Assembly	Primers
1	1/2/3/4	57-GR
2	1/2	1577-1898
3	2/3	4965-5260
4	3/4	6638-6714
5	1/2/3/4	57-GR nest
6	1/2/3/4	1577-1898
7	1/2/3/4	4965-5260
8	1/2/3/4	6638-6714

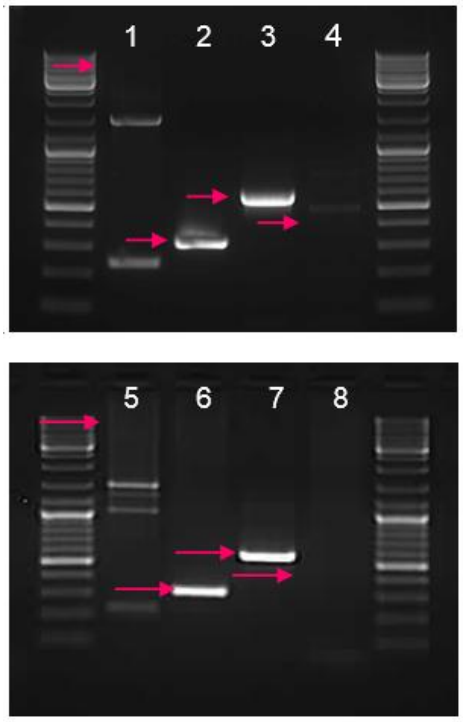

Figure 27: PCRs testing the success of the assembly reaction, using the primers in the table to amplify the overlapping regions of the assembly mixes. The pink arrows indicate the appropriate size products. Amplicons of the appropriate size were extracted from lanes 2, 3, 6 and 7.

3.1.2.4 VP1 protein amplification

In total, 9 VP1 (3650-3940) sequences were generated from diarrhoeic calves on farms in Ayrshire and Dumfries. These included calves 1 and 8 that have been previously mentioned, along with 7 others from the other farms in South West Scotland. These were compared to the VP1s of the U-1 strain (NC_004421.1), other bovine KoVs (from the Chang *et al.* 2014 study)(KF728711.1; KF728712.1; KF728713.1; KF728714.1; KF728715.1; KF728715.1; KF728716.1; KF728717.1; KF728718.1; KF728719.1; KF728720.1; KF728721.1; KF728722.1; KF728723.1; KF728724.1; KF728725.1; KF728726.1; KF728727.1) and porcine KoVs (JX401523.1; JX177612.1; NC_011829.1; NC_016769.1; JQ692069.1; JN630514.1) (Figure 28). The maximum likelihood tree shows the expected separation of bovine KoVs from the other kobuvirus species. The bovine KoVs from the Chang *et al.* study can be divided into 2 lineages, with an additional VP1 forming a separate lineage with the U-1 strain and VP1s from this study.

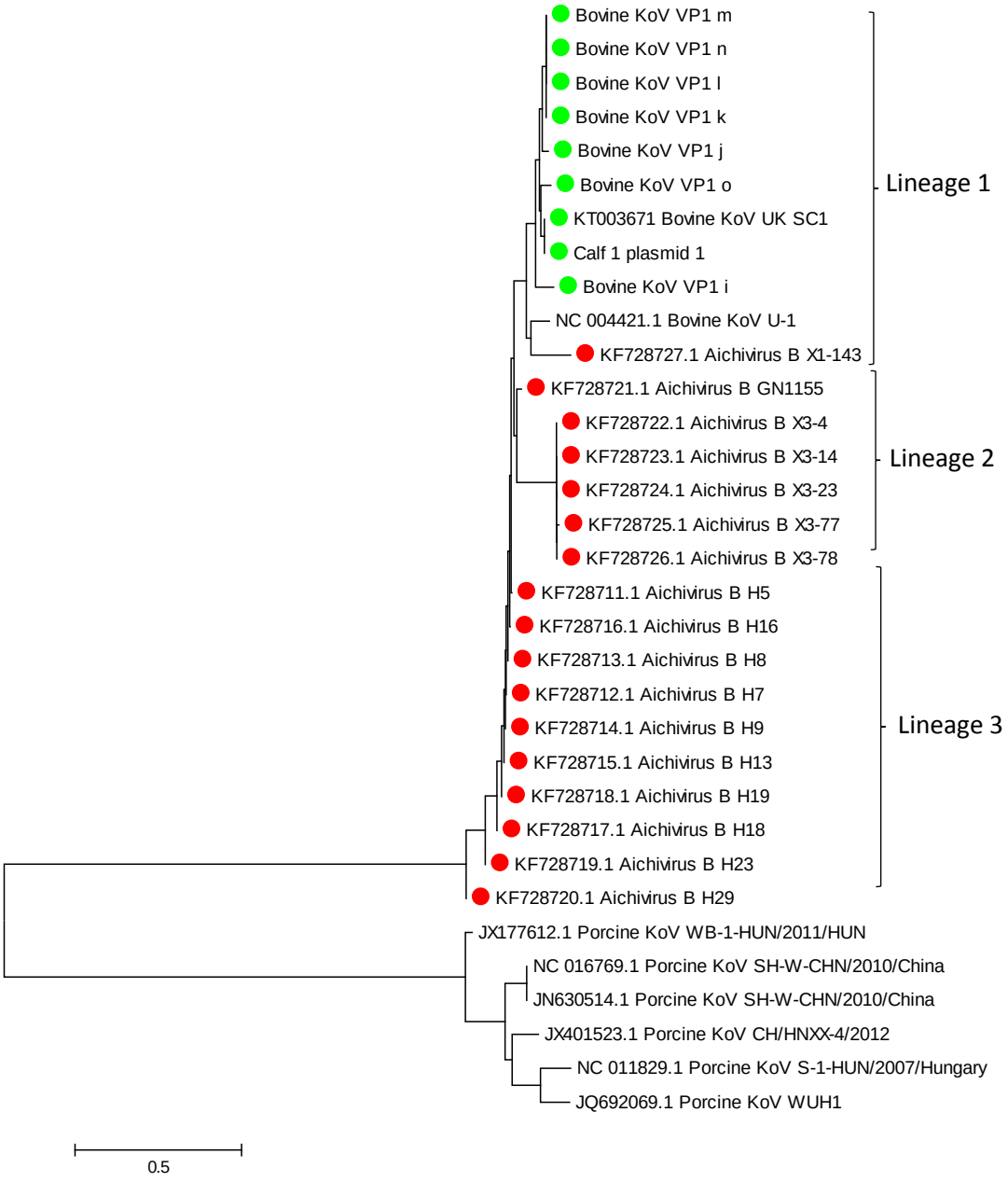

Figure 28: VP1 comparison between the 9 VP1s in this study, the U-1 strain and other bovine KoVs from Chang *et al.* 2014. This maximum likelihood tree is based on the 3650-3940 region of the VP1 protein. Circles in green indicate novel sequences generated in this study, while those in red are from other bovine kobuvirus studies.

3.2 Discussion

3.2.1 cDNA synthesis and cloning of bovine KoV genome

Of the total 64 KoV positive calves in the Gregory *et al.* study (2015, manuscript submitted), 8 diarrhoeic calves' samples were selected for genome amplification in order to generate and assemble a full genome from an infected individual. Specific PCRs were designed to target four overlapping regions of the ~8Kb genome. The number of fragments to divide the genome was decided to enable quick, efficient optimisation of PCRs to generate the products, while also remaining low to aid the assembly of the genome. Excluding fragment 1577-5260, the PCRs were nested or hemi-nested to improve specificity, having found a one round PCR not to be sufficient to produce the appropriate size product. Primer sites were selected to share large overlapping regions between fragments, allowing the genome assembly from generated sequences to produce a sequence representative of a single virus, rather than a chimeric one due to a mixed infection. Large overlaps would also allow a greater range of assembly cloning kits to potentially be used. The primer pairs chosen for PCR, shown in Figure 10, were designed from the bovine, some porcine and/or ovine full KoV genome sequences available from GenBank. Where possible, the primers were designed according to the criteria set out, to ensure time and resources were not wasted on primers that were unlikely to amplify the template. Due to the nature of bovine KoV, in that there is only one reference genome, sometimes primers did not work effectively, despite fitting the criteria previously indicated.

Originally, the 1-1597 fragment was targeted for amplification, although this primer set failed to amplify the appropriate product (Figures 12-14). The colony screen produced by the three weak amplicons in Figure 12, was repeated due to the poor intensity of the appropriate sized amplicon and the high intensity of another amplicon. As there should only be one product present in a colony screen if the ligation and transformation has worked effectively, the extracted products were ligated and transformed again. This repeated colony screen did produce intense amplicons of appropriate size, suggesting the problem that had occurred with the first colony screen had been resolved. Once extracted and sequenced the two amplicons

from the second colony screen (11 and 12) either produced failed sequences, or were positive for bovine KoV U-1 strain but matched a region of the genome of approximately 5.1Kb. For this reason a new PCR was designed to ensure the appropriate product covering the first ~2Kb of the genome would be amplified.

The four PCRs (described as 57-1898, 1577-5260, 4695-6714 and 6638-GR nest) were carried out on cDNA, after the reverse transcription of RNA extracted from 8 calves' faecal samples. When the 57-1898 PCR was designed to replace the 1-1597 PCR, the 57s primer covered the first available sequence without multiple base repeats and that was not as GC rich as the 1s primer sequence. The antisense primer for this fragment was also designed to have a large (~300bp) overlap with the second fragment, to enable virus-specific assembly of the genome at a later date. The PCR conditions used to amplify the fragments were initially based on the manufacturers' protocols (found in the methods section), with small changes made throughout the course of the study to optimise the conditions for each fragment PCR. The LongAmp taq polymerase worked for the 57-1898, 1577-5260 and 4695-6714 fragments. However, the 6638-GR nest fragment required optimisation with SequalPrep taq polymerase to amplify the product (Figure 16). From these gel images, the calves with the most successfully amplified fragments were AstVs from calves 1, 7 and 8. Therefore these became the focus for completing the genome. LongAmp and SequalPrep taq polymerases were most commonly used in this project as they have been the preferred taqs used in this lab. LongAmp taq polymerse was used first to generate each fragment, then SequalPrep was used if this was unsuccessful.

Following insertion of these PCR products in pGEM-T-Easy or pCR-blunt II-TOPO vector, internal sequencing was carried out with M13 primers and the original primers used to generate the fragments. Internal primers were then designed from the sequences obtained (Table 5) allowing full sequencing of the 1.9-3.8Kb fragments. Numerous primers were designed to overlay throughout the genome, particularly in the overlapping regions, to ensure the base and amino acid sequence was without sequencing errors and to allow primer walking. Initial generation of fragments did not produce all four for the calves 1, 7 and 8. The weaker amplicons

observed in Figures 15-17 for calves 7 and 8 may have been as a result of mispriming or variations in sequence, not taken into account with primer design. The selection of primers to target a region, which in most cases had only one bovine reference sequence, meant unexpected variations in these regions would either reduce the number of copies produced or prevent amplification altogether. This could be the reason calves 2-6 were often unsuccessful. Repeat PCRs of these fragments were difficult with only small amounts of original cDNA available and calf-specific primers required to target the varied regions. For this reason calf 1 became the main focus for the full genome.

The Generacer™ Kit (Invitrogen) was used to generate the 5' end with bovine-specific primers (conserved from the bovine U-1 strain), however, this was unable to amplify the appropriate product (Figure 19). Secondary and tertiary structures have been detected at the 5' end of other KoV species. The GC-rich nature has previously made it difficult for other researchers to amplify and sequence this region (Sasaki *et al.* 2001; Yamashita *et al.* 2003; Martínez-Salas *et al.* 2015). This has not, however, been verified in bovine KoVs to date. If this method was to be repeated, a proteinase K digestion would be carried out before the kit protocol to remove the VPg and any other unknown structures, enabling the kit to work more effectively. As this 1-50bp region is required for virus replication, it will be synthesised separately and ligated onto the completed genome to function as an infectious clone at a later date.

The proof-reading Herculase taq polymerase was used with the aim of reducing the likelihood of sequences containing errors, compared to those found in sequences produced by the other taq polymerases used (Table 7), thus producing a KoV genome with the highest amino acid identity to the one originally detected in the faecal sample. Maintaining a high amino acid identity is important when producing an infectious clone, to ensure it will replicate and interact in the same way in virus challenge studies as in the infected animal. While the 57-1898 and 4695-6714 calf 1 fragments were able to be amplified with Herculase taq polymerase, 57-1898 and 4695-6714 from other calves and the other two fragments from calf 1 were unsuccessful.

3.2.2 Genome assembly and comparison of VP1 regions

Phylogenetic identity to the known bovine KoV strain (U-1) and other mammalian KoVs was calculated, the results of which can be seen in Figure 23. The Aichivirus B group (highlighted in blue) includes the U-1 strain, ovine and ferret KoVs, thought as candidate species to be in the Aichivirus B group (Cho *et al.* 2015). The Aichivirus A and C groups also include the expected species, with murine KoV, canine KoV, feline KoV and Aichi virus in the former, and porcine KoV in the latter (Ribeiro *et al.* 2013; Cho *et al.* 2015). The recent discovery of caprine KoV, situated between Aichivirus groups B and C, has not yet been classified. The KoV genome from this study was submitted to GenBank with the title BKoV_UK_SC1 and accession number KT00367. As shown in Figure 23, the BKoV_UK_SC1 genome is most closely related to the U-1 bovine KoV strain (GenBank number: NC_004421.1). While this may not seem surprising as several of the primers were designed using the U-1 strain, BKoV_UK_SC1 showed a 91% similarity to the U-1 strain, which is less than that found between other species such as porcine and canine KoV. This suggests there is great variation in bovine KoVs compared to KoVs of any other species. The second closely related KoV is the ovine KoV (ADG03747.2) with a similarity of 81%.

Using an SSE alignment containing the bovine U-1, six porcine and six Aichi virus genomes, pairwise-distance graphs were generated showing the a) amino acid difference within each Aichivirus species compared to each of the other two groups (Figure 24), b) amino acid difference between the BKoV_UK_SC1 genome and the U-1, porcine and Aichi virus genomes individually (Figure 25). Although there are several more than six genomes available from GenBank for Aichi virus and porcine KoV, they are shown to group closely together in Figure 23 so six genomes would be representative of the species. From the first comparison (Figure 24), the bovine and porcine genome amino acid sequences are more closely related than the bovine and Aichi virus genomes, which are the most diverse out of the Aichivirus species. This is consistent among the three groups. There are two main regions where this can be seen are: ~350-400aa and ~1500aa. Using Figure 10 as a reference for the genome, positions 1, 3 and 5 on Figure 24 are the most diverse regions of the genomes for all

three species. These regions correspond to the L protein, VP1 and 3B regions, respectively, which have been previously acknowledged as the most diverse regions of the KoV genome. The L protein is thought to play a role in RNA replication and encapsidation; the VP1 has been found it be most immunogenic; while the exact function of the 3B protein is currently undetermined (Sasaki *et al.* 2003). The points 2, 4 and 6 correspond to the VP0, 2C and 3D regions, respectively, which are typically the most conserved KoV proteins. VP0 is cleaved into VP4 and VP2 situated in the capsid; 2C is vital for the formation of viral replication vesicles; and the 3D protein codes for a RNA polymerase (common among *Picornaviridae*) (Yamashita *et al.* 1998; Dietz *et al.* 2000).

The second comparison graph (Figure 25) shows the amino acid difference between the BKoV_UK_SC1 genome and the bovine U-1, porcine KoV and Aichi virus genomes. The comparison between the U-1 and BKoV_UK_SC1 genomes show they are very similar in terms of amino acid sequence, with the L and 2B proteins showing the greatest diversity. The trend of this graph and that of Figure 24 is similar for the BKoV_UK_SC1 vs. porcine KoV and Aichi virus, with the same regions showing the highest and lowest regions of diversity. There is, however, a point at approximately 500aa which shows a higher amino acid diversity between the BKoV_UK_SC1 and porcine KoV genomes, which corresponds to the 2B protein. The 2B protein is known to alter host cell membrane permeability (Dietz *et al.* 2000), which could equate to one of either BKoV_UK_SC1 or the U-1 strain playing a greater role in diarrhoeal disease than the other. Having few bovine KoV genomes available for analysis, or even partial genomes covering the P1 and P2 segments of the genome, means comparisons of pathogenicity are difficult to confirm. The source of the U-1 strain as a contaminant of Vero cell culture, possibly from the fetal bovine serum used, means there is no background information on the animal or group of animals from which it originated.

Comparison of the two bovine KoV genomes in both Figures 24 and 25, proves to be diverse from the Aichi viruses included in the sample group for comparison. There has not at present been any cause for concern regarding the zoonotic potential of

bovine KoV, and the distance between the Aichi viruses seen here and current bovine genomes available supports this.

In preparation for the assembly of the full genome, each of the four fragments underwent a restriction digest at both ends to produce a linearised KoV fragment separated from the vector. The NEBuilder® HiFi DNA Assembly Cloning Kit was chosen to assemble the genome in preference over other cloning methods such as the Gibson Assembly® Cloning Kit. Reasons for this decision include the higher accuracy and more colonies associated with using the NEBuilder® HiFi DNA Assembly Cloning Kit. Also, this master mix can join DNA fragments together more efficiently, even with larger fragments of low DNA inputs, and is a high-fidelity assembly. It is important a high fidelity kit was used to prevent errors being introduced at this later stage, having undertaken measures to reduce errors throughout the study. When selecting the most appropriate sites (see Table 10), the optimum overlap criteria included in the NEBuilder® HiFi DNA Assembly Cloning kit manual was taken into account, therefore overlaps were cut back accordingly.

The restriction digest was initially successful for the 57-1898, 1577-5260 and 6638-GR nest fragments (Figure 26). The 4695-6714 fragment may not have been cut on the first attempt as two incubation temperatures were required for both of the restriction enzymes selected to function. Once repeated with an overnight incubation the digest was successful, suggesting the previous 2 hour incubation was insufficient to cut the desired product.

Assembly of the genome using the NEBuilder® HiFi DNA Assembly Cloning Kit was effective for ligating fragments 1 and 2, and 2 and 3 together (shown in Figure 27). For both the assembly reactions using the 3/4 and 1/2/3/4 assembly mixes, the 3/4 PCR was unsuccessful as were the 57-GR and 57-GR nest PCR reactions. The GR nest primer was used in the second set of assembly reactions as the 6638-GR nest PCR was a hemi-nested PCR with the GR nest primer in the second round. It was originally thought the first 57-GR assembly reaction failed for this reason. However, the second assembly reaction proves that was not the case. The reason this assembly failed would appear to be as a result of the 3/4 assembly also failing to ligate, as this assembly reaction did not work with either of the two assembly mixes used. The 57-

GR was incubated at 50°C for 60 minutes as detailed in the manufacturer's protocol for 4-6 fragments, however, due to the size of the final assembled fragment a longer incubation period may have also been necessary. The 4695-6714 and 6638-GR nest fragment sequences were checked for the necessary restriction sites which were present, and at the expected bases in the sequence. The restriction buffers and conditions were also checked. As the LongAmp 4695-6714 1.1 plasmid was originally used in the restriction digest, unsuccessfully, the 1.1 plasmid may have accidentally been used in the assembly mix rather than the 1.2 plasmid that was used to repeat the restriction digest and produce the appropriate products.

The VP1 region of the KoV genome is a logical choice for measuring diversity within and between the species, as it is recognised as the most diverse of the three capsid regions. Capsid diversity can reveal crucial information about both host-pathogen interactions and their ability to survive in the environment, which can lead to better control measures and animal management to reduce the impact of viral disease. The neighbour-joining method was used to generate the VP1 comparison tree (Figure 28) because it was appropriate to show the diverse lineages between the bovine KoVs and porcine KoVs. While whole genome comparisons are difficult for the reasons previously explained, one Chinese study by Chang *et al.* (2014) investigated VP1 diversity in their sample set and were able to sequence 17 VP1 proteins. From the tree, two main lineages can be observed from the published 17 VP1 regions of bovine KoVs, and a further lineage including 1 VP1 from the Chang *et al.* study, the U-1 VP1 and the 9 VP1s, from this study. While these sequences have been separated into lineages, the diversity between the bovine VP1 sequences is much lower than expected. This may be due to the relatively low number of VP1 sequences available or that bovine KoVs have more conserved VP1 regions compared to other KoV species. It is also important to consider the other proteins in the KoV genome which have been found to be highly diverse among porcine KoVs and Aichi viruses: the L protein and 3B protein. As most published bovine KoV sequences are from the detection of the virus, they are usually fragments of the RdRp region and are therefore highly conserved. The sequencing of more full bovine KoV genomes would provide more insight into the regions of highest diversity in the bovine KoV genome.

4 Astrovirus

The aims for the AstV section of this project were to sequence and prepare bovine AstV capsids, in order to express their proteins in baculovirus. An ELISA could then be developed to enable detection of AstV antibodies in bovine serum, useful for further investigation into their role in calf diarrhoea.

4.1 Results

4.1.1 RdRp and Capsid Analysis

4.1.1.1 Bovine astrovirus PCRs targeting the RdRp and capsid regions

Two PCRs were designed to sequence the whole genome (56-_AS, nested from 56-3862 (from Sharp *et al.* 2015), and 2312-GR nest, nested from 2312-GR, see Figures 29-32). The 56-_AS PCR and 2312-GR nest PCR reagent quantities and conditions can be found in Tables 10 and 11 in the Appendix, respectively. The 2312-GR nest PCR aimed to amplify the RdRp and capsid proteins, while the 56-_AS PCR targeted the ORF1a and RdRp segments. The 56S and 2312S primers were designed from four published bovine AstV sequences: B170/HK, B76/HK, B18/HK and B76-2/HK (GenBank numbers: NC_023632.1; NC_023629.1; NC_023631.1; NC_023630.1), as shown in Figure 30. The 3862s and 3882AS primers were designed from the 52 nucleotide conserved region found immediately upstream of the ORF1b/ORF2 junction identified in the Finkbeiner *et al.* study (2008).

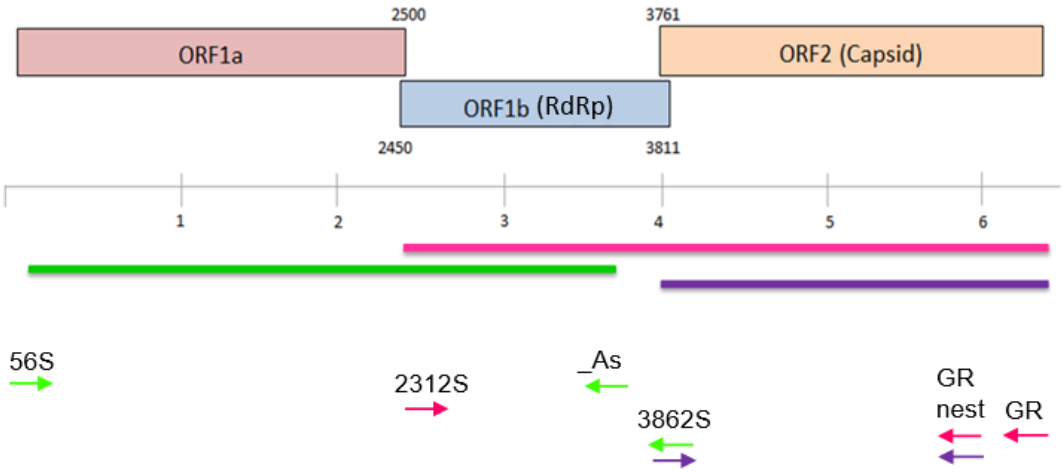

Figure 29: PCR regions in relation to the bovine AstVs genome: 56-_AS (green); 2312-GR nest (pink) and 3862-GR nest (purple).

B170/HK	TACATCAATCGTGCTGACGAGGC
B76/HK	TACATCAACCGTGCTGACGAGGC
B18/HK	TACATCAACCGTGCTGACGAGGC
B76-2/HK	TACATCAACCGTGCTGATGAGGC
56S	TACATCAACCGTGCTGAYGAGGC

B170/HK	AACAACGGCCCCAGAAATCCAC
B76/HK	AACAACGGCCCCAGAAATCCAC
B18/HK	AACAGCGGCCCCAGAAATCCAC
B76-2/HK	AACAGCGGCCCCACAAATCCAC
2312S	AACARCGGCCCCAGAAATCCAC

Figure 30: Primer design for primers 56S and 2312S.

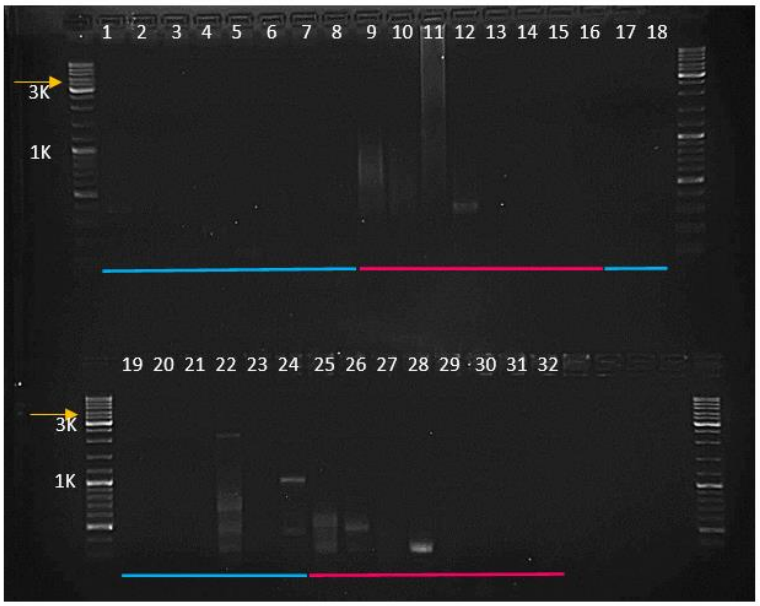

Figure 31: LongAmp 56-3862 (blue) and 2312-GR nest (pink) 1st (lanes 1-16) and 2nd (lanes 17-32) round PCR, on calves 1-8. The 56-3862 PCR was nested with 56s and _AS, and the 2312-GR nest PCR was nested with IAS1, IAS2 and GR nest.

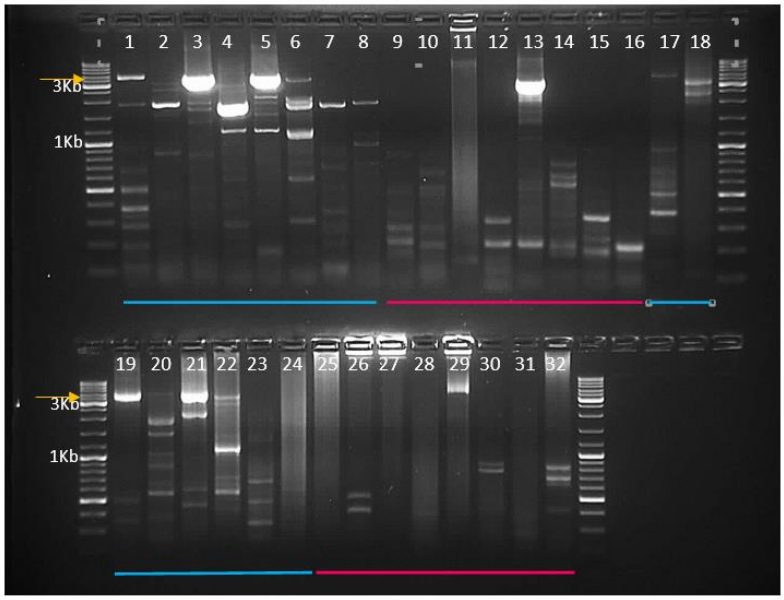

Figure 32: SequalPrep 56-_AS (blue) and 2312-GR nest (pink) 1st (lanes 1-16) and 2nd (lanes 17-32) round PCR, on calves 1-8.

The LongAmp 56-_AS PCR in Figure 31 was unable to amplify the appropriate size product, therefore the focus turned to optimising the 2312-GR nest PCR to include the RdRp and capsid proteins within the same PCR product (Tables 18 and 19 in the Appendix). This would allow RdRp sequences previously amplified from this sample set to be analysed and separated into lineages showing the extent of diversity of the AstVs detected. The SequalPrep 2312-GR nest PCR produced 2 amplicons of

the appropriate size, one 1st round (lane 13) and one 2nd round (lane 29), while the 56-_AS PCR produced 8 amplicons of expected size, 4 1st round (lanes 1, 3, 5 and 6) and 4 2nd round (lanes 17, 19, 21 and 22) (Figure 32). These products were extracted and purified. Sequencing confirmed that the 56-_AS amplicons 3, 5, 13 and 19 were bovine AstV. Sequencing reactions were also performed on 2312-GR nest amplicons but did not produce readable data. Amplicons 13 and 29 were cloned into pGEM®-T easy vector but only amplicon 29 produced colonies with an appropriately sized screening PCR (methods 2.7) product. Sequencing of this product with M13 primers proved it to be a bacterial contaminant.

Following this difficulty generating the 2312-GR nest fragment, it was decided that previously cloned capsid sequences would be used for the generation of expression constructs.

4.1.1.2 Neuro AstV

During this project some pig brain samples became available to test for neuro AstVs, with an additional cow brain sample positive for bovine neuro AstV used as a control. The PanAstro primers (used in Sharp *et al.* 2015) were used in a PCR with the conditions from the Chu *et al.* 2008 paper. Details can be found in Table 20 of the Appendix. While the appropriate size amplicon for the pig sample in lane 3 was negative for AstV (Figure 33), the bovine neuro AstV positive control was extracted and cloned for restriction digest and ligation into FastBac at a later date.

Figure 33: Neuro AstVs PCR targeting pig samples (lanes 1-3) and using a bovine neuro AstV as a positive control (lane 4). The products in lanes 3 and 4 were extracted, purified, cloned and sent for sequencing (methods sections 2.5-2.7). The pig sample in lane 3 was negative for AstVs.

4.1.2 Capsid analysis and cloning into FastBac

4.1.2.1 Capsid analysis

Prior to this project, another member of the research group (Bill Gregory) had developed a capsid PCR to gain as many AstV capsids from the diarrhoeic and non-diarrhoeic calves as possible. He then cloned the capsids using the methods detailed earlier in this thesis (methods 2.7).

In Figure 34, the RdRp lineages (assigned in the Sharp *et al.* 2015 study) and the capsid lineages from the same sample set can be compared. While the coloured lines show the RdRp sequences matched to the calf cDNA they originated from, they cannot be matched to individual capsid clones as these were generated at a later date. None of the capsid sequences matched with RdRp sequences from lineage 2. As more calves were targeted for RdRp sequences previously, there are more calves represented in the RdRp tree than the capsid tree which explains why there are no capsid sequences matched to RdRp lineage 2.

The calf 2 AstV capsids (lineage 4), in Figure 35, appear to be diverse from the other AstV capsids from other calves, which is also suggested in Figure 34. The AstV capsids appeared to form four lineages as indicated in Figure 35, however, the pairwise distance comparison of capsid amino acid sequences suggested there were just two distinct capsid lineages, one of which could be separated into three clades. From the pairwise-distance comparison in Figure 36, lineage 4 is the most diverse compared to the other three lineages as the neighbour-joining tree in Figure 35 suggests. According to ICTV's 2011 AstV report, the mean amino acid genetic distances (p-dist) requires a range of 0.378-0.750 between groups to be counted as separate species. Lineage four has a mean pairwise distance of between 0.562-0.644 making it a separate species compared to the pairwise distances of the other three lineages by the ICTV criteria. However, mean pairwise distances for the other lineages are as follows: 1 v 2 is 0.356; 1 v 3 is 0.347 and 2 v 3 is 0.305. Consequently, none of the other three lineages have a large enough mean pairwise distance to qualify as being separate species according to the ICTV criteria. Figure

36 suggests capsids labelled lineages 1 and 2 are more closely related to each other than either of these lineages to lineage 1, although this is not a significant result.

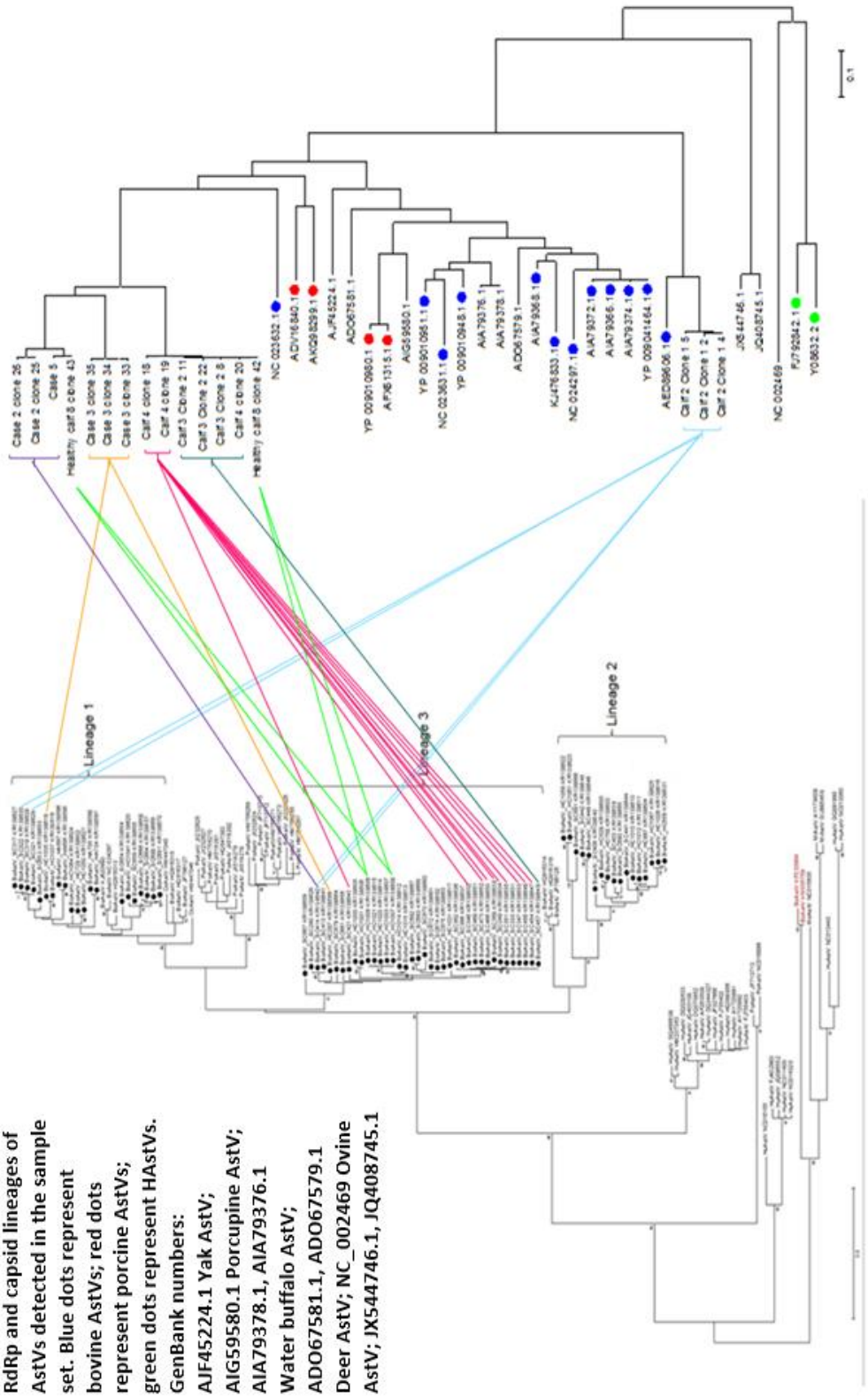

Figure 34 Neighbouring-joining trees comparing the RdRp and capsid lineages of AstVs detected in the sample set. Blue dots represent bovine AstVs; red dots represent porcine AstVs; green dots represent HAstVs. GenBank numbers: AJF45224.1 Yak AstV; AIG59580.1 Porcupine AstV; AIA79378.1, AIA79376.1 Water buffalo AstV; ADO67581.1, ADO67579.1 Deer AstV; NC_002469 Ovine AstV; JX544746.1, JQ408745.1

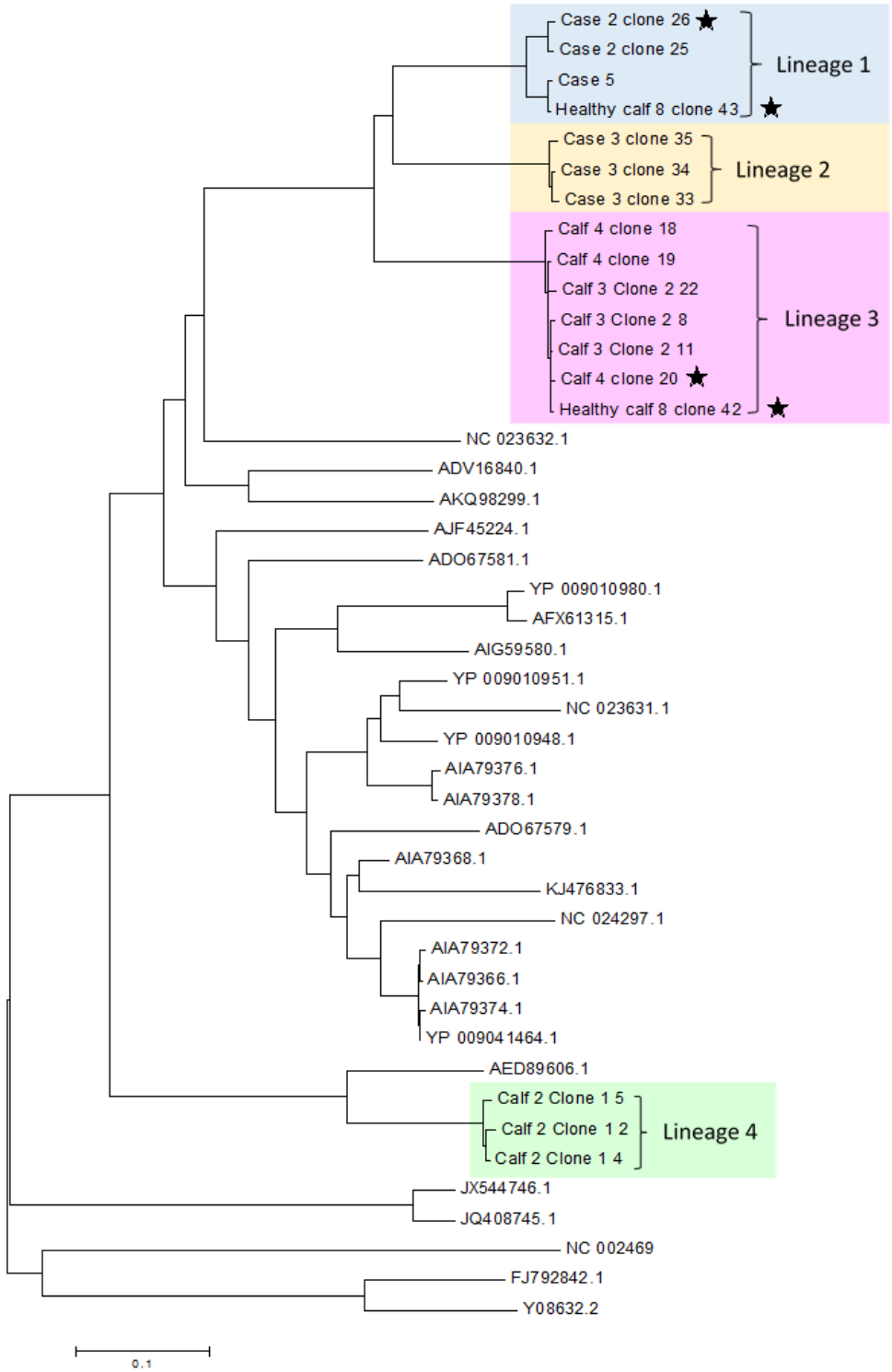

Figure35: Phylogenetic relationships between AstV capsids sequenced in this study and a selection of other mammalian AstVs. The stars indicate the capsids that were successfully cloned into FastBac.

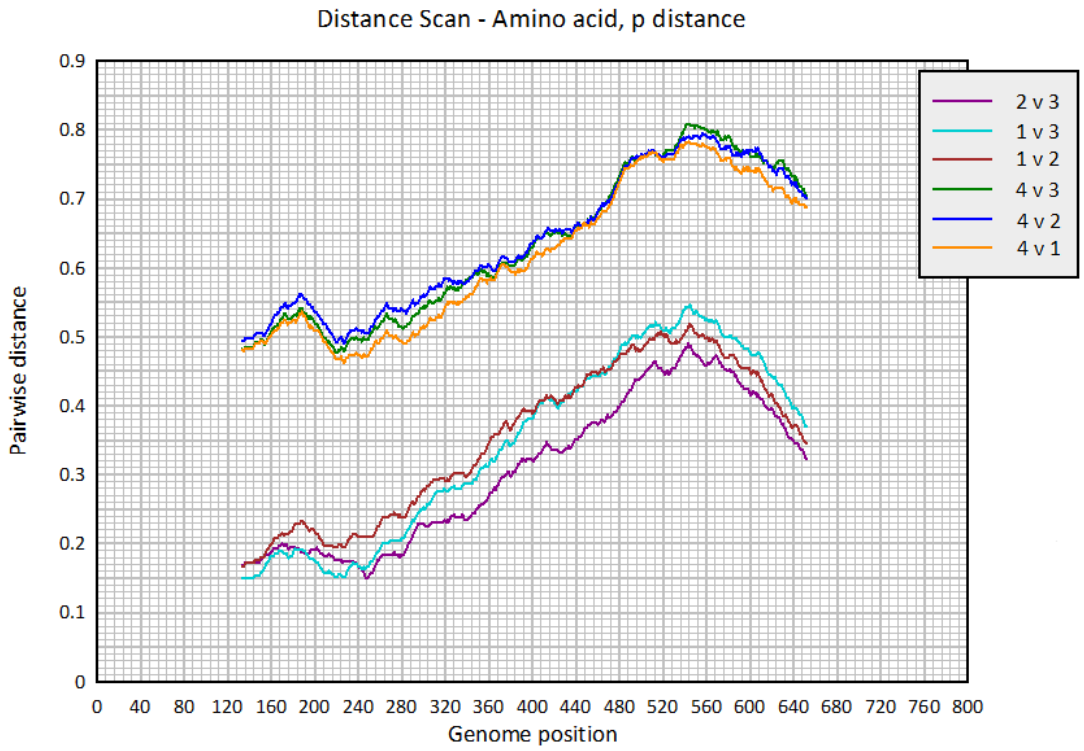

Figure 36: Pairwise-distance comparison of the four lineages in Figure 31, using amino acid sequences.

4.1.2.2 Cloning AstV capsids into FastBac

Primers were designed to add restriction sites to the capsids using the capsid plasmids as the PCR template (Table 11). Due to the previous design of primers, the capsid sequences were 13 nucleotides short of the full capsid seen in the B18/HK, B170/HK and BAstGX-G1 sequences (Figure 37). Therefore this conserved short sequence was to be added to the forward primer and presumed the same for the capsid sequences in this study. Using the NEB cutter tool V2.0 (NEB), the RsrII and SalI restriction sites were identified either side of the insert region in the FastBac vector. The PCR primers, 3748s and 5970AS, were designed to include the missing 13 nucleotides and the RsrII and SalI restriction sites attached at the 5' and 3' ends, respectively. Long and short versions of the 3748s+RSrlI were designed to ensure there was enough capsid sequence overlap before the restriction site to allow binding. Details of these primers can be found in Table 12.

Herculase II Fusion DNA Polymerase (Agilent) was used to generate this product in a reaction detailed in Table 21 in the Appendix. In total, 16 capsids from 7 different

calves were included in the restriction site PCR. Details of the capsids used can be found in Table 11.

Table 11: The clones produced from the healthy and diarrhoeic samples, with their capsid names for reference. The clones in red were not available in sufficient quantities for restriction digest.

Calf number	Clone names
Calf 2	1.2, 1.4, 1.5
Calf 3	2.11, 2.22, 2.8
Calf 4	18, 19, 20
Case 2	25, 26
Case 3	**33**, 34, 35
Case 5	3
Healthy calf 8	**41**, 42, 43

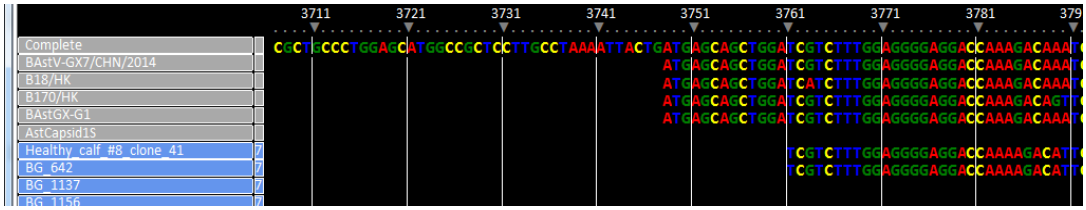

Figure 37: SSE alignment showing the missing 13 nucleotides from the capsid sequences that were added to the primer sequences.

Table 12: Sequence details for the primers used in the 3748-5970 restriction site PCR. In bold is the sequence added from Figure 33.

Primer	Direction	Length (bp)	Sequence
BAstVCap 3748s+RsrI I short	Sense	31	GGCGGTCCG**ATGAGCAGCTGGA**TCG TCTTTG
BAstVCap 3748s+RsrI I long	Sense	40	GGCGGTCCG**ATGAGCAGCTGGA**TCG TCTTTGGAGGGGAGG
BAstVCap 5970AS+Sa l1	Antisense	28	CCGTCGACCTACAGCCCCATGGCATA GC

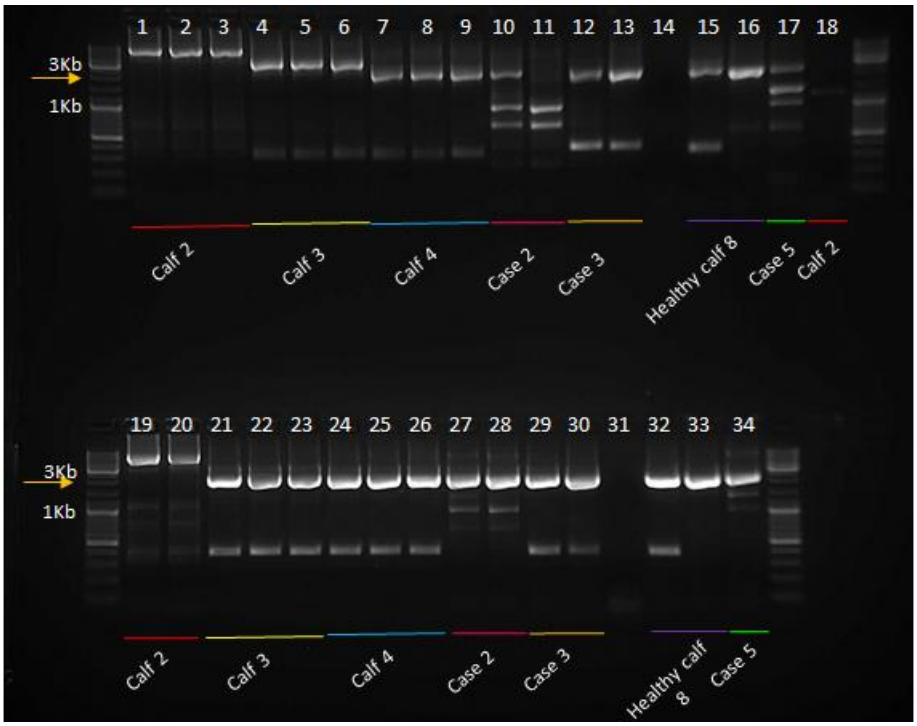

Figure 38: Herculase 3748s-5970 capsid region PCR with RsrII and SalI restriction sites. Lanes 1-17 represent the reactions carried out with BAstVCap 3748s+RsrII short primer, and lanes 18-34 represent those with BAstVCap 3748s+RsrII long primer. Lanes 14 and 31 were negative controls.

The 3748s+RsrII long primer was able to amplify the fragment more effectively than the shorter version of the primer and therefore it was these products that were extracted (Figure 38). Amplicons in lanes 21-30 and 32-34 were extracted, purified and ligated into the pCR™-*Blunt II-TOPO*® vector. The calf 2 products were also extracted, purified and sequenced. When the BLAST analysis was first carried out only 20bp of the ~350bp sequence was positive for bovine AstV, and so was not included in the restriction digest. These sequences were checked again at a later date and recently added sequences to GenBank were a match for the full ~350bp sequence analysed. These capsids can be processed further at a later date to be included in the baculovirus expression.

The capsids cloned from Figure 38 underwent the restriction digest with RsrII and SalI. Details of the restriction digests can be found in Table 22 in the Appendix. Initially the digest was carried out as a double-cut with both restriction enzymes in the same mix, however, this was unsuccessful as Figure 39 shows the plasmids were

uncut. The digest was repeated by first cutting with SalI in buffer 3.1 with a 100µl reaction, then extracting and purifying the products (eluting with water rather than elution buffer). The purified products were cut with the RsrII enzyme, the results of which are shown in Figure 40. Case 2 plasmids 25 and 26; calf 4 plasmid 20 and healthy calf 8 plasmids 42 and 43 were successfully cut with RsrII and SalI. The FastBac vector also underwent a restriction digest with RsrII and SalI

These five capsids were cloned into the cut FastBac vector, and the colony screen was successful for case 2 plasmid 26; calf 4 plasmid 20 and healthy calf 8 plasmids 42 and 43. These four plasmids were then extracted from cultures of these positive clones using the QIAprep Miniprep kit (Qiagen). Figure 35 shows the positions of the four capsids within the four lineages containing the capsid amino acid sequences from this study.

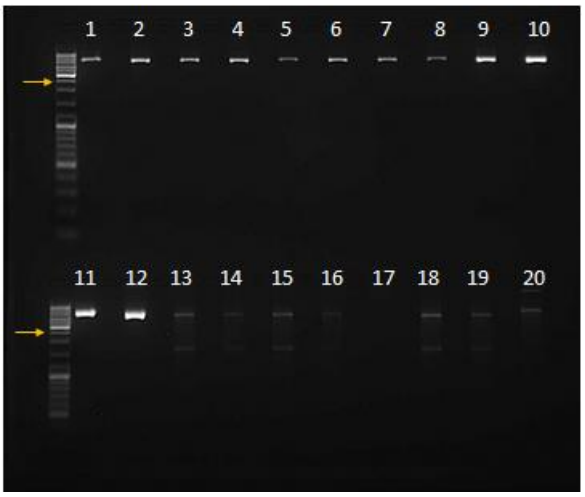

Figure 39: Restriction digest of capsid plasmids with RsrII and SalI in buffer 3.1. Capsids.

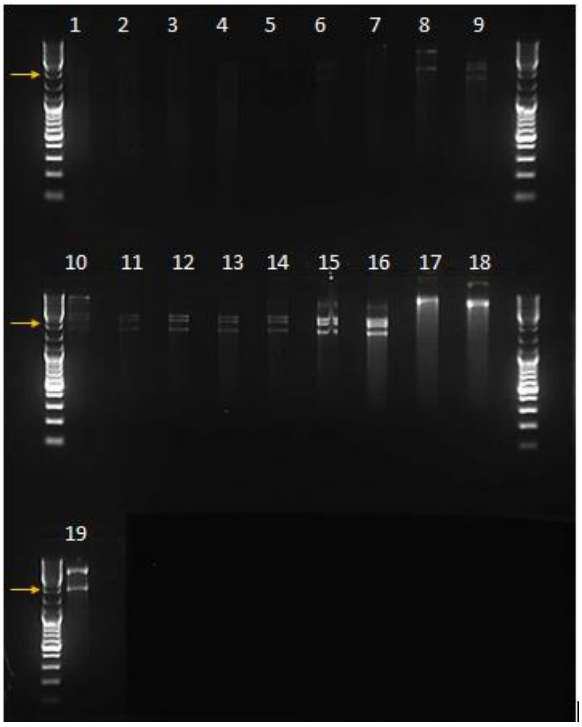

Figure 40: Sequential digest with SalI then RsrII. The appropriate size products (middle product indicated by the arrow) were extracted from lanes 11-14.

Table 13: PCR primers used to target the complete bovine KoVs and AstVs genomes.

Sequence Name	Orientation	Length (bp)	Sequence
Kobuvirus			
1S	Sense	27	TTTGAAAAGGGGGTGGGGGGGCTTCGG
156AS	Antisense	22	GGCGAGAAAGTACCAAGAGGAC
92AS	Antisense	22	ACGCAGTAGCACTTCCGCACCG
1577S	Sense	23	AAMCAYTGGTGGGAACCTGCTGC
1597AS	Antisense	23	CGTCGTCCAAGGGTGGTYACMAA
1898AS	Antisense	21	GGCTCCTGGACACTTTCGAGT
4695S	Sense	25	TGGTSATMATCTGTGCKGATCTKGC
5260AS	Antisense	25	AGCCAAGCAGGGATTTCCCAGTGCC
57S	Sense	22	GTTCGCGTCGTCGTAAGTCTGG
6638S	Sense	25	CYTYGAGGGTMTSTGTGGTGCTWCC
6714AS	Antisense	21	TCTTCCTTAAACACAACAAGG
GR	Antisense	25	GCTGTCAACGATACGCTACGTAACG
GR nest	Antisense	23	CGCTACGTAACGGCATGACAGTG
PanKobu_IAS	Antisense	24	ATGATGGTGTTRAKGATRGARGTG
PanKobu_IS	Sense	22	TGGAYTACAAGTGYTTTGATGC

PanKobu_OAS	Antisense	23	GTGTCNGGRTCCATSACHGGGTG
PanKobu_OS	Sense	21	GTGGTTGAGGCAGCTCCAGTG
Astrovirus			
2312S	Sense	22	AACARCGGCCCCAGAAATCCAC
56S	Sense	23	TACATCAACCGTGCTGAYGAGGC
3862s	Sense	21	TCRTCTTTGGAGGGGAGGACC
3717S	Sense	20	GAGCATGGCCGCTCCTTGCC
5990AS	Antisense	20	CTGAAACCAGGGTGAATTGC
BAstVCap 3748S+RsrII long	Sense	40	GGCGGTCCGATGAGCAGCTGGATCGTCTTTGGAGGGGAGG
BAstVCap 3748S+RsrII short	Sense	31	GGCGGTCCGATGAGCAGCTGGATCGTCTTTG
BAstVCap 5970AS+Sal1	Antisense	28	CCGTCGACCTACAGCCCCATGGCATAGC
PanAstro_IAS1	Antisense	20	CGKTAYGATGGKACKATHCC
PanAstro_IAS2	Antisense	20	AGGTAYGATGGKACKATHCC
PanAstro_IS	Sense	22	TGGAYTACAAGTGYTTTGATGC
PanAstro_OS1	Sense	23	GARTTYGATTGGRCKCGKTAYGA
PanAstro_OS2	Sense	23	GARTTYGATTGGRCKAGGTAYGA

4.2 Discussion

AstV diversity has been well recognised within the literature with the capsid proteins showing highest diversity compared to the ORF1a or ORF1b (RdRp) regions. As with KoV and other viruses, the capsid is often the most diverse segment of the genome in order to be able to adapt to the host environment. Studying capsid diversity gives insight into the host-pathogen interactions resulting in infection; potential differences in pathogenicity between AstVs and how adult cattle gain acquired immunity to the AstVs they come into contact with.

4.2.1 RdRp Capsid diversity

Initial PCRs targeted the complete coding region of the genome for amplification. As this would be able to highlight all regions of diversity throughout the genome, any highly diverse regions in the ORF1a or RdRp segments may explain why some AstV co-infections result in more severe diarrhoea than others. Another focus for targeting the complete coding region was to get the RdRp and capsid from each AstV. Previous RdRp from the Sharp *et al.* study (2015) and capsid sequences, were obtained from multiple animals but it could not be assumed the RdRp and capsid sequences were from the same AstV, with many calves being co-infected. Difficulty generating the 2312-GR nest fragment with both LongAmp and SequalPrep taqs meant this was unfortunately not possible with the PCR reagents and conditions used. Following sequencing with M13 primers, the SequalPrep colony screen amplicon produced in Figure 32 was found to be bacterial contamination. Although the amplicon in Figure 31 extracted for the colony screen was the expected amplicon size, using faecal samples for PCR can lead to bacterial contaminants from the sample being amplified and cloned instead of the targeted AstV, particularly when using degenerative primers as is the case here. SequalPrep taq polymerase was used after the LongAmp PCR was unsuccessful, because it has a high amplification success rate for long range PCR and is reported to require less optimisation than the LongAmp taq polymerase. This PCR may have been unsuccessful for several reasons, for example the PCR spanned a 4Kb region of the genome of both highly conserved and highly diverse sequence. While the RdRp region is the most conserved AstV fragment, this is relative to the remaining of the AstV genome, with

few 18-25bp sections with appropriate GC content at the start of the RdRp for a primer to target. The 2312 site was the most suitable choice for a primer, without making the PCR product much larger. The decision was then made to focus on the capsid PCR as this would still enable diversity between capsids, and then between RdRp and capsid groups, to be compared.

Matching up the RdRp and capsid sequences in Figure 34 demonstrates the diversity between the RdRp and capsid lineages from the AstV capsids sequenced. It is worth noting there are more RdRp than capsid AstV sequences, which is due to the success of the RdRp PCR for more calf samples than the capsid PCR. The capsids (excluding calf 2) primarily match with the RdRp lineage 3, with just one capsid sequence from case 3 matching with an RdRp from lineage 1. The diversity of calf 2 capsids to the AstVs of other calves suggests these capsid sequences would match RdRp lineage 1. The main purpose of generating these two neighbour-joining trees was to ensure capsids representative of multiple viral lineages (based on RdRp phylogeny) were selected for use in the baculovirus expression system.

The neuro AstV PCR in Figure 33 produced the appropriate size amplicon for one of the pig samples and the bovine positive control using the reagents and conditions in Table 20 of the Appendix. While the porcine amplicon sequence did not match any AstV sequences when entered into BLAST, the bovine positive control was sequenced and matched the Bovine AstV NeuroS1 sequence with 94-99% nucleotide identity. The 3129-GR nest capsid PCR will be performed on the bovine neuro AstV positive sample, then cloned and treated the same as the other AstV capsids. With the recent discovery of neuro AstVs, little is known about epidemiology and geographical distribution of this group of AstVs in the UK. The bovine neuro AstV capsid will also be expressed in baculovirus to produce proteins that can be used to test bovine serum for antibodies against the virus.

4.2.2 Capsid selection for cloning with restriction sites

Cloning capsids into baculovirus using the Bac-to-Bac® baculovirus expression system would allow the production of virus-like particles, which can be used to detect the presence of antibodies in bovine serum. This technique has been effective for human and avian AstVs, and has led to the production of a diagnostic ELISA and potentially antivirals for avian AstV in chickens (Caballero *et al.* 2004; Lee *et al.* 2013; Skibinska *et al.* 2015). Restriction sites for the capsids were selected from those available in the FastBac vector, at the 5' and 3' ends of the insert site, and the sites were searched for in the AstV capsid sequences to ensure the capsid would not be cut during restriction digest.

Initially, the 3748-5970 restriction site PCR was carried out on all of the capsids in Table 11, excluding capsids 33, 41 and 1 as an insufficient amount of these plasmids could be recovered. Multiple clones were included from each calf to increase the likelihood of at least one of the clones for each calf producing the appropriate amplicon. The calf 2 capsids produced amplicons much larger than the expected size (approximately 1.5Kb larger). However, as mentioned previously in the results section, GenBank sequences later became available which had a high identity to the ~350bp capsid sequence analysed. The 3748-5970 PCR was repeated on calf 2 capsids, and the result was the same. As plasmid DNA was used as template in these PCRs and the inserts had been sequenced in full, the reason for this is unknown. The restriction digest of the capsids with RsrII and SalI, was originally carried out with both enzymes in the same reaction with buffer 3.1. However, the capsids were only cut once indicating one of the enzymes were not working effectively (Figure 39). This may have been unsuccessful because the CutSmart buffer is recommended for use with the RsrII enzyme, rather than the buffer 3.1 used. Repetition using a sequential digest, with SalI first, was successful for 5/18 capsids from 3 calves (2 diarrheic, 1 healthy).

5 Future work

Progress made during this project in the genetic characterisation and analysis of bovine KoV and bovine AstV, has both continued the work of previous studies and provided the foundation data for future exploration into the replication and pathogenesis of these two viruses. The project aimed to sequence the full bovine KoV genome from one animal and produce an infectious clone to be used in future challenge studies. While bovine AstV capsids required full sequencing and preparation for use in the baculovirus expression system, in order to develop serological techniques.

Continuing on from this study there are both virus-specific and, more generally, calf diarrhoea developments to be made in order to broaden the knowledge of these viruses and their role as a primary pathogen or a co-factor in diarrhoeal disease. Firstly, the KoV part of the project, has additional work to be done on the production of a complete infectious clone. At present the 57-1898, 1577-5260 and 4695-6714 fragments are assembled, but the 6638-GR nest fragment and first 50bp of the genome are not ligated to the 5' and 3' ends of the assembled sequence. Virus produced from this infectious clone can be used in challenge studies to investigate its impact and role in calf diarrhoea. It is important to find out if bovine KoV is truly pathogenic in the absence of other known enteric pathogens.

Finishing the genome of viruses from calves 7 and 8 and any gaps in these genomes would increase the number of reference genomes available for comparison. Producing multiple infectious clones, including those from healthy animals, would allow for differences in pathogenicity depending on strain of bovine KoV to be studied. Detection of bovine KoV neutralising or recognising antibodies in cattle would also confirm whether immunity is acquired in infected calves and determine whether this persists to adulthood.

Following the previous work comparing VP1 proteins in KoVs extracted from infected calves in the Chang *et al*, 2014 study and the attempts to amplify the same region in this study, this PCR should be optimised and performed on the cDNA of the 99 KoV-positive calf faecal samples stored. This would enable the comparison

of a much larger sample set, producing a more representative conclusion of the lineages formed and the true diversity of bovine KoVs in the field. Epidemiology studies could also use this information to examine the prospect of geographical subtypes of the virus and possible recombination events.

Further work on the AstV part of the project would include cloning broader diversity of capsids (including those from neurotropic viral species), and using the virus-like particles in ELISAs to detect the presence of antibodies in bovine serum. This would enable more representative epidemiological studies of AstV in the UK, as well as investigating how healthy calves shed AstV in their faeces without any clinical signs. Also, there may be differences in the prevalence of each capsid lineage in different areas of the UK, or some lineages more associated with aiding other enteric pathogens during co-infections, e.g. rotavirus.

The 3748-5970 capsid PCR should be performed on the bovine neuro AstV positive control used with the pig samples, in order to fully sequence the capsid of this AstV. As the prevalence of neuro AstVs across the world is unknown, testing for antibodies in cattle blood serum would provide a foundation for further study in transmission, replication and pathogenesis of this virus.

6 Conclusion

This project used genetic characterisation and sequence analysis to investigate the role of bovine KoVs and AstVs in calf diarrhoea using a sample set from diarrhoeic and non-diarrhoeic calves. A key aim was to produce outcomes which could then be used to develop serological assays and virus clones in preparation for further investigation of prevalence and pathogenicity.

The full KoV genome (BKoV_UK_SC1) was sequenced and submitted to GenBank under the accession number KT003671. Following pairwise distance analysis, this genome had a high amino acid identity to the bovine U-1 strain and the VP1 protein could be identified as the principal region of the highest diversity between these genomes. Partial VP1 sequences of 8 diarrhoeic animals were successfully generated and compared to the BKoV_UK_SC1 and U-1 genomes. A high nucleotide identity between these VP1 sequences compared to those from the Chang *et al.* study was unexpected, although additional full VP1 sequences should be generated before a reliable conclusion can be formed. At present, the 57-6714 section of the KoV genome has been successfully assembled with the 1-56 and 6638-GR nest fragments to be added at a later date.

AstV capsid clones from 7 diarrhoeic and non-diarrhoeic calves were sequenced, and compared to other bovine AstV capsids by phylogenetic analysis. The generated capsid clones were representative of two RdRp and two capsid lineages by ICTV criteria. Attempts to clone the AstV capsids into the FastBac vector were successful for 4 capsid clones (20, 26, 42 and 43), which will be sub-cloned and expressed in baculovirus for the expression of capsid proteins. The bovine neuro AstV clone and calf 2 capsid clones will be cloned into the FastBac vector to enable further studies of AstV diversity and prevalence in the British cattle population.

In conclusion, this project has provided some fundamental tools to investigate KoV prevalence and pathogenicity in diarrhoeic animals; AstV diversity and its role in mixed enteric pathogen infections and the prevalence of the recently discovered bovine neuro AstV and its potential threat to the animal and human health.

7 Appendix

Table 14: The quantities of reagents used in the 1-1597 PCR with the relevant conditions. *the 1597AS primer was used in the second round PCR.

Reagent	Amount (µl)	Conditions
Nuclease free water	15.25	
LongAmp buffer	5	94°C for 1 minute
dNTPs	0.75	
1S primer (10mM)	1	94°C for 30 seconds
2968AS primer (10mM)*	1	55°C for 4 minutes } X35 cycles
LongAmp Polymerase	1	
N6 cDNA template	1	60°C for 10 minutes

Table 15: The quantities of reagents used in the 57-1898, 1577-5260 and 4695-6714 PCRs with the relevant conditions for each.

Reagent	Amount (µl)
Nuclease free water	15.25
LongAmp buffer	5
dNTPs	0.75
Forward primer (10mM)	1
Reverse primer (10mM)	1
LongAmp Polymerase	1
cDNA template*	1

57-1898 Conditions:

94°C for 1 minute
94°C for 1 minute
50°C for 1 minute } x35 cycles
65°C for 2 minutes
65°C for 10 minutes

1577-5260 Conditions:

94°C for 1 minute
94°C for 30 seconds
55°C for 4 minutes } x35 cycles
60°C for 10 minutes

4695-6714 (1st round) Conditions:

94°C for 1 minute
94°C for 30 seconds
60°C for 5 minutes } x35 cycles
65°C for 10 minutes

4695-6714 (2nd round) Conditions:

94°C for 1 minute
94°C for 1 minute
50°C for 1 minute } x35 cycles
65°C for 5 minutes
65°C for 10 minutes

Table 16: The reagent quantities and conditions required to produce the 6638-GR nest fragment.

Reagent	Amount (µl)
Nuclease free water	13.24
10x buffer	2
DMSO	0.9
Enhancer A	1
Forward primer (10mM)	1
Reverse primer (10mM)	1
SequalPrep Long Polymerase	0.36
GR cDNA template	1

6638-GR Conditions:

94°C for 2 minutes
94°C for 10 seconds
55°C for 30 seconds
68°C for 4 minutes
} x9 cycles

94°C for 10 seconds
55°C for 30 seconds
68°C for 4 minutes 40 seconds
72°C for 5 minutes
} x29 cycles

Table 17: Conditions for the 57-1898 and 4695-6714 Herculase PCRs.

Conditions:

94°C for 2 minutes
94°C for 18 seconds
50°C for 21 seconds
72°C for 3 minutes
} x35
72°C for 5 minutes

Table 18: Details of the reagent quantities and conditions for the 56-_AS PCR.

Reagent	Amount (µl)
Nuclease free water	15.25
5x buffer	5
dNTPs	0.75
Forward primer (10mM)	1
Reverse primer (10mM)	1
LongAmp Polymerase	1
N6 cDNA template	1

Conditions:

94 ˚C for 2 minutes
72 ˚C for 15 seconds
68 ˚C for 4 minutes
} x3

94 ˚C for 15 seconds
68 ˚C for 4 minutes
} x3

94 ˚C for 15 seconds
60 ˚C for 15 seconds
68 ˚C for 4 minutes
} x18

Table 19: The quantities of reagents and relevant conditions used in the 2312-GR nest PCR. *GR primer used in first round, and GR nest primer used in the second round.

Reagent	Amount (µl)
Nuclease free water	16.24
10x buffer	2
DMSO	0.4
Enhancer A	1
2488s primer (10mM)	0.5
GR primer (10mM)*	0.5
SequalPrep Polymerase	0.36
GR cDNA template	1

Conditions:

94°C for 2 minutes
94°C for 10 seconds
55°C for 30 seconds
68°C for 4 minutes
} x9
94°C for 10 seconds
55°C for 30 seconds
68°C for 10 minutes 40 seconds
} x29
72°C for 5 minutes

Table 20: Reagent quantities and conditions used to perform the neuro AstV PCR. *40 rounds of amplification were used for the second round PCR.

Reagent	Amount (µl)
Nuclease free water	20.4
10x AccuPrime™ buffer	2.5
Forward primer (10mM)	0.5
Reverse primer (10mM)	0.5
AccuPrime™ Taq Polymerase	0.1
N6 cDNA template	1

Conditions:

94°C for 1 minutes
94°C for 30 seconds
50°C for 30 seconds
68°C for 30 seconds
} x30*

Table 21: The reagent quantities and conditions used in the 3748-5970 restriction site PCR.*Plasmid was diluted 1 in 10, of which 1µl was used in the reaction.

Reagent	Amount (µl)
Nuclease free water	31
5x buffer	10
dNTPs	5
Forward primer (10mM)	1
Reverse primer (10mM)	1
Herculase DNA Polymerase	1
Plasmid template*	1

Conditions:

94°C for 2 minutes
94°C for 18 seconds
45°C for 21 seconds
72°C for 3 minutes
} x10
94°C for 18 seconds
60°C for 21 seconds
72°C for 3 minutes
} x20
72°C for 5 minutes

Table 22: The buffers and conditions used for the restriction digests, and the estimated product sizes. The product size in bold is the appropriate size for extraction and cloning.

Restriction enzyme	Buffer	Incubation temperature	Incubation time	Product sizes (Kb)
SalI	3.1	37°C	16 hours	2.5, **2.2**, 2
RSrII	CutSmart	37°C	16 hours	

8 References

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