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Investigating the role of bovine herpesvirus-1 in abortion and systemic disease in cattle

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Author Declaration

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List of abbreviations

α-TIF α gene trans inducing factor
ANOVA Analysis of Variance
BoHV-1 Bovine herpesvirus-1
BoHV-5 Bovine herpesvirus-5
BRD Bovine respiratory disease
BT Bovine Turbinate
CD Cluster of differentiation
CNS Central nervous system
CPE Cytopathic Effect
Ct Threshold cycle
Cyt Cytokeratin
DAB 3,3'-Diaminobenzidine
DAPI 4',6-diamidino-2-phenylindole
DIVA Differentiating Infected from Vaccinated Animals
DMEM Dulbecco's Modified Eagle Medium
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid
dNTP Deoxyribonucleotide triphosphate
dUTPase Deoxyuridine triphosphate nucleotidohydrolase
EDTA Ethylenediaminetetraacetic acid
EHV-1 Equine herpesvirus-1
ELISA Enzyme linked immunosorbent assay
FBS Foetal bovine serum
FRET Fluorescence resonance energy transfer
g Glycoprotein
GLM General Linear Model
GNUMAP Genomic Next-generation Universal MAPper
H&E Heamatoxylin and Eosin
HBSS Hank's buffered saline solution
HIV Human immunodeficiency virus

HS Hepearan Sulphate

HSV-1 Herpes simplex virus-1

IBR Infectious bovine rhinotracheitis

IEtu Immediate early transcription unit

IFAT Immunofluorescent antibody test

Ig Immunoglobulin

IHC Immunohiostochemistry

IL Interleukin

IMDM Iscove's modified Dulbecco's medium

IPV Infectious pustilar vulvovaginitis

IPB Infections pustular balanoposthitis

IR Internal repeat region

KSHV Karposi's Sarcoma-associated herpesvirus

LANA Latency associated nuclear antigen

LAT Latency associated transcript

LR Latency related

MAQ Mapping and Assembly with Qualities

MHC Major histocompatability complex

ORF Open reading frame

PBS Phosphate buffered saline

PCR Polymerase Chain Reaction

pi Post infection

PrV Pseudorabiesvirus

REML Residual maximum likelihood estimation

RE Restriction enzyme

RFLP Restriction fragment length polymorphism

RNA Ribonucleic acid

RR Ribonucleotide reductase

RT-PCR Real-time PCR

SNP Single nucleotide polymorphism

SNT Serum neutralization test

SOC Super optimal broth with catabolite repression

TCID50 50% tissue culture infective dose

TAE Tris-acetate-EDTA

TBS Tris Buffered Saline

TE Tris EDTA

TK Thymidine kinase

TNE Tris, NaCl, EDTA

TNF-α Tumor necrosis factor-alpha

TR Terminal repeat region

UDG Uracil-DNA Glycosylase

U_L Unique long region

Us Unique short region

UV Ultra-violet

VP Virion protein

VTM Virus transport medium

vWF von Williebrand factor

Abstract

Bovine herpesvirus-1 (BoHV-1) is a pathogen of cattle, which most commonly affects the upper respiratory tract to cause infectious bovine rhinotracheitis (IBR). It can also spread systemically to cause fatalities in calves and abortion in pregnant cattle. The virally encoded mechanisms of this systemic spread are poorly understood and therefore have been addressed by comparing isolates from the respiratory form of disease with isolates that have previously demonstrated systemic spread.

A survey of 400 bovine abortions in Scotland from 2007-2009 demonstrated a BoHV-1 prevalence of 2.5%. It also demonstrated the importance of real-time PCR as a diagnostic technique when analysing samples from natural cases. The study of BoHV-1 distribution in the placenta and foetal tissue provided support for a haematogenous route of viral spread.

Whole genome sequencing of 11 BoHV-1 isolates using Illumina Solexa technology was completed and added significantly to the sequencing data of BoHV-1. In terms of identifying genetic variation between isolates causing respiratory infection and those causing systemic infection, no differences were observed by SNP or phylogenetic analysis. However, there were significant differences in the extent of variation between essential and non-essential genes, which may reflect the evolution of BoHV-1.

An *in vivo* challenge of the natural host to compare two isolates representing the respiratory and systemic forms of infection showed differences in clinical presentation, histopathological analysis, viral distribution and viral transcript expression, measured throughout the infection period. In particular, it was noted that a more severe ocular infection, rather than respiratory based infection was caused by infection with the ‘systemic’ isolate. Differences in the tropism of the virus were observed early in the infection with the ‘systemic’ isolate showing more association

with the nasal mucosa than the trachea. The tonsils demonstrated different responses to the virus and differences in viral transcript expression. However, this may simply represent different stages of virus infection. Both isolates demonstrated spread to the brain at day 10 post infection.

In vitro methods were used to study the differences in transcript expression in more detail. In a bovine turbinate cell infection faster replication of the respiratory isolate was observed by a significantly faster development of cytopathic effect. This was also reflected in the higher gene expression levels of the respiratory isolate in the first 12 hours of infection. More isolates were studied to investigate whether these differences were consistent, or as suggested by the sequencing, random differences between isolates. Six isolates were used to infect bovine lung slices. Differences in transcript expression were minimal between the two isolate groups. Immunofluorescence did not provide the sensitivity to detect virus in all samples where PCR showed replication. This compromised the study of co-localization but did show promise as a model to study the tropism of respiratory viruses.

Overall, this work has showed that systemic spread of BoHV-1 does not appear to be controlled by virally encoded mechanisms. The *in vivo* experimental infection suggested host factors may play a large part. Further work is also needed to consider any differences that may exist between reactivated virus and the original infecting isolate.

Chapter One: Introduction

1.1 General Introduction

Bovine herpesvirus-1 (BoHV-1) is a pathogen of cattle which most commonly infects the respiratory tract, resulting in a disease called infectious bovine rhinotracheitis (IBR). The virus is a member of the herpesvirus family, an established virus group with hosts in every order of living organisms. The disease can have a significant economic impact on the farming industry, particularly when more serious disease such as systemic spread and abortion occurs. Viral genetic factors and host mechanisms may be at play in the systemic dissemination of the virus. The first of these possibilities will be investigated in this project. This section will begin by introducing the background to the virus and the disease before detailing the specific objectives of the project.

1.2 Herpesviruses

The *Herpesvirales* order consists of three herpesvirus families that includes the *Herpesviridae* family that infect mammals, reptiles and birds, and also the two more distantly related new additions to the order, *Alloherpesviridae* which infect fish and amphibians and finally the *Malacoherpesvridae* that infect bivalves (Pellet & Roizman 2007). Herpesviruses are diverse, widely successful viruses able to infect a wide range of animals from mussels to humans, with most animal species having at least one associated herpesvirus. Two hundred had been identified up to 2007 but it is expected that many more exist and are yet to be discovered (Pellet & Roizman 2007).

1.2.1 General properties

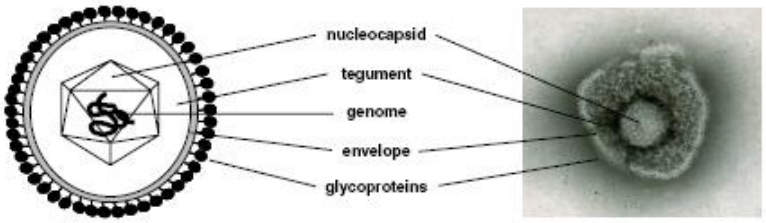
Herpesviruses are traditionally classified by their structure (figure 1.1). A core of linear double stranded DNA is surrounded by an icosahedral capsid, coated by the

tegument (Thiry *et al.* 2006b). The whole virus is surrounded in an envelope studded with glycoproteins, which are important for cell-cell spread and cell entry (Mettenleiter 1994). DNA in the core has been reported to be arranged in the form of a toroid, possibly supported by a spindle made up of proteinaceous fibres embedded in the capsid, although the precise arrangement of the DNA is not known (Pellet & Roizman 2007; Roizman & Baines 1991). The capsid consists of 162 capsomeres of 9.5x12.5nm with a total diameter of ~100nm. During a cell infection non-enveloped capsids exist in 3 forms: A-capsids have no core structure, B-capsids contain a scaffold but no genome and C-capsids contain the DNA genome that replaces the scaffold (Pellet & Roizman 2007).

The tegument refers to the structure between the capsid and envelope that has varying thickness depending on the infection stage and was reported to have no distinctive features (Roizman & Baines 1991). Despite this, evidence of structural polarity suggests it is an ordered structure. Layers are added as the virion moves through the cytoplasm after nuclear egress. The amount of tegument is thought to depend on the location within the cell, with more tegument observed in cytoplasmic vacuoles compared to perinuclear space, and is considered to be determined by the virus rather than the host (Pellet & Roizman 2007). One of the important roles of the tegument is to provide pre-synthesized proteins that can influence the host environment on cell entry to suit the virus, including shutting down host protein synthesis and inhibiting infection defences (Pellet & Roizman 2007).

The envelope is made up of lipids and virus-specific glycoproteins in greater numbers than other enveloped viruses. It has a trilaminar appearance and is thought to be derived from altered cell membranes (Pellet & Roizman 2007).

Figure 1.1 Herpesvirus structure (Thiry *et al.* 2006b).



Although highly divergent, all the herpesviruses have four common characteristics:

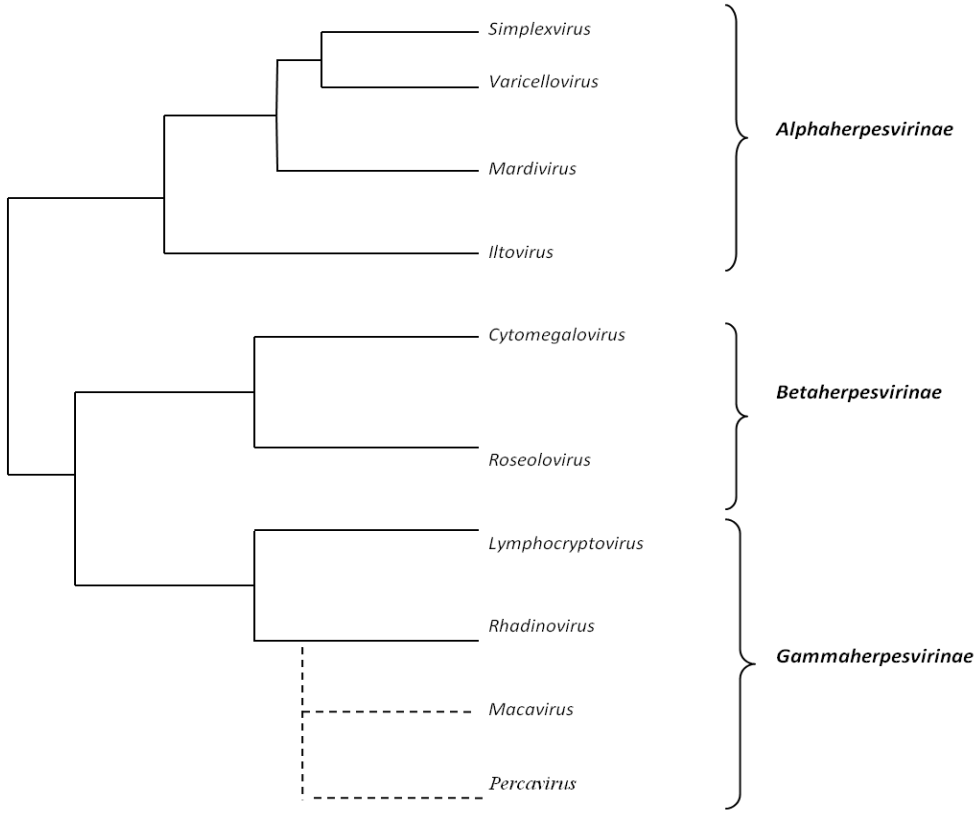
1) They encode a range of enzymes involved in nucleic acid metabolism, DNA synthesis and processing of proteins.
2) The initial synthesis of viral DNA and capsid assembly occurs in the cell nucleus, whereas the final processing of the virion occurs in the cell cytoplasm.
3) When infectious progeny are produced by the parent virus the cell is destroyed.
4) The most well known property of the herpesviruses is their ability to persist in latent form for the host's lifetime, from this latent state the virus can be reactivated and the animal becomes infectious again (Pellet & Roizman 2007; Roizman *et al.* 1992).

The variation of the *Herpesviridae* is represented by their latency sites, their host cell ranges and the length of replicative cycles to result in a wide range of host clinical signs and disease, individual to each virus and its preferred host.

1.2.2 *Herpesviridae* subfamilies

To further classify some of the differences observed between members of the *Herpesviridae*, the family is split into 3 subfamilies, based originally on biological properties (Roizman *et al.* 1992). This classification was made before the widespread use of viral DNA sequencing, but it has been supported by new information gained from viral DNA. They are defined as *alpha*, *beta* and *gamma herpesvirinae*, each containing at least two genera (figure 1.2) (Fauquet *et al.* 2005).

Figure 1.2. Phylogenetic diagram to show relationships within the *Herpesviridae* family that infect mammals and birds. Adapted from (Fauquet *et al.* 2005).



The subfamily *Alphaherpesvirinae* is distinguished from the other two groups by their ability to target a wide range of natural hosts, having a short reproductive cycle, rapid spread in culture, destruction of infected cells and latency usually being established in sensory ganglia (Pellet & Roizman 2007). The wide host range is thought to be due to their low nucleotide substitution rate, resulting in relatively slow evolution and high levels of genetic conservation between the species (Thiry *et al.* 2006a). Characteristic genes are found in the unique long region and both of the repeat regions of the genome. Alphaherpesviruses can be cultured in fibroblasts *in vitro* and in epithelial cells *in vivo* and usually cause visceral epithelial lesions in their natural hosts (Fauquet *et al.* 2005).

Alphaherpesvirinae consists of four genera: *simplexvirus, varicellovirus, mardivirus and iltovirus*. Simplexviruses mainly affect primates, have a rapid cytolytic productive cycle and establish latency in neurons; for example herpes simplex virus-1 (HSV-1) which causes cold sores in humans. Members of the *varicellovirus* genus have been found in a wide-range of mammalian hosts. They frequently cross-react

serologically and establish latency in the sensory nervous system. They include varicella-zoster, the cause of chickenpox and shingles in humans and BoHV-1. Mardiviruses affect only birds, and are associated with malignancy; for example Marek's disease virus that causes a lymphoproliferative disease in chickens. Cell free virus is shed from the feather follicle epithelium, after replication in lymphocytes and possibly macrophages. The *Iltovirus* genus also only affects birds and includes only one species, *gallid herpesvirus-1* that causes avian infectious laryngotracheitis. It was designated a separate genus due to a distinct predicted amino acid sequence (Fauquet *et al.* 2005). Reptilian herpesviruses also belong to the alphaherpesvirus subfamily but have not been assigned to one of the current genera (Pellet & Roizman 2007).

In contrast to the *alphaherpesvirinae,* the *betaherpesvirinae* demonstrate a much smaller host range, a longer reproductive cycle and a slower infection in cell culture. Cytomegaly (the enlargement of cells) is frequently observed along with the establishment of carrier cultures. A wide range of latency sites have been observed including secretory glands, lymphoreticular cells and kidneys among others. The subfamily includes *Cytomegalovirus*, *Muromegalovirus*, *Roseolovirus* and *Proboscivirus* (Pellet & Roizman 2007). The viruses in this subfamily mainly affect humans and primates with the exception of the *Muromegalovirus* genera which represents murine cytomegalovirus. Human cytomegalovirus is responsible for largely asymptomatic persistent infections. However, without the presence of an adequate immune response they can pose a problem and therefore incidence has increased in immuno-compromised people such as organ transplant and HIV patients. It is also the leading infectious cause of birth defects (Landolfo *et al.* 2003).

The host range of *gammaherpesvirinae* is restricted to members of the family or order of the natural host. All gammaherpesviruses replicate in lymphoblastic cells *in vitro*. They are usually specific for either T or B lymphocytes and latency is established in lymphoid tissue. The two established genera of the *gammaherpesvirinae* are *Lymphocryptovirus*, including Epstein-Barr virus and *Rhadinovirus* that historically contain viruses hosted by primates. However, it has

been suggested that new genera, *Macaviruses* including the malignant catarrhal fever virus affecting ruminants and *Percaviruses* of the perssodactyls and carnivores should also be included (Pellet & Roizman 2007; McGeoch, Rixon & Davison 2006).

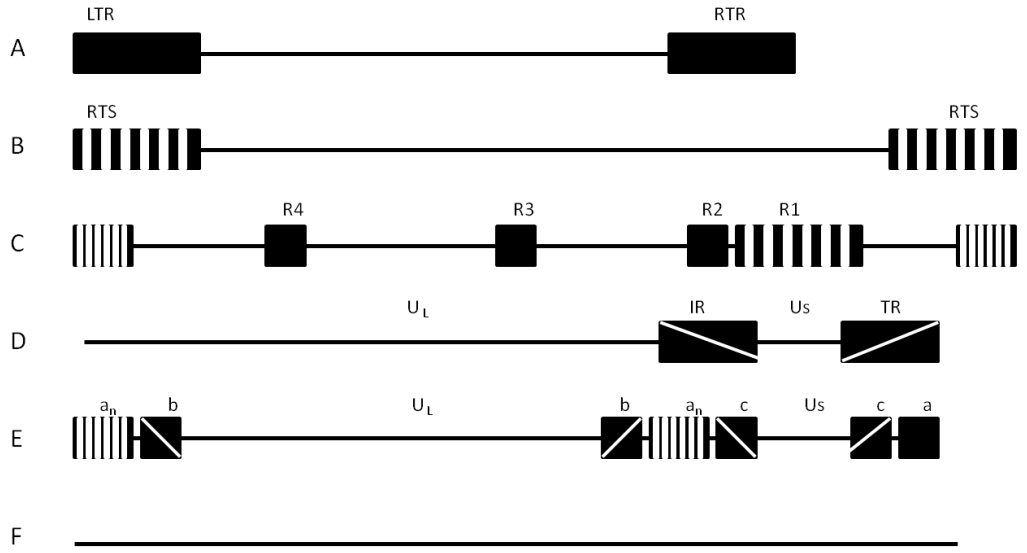
1.2.3 Herpesvirus genetics

All herpesviruses have a double stranded DNA genome that exists in a linear form, except when released from the capsid into the nucleus of an infected cell, when the genome has been shown to circularise (Pellet & Roizman 2007). However, beyond that similarity, extensive diversity can be observed in the genome length, GC content and sequence arrangement of the herpesviruses. The length of herpesvirus genomes ranges from the 124kbp of the simian varicella virus to the 241 kbp of the chimpanzee cytomegalovirus (McGeoch *et al*. 2006). They all contain several key genes (table 1.1), but differences in genome length can be observed between individual viruses of the same species due to repeated sequences at both terminal and internal loci that vary in the number of repetitions. The herpesvirus genomes also have highly variable G and C nucleotide content ranging from 31-77% that fluctuates across the length of the genome with the highest G and C content generally observed in the terminal repeat region (Pellet & Roizman 2007; Roizman *et al.* 1992). Genomes contain unique regions flanked by repeat regions and, can occur in 6 sequence arrangements identified for the herpesviruses discovered so far (figure 1.3). Different isomers are thought to occur by recombination between repeats in concatameric DNA but they do not appear to affect functionality (McGeoch *et al.* 2006). For example sequence arrangement E representing HSV-1 is known to have four isomers (Pellet & Roizman 2007).

Table 1.1 Genes conserved in all herpesvirus subfamilies. Adapted from (Pellet & Roizman 2007).

Gene function category	**Gene function**	**HSV homolog**
Gene regulation	Multifunctional regulatory protein	UL54
Nucleotide metabolism	Ribonucleotide reductase, large subunit	UL39
	Uracil DNA glycosylase	UL2
	dUTPase	UL50
DNA replication	Helicase/primase complex (3 subunits)	UL5, UL8, UL52
	DNA polymerase	UL30
	ssDNA binding	UL29
	DNA polymerase processivity factor	UL42
Virion maturation	Alkaline exonuclease	UL12
	Genome cleavage/packaging	UL28, UL32,
	Terminase/packaging	UL15a
	DNA packaging	UL25
	Scaffold protease	UL26
	Scaffold	UL26.5
	Capsid nuclear egress	UL31, UL34
	Virion	UL16
Capsid	Major capsid protein	UL19
	Minor capsid protein, portal protein	UL6
	Capsid triplex	UL18, UL38
	Hexon tips	UL35
Tegument	Large tegument protein	UL36
	Tegument protein	UL7
	Protein kinase	UL13
	Myristoylated	UL11, UL14,
Envelope	Glycoprotein B	UL27
	Glycoprotein H	UL22
	Glycoprotein L	UL1
	Glycoprotein M	UL10
	Glycoprotein N	UL49.5
Other	Cell-cell fusion	UL24

Figure 1.3. Herpesvirus sequence arrangements adapted from (Pellet & Roizman 2007). Lines represent unique genome regions, blocks represent repeat regions, and broken blocks represent reiterations of the same sequence. LTR/RTR = left and right terminal repeats. RTS = reiterated terminal repeats. IR/TR = internal/terminal repeat. Sequence E has several repeat regions with sequence a reiterated n times associated with either sequence b or c. Sequence arrangement F has not yet been described. Only one isomer is found for arrangements A, B, C and F. There are four possible isomers for arrangement D with the 2 forms of inversions of the Us region most commonly observed. Four isomers have also been identified for arrangement E. Examples of viruses of each arrangement are: A-Human herpesvirus-6, B- Herpesvirus saimiri, C- Epstein-Barr virus, D- Varicella-Zoster virus, E- Herpes simplex virus, F- Tupaia herpesviruses.



Complete genome sequences are available for 47 species (RefSeq 2010). Herpesvirus genomes encode between 70 and 200 genes as well as non-coding RNAs. Most herpesvirus genes are transcribed by RNA polymerase II and transcription is initiated by a promoter sequence 50-200bp upstream of a TATA box. Downstream of the TATA box a transcription initiation site, 5' non-translated leader sequence and a translation initiation codon within a single major open reading frame also facilitate DNA replication (Pellet & Roizman 2007). Introns are generally rare but can be found in up to 10% of genes in some species and are most common in the beta- and gamma herpes viruses (McGeoch *et al.* 2006). Gene functions are still being identified and are mainly compared to the findings in the well-studied HSV-1. Several gene products have been found to interact with numerous proteins, therefore establishing one function of a gene is rarely a definitive result. The role of viral-encoded proteins has been identified as important although 50% of genes are

considered ‘non-essential’ for growth in cell culture. However, many of these proteins are required for viral replication or reactivation from latency. 140 microRNAs have been identified across all 3 subfamilies of virus. MicroRNAs are ~22bp short RNAs that regulate gene expression post transcriptionally, so far they have been identified to have roles in both cellular and viral processes including immunity, angiogenesis, apoptosis, herpesvirus latency and reactivation (Boss, Plaisance & Renne 2009)

1.2.4 Herpesvirus replication

For the replication of herpesviruses, key proteins include those involved in cell entry, viral gene expression regulation, nucleotide metabolism, synthesis of viral DNA, structural proteins and virion assembly (table 1.1). It is also necessary for the virus to avoid the host response to viral gene expression. This will usually involve programmed cell death, which the virus needs to delay long enough to allow viral replication(Pellet & Roizman 2007). Upon the infection of a cell and release of viral DNA into the nucleus, viral replication depends on ‘temporal’ phasic gene expression of 4 key groups of genes. The α (or immediate-early gene) expression is stimulated by tegument proteins such as the α-TIF (alpha gene trans inducing factor). The resulting proteins initiate viral gene replication by stimulating expression of β (or early genes) including genes important in DNA replication such as DNA polymerase. γ (or late genes) are then expressed, sometimes as two groups, ‘leaky-late’ genes that just require the onset of viral DNA synthesis and ‘true’ late genes that are entirely dependent on prior viral DNA synthesis. This final phase of gene expression includes the synthesis of many of the structural proteins such as the glycoproteins, allowing the formation and assembly of progeny virions as capsids containing the viral genome core. Progeny virions are released from the nucleus into the cytoplasm where final processing takes place and the tegument and envelope is formed before release from the cell (Pellet & Roizman 2007). The specific genes and mechanisms involved with specific reference to BoHV-1 replication will be detailed in section 1.3.4.

1.2.5 Herpesvirus latency

The latent state of herpesviruses is exemplified by failure to isolate infectious virus in latently infected cells (Engels & Ackermann 1996). As has been described above, latency is an important characteristic of all herpesviruses; however the sites of latency vary widely between the different subfamilies and species. The latency mechanisms of BoHV-1 will be described in detail in section 1.3.5. The latency of alphaherpesviruses has been well-studied, with HSV-1 frequently being used as the model. Typically, they establish latency in sensory neurons such as the trigeminal ganglia, and a latency-associated transcript (LAT) is expressed which prevents host cell apoptosis, viral replication and infection (Perng & Jones 2010; Quinn, Dalziel & Nash 2000). The process of establishing latency can be more challenging for the gammaherpesviruses, which most commonly establish latency in dividing cells such as B cells. This means they must find a way to stop the viral genome from being diluted by the process of mitosis in the host cells. In KSHV the viral genome is thought to replicate before mitosis and segregate to the daughter cells. A protein known as latency associated nuclear antigen (LANA) is consistently expressed throughout latency (Coscoy 2007). However, it is thought to restrict its own translation and prevents proteasomal degradation to reduce the production of viral epitopes that could be made available for MHC 1 molecules on the cell surface. This also occurs in Epstein-Barr and murine gammaherpesvirus 68. This demonstrates possible conservation of an immune evasion mechanism between several gammaherpesviruses (Coscoy 2007). After the initial infection the virus will enter the cell types associated with latency for that species. Regulation of this state and reactivation of the virus when viral DNA replication reoccurs usually involves some genetic component, but there are no genetic factors known to control maintenance in a latent form or reactivation from latency common to all herpesviruses (Pellet & Roizman 2007).

1.2.6 Herpesvirus evolution

The three subfamilies of *Herpesviridae* are estimated to have arisen between 180 and 220 million years ago (McGeoch *et al.* 1995). Studying sequence homology from the different genomes is key to identifying conserved functions and learning more about

herpesvirus evolution (Alba *et al.* 2001). The core genes identified as common across all 3 subfamilies (table 1.1), are mainly located in the central region of the genomes with non-core, more recently evolved genes located at more terminal locations (McGeoch *et al.* 2006). Genetic diversity has been gained by gradual mutation by methods including nucleotide substitution, insertion and deletion which can lead to changes in gene function, or whole genes are gained or lost. Recombination mechanisms have also been shown to play a role in creating genetic diversity (Davison 2002). Gene duplication has occurred in the herpesviruses, particularly in the beta subfamily. Lateral transfer from host cells is also possible, as demonstrated by the capture of β-1,6-N-acetylylglucosaminyltransferase-mucin gene by bovine herpesvirus-4. Lateral transfer between viruses is also thought to have occurred, resulting in distinct genotypes with geographical associations, for example in genes associated with latency in Epstein Barr virus (McGeoch *et al.* 2006).

Phylogenetic studies have been used to document the history of herpesvirus evolution. The separation of the three subfamilies can clearly be demonstrated, with further divisions indicating the different genera showing that the biological properties used to define the different subfamilies and genera are supported by sequencing data. Another of the main findings from this work has been that features of the phylogeny correspond with host organisms, proving evidence of co-evolution of viruses alongside their hosts (McGeoch *et al.* 1995; McGeoch, Dolan & Ralph 2000). Models of co-evolution can be particularly useful to calculate a time scale for virus evolution, as there is no fossil record available. Current evidence shows herpesviruses have existed for longer than phylogenetic trees can measure, and generally they evolve faster than their hosts (Davison 2002). Another study used a whole genome approach to study molecular functions of homologous protein families from 19 herpesviruses to create genome-wide phylogenetic trees and determine conserved functions, which were found to agree with molecular sequence derived trees (Alba *et al.* 2001). This is a growing field of virology and as more herpesvirus sequences become available further information will be derived about their evolution.

1.3 Bovine herpesvirus-1 (BoHV-1)

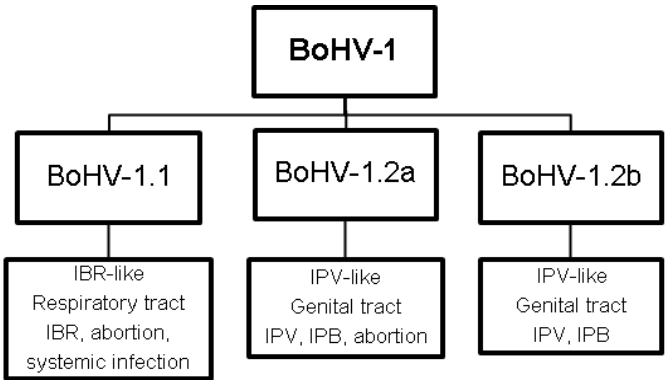
1.3.1 BoHV-1 Classification

BoHV-1 was first isolated in bovine embryonic kidney cells in 1956 from outbreaks of infectious bovine rhinotracheitis (IBR) in California where the disease was first reported 3 years earlier (Madin, York & McKercher 1956). BoHV-1 is in the *Varicella* genus of the alphaherpesvirus sub-family, and is closely related to *suid herpesvirus-1* (pseudorabiesvirus), *equine herpesvirus-1* and *human herpesvirus-3* (varicella-zoster virus). It has a typical alphaherpesvirus structure and a type D genome as described in 1.2.1 and 1.2.3. Its main host is cattle, however as is typical of an alphaherpesvirus it has the potential to cause cross-species infection. Sheep and goats have shown mild symptoms, and latency has been demonstrated after an experimental infection, although reactivation from latency was not observed (Engels *et al.* 1992). Red deer and reindeer have been found to be positive for BoHV-1 after experimental challenge but titres of virus excreted were very low and considered unlikely to be sufficient to facilitate further spread of the virus (Thiry *et al.* 2006b).

BoHV-1 isolates have been divided into 3 subtypes based on genetic variation and on differences in the clinical aspects of the resulting infection (figure 1.4). BoHV-1.1 is most commonly found in respiratory cases of IBR, but can also cause abortion and systemic infection. This subtype was originally seen in the USA in the 1960's but is now also the most common subtype found in the UK (Graham 2007). BoHV-1.2a is usually found in genital cases causing infectious pustular vulvovaginitis (IPV) in cows and heifers or infectious pustular balanoposthitis (IPB) in bulls and has also been isolated in cases of abortion (Miller, Whetstone & Van Der Maaten 1991). This subtype is not currently present in the UK. BoHV-1.2b is also associated with genital disease but has not been found in abortion cases. BoHV-1.2b was the subtype most common in the UK when BoHV-1 was first observed in the UK in 1961, however since 1977 the genital form of BoHV-1 has been less common and most outbreaks are associated with BoHV-1.1, causing the respiratory form of the disease (Graham 2007). Despite the subtypes usually being associated with different routes of infection, all 3 subtypes can cause infection of both the respiratory and genital tracts

(Spilki *et al.* 2004). Historically, bovine herpesvirus-5 (BoHV-5) was originally grouped as subtype BoHV-1.3 which showed neuropathogenic characteristics and has since been reclassified as BoHV-5 (Edwards, White & Nixon 1990). The subtypes can be distinguished using restriction enzyme digestion with *Hind III* (Misra, Babiuk & le Q Darcel 1983; Mayfield et al. 1983; Edwards et al. 1990).

Figure 1.4. Diagram showing subdivision of BoHV-1. Adapted from (Graham 2007).



1.3.2 BoHV-1 history and epidemiology

BoHV-1 is found worldwide with high prevalence of infection but a low incidence of disease. Most direct economic losses are from death and abortions in more serious incidences of disease, but it also has an impact on international trade. Seroprevalence varies extensively from 10-50% or even higher depending on geographical area, herd vaccination practices and frequency of mixing of animals with different infection statuses. Due to the more frequent introduction of susceptible individuals, morbidity and mortality have been reported to be higher in feedlot cattle (20-30% and 1-10% respectively) compared to dairy herds (8 and 3 % respectively) (Radostits *et al.* 2000). Several risk factors have been identified for seroprevalence including age, sex (with more positive males), herd size, contact with animals from other herds, farm and cattle density (Muylkens *et al.* 2007).

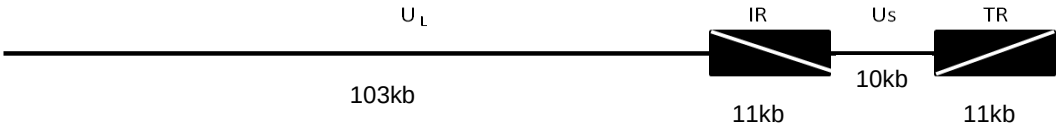
While a worldwide disease, it is most common in North and South America and Europe. The genital form of the disease was first reported in Germany in the 1920's (Ackermann & Engels 2006). This was the most common form of BoHV-1 infection

in the UK until the 1970's when stricter measures were introduced in artificial insemination centres (Radostits *et al.* 2000). The more serious respiratory disease was first reported in the 1950's, and the virus was subsequently isolated in California, from where it spread to other parts of North America. In 1977 the first outbreaks of the respiratory disease occurred in the north east of Scotland, from where it spread to other parts of the UK. Throughout the 1990's several countries in Europe have attempted to eradicate the virus, so far Denmark, Sweden, Switzerland, Finland and Austria have been successful (Ackermann & Engels 2006; Graham 2007; Radostits *et al.* 2000). However, there is still some debate over whether eradication is the best long term solution due to the losses of healthy virus carriers and high associated costs (Ackermann & Engels 2006).

1.3.3 BoHV-1 genetics

BoHV-1 has a 135kb double stranded DNA genome sequence containing 73 known open reading frames (ORFs) (table 1.2) (Robinson *et al.* 2008). The genome, fully sequenced in 1995, demonstrated the 73 ORFs represented 69 unique genes and two genes replicated in each of the repeat regions (Schwyzer & Ackermann 1996). The BoHV-1 genome represents sequence arrangement D of the 6 observed herpesvirus genome sequence arrangements (figure 1.3). This means it consists of two unique coding regions, a long segment U_L and a short segment U_S. These regions are separated by an internal repeat region, which is the inverted sequence of that found in the terminal repeat region found on the U_S end (figure 1.5).

Figure 1.5. Diagram representing basic genome structure of BoHV-1. IR= internal repeat; TR= terminal repeat.



Most of the genes in BoHV-1 have counterparts in HSV-1, and were therefore named after the homologs (table 1.2). However, some novel genes have been identified, including UL0.5, UL0.7, UL3.5, *circ* and US 1.67, which do not have homologs in

HSV-1. At least 10 genes encode the glycoproteins which are part of the virion envelope (figure 1.1) and are therefore mainly associated with the initial interaction with host cells and also with the host's immune response. There are several enzymes important in genome replication including ribonucleotide reductase, thymidine kinase, dUTPase and DNA polymerase. Other proteins are involved in virion assembly, structure and tegument proteins. The final group of genes are the regulatory genes that control both lytic and latent replication (Schwyzer & Ackermann 1996). Since the sequencing of the whole genome, much analysis of individual and groups of genes has been conducted to learn more about gene function. In particular, there has been a focus on identifying whether genes are essential or non essential for replication in cell culture (Robinson *et al.* 2008). A full study of BoHV-1 genes found 33 essential and 36-non essential genes. Most were consistent with HSV-1 homolog requirements. However, UL54, UL53, UL49.5, UL49, UL35, UL20, UL16 and UL7 differed in their requirement, the significance of this is as yet unknown. There was also a difference found in the requirement of *circ*, which had previously been identified as non-essential in the Jura strain, but in 1.2b it does appear to be essential (Robinson *et al.* 2008). This study also highlighted that demonstration of cytopathic effect may not be sufficient to identify essential or non-essential genes as real-time PCR showed presence of viral DNA when no cytopathic effect was observed.

The lytic phase of viral replication involves many of the regulatory genes by the process of a temporal cascade of gene expression (Misra, Blumenthal & Babiuk 1981; Wirth *et al.* 1989; Seal, Irving & Whetstone 1991). It is initiated by activation of immediate early genes, the first of the temporally controlled phases of gene expression that are largely associated with gene regulation, followed by early and late gene expression phases (table 1.2).

In addition to the standard cascade expression of genes in the lytic phase of the virus there have recently been discoveries of microRNAs encoded and expressed by BoHV-1 during infection. A recent study showed that of 10 microRNAs encoded by the BoHV-1 genome, four of these are found in repeat regions and therefore the total

number could be considered 14. Similar findings have been demonstrated in other herpesviruses and may be a characteristic of the group (Glazov *et al.* 2010). More research is required to establish the importance of these microRNAs in the regulation of gene expression. In other herpesviruses they have been identified as potentially important in the transition between the lytic and latent replication (Cullen 2009).

Table 1.2. Products and functions of the genes of BoHV-1 (IE – immediate early, E- early, L-late, Essential refers to whether they are required for growth in cell culture). Created with reference to Refseq NC_001847 and (Robinson *et al.* 2008).

ORF	Product	Expression phase	Function	BoHV-1 specific	Essential
Circ	Circ protein	IE	Unknown, potentially envelope associated	Y	Y
UL54	Multifunctional expression regulator (ICP27)	E	Gene regulation/RNA transport and metabolism	N	N
UL53	Envelope glycoprotein K	L	Virion morphogenesis/membrane fusion	N	Y
UL52	Helicase-primase primase subunit	E	DNA replication	N	Y
UL51	Tegument protein	L	Virion morphogenesis	N	N
UL50	dUTPase	E	Nucleotide metabolism	N	N
UL49.5	Envelope glycoprotein N	L	Virion morphogenesis	N	N
UL49	Tegument protein VP22	L	Virion morphogesis/possible RNA transport to uninfected cells	N	N
UL48	Transactivating tegument protein VP16	L	Gene regulation/virion morphogenesis	N	N
UL47	Tregument protein VP8	L	Possible gene regulation	N	N
UL46	Tegument protein VP11/12	L	Possible gene regulation	N	N
UL44	Envelope glycoprotein C	L	Cell attachment	N	N
UL43	Envelope protein	L	Possible membrane fusion	N	N
UL42	DNA polymerase processivity subunit	E	DNA replication	N	Y
UL41	Tegument host shut off protein	L	Cellular mRNA degradation	N	N
UL40	Ribonucleotide reductase subunit 2	E	Nucleotide metabolism	N	N
UL39	Ribonucleotide reductase subunit 1	E	Nucleotide metabolism	N	N
UL38	Capsid triplex subunit 1	L	Capsid morphogenesis	N	Y
UL37	Tegument protein	L	Virion morphogenesis	N	Y
UL36	Large tegument protein	L	Capsid transport	N	Y
UL35	Small capsid protein	L	Capsid morphogenesis/possibly capsid transport	N	N
UL34	Nuclear egress membrane protein	Unknown	Nuclear egress	N	Y
UL33	DNA packaging protein	Unknown	DNA encapsidation	N	Y
UL32	DNA packaging protein	L	DNA encapsidation	N	Y
UL31	Nuclear egress lamina protein	L	Nuclear egress	N	Y
UL30	DNA polymerase catalytic subunit	E	DNA replication	N	Y
UL29	Single-stranded binding protein	E	DNA replication/ possible gene regulation	N	Y
UL28	DNA packaging terminase subunit 2	L	DNA encapsidation	N	Y
UL27	Glycoprotein BALF4	L	Cell entry/cell-cell spread	N	Y
UL26	Capsid maturation protease	L	Capsid morphogenesis	N	Y
UL26.5	Capsid scaffold protein	L	Capsid morphogenesis	N	Y
UL25	DNA packaging tegument protein	L	DNA encapsidation	N	Y

Table 1.2 (cont)

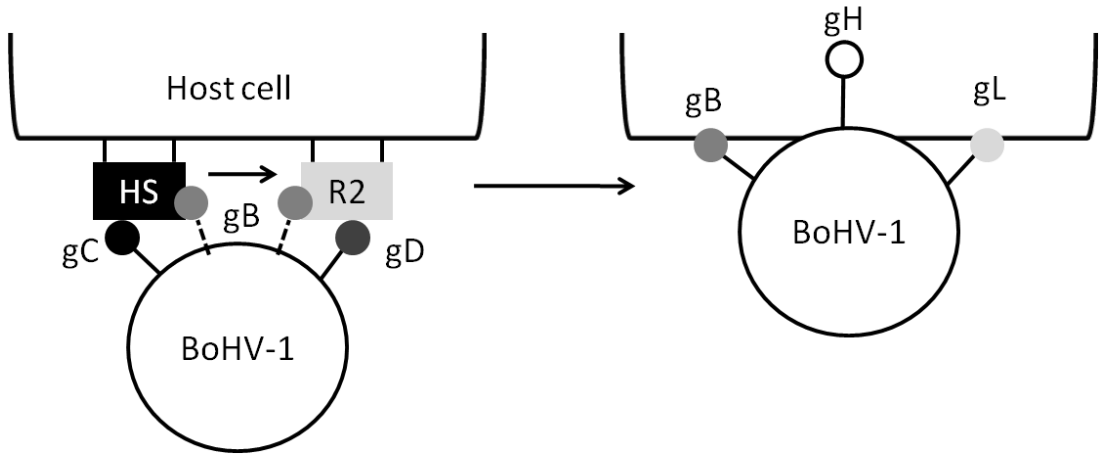
ORF	Product	Expression phase	Function	BoHV-1 specific	Essential
UL24	Nuclear protein	L	Unknown	N	N
UL23	Thymidine kinase	E	Nucleotide metabolism	N	N
UL22	Envelope glycoprotein H	L	Cell entry/cell-cell spread	N	Y
UL21	Tegument protein	Unknown	Virion morphogenesis	N	N
UL20	Envelope protein	L	Virion morphogenesis/membrane fusion	N	Y
UL19	Major capsid protein	L	Capsid morphogenesis	N	Y
UL18	Capsid triplex subunit 2	L	Capsid morphogenesis	N	Y
UL17	Tegument protein	L	DNA encapsidation/capsid transport	N	Y
UL16	Tegument protein	L	Possible virion morphogenesis	N	Y
UL15	DNA packaging terminase subunit 1	L	DNA encapsidation	N	Y
UL14	Tegument protein	L	Virion morphogenesis	N	Y
UL13	Tegument serine/threonine protein kinase	L	Protein phosphorylation	N	N
UL12	Deoxyribonuclease	E	DNA processing	N	N
UL11	Alkaline exonuclease	L	Virion morphogenesis	N	N
UL10	Envelope glycoprotein M	L	Virion morphogenesis	N	N
UL9	DNA replication origin-binding helicase	L	DNA replication	N	Y
UL8	Helicase-primase primase subunit	E	DNA replication	N	Y
UL7	Tegument protein	E	Virion morphogenesis	N	N
UL6	Capsid portal protein	E	DNA encapsidation	N	Y
UL5	Helicase-primase subunit BBLF4	E	DNA replication	N	Y
UL4	Nuclear protein	Unknown	Unknown	N	N
UL3.5	Protein V57	L	Possibly virion morphogenesis	Y	Y
UL3	Nuclear protein UL3	L	Unknown	N	N
UL2	Uracil DNA glycosylase	E	DNA repair	N	N
UL1	Envelope glycoprotein L	L	Cell entry/cell-cell spread	N	Y
UL0.7	Unknown	Unknown	Unknown	Y	N
UL0.5	Unknown	Unknown	Unknown	Y	N
BICP4	Transcriptional regulator ICP4	IE	Gene regulation	N	Unknown
BICP0	Ubiquitin E3 ligase ICP0	IE	Gene regulation/cellular protein	N	N
BICP22	Regulatory protein	IE	Gene regulation/cell-cycle regulation	N	Unknown
US1.67	Virion protein V67	Unknown	Unknown	Y	Unknown
US2	Virion protein	Unknown	Unknown	N	N
US3	Serine/threonine kinase	E	Protein phosphorylation/nuclear	N	N
US4	Envelope glycoprotein G	L	Cell to cell spread	N	N
US6	Envelope glycoprotein D	L	Cell attachment	N	Y
US7	Envelope lycoprotein I	L	Cell to cell spread	N	N
US8	Envelope glycoprotein E	L	Cell to cell spread	N	N
US9	Membrane protein	E	Axonal transport	N	N

1.3.4 BoHV-1 pathogenesis

Transmission of infection usually occurs by direct contact, either nose to nose, direct genital contact, or via virus infected semen during mating (Muylkens *et al.* 2007). Aerosol transmission has also been demonstrated over short distances (Mars *et al.* 2000). In the case of the respiratory form of the infection, the virus enters the nasal passages and upper respiratory tract where it targets epithelial cells and the first round of viral replication occurs (Engels & Ackermann 1996).

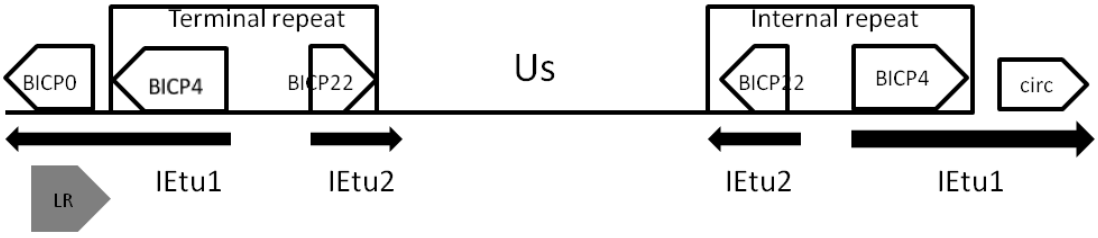
When infection occurs through the respiratory route, and following exposure of respiratory epithelium, cell entry is a multistep process primarily involving the glycoproteins found in the viral envelope. It is well documented that glycoprotein C binds to heparan sulfate (HS) proteoglycans located on the cell surface of many cell types (Okazaki *et al.* 1991), glycoprotein D then completes the attachment by binding to the second cellular receptor. It has also been found that glycoprotein B plays an important role and binds initially to HS before binding to the secondary non-HS receptor (Li *et al.* 1995). Interactions between glycoproteins B, H and L then allow fusion of the viral envelope with the cell membrane, to ultimately release the capsid into the cell cytoplasm (Nandi *et al.* 2009) (figure 1.6).

Figure 1.6. Schematic diagram showing mechanisms of BoHV-1 cell entry. HS = heparan sulfate, R2 = cell receptor 2, g = glycoprotein.



Upon cell entry, a dynein motor complex, together with microtubules, transports virus particles through the cytoplasm to the cell nucleus where the viral DNA is released. Tegument proteins are released in the cytosol at this stage and are likely to play a role in the early stages of viral infection such as shut down of host cell protein synthesis by the host shut off (vhs) protein, common to all alphaherpesviruses, which degrades cellular mRNA by endoribonucleolytic cleavage (Elgadi, Hayes & Smiley 1999). The largest BoHV-1 virion protein (VP) 8 (UL47), a homologue of VP13/14 in HSV-1, is also released at this stage and a nuclear localization signal causes its abundance in the nucleus soon after infection, although its function is unknown (Muylkens *et al.* 2007). VP 16 (UL48/α-trans inducing factor) is also released at this stage which initiates the cascade of viral DNA replication. VP16, combined with cellular proteins, binds to a specific motif in the promoter sequence of two immediate-early transcription units (IEtu1 and IEtu2) (Wirth, Vogt & Schwyzer 1991; Misra *et al.* 1995). IEtu1 encodes bICP0 and bICP4 that have homologs in HSV-1, but it also activates *circ* that is specific to BoHV-1 and is present throughout the viral cycle, IEtu2 encodes BICP22 (figure 1.7) (Schwyzer & Ackermann 1996). Regulation of immediate-early genes in BoHV-1 potentially differs from those found in HSV-1 as they are found to accumulate throughout the replication cycle rather than degrade after a peak, 3 hours post infection (Seal *et al.* 1992).

Figure 1.7. Schematic view of immediate early genes (blue) and latency related transcript (LR) (red). Us – unique short region of genome. Adapted from (Muylkens *et al.* 2007).



After the synthesis of bICP0, bICP4 and bICP22 proteins, early gene expression is activated encoding proteins necessary for viral DNA replication such as DNA polymerase, although the exact mechanisms stimulating this are not known. The process of DNA replication is still being researched, but work on HSV-1 suggests circularised DNA undergoes theta replication from two origin of replication sites

(*ori*), within the inverted repeat sequence (Chew, Choi & Leung 2005). Bidirectional replication then forms concatameric DNA with genomes arranged head to tail, and would allow recombination events to form the four possible isomers seen in HSV-1. However, BoHV-1 genomes are rarely found in the inverted U_L region isomer. Experiments have shown that this is due to the cleavage and packaging of DNA, rather than the U_L region not inverting, as adjacent U_L regions are found in abundance in concatameric DNA (Schynts *et al.* 2003a). As viral DNA replicates, capsid shell proteins assemble containing a scaffold that the DNA genome then replaces during packaging. The first wave of late gene expression, sometimes referred to as 'leaky-late' or γ_1 genes are detected during DNA replication but the second phase of late gene expression is entirely dependent on the completion of DNA synthesis. Late gene expression controls virion assembly and release of the progeny viruses. The late genes largely consist of genes associated with the structure of the virus such as the glycoprotein and envelope proteins that are needed to complete the production of progeny viruses in the cytoplasm of the host cell (table 1.2) (Muylkens *et al.* 2007).

The capsids containing the genome are released into the cytoplasm for the final phase of gene expression, which translate proteins to complete the virion structure and the final stages of assembly take place (Muylkens *et al.* 2007). Currently the view of capsid release and acquisition of the envelope is not fully determined. Capsid egress from the nucleus is a three step process, beginning with the acquisition of a primary envelope by budding through the inner nuclear membrane. Secondly, the virus fuses the primary envelope with the outer membrane of the nucleus to gain entry to the cytoplasm; this process has been shown to require viral kinase activity. Finally, virions must gain their tegument layer in the cytoplasm and the secondary envelope thought to be acquired by budding into the trans Golgi compartment (Wild *et al.* 2005; Leuzinger *et al.* 2005). However, it should be noted this mechanism of capsid egress does have two alternative theories. One suggests budding through the inner nuclear membrane contributes to the primary envelope, and the virion is then transported to the endoplasmic reticulum and Golgi secretory pathway by intra-luminal transport (Muylkens *et al.* 2007). The second theory, supported by microscopic observations suggests dilation of nuclear pores allows the capsids direct

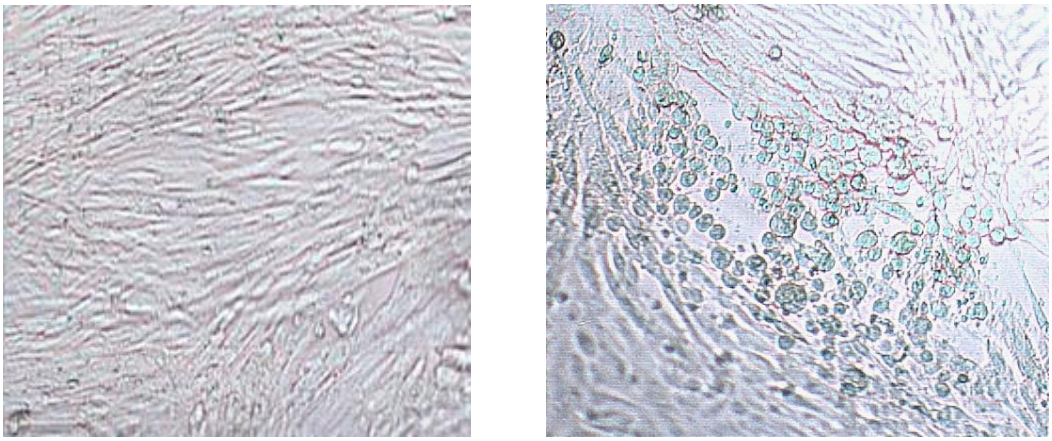
egress to the cytoplasm, and envelopes are gained by budding at any cell membrane (Wild *et al.* 2005).

In a natural infection, the new progeny viruses are then shed in the nasal, tracheal and ocular mucus. Progeny viruses may also spread to other cells within the host by two mechanisms. Viruses released into the extracellular matrix are fully enveloped and able to interact with cell receptors to enter via the epithelial surface by the route previously described (Muylkens *et al.* 2007). Viral particles can also spread directly from cell-cell across cell junctions, avoiding the host immune response, which requires glycoproteins B, D, G, H, L, E and I (Johnson & Huber 2002). VP22 coded for by UL49 has been identified as essential for this process in a study of gE and VP22 mutant viruses (Kalthoff *et al.* 2008).

In vitro the cytopathic effect (CPE) can be observed in infected bovine cells in culture, in the form of the rounding, shrinking and clumping of cells first described in 1956 (figure 1.8) (Madin *et al.* 1956). Its ability to grow well in culture has resulted in BoHV-1 becoming a well studied virus, often used as a model for studying alphaherpesviruses.

Figure 1.8. BoHV-1 infection in cell culture. A) Uninfected cells; B) BoHV-1 infected cells.

A) B)



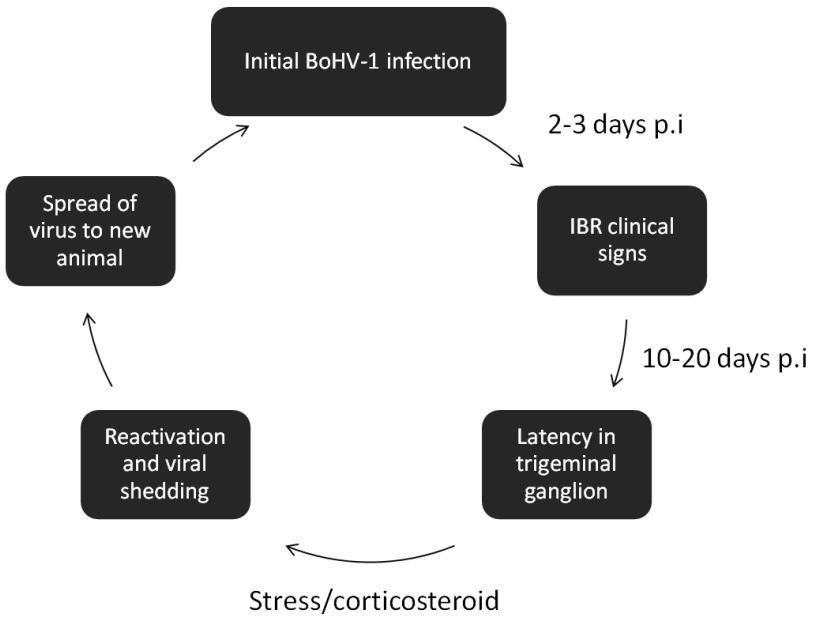
In vivo, the process of viral infection of a cell and production of progeny viruses results in cell death due to necrosis and apoptosis (Muylkens *et al.* 2006). This cell

death triggers an inflammatory response that leads to the clinical signs observed during a BoHV-1 infection (Nandi *et al.* 2009). These effects may be increased by prevention of repair of the airway epithelium by inhibition of new epithelial cell migration to damaged sites, shown in a sheet-migration assay (Spurzem *et al.* 1995).

1.3.5 BoHV-1 latency and reactivation

After the initial infection process, or vaccination with an attenuated strain, the virus is able to establish latency (figure 1.9), when viral genomes can remain in an inactive non-replicative state for up to the lifetime of the animal. In BoHV-1 this occurs most commonly in the trigeminal ganglion. Neuroinvasion is thought to occur through the olfactory and trigeminal nerves, by infection of the nerve endings at the oro-nasal mucosa; this route is also used by closely related neurotropic viruses suid herpesvirus-1 and BoHV-5. BoHV-1 predominantly uses the trigeminal route and usually stops in the cell body of the sensory neuron, in the trigeminal ganglia, where latency is established rather than continuing on to cause neurological clinical signs, as seen in BoHV-5 and PrV (Enquist *et al.* 1998). Past research has also demonstrated evidence of latency and reactivation of BoHV-1 in the lymphoid tissue of tonsils (Winkler, Doster & Jones 2000).

Figure 1.9. BoHV-1 infection cycle.



The latent state of the virus is maintained by virally encoded genes, primarily by the expression of the BoHV-1 latency related (LR) transcript (figure 1.7). The LR transcript is antisense to the BICP0 regulatory gene and is thought to be a key component. The LR transcript shows similar properties to the latency associated transcript (LAT) identified in HSV-1; in particular it inhibits BICP0 expression during latency, which prevents initiation of the viral replication cycle (Jones *et al.* 2006; Jones 2003). Although this relationship between LAT and ICP0 in HSV-1 latency and reactivation has been questioned in studies of LAT and ICP0 mutant viruses (Quinn *et al.* 2000). The process can be divided into three main steps; firstly the establishment of latency by the entry of the genome into the sensory neurons where replication will continue to occur during infection. Secondly, the viral gene expression described above is then replaced by LR expression in BoHV-1 or LAT expression in HSV-1, which marks the transfer into the second stage, maintenance of latency, during which no viral DNA replication or lytic viral gene expression occurs (Jones 2003). The host immune system is also thought to play a role in confining the virus to sensory ganglia in its latent state. For example CD8+ T cells were shown to be present in close proximity to neurons containing latent HSV-1 in a mouse model (Liu *et al.* 2000). The third stage refers to the reactivation of the virus.

Reactivation has been shown to be stimulated by situations of stress such as parturition (Thiry *et al.* 1985) and transport (Thiry *et al.* 1987). It can also be stimulated experimentally by a large dose of corticosteroids (Graham 2007). This final stage of latency initiates viral DNA replication as described previously, temporal gene expression and production of infectious virus (Jones 2003). This switch from latency to lytic gene expression allows re-release of virus. The virus is transported intra-axonally back down the sensory neurons to replicate in epithelial cells, and virus can be shed from the initial point of entry (Engels & Ackermann 1996). Whether infectious virus is shed in detectable titres that can spread to other animals and cause recrudescence of disease depends on the immune status of the animal and progeny virus phenotype (Muylkens *et al.* 2007). Reactivated virus from latently infected animals can infect susceptible animals at birth, following transport, and on introduction of naïve animals to the herd. Identifying latent carriers, usually by serology, is vital for the local control of disease (Muylkens *et al.* 2007).

1.3.6 Genetic variation in BoHV-1 and closely related viruses

Several of the genetic differences found between BoHV-1 and other species in the group have been connected to variations in the glycoproteins (g). For example differences in gC have been found between BoHV-1 and BoHV-5 (Esteves *et al.* 2008; Ros & Belak 2007); this could potentially represent differences in cell tropism as gC is strongly associated with cell entry and the two viruses represent different cell entry patterns, given the neurotropic characteristics of BoHV-5. A comparison between five ruminant alphaherpesviruses including BoHV-5, caprine herpesvirus-1, cervine herpesvirus-1, rangiferine herpesvirus-1 and BoHV-1 showed gB to be highly conserved but that gD was much more variable, with identity ranging from 71.3-98.9% (Ros & Belak 2007).

DNA polymerase and thymidine kinase have been the focus of other studies relating genetic variation to clinical outcome of disease. In equine herpesvirus-1, closely related to BoHV-1, a single point mutation in the DNA polymerase coding region was found to have an effect on the neuropathogenicity of the infection (Goodman *et al.* 2007; Nugent *et al.* 2006). The differences were determined by sequencing of two strains, Ab4, which frequently caused severe clinical disease including abortion and signs of neurological damage, and strain V592 which caused less serious clinical signs of fever and mild respiratory illness. After sequencing it was possible to identify the same differences in field isolates by PCR and sequencing methods at nucleotide 2254 of ORF30 that encodes DNA polymerase (Nugent *et al.* 2006). An experimental infection also comparing these strains found a higher and longer duration of leukocyte associated viraemia in isolates with neuropathogenic potential (Allen & Breathnach 2006). These studies are also supported by a study investigating this mutation in terms of its pathogenic potential (Goodman *et al.* 2007). However, it is not entirely clear what mechanism this mutation acts on as it was found not to have any effect on growth kinetics of the virus in cultured murine neuronal cells (Yamada *et al.* 2008).

Thymidine kinase (TK) is thought to be related to BoHV-1 virulence and has been specifically associated with abortion in experimental infection studies. An intravenous infection showed a thymidine kinase deletion mutant significantly

reduced abortifacient activation of BoHV-1 compared to the wild type Cooper isolate (Miller *et al.* 1991). However, the intravenous route of inoculation does not represent a natural route of infection, which resulted in the Cooper isolate (control) infected group showing more abortifacient activity than would usually be expected from a BoHV-1 infection. The differences seen could therefore be due to other factors such as speed of replication. Little work investigating this finding further has been conducted since.

There have also been reports of differences in BoHV-1 gene expression between different BoHV-1 subtypes. The subtype 1.1 Jura strain was found to show earlier transcript expression in much higher abundance than the 1.2b strain K22 in a study to spatially and temporally classify BoHV-1 transcripts using northern blot analysis (Wirth *et al.* 1989).

Recombination has been shown to be an important source of variation in herpesviruses and BoHV-1 is no exception. An *in vivo* infection with two strains of BoHV-1 has demonstrated that recombination is a frequent event (Schynts *et al.* 2003b). A later study showed this was restricted to two very closely related viruses, and the infections have to be simultaneous or very close to allow recombination to occur. It is thought that this can result in both a mechanism for long term evolution, as well as immediate strain diversity that can play a role in strain virulence (Thiry *et al.* 2006a).

1.3.7 Clinical presentations of BoHV-1

The main disease associated with BoHV-1 is the respiratory disease IBR, which can be recognized by the presence of typical clinical signs. A high fever is usually observed within 24 hours of infection, although if not closely monitored, this stage of the infection may be missed. This is followed by nasal discharge containing infectious virus, conjunctivitis and dry coughing due to tracheal lesions. In dairy cattle, a drop in milk yield is often seen two to three days post infection. The clinical signs usually peak on day 4 post infection, and where there are no complications, disappear within 8-10 days, as the virus enters its latent phase (Graham 2007; Muylkens *et al.* 2007).

Complications of BoHV-1 infection can cause other clinical presentations, generally more severe and in some cases with fatal consequences, such as encephalitis, abortion or systemic infection. Secondary bacterial infections are particularly common in calves infected with BoHV-1, as it increases susceptibility to *P.multocida* and *M. haemolytica* due to lysis of ciliated respiratory epithelium, disrupting the normal bacterial clearance mechanisms and also suppression of the immune response, which can lead to pneumonia. These mixed infections are known as BoHV-1 associated bovine respiratory disease (BRD) that results in severe lower respiratory tract disease (Ellis 2009; Jones & Chowdhury 2007; Jones & Chowdhury 2010; Srikumaran, Kelling & Ambagala 2007). The effect of a combined BoHV-1 and *P. haemolytica* has been demonstrated in experimental coinfections, where BoHV-1 destroyed the mucociliary apparatus, which facilitates the proliferation of the bacteria and increased the endotoxin released by *P. haemolytica*, found in the bronchoalveolar lavage fluid (Narita *et al.* 2000a). There have also been sporadic reports of BoHV-1 being isolated from cases of encephalitis (Penny *et al.* 2002; Higgins & Edwards 1986; Straub 1991). It appears the neuroinvasion may extend beyond latency in the trigeminal ganglia in these cases, but it is considered to be due to differences in host susceptibility to CNS infection rather than a tropism for the CNS in particular strains (Muylkens *et al.* 2007). In young calves and pregnant cows the virus has also been found to spread systemically causing systemic infection or abortion (Higgins & Edwards 1986; Ormsbee 1963; Miller *et al.* 1991).

1.3.8 BoHV-1 systemic infection and abortion

Research by Ormsbee recorded the first abortion in which BoHV-1 was considered to be the causal agent in 1963 (Ormsbee 1963). This was several years after IBR was first observed. It may simply have not been recognised and recorded until this point, or it could indicate adaptation of the virus to enable systemic spread. Clinical signs of IBR disease are not always observed in the dam and abortion usually occurs in the third trimester of pregnancy (Straub 1991). Abortions due to natural BoHV-1 infections are generally observed between 4 and 8 months gestation (Smith 1997). From the time of foetal infection, foetal death is thought to occur within 24-48 hours, but the foetus may be retained for up to 7 days before abortion (Kendrick, Schneider & Straub 1971).

Surveys conducted to establish the prevalence of BoHV-1 as an abortifacient agent have produced mixed results. In a survey of 149 bovine abortions between 1984 and 1986 in the north west of England 13% were estimated to be related to BoHV-1 using histopathology and paired serology (Murray 1990). A 10 year study in North America investigating viral causes of abortion found BoHV-1 to be the most common cause. A prevalence of 5.41% in bovine abortion cases was found based on gross and histo- pathology, virus isolation and immunofluoresent antibody test (Kirkbridge 1992b). However, currently in the UK it is not considered a common cause for abortion. A study of abortions in Scotland in 1995 did not identify any cases of BoHV-1, but as the authors highlight it was most likely due to the reliance of detection of foetal antibody (Caldow *et al.* 1996). The most recent survey of bovine abortions in the UK found less than 2% of bovine abortions were related to BoHV-1 between 2004 and 2008 in Scotland (SAC VS 2009).

Several studies have been conducted describing the pathology of foetal infection leading to several key findings (Owen, Chow & Molello 1964; Molello *et al.* 1966; Kendrick *et al.* 1971; Rodger *et al.* 2007). All studies consistently report extensive multifocal necrotic lesions in the foetal liver. However, observations in the placenta have been more varied. An early study focussing on placental changes during infection suggested placental infection was secondary to foetal infection as a result of failure of foetal circulation (Molello *et al.* 1966). However, the spread of infection has been described in other studies to involve a slow cell-cell spread of the virus between placentomes, explaining the time lapse between initial infection and foetal death (Kendrick *et al.* 1971). There have also been reports of association of BoHV-1 with the endothelium of blood vessels in the placenta and foetal tissues (Rodger *et al.* 2007). However, these last results should be interpreted cautiously as this was as a result of an intravenous experimental challenge. A haematogenous route via the umbilical vein has been suggested as a likely route of spread to the foetus due to the consistent liver lesions observed in foetuses (Smith 1997; Rodger *et al.* 2007). However, this does not explain the initial systemic infection from the respiratory infection.

There have also been reports of systemic spread of the virus in neonatal calves causing a severe systemic infection with a 31% mortality rate (Higgins & Edwards 1986). In a more recent review, the fatality rate has been reported to be nearly 10% in newborn calves (Radostits *et al.* 2000). These incidents are mainly reported to be related to calves born to newly introduced heifers (Higgins & Edwards 1986; Ross *et al.* 1983). Noticeable pathology has been reported in the liver in the form of multifocal necrosis, similar to that observed in foetal livers in the abortive presentation. In these cases of systemic spread, virus showed an extensive dissemination, and was isolated from liver, spleen, lymph nodes, pharyngeal mucosa and brain (Higgins & Edwards 1986).

Despite the evidence of systemic infection, very little is known about the mechanisms associated with the systemic spread of the virus. There is extensive evidence of cell-associated mechanisms of viraemia in the closely related pseudorabies virus (Nauwynck & Pensaert 1992) and equine herpesvirus-1 (Allen & Breathnach 2006). However, so far attempts to clearly demonstrate the same process in BoHV-1 infection have been unconvincing. After intranasal infection with a highly virulent strain, one study demonstrated presence of virus in serum for several days post infection, indicating a cell-free viraemia 4-9 days post infection (Kaashoek *et al.* 1996). However, this was only observed in calves infected with the Iowa strain and has not been reported in other experimental infections via the intranasal route since. There has also been some evidence of cell association with lymphocytes and monocytes *in vitro* (Nyaga & McKercher 1979). Reports of isolation of BoHV-1 from blood buffy coats have predominantly been reported in studies using an intravenous method of inoculation (Miller *et al.* 1991). Only one study has isolated BoHV-1 in the blood buffy coat following intranasal experimental infection, although this was during a multiple virus infection in which a bovine viral diarrhoea virus challenge had been given intravenously. Under these circumstances, the immunosuppression associated with the bovine viral diarrhoea infection, may have influenced the IBR infection (Castrucci, Ferrari & Tartaglione 1992). Therefore the main mechanism of systemic spread still remains unclear.

1.3.9 Host immune response to BoHV-1

The host immune response has to be fast acting due to the rapid infection by the virus; viral antigen expression on the cell surface occurs within 2-4 hours post infection. This is the point in a primary infection when the host starts to initiate an immune response. The virus replication initially triggers a non-specific or innate immune response including complement activation and the secretion of interferon-α and interferon-β by the infected cells. Specifically this α and β IFN activity has been shown to inhibit herpesvirus thymidine kinase activity (Otsuka, Qavi & Kit 1982). Macrophages, neutrophils and natural killer (NK) cells in the form of large granular lymphocytes are recruited and activated at the site of infection, which act in the infected epithelium by killing virus infected cells after cytokine activation. Research has demonstrated that the primary viral targets for the NK cells are gB and gD (Palmer *et al.* 1990). These cells induce an early wave of cytokines are induced, such as IL-1 and IL-6 (Babiuk, van Drunen Littel-van den Hurk & Tikoo 1996; Muylkens et al. 2007). IL-1 and IL-6 stimulates parenchymal cells and lymphocytes to produce GM-CSF, which in turn stimulates macrophages to produce molecules such as TNF-α (Heidenreich *et al.* 1989).

After this first response of the innate mechanisms, there is a specific immune response mediated by the recognition of the antigen that usually coincides with the recovery from clinical signs. Antigen presentation occurs by both the MHC class 1 and 2 pathways. A cell mediated response is vital to tackle a virus that initially spreads via a cell-cell route (Deshpande *et al.* 2002). This phase is mainly driven by the Th1 specific cytokine production (CD4 Th1 cells), which activates the direct cytotoxicity of CD8 T cells and result in the production of specific late cytokines including IL-2, IL-12 and IFN-γ. In this response, CD8 or cytotoxic T cells play a major role, both killing infected cells and secreting cytokines (Babiuk et al. 1996). *In vitro* work in bovine cells has suggested gC and gD are the main target antigens for the cytotoxic T cells, although the *in vitro* method used was shown to influence these results (Denis *et al.* 1993). The Non-MHC-restricted cytotoxicity via the monocyte and macrophages described earlier is considered to be the more abundant cell mediated response but requires the IFN-γ produced by the T-lymphocytes to help activate the macrophages (Babiuk et al. 1996). CD4 or helper T lymphocytes also

migrate into the site of lesions and secrete the second or late wave of cytokines, such as IFN-γ (Babiuk, van Drunen Littel-van den Hurk & Tikoo 1996; Turin, Russo & Poli 1999).

The adaptive immune response is most active between 5 and 10 days post infection and plays a major role in resolving the infection and remission of any clinical signs of the disease. This includes a Th2 response, most relevant towards the late phases of the infection and regulated by a different range of late cytokines including IL-4, IL-5 and IL-6. IgM, IgG and IgA antibody production is stimulated, which neutralize any extra-cellular virus believed to be important in preventing reactivation and shedding of latent virus (Graham 2007; Babiuk et al. 1996; Engels & Ackermann 1996; Turin et al. 1999). The antibody response is considered more important in preventing an infection than in recovery of a current infection. It is particularly effective when high levels of antibody are found in the nasal passages during a reactivation event, which immediately neutralize the infecting virus and prevents further spread or clinical signs to develop (Babiuk et al. 1996). A study investigating antibody isotypes during an initial infection and reactivation found that IgM was detectable first from 9 days pi followed by IgA from day 12 and IgG from day 13 pi. IgA was only detectable for 3 weeks in the sera but persisted in the mucosal secretions and increased significantly in nasal and ocular titres after reinfection and reactivation as well as rapidly appearing in the serum. This led to suggestions that IgA would offer a sensitive indicator at reinfection (Madic *et al.* 1995a; Madic *et al.* 1995b). Intranasal immunization has been found to be an effective way of stimulating a muscosal IgA response (Papp, Babiuk & Baca-Estrada 1998).

Due to this extensive immune response by the host, the virus has evolved several ways to try to evade the immune response. For example, gG binds to chemokines to prevent their action and Type I-IFN expression is down regulated by the BICP0 gene product by inhibition of transcription. Glycoprotein C interacts with the third component of the complement to reduce complement activation, an immune evasion factor conserved in other related alphaherpesviruses. During the acute phase of the disease BoHV-1 infects and causes apoptosis of epithelial cells to ensure efficient virus release, but later during the latency phase, the latency related gene inhibits

apoptosis in latently infected cells. BoHV-1 infection can cause apoptosis of CD4 T lymphocytes in peripheral blood and lymph nodes which are important in the specific immune response (Srikumaran et al. 2007) and (Winkler et al 1999). The UL49.5 protein acts to down-regulate the expression of the MHC I by the virus infected cells by blocking the transporter associated with antigen processing (Koppers-Lalic *et al.* 2005). This means these cells are not recognised and removed by the cytotoxic T lymphocytes (CD8) (Muylkens et al. 2007). *In vitro* it has been reported to reduce the proliferation of leukocytes by suppressing the mitogen that usually stimulates cell division (Srikumaran et al. 2007). These actions allow the survival of the virus, and also lead to a state of immune suppression that leaves the host vulnerable to secondary bacterial infection.

1.3.9 Diagnosis and control

Diagnosis of IBR is traditionally achieved by virus isolation from nasal swabs, broncho-alveolar lavage or tissue samples in cultured bovine cells. The presence of virus is detected by the characteristic cytopathic effect within 3 days of inoculation characterized by grape-like clustering of rounded cells around a microplaque (figure 1.8) (Nandi *et al.* 2009; Beer 2010). A quicker test, known as IFAT, involves staining the viral antigen within smears or cytospin respiratory sample cells. This can be done either directly using monospecific antiserum conjugated with a fluorescent dye, or indirectly with the anti-species secondary antibody conjugated to a fluorochrome. While virus isolation is more sensitive and specific than the fluorescent antibody technique, it is a more time consuming method (Beer 2010).

Histopathology can also be used for diagnosis in cases where abortions or deaths have occurred. Multifocal necrosis of the liver is common in both neonatal systemic infection and abortion, and can be confirmed to be caused by BoHV-1 by immunohistochemical labelling using a BoHV-1 specific monoclonal antibody (Rodger *et al.* 2007). However, the usefulness of histopathology, as well as virus isolation, can be limited by the autolysis, especially in abortion material.

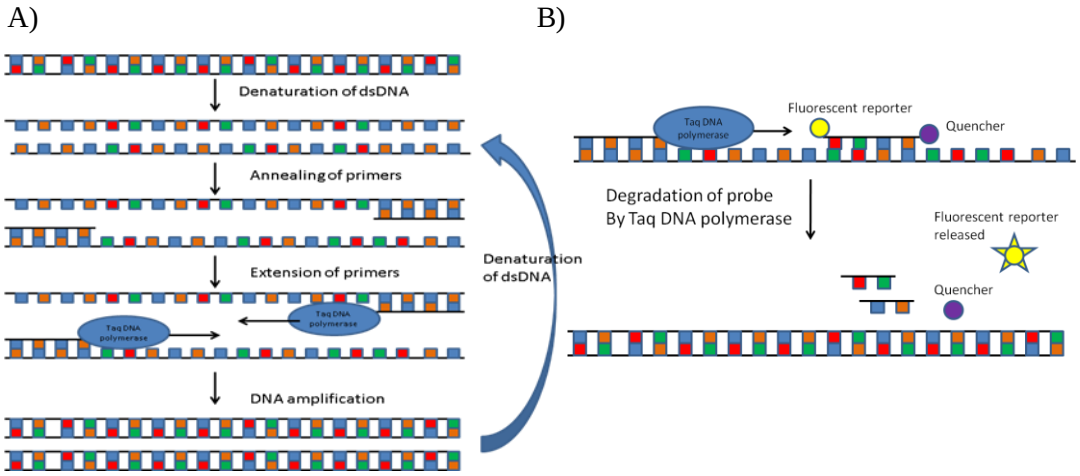
More recently, molecular methods are being favoured for virus detection which provides fast, sensitive and accurate detection from a range of sample types. They

also allow the detection of latently infected cells in tissue samples, which cannot be achieved by virus isolation of histopathology/immunohistochemistry. Conventional PCR can detect virus in tissue following DNA extraction, and adding it to the PCR assay. Heat is used to denature the DNA, primers bind to specific regions of the viral DNA and *Taq* DNA polymerase extends the primers to produce another copy of this region (figure 1.10A). This process is repeated by thermal cycling to amplify the specific region of viral DNA, enabling detection of low virus titres (Mweene, Okazaki & Kida 1996; Takiuchi *et al.* 2005; Vilcek *et al.* 1994; Beer 2010). There have also been multiplex PCRs, where several sets of primers recognising different sequences are used simultaneously, combined with a restriction typing assay that allow distinction between the different bovine herpesviruses (De-Giuli *et al.* 2002; Vilcek *et al.* 1994; Claus *et al.* 2005). However, amplifying DNA for the purposes of diagnosis can have associated problems. Post PCR product handling is necessary to analyse the results visually on an agarose gel using electrophoresis. This provides many opportunities for viral DNA to contaminate other samples (Mackay, Arden & Nitsche 2002). These problems explain the welcome response to the prospect of using real-time PCR, in which assays take place in a single sealed tube.

Real-time PCR monitors the amplification of the targeted DNA region throughout the reaction in an enclosed system. In addition to specific primers a fluorescent labelled probe is also designed to bind to the target sequence (Klein 2002). There are five chemistries in use which I will briefly describe here. DNA binding fluorophores such as SYBR green, a fluorescent dye that binds DNA, can also be used instead of a probe but this is not specific to the target sequence, and therefore will bind to all double stranded DNA produced in the reaction, which may produce some false positives due to primer/dimer combinations, making it a less suitable tool for diagnostics. Hybprobes are linear oligoprobes containing a donor fluorophore on the upstream probe 3'end and an acceptor fluorophore on the downstream probe 5' end. Upon hybridization these fluorophores interact to produce a FRET signal. 5' nuclease oligoprobes such as Taqman probes use a fluorophore reporter and quencher remain in proximity of the probe, no fluorescence occurs, but following specific binding to the target sequence 5'-3'endonuclease activity of Taq polymerase degrades the probe allowing the fluorophore reporter and quencher to become

separated, leading to fluorescence to confirm detection and quantification of the target sequence (figure 1.10B). Hairpin oligoprobes have a quencher and fluorophore on terminal ends and remain in close proximity until hybridization with the target sequence when the hairpin structure is shifted to an open configuration to allow a FRET signal to be released. Similar to the hairpin oligos are self fluorescing amplicons that include scorpion and sunrise primers, which become incorporated into the PCR products (Mackay *et al.* 2002). The lack of any post PCR product handling and the improved technology resulting in reduced cycle times means that real-time PCR can also decrease time taken for diagnosis (Mackay *et al.* 2002). Using real-time PCR, it is also possible to accurately quantify viral load in samples, which is useful for monitoring for clinical purposes and research (Niesters 2001). Disadvantages of real-time PCR include being unable to establish the size of the amplified DNA fragment without opening the system and there being some limitations of current multiplex possibilities due to the number of detection channels available (Mackay *et al.* 2002). Recently, a protocol for the detection of BoHV-1 using real time PCR was developed focussing on detecting the target sequence in gB of BoHV-1 in bull semen, which included an international ring trial (Wang *et al.* 2007; Wang *et al.* 2008). This method is rapidly becoming the preferred method of diagnosis where facilities are available.

Figure 1.10. A) Standard PCR process. B) Extension step of real-time PCR using a 5'nuclease Taqman probe.



Serological tests such as antibody detection ELISAs and serum neutralization tests (SNTs) are used to identify seropositive animals, using specific BoHV-1 antigens which are recognized by specific serological antibodies in the serum. However, they cannot distinguish between a distant and recent infection unless paired samples are taken. A rising BoHV-1 antibody titre is an indication of a recent BoHV-1 infection (Nandi *et al.* 2009). There are several ELISA kits available commercially that use both blocking and indirect mechanisms, the antigen targets are often glycoproteins such as gB, gE and gC (Das Neves *et al.* 2009). The use of gB as a target antigen is a standard ELISA test that was found to be more sensitive than the gE ELISA and indirect ELISA in a ring trial (Kramps *et al.* 2004). The gE ELISA is used to identify between vaccinated and unvaccinated animals in eradication schemes (Kaashoek *et al.* 1995). The gC ELISA can be used to discriminate between subtypes (Spilki *et al.* 2005). SNTs look at the immune response to the whole virus rather than specific proteins. This does not enable any discrimination between subtypes but provides a good initial screening option and can be used to confirm ELISA test results (Varela *et al.* 2010).

These serological tests can be important in monitoring prevalence and identifying latently infected animals, which should test seropositive. This is important as latently infected animals pose risk to the rest of the herd, especially after transportation or during parturition. However, serology is not always reliable, as some young calves may become infected while still possessing some maternally derived antibody and do not produce an active humoral response, allowing them to go unnoticed in serological tests (Graham 2007).

Control is mainly in the form of vaccination to prevent initial infection. Eradication schemes have been successful in several European countries using vaccination methods or culling of seropositive animals in regions with low seropositivity (Ackermann & Engels 2006). Many vaccines are available and are widely used to control outbreaks of IBR in the form of modified live, inactivated, marker and multivalent vaccines (Radostits *et al.* 2000). Vaccines containing live virus produce latent infection and may induce viral shedding of reactivated vaccine virus when given by the intranasal route (Graham 2007). There are also reports of abortion in

pregnant animals of unknown vaccine status given modified live vaccines (McFeely, Merritt & Stearly 1968). Inactivated vaccines consist of an adjuvant to initiate an immune response combined with high levels of inactivated virus or parts of the virus such as glycoproteins (Beer 2010). A recent study suggests inactivated forms of the vaccine provide similar efficacies as modified live vaccines, but may be safer to give during gestation (Zimmerman *et al.* 2007).

Marker or DIVA vaccines were developed to differentiate between a wild-type infection and a vaccination by using a gE deletion mutant version of the virus in the vaccine (Beer 2010). Antibodies produced as a result of the vaccination do not recognise gE and therefore in a specific gE ELISA, vaccinated animals will give a negative result while remaining positive in other assays such as gB or whole virus based ELISA tests or SNTs (Kaashoek *et al.* 1995). These vaccines have been most heavily used as part of successful eradication schemes in several European countries. These gE deleted vaccines do offer the advantage that re-excretion is reduced if the virus is reactivated from latency (Mars, de Jong & Van Oirschot 2000). However, they have been criticised for a number of reasons; while vaccines do reduce virus replication and transmission, they do not prevent infection; therefore seroprevalence often remains high in areas where eradication with the aid of vaccination has been attempted (Ackermann & Engels 2006). As they are live vaccines they have been criticised as being contradictory to the aim of eradication (van Drunen Littel-van den Hurk 2006).

In neonates, often the most vulnerable to infection, modified-live and killed vaccines show a low efficacy due to the immaturity of the immune system and interference of maternal antibodies. New approaches are using DNA vaccines to induce immunity as part of the vaccine strategy to address this problem. These new vaccines, described as antigen-encoding bacterial plasmids, are being tested to address this problem of BoHV-1 outbreaks in neonates (van Drunen Littel-van den Hurk 2006). There is also pressure to reduce the need for intramuscular delivery of vaccines due to damage to tissue resulting in rejected meat, therefore intranasal methods are favoured when possible (van Drunen Littel-van den Hurk 2006).

None of the current vaccine strategies are specifically aimed at stopping systemic spread of the virus as so little is known about the mechanisms resulting in systemic spread. In vaccinations against the closely related EHV-1 virus, research into the immune response during infection has suggested that an effective vaccine to prevent systemic dissemination would require activation of a high virus neutralizing antibody titre combined with increased cytotoxic immune responses (Kydd, Wattrang & Hannant 2003; Kydd, Townsend & Hannant 2006).

1.3.10 Use of model systems to investigate host-pathogen relationships of BoHV-1

Model systems are frequently used in the study of viruses, as studies in the natural host are expensive and often ethically complicated. They can also be limited in terms of statistical significance due to the small numbers of animals that can be used. BoHV-1 is no exception, with many experiments being conducted *in vitro* using bovine cell cultures. However, the question always remains how well cell culture represents the *in vivo* environment, with only one cell type and no host immune response that is such an important part of a viral infection. There have also been several studies using alternative animal models to cattle such as the rabbit, which appears to provide a reliable infection model (Rock & Reed 1982; Brown & Field 1990; Lupton, Barnes & Reed 1980; Valera *et al.* 2008). An evaluation of the rabbit model concluded that infection with BoHV-1 produced a wide range of clinical signs associated with IBR including respiratory tract infection and systemic spread, although BoHV-1 induced abortion in rabbits differed significantly to that found in cattle as no evidence of virus infection of the foetus was observed (Lupton *et al.* 1980). Experimental infection in rabbits has also been shown to be able to model latency of the virus with experimental reactivation using corticosteroids, as can be achieved in cattle (Brown & Field 1990).

More recently, an alternative option re-emerged and has been increasingly used as an infection model, using *ex vivo* thin tissue sections originating from the natural host, culturing them for a time period, to allow infection using the chosen infectious organism. This provides the advantage of a mixed cell population and maintenance of tissue structure that can be considered more representative of the host environment than cell culture of a single cell-type. Examples of the successful use of

these models include adenovirus infection in a lung slice model (Booth *et al.* 2004) and Maedi visna infection of both lung and trachea tissue with discs of tissue being placed in tissue culture dishes and maintained for 7 days in supplemented media (McNeilly *et al.* 2007). A similar system, described as an air-liquid interface bovine respiratory organ culture system has been used to culture canine tracheal tissue infected with *Bordetella bronchoseptica* (Anderton, Maskell & Preston 2004). This system has also been evaluated for its suitability as a model for BoHV-1 infection. Results demonstrated that as well as studies in pathogen adherence, these systems may also be suitable to measure host responses at the molecular and morphological level. Ciliary function and structural integrity was maintained for 72 hours and frequent, detailed sampling made this a reliable model that required no use of experimental animals with the exception of donors (Niesalla *et al.* 2009).

1.4 Aims and Objectives

As this literature review has demonstrated, extensive work has been conducted on BoHV-1, and the genetics, replication and latency strategies of the virus are reasonably well understood. This PhD project first studies the importance on BoHV-1 as a cause of abortion in Scotland and analyzes the current diagnostic methods used to investigate abortion cases. Then, it focuses on the study of virus encoded variation between isolates which is hypothesized to influence dissemination of virus from the primary respiratory infection to cause systemic infection and abortion. Differences in clinical outcome of closely related viruses such as EHV-1 have been related to single point mutations in the viral genome. Genetic variation between BoHV-1 subtypes has been identified but there has been no research into the potential role of viral genetic variation in systemic spread and abortion. To test the hypothesis that viral factors such as genetic variation are important in the systemic spread of BoHV-1, differences between BoHV-1 isolates were analysed using *in vitro* and *in vivo* infections, whole genome sequencing and a prospective study of natural cases of abortion.

Specifically this project aimed to:

- Establish the prevalence of BoHV-1 in aborted foetal material and identify whether current diagnostic methods were producing representative results of the importance of BoHV-1 as an abortive agent.
- Investigate the distribution of BoHV-1 in foetal infection leading to abortion in natural conditions.
- Identify any genomic differences that may contribute to the tissue tropism or biological behaviour of BoHV-1.
- Carry out an experimental infection in the natural host to identify any differences between an isolate identified previously as causing a systemic infection and one resulting in IBR only, by measuring clinical host response, spread of the virus and viral expression throughout the infection.
- Test the behaviour of different isolates in model infection systems and test the validity of lung slice culture as a BoHV-1 infection model.

Chapter Two: Materials and Methods

This chapter will cover the basic general materials and methods used throughout the work included in the results chapters and will be referred back to in the results chapters. More detail or any alterations specific to certain experiments will be detailed within the results chapters.

2.1 Samples

2.1.1 Virus isolate sources

BoHV-1 isolates were all sourced from submissions from across the UK to the virus surveillance unit (VSU) at Moredun Research Institute (table 2.1) with the exception of the reference strains. Isolate Colorado-1 (Cooper strain) was purchased from ATCC. IBR6660 was isolated at Moredun in 1978 from an Aberdeenshire case (Nettleton & Sharp 1980). In each case received by the VSU the virus was isolated from fresh tissue or swab sample, for cases received pre-2007 the tissue was homogenized using a mortar and pestle in 4ml virus transport medium (VTM) with sterile sand. For tissue received post-2007 tissue was homogenized in 4ml of VTM in gentleMACS M tubes (Thistle Scientific) on a dispomix machine. The homogenized tissue from either method was then centrifuged at 2000 x g for 10 minutes at 5°C and supernatant was collected for use in virus isolation. Swab samples were sonicated in VTM for 1 minute to release virus particles from the swab into the VTM. An aliquot of VTM was collected for testing and the swab discarded. All the isolates subsequently used in this work were identified as BoHV-1 using the virus isolation method. For the purposes of this work they were subsequently classified into respiratory, systemic and abortion groups determined by the tissue type the virus was isolated from and presenting clinical signs reported by the submitter (table 2.1).

2.1.2 Abortion study samples

400 samples from bovine abortions were received from veterinary diagnostic centres across Scotland and Northern England between 2007 and 2009. These samples were used to retrospectively detect BoHV-1. No further selection of samples was carried out, therefore samples represented those considered to warrant further investigation by local vets. Foetal liver, lung, heart, brain, placenta and foetal fluid were all requested for each case but all tissues were not always available. Diagnostic testing was conducted including real-time PCR, virus isolation and histopathology as described in sections 2.2.2 and 2.7 below. Background information was also available for samples on previous clinical and diagnostic findings.

Table 2.1 Virus isolate sources.

Isolate no.	Year	Location	Tissue	Classification
07/267	2007	Perth	Lung	Respiratory
07/282	2007	Perth	Organs	Systemic
07/1003	2007	Loughborough	Trachea and lung	Respiratory
06/134	2006	Dumfries	Spleen	Systemic
05/898	2005	Dumfries	Spleen	Systemic
K739	2004	Penrith	Placenta	Abortion
K514	2004	St Boswell	Tissues	Systemic
08/68	2008	Inverness	Spleen	Systemic
06/108	2006	Penrith	Trachea and lung	Respiratory
05/1372	2005	Winchester	Trachea	Respiratory
K1468	2004	Aberdeen	Nasal Swabs	Respiratory
K303	2004	Dumfries	Nasal swab	Respiratory
06/1889	2006	Thurso	Trachea and lung	Respiratory
06/1093	2006	Penrith	Nasal swabs	Respiratory
07/1530	2007	Dumfries	Abortion	Abortion
06/068	2006	Penrith	Trachea	Respiratory
K1527	2004	St Boswell	Trachea	Respiratory
05/1570	2005	Penrith	Trachea	Respiratory
07/184	2007	Edinburgh	Nasal swab	Respiratory

2.2 Virus culture in cells

2.2.1 Cell culture

Virus stocks were cultured in a semi-continuous cell line of bovine turbinate (BT) cells from the cell line BT1802/1/4. Cells were cultured in monolayers using

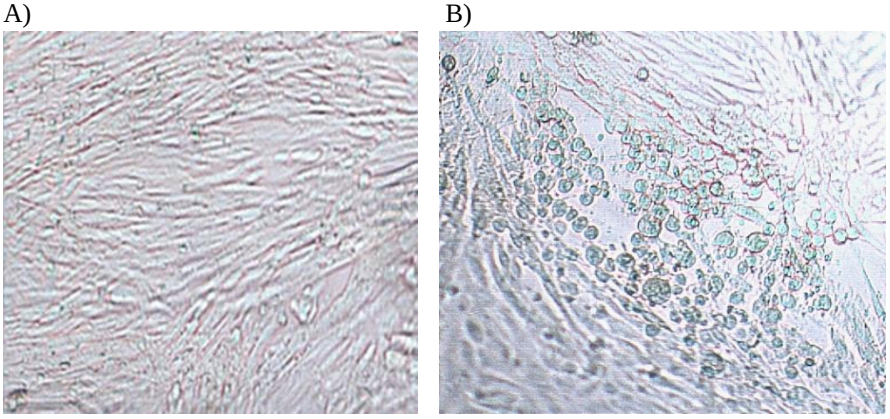
standard methods (Peterson & Goyal 1988), in Iscoves Modified Dulbecco's Medium (IMDM) (Sigma) supplemented with 10% FBS and 8mM glutamine. Briefly, they were raised from stocks stored in liquid nitrogen in v/v 10% DMSO and 90% FBS, washed in IMDM, and then maintained by splitting twice weekly to produce a seeding rate of ~$1x10^5$ cells/ml. To split cells, the cell monolayer was washed in PBS and the cells were trypsinized to detach them from the flask. If necessary, cells were counted by staining with 0.2% nigrosin to stain non-viable cells. Viable cells could then be counted using a modified-fuchs haemocytometer. After calculation of cell concentration the appropriate amount of IMDM could be added to achieve the desired cell concentration. Cells were incubated at 37°C.

2.2.2 Virus isolation

To isolate the virus, cell monolayers were washed twice in Hanks balanced salt solution (HBSS). The tissue or swab supernatant suspected to contain virus particles was added to cover the cell monolayer in the tubes or flask (2ml supernatant plus 2ml HBSS for a 150cm^2 flask). A control flask was also set up containing HBSS only, to check for contamination. The flasks or tubes were incubated at 37°C for 1 hour, 199 maintenance media (199 (Sigma) with 5% bovine serum albumin, tryptose phosphate broth, 20mM Hepes, 3.75mM sodium hydroxide, sodium bicarbonate, 10% yeast extract, supplemented for use with 2% FBS, 1mM glutamine, 1% penicillin and streptomycin and 0.5% amphotericin) was added and the flasks or tubes were returned to 37°C and checked daily for cytopathic effect (figure 2.1). From the initial infection in tubes it was possible to identify these cases as BoHV-1 from the characteristic cytopathic effect (CPE). From these cases stocks were produced in flasks cultured until 100% CPE was observed to get a larger yield of virus that could be used for further experiments. The virus was harvested by freezing the flasks at -70°C and thawing twice. The media containing the cell debris and virus was decanted and centrifuged at 2000 x g for 10 minutes at 5°C. The supernatant was collected and cell debris discarded.

Figure 2.1. BoHV-1 cytopathic effect.

A) B)



For high-throughput sequencing and restriction enzyme analysis it was necessary to purify the virus to minimize the presence of cell debris. This was achieved using a 27% w/v sucrose cushion in TNE buffer (0.1M NaCl, 10mM Tris-HCl, 1mM EDTA in dH_2O). 5ml of the sucrose cushion was added to the bottom of each centrifuge tube. The virus supernatant, harvested as described above, was carefully added on top of the sucrose cushion. Tubes were placed in SW32ti centrifuge rotor (Beckman-Coulter) and centrifuged for 1 hour at 100,000 x g at 4°C using an Optima L-90k ultracentrifuge (Beckman-Coulter) (Thiry *et al.* 2007; Lobanov *et al.* 2010). The supernatant was discarded and the virus pellet was resuspended in 200µl TNE buffer.

2.2.3 Time course infection

To study two virus isolates at different stages of infection virus was cultured in $25cm^2$ flasks as described in 2.2.2. Cytopathic effect was recorded and the infected cells were harvested at 3 hourly intervals from 3-48 hours post infection to enable analysis of viral gene expression at different time points during the infection. When the flasks were harvested the cells were collected by using a cell scraper to remove cells from the flask surface and decanting the flask contents into a 50ml centrifuge tube which was then centrifuged at 2000 x g for 10 minutes at 4°C to form a cell pellet, the supernatant was discarded and the pellet of infected cells were frozen for RNA extraction at a later date.

2.2.4 Virus Titration

To quantify the virus for infections the virus stocks were titrated out on 96 well plates containing BT cells. 10 fold dilutions of the virus stock were made up in 199 maintenance media from 10^{-1} to 10^{-8}. 50µl of 199 maintenance media was added to 4 wells rows A-G and 100µl to row H, the cell control containing no virus dilutions. Virus dilutions were added in rows A-G from 10^{-2} in row A down to 10^{-8} in row G. 100µl of BT cell suspension ($2x10^5$ cells/ml) was added to each well. The plate was then sealed and incubated at 37°C and 5% CO_2. After 2-3 days the cytopathic effect for each well was recorded as positive or negative. The TCID50 was then calculated using the Spearman-Karber calculation (Hierholzer & Killington 1996).

$$TCID_{50} = (\text{Highest dilution giving 100\% CPE} + 0.5) - \frac{\text{No. test units showing CPE}}{\text{No. of test units per dilution}}$$

N.B No. test units showing CPE only includes the highest dilution showing 100% and any positives at higher dilutions.

2.2.5 Serum neutralization test

The serum neutralization test (SNT) was used to identify animal BoHV-1 serological status. Serum was collected from a clotted blood sample and was heat inactivated at 56°C for 30 minutes. A doubling dilution of the heat-inactivated serum was made down the plate using 199 media as a diluent, with each sample in duplicate with a control row containing no serum to check for cytotoxicity of diluent. On the first plate a virus titration was set up using reference isolate, IBR6660 with a 10 fold dilution in 199 media series to check the TCID 50 of the virus added to the other wells was within the range of 30-300 TCID50 per well. Virus at this working dilution was added to each well except the serum and cell controls. Plates were covered and incubated at 37°C in 5% CO_2 overnight. 100µl of BT cell suspension at a concentration of $2x10^5$ cells/ml in IMDM, containing 10% heat inactivated FBS was added to each well. Plates were then sealed and incubated at 37°C in 5% CO_2 for 2-3 days. Results were recorded by looking for cytopathic effect using a light microscope. Wells containing CPE showed the serum did not contain enough antibody to neutralize the virus. Results are expressed as the reciprocal of the highest serum dilution that neutralises viral CPE in 50% of the wells.

2.3 Virus culture in lung slices

2.3.1 Lung slice preparation

A BoHV-1 antibody negative cow involved in another experimental procedure was identified using a serum neutralization test as described in 2.1.5. For the purposes of the other experiment the animal was euthanased according to Home Office legislation. Following euthanasia the trachea was clamped and the lungs taken out whole. The right apical lobe of the caudal segment was identified and clamped off from the rest of the lung. A suitable bronchiole was found to fill the lung segment with 150ml of 2% low melting point agarose in HBSS at 37°C. It was then separated from the rest of the lungs and placed in HBSS on ice for 1 hour. Lung slices were then cut by taking circular cores of lung filled with agarose and outing these into the Krumdieck tissue slicer that produced ~600µm thick slices of lung which were then washed in HBSS buffer.

2.3.2 Lung slice maintenance and infection

Lung slices were plated out in 24 well plates, with one slice in each well. On each plate there were also control wells containing no lung slices. Lung slices were washed 3 times in Dulbecco's Modified Eagle Medium supplemented with glutamine, penicillin, streptomycin, genotomycin and amphotericin (DMEM+). 0.5ml of the supplemented DMEM+ was added to each well and the plates containing the lung slices were placed on a rotor set to 1 rpm overnight at 37°C in 5% CO_2.

After overnight incubation lung slices were washed twice with 0.5ml DMEM+ before being infected with 100µl of one of 6 BoHV-1 isolates that had previously been titrated and diluted in DMEM+ to a concentration of 10^5 $TCID_{50}$/100µl. DMEM+ containing no virus was used for control wells. Lung slices were incubated for 2 hours on a rotor at 37°C in 5% CO_2, then washed 3 times in DMEM+ and 0.5ml of DMEM+ was added before returning plates to the incubator. Lung slices were harvested at 24, 48 and 72 hours post infection. Harvesting involved taking out the lung slice and placing it either in 10% buffered neutral formalin for histopathology,

RNAlater for DNA or RNA extraction and snap-frozen in liquid nitrogen for immunofluorescence work by placing it on a disc of gelatine to maintain structure. Supernatant was also collected for DNA extraction to quantify virus produced on each day of infection. If not scheduled for harvest at that time point lung slices were washed in DMEM+ each day.

2.4 Experimental infection

2.4.1 Animals

27 colostrum deprived male calves were sourced from Scottish farms and fed BoHV-1 antibody free colostrum (CER Groupe, Belgium) to ensure they remained susceptible to a BoHV-1 challenge. Blood samples were taken on arrival and serum was tested using the SNT method described in 2.2.4 to ensure their BoHV-1 antibody negative status. They were also screened for the presence of *Pasturella spp*. by taking a bacterial nasopharyngeal swab. The animals were housed in individual pens for the first 8 weeks before being randomly assigned to one of 3 treatment groups. Each treatment group consisted of a total of 9 animals and was split into smaller groups of 3 and 6 to reduce stress later in the experiment. All animals were fed the same diet and were housed in identical rooms $20m^2$ maintained at an average temperature of ~15°C and ~68% humidity. Two weeks acclimatization after grouping was allowed before the experiment began.

2.4.2 Virus infection

Two BoHV-1 isolates and an uninfected cell culture control were used to infect the different treatment groups respectively. Isolates 05/1570 and 07/1530 (table 2.1) were cultured and harvested as described in section 2.2.2. Both virus isolates were used on the third pass through cell culture and diluted to a concentration of $1x10^7$ $TCID_{50}$/ml, based on results from a virus titration in BT cells. The control inoculum was collected from an uninfected flask of cells to ensure none of the observations were caused by cells or media. Inoculation was carried out using an intranasal spray with 1ml of inoculum sprayed up each nostril with a syringe attached to a fine nozzle to create a fine mist representative of an aerosol transmission (Reddick 2009).

2.4.3 Sampling

Sampling was conducted two weeks pre-challenge, daily for the first 11 days post infection, then twice weekly until 24 days post infection (figure 2.2). Sampling consisted of a blood sample taken from the jugular vein using vacutainer tubes, one heparinised to collect buffy coat and one allowed to clot for serum collection. Nasal and ocular swabs were taken to measure viral load using flocked swabs in universal transport medium (Cat no. 328C, Sterilin). In accordance with project licence no. PPL 60/3976 clinical scores were recorded according to table 2.2, and was carried out by one of two people assigned a group of animals randomly each day. Transfer of the virus between groups was controlled for by all personnel showering and changing clothes/boots between entering rooms containing different treatment groups. Control animals were always inspected first. Usually two teams were available to avoid the same team sampling the two infected groups.

At post mortem, animals were euthanased according to Home Office legislation by barbiturate overdose. Post mortem examination involved collection of 16 tissues: turbinates, trachea, palatine tonsil, nasopharyngeal tonsil, retropharyngeal lymph node, lung- cranial, and caudal, brain – forebrain, cortex, hindbrain, trigeminal ganglia, liver, kidney, spleen, testicle and mesenteric lymph node. Four samples of each tissue were placed in RNA later, VTM, formal saline and zinc salts for nucleic acid extraction, virus isolation and immunohistochemistry respectively.

Figure 2.2. Time scale of experimental infection.

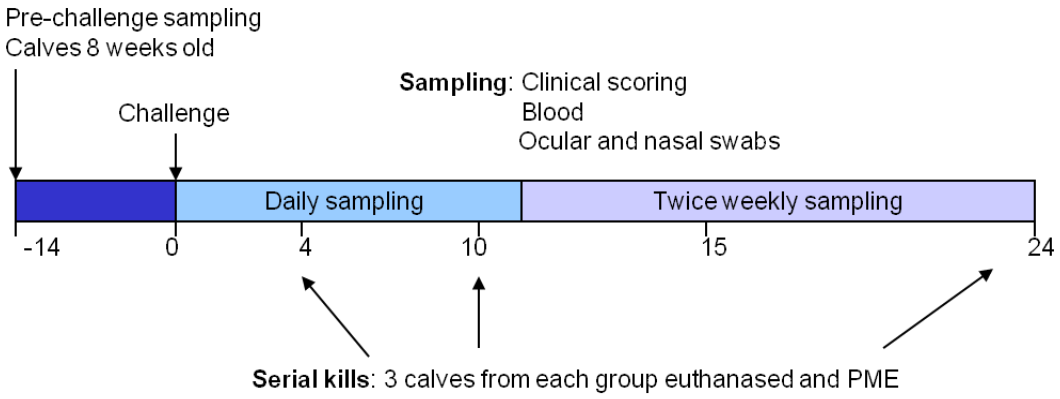


Table 2.2. Clinical score guidelines.

Clinical examination	**0** **Absent**	**1** **Mild**	**2** **Moderate**	**3** **Severe**
Respiratory Effort	Normal respiration rate (eupnoea) RR: <40	Slightly increased respiration rate RR: 41-60	Increased respiration rate, obvious abdominal breathing RR: 61-100	Increased respiration rate, severe abdominal breathing, mouth breathing, two-staged expiration RR:>100
Depression/ demeanour	Bright, alert, responsive. Normal behaviour	Reduced responsiveness. Otherwise normal behaviour	Depressed Separates from group Extended resting periods, reluctant to stand, lethargic	Unable to stand without assistance
Appetite	Normal appetite	Slightly decreased appetite	Decreased appetite	Total anorexia for longer than 2 days
Cough	No coughing	Occasional spontaneous (dry) cough and/or unproductive induced cough	Frequent spontaneous cough and/or easily induced unproductive cough	Frequent spontaneous cough at rest and/or prolonged, productive induced cough
Nasal discharge	Quantity: No nasal discharge Quality: Serous	Quantity: Presence of nasal discharge Quality: Intermittent, mucoserous	Quantity: Increased nasal discharge Quality: Persistent mucoid-mucopurulent discharge	
Rhinitis, lesions of oro-nasal mucosae	Absent	Reddening of the muzzle and/or formulation of plaques on the mucosae	Plaques and/or erosion of the mucosae	
Ocular discharge, Conjunctivitis	Quantity: No discharge Quality: Eyes appear normal	Quantity: Ocular discharge forming a streak down the cheek Quality: Eyes appear normal, serous discharge	Quantity: Ocular discharge wets the side of the face Quality: Abundant serous discharge or/and amount of mucopurulent discharge	
Pyrexia	absent		>40.0	
Dehydration				Skin tenting or other signs of dehydration
Body condition	normal		thin	Very thin (ribs and spine prominent)

2.4.4 Sample handling

Blood samples were centrifuged at 2000 x g for 10 min at 5°C. Serum was collected from the clotted samples and stored at -20°C. Buffy coat layers were collected from the heparinised samples. Buffy coats were purified by lysing red cells by vortexing sample with tris ammonium chloride and centrifuging at 12000 x g for 1 minute, supernatant was removed and the process was repeated if red cells were still present on the buffy coat pellet. When all red cells were removed buffy coat was washed in PBS and then resuspended in fresh PBS ready for nucleic acid extraction.

Swab samples were sonicated in a bath sonicator for 1 minute to release viral particles into the universal transport media. 2ml of the media was collected for nucleic acid extraction or virus isolation.

Tissues collected in RNA later or VTM were stored at -70°C until needed for nucleic acid extraction or virus isolation. Tissues in formal saline were allowed to fix for several days before they were processed as described in the histology methods.

2.5 Molecular biology methods

2.5.1 DNA isolation from virus supernatant from cell culture or lung slice culture supernatant

DNA from BoHV-1 grown in cell culture was extracted from the supernatant of the harvested flasks after cell debris had been removed by centrifugation. The QIAamp DNA minikit (Qiagen) was used to isolate the DNA following the manufacturer's instructions for DNA purification from blood or body fluids protocol. DNA was eluted in 100µl AE buffer.

2.5.2 DNA isolation from fresh tissue, swabs, buffy coats

The QIAcube (Qiagen) was used to extract DNA from tissue collected for the abortion study and from the tissues and swabs collected during the experimental challenge. Tissue samples were homogenized in VTM or PBS in gentleMACS M tubes (Thistle Scientific) on a dispomix machine (Medic Tools AG). Swabs were

sonicated in the VTM they were collected in and aliquoted for later use. Buffy coats were prepared from heparinised blood samples and resuspended in PBS. 100µl of sample was added to sample tubes and set up for the DNeasy animal blood and cells protocol according to the manufacturer's instructions. DNA was eluted in 50µl of the supplied AE buffer.

2.5.3 DNA from purified virus

It was important to get the maximum DNA yield from the purified virus resuspended in TNE buffer for restriction enzyme digestion and Solexa sequencing. The Easy DNA kit (Invitrogen) was found to be the most suitable for this purpose. Briefly, solution A was added to the sample and incubated at 65°C to lyse cells, solution B and chloroform was added to precipitate the lipids and proteins. Centrifugation separated the different phases and DNA was isolated in the aqueous phase. This phase was removed from the solid phase and the chloroform, to be precipitated with ethanol. The resulting DNA pellet after centrifugation was resuspended in the TE buffer provided.

2.5.4 RNA isolation from virus in cell culture, tissue and lung slices

RNA was extracted from pellets of virus infected cultured cells resuspended in RLT buffer and tissues or lung slices stored in RNA later were homogenized in RLT buffer (Qiagen) in gentleMACS M tubes (Thistle Scientific) on a dispomix machine (Medic Tools AG). Both cell and tissue samples underwent further disruption to ensure RNA was released from the cells by centrifuging samples in qiashredder tubes (Qiagen). The RNeasy (Qiagen) animal cells spin protocol was then followed according to the manufacturer's instructions. RNA was eluted in 35µl double distilled water.

All RNA was treated with DNase to reduce DNA contamination which would affect interpretation of viral gene expression. Briefly, 10u of DNase recombinant I and 5x incubation buffer (Roche) was added to RNA samples and made up to 50µl with ddH_2O and incubated at 35°C for 20 minutes. EDTA was added to a final concentration of 8mM and heated to 75°C for 10 minutes to stop the reaction.

2.5.5 Nucleic acid quantification

The nanodrop spectrophotometer (Applied Biosystems) was used to quantify and quality check any DNA and RNA. Briefly, after blanking the machine with the elution buffer, 1.5µl of the sample was placed on the fluorescence reader. The nucleic acid yield was recorded in ng/µl and the 260:280 ratio was recorded enabling rejection of any failed or poor quality extractions. The quantification was also used to check for gross contamination in the negative control.

2.5.6 Cloning to produce standards for absolute quantification real-time PCR

Cloning methods were used to produce plasmid DNA containing the inserted DNA fragment of interest. A BoHV-1 glycoprotein B DNA fragment was amplified using platinum taq DNA polymerase PCR (Invitrogen) following manufacturer's instructions. Briefly, each 50µl PCR reaction was made up of PCR buffer (Invitrogen), 0.8mM dNTPs (Bioline), 1.5mM $MgCl_2$, 1U platinum taq DNA polymerase (Invitrogen), 200nM of each primer, gB-F and gB-R (table 2.3), 10ng DNA and ddH_20. The reactions were subjected to the following conditions on a thermal cycler (MBS, Thermo), 2 minutes at 94°C, followed by 40 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. The reaction was maintained at 4°C after cycling. PCR products were run using electrophoresis on a 2% agarose gel in Tris acetate EDTA (TAE) buffer containing ethidium bromide to bind the amplified DNA at 80v for 45 minutes. The DNA bands were visualised using a UV transilluminator to check the size of the bands and that only a single product had been produced (figure 2.3).

The PCR product was cloned using the TOPO TA cloning kit for sequencing (Invitrogen). The cloning reaction was made up of 2 µl PCR product, salt solution made up to a volume of 5µl with ddH_2O before adding 1µl of the pCR4-TOPO vector (figure 2.4). The reaction was incubated at room temperature for 5 minutes then placed on ice. Two controls were included: vector only and supplied PCR product. TOP10 *E. coli* cells were transformed by adding 2µl of the cloning reaction to the vial of competent cells and incubating on ice for 5 minutes. The cells were then heat-shocked for 30 seconds at 42°C and immediately transferred back to ice.

250µl of room temperature SOC medium was added and the cells were shaken horizontally for 1 hour at 37°C. Cells in SOC medium were spread on LB agar plates containing 100µg/ml ampicillin, 2 plates were set up for each transformation, with 10 and 100 µl to ensure one of the plates produced evenly spaced colonies. Plates were incubated overnight at 37°C overnight. All colonies that grew were considered successful transformations as the inserted DNA disrupts the lethal *E.coli ccdB* gene (figure 2.4).

To check successful cloning of the gB DNA fragment, a PCR reaction was set up as described above using the supplied T7 or T3 primers paired with either the gB-F or gB-R with a small amount of the colony added to the PCR reaction mix instead of template DNA. Finally, to confirm plasmids contained the correct DNA insert, DNA was purified using Wizard Plus SV Minipreps DNA purification System (Promega), according to manufacturer's instructions and eluted into 100µl of double distilled water before sending off for sequencing with GATC.

Figure 2.3. Gel image showing 100bp BoHV-1 gB fragment PCR product. Lanes 1-3 IBR6660, Lane 4 no template control. (M – marker lane)

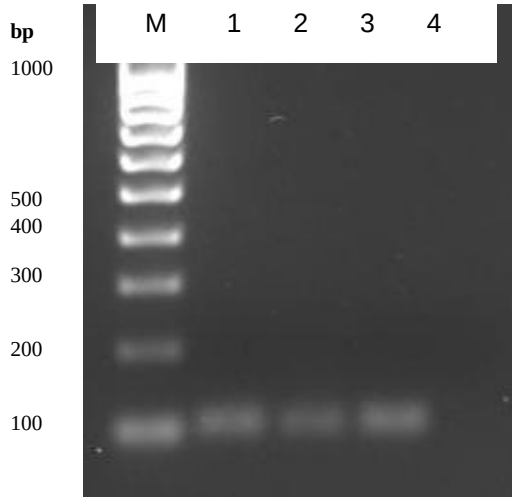
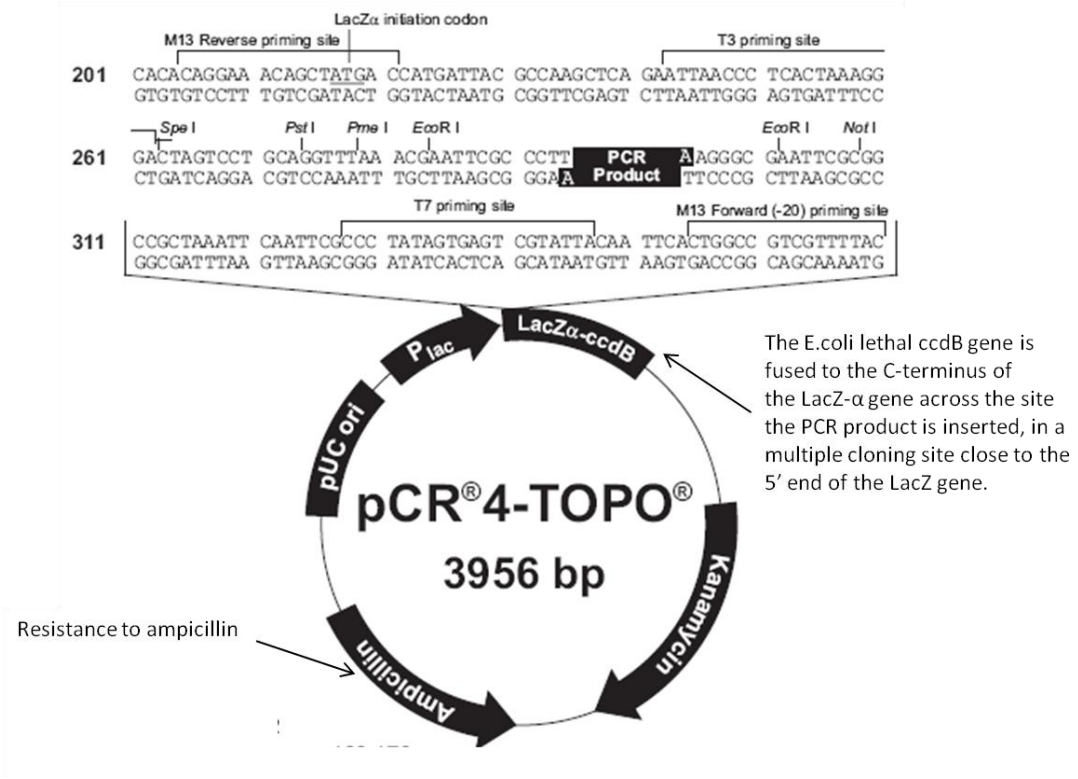


Figure 2.4 pCR4-TOPO vector map. Adapted from Invitrogen Manual.



2.5.7 Restriction endonuclease analysis

Restriction enzymes *Hind III* and *EcoRI* (Promega) were used to cleave DNA extracted from purified viral isolates to identify the BoHV-1 subtype. A restriction enzyme digestion containing 2µl restriction enzyme buffer, 2µl acetylated BSA (1µg/µl), 1µg sample DNA and 20 units of restriction enzyme made up to 20µl with ddH_2O was incubated overnight at 37°C. Control reactions, one containing no enzyme and one containing lambda DNA were also set up for each digestion.

After the incubation, gel loading buffer was added to the samples and along with marker X (Roche) and lambda *Hind III* digested DNA (Sigma) were loaded into a 0.75% agarose gel in TAE buffer. The gel was run overnight at 12v and stained using SYBRGold (Invitrogen) in TAE buffer according to manufacturer's instructions for 40 minutes. The gel was scanned using a laser imaging system (Biorad) to visualise bands of cleaved DNA to produce an image that could then be analysed.

2.6 Real time PCR

2.6.1 Primers and probes

With the exception of the glycoprotein B primers and probe (Wang et al., 2007) all primers and probes were designed using Primer Express 2.0 (Applied Biosystems) and used minor groove binding taq man probes labelled with a reporter fluorescence marker and a non-fluorescent quencher. For each primer/probe combination working concentrations were optimised and reaction efficiency evaluated to ensure optimum conditions were used. This involved a cross tabulation design to test 3 primer concentrations for both the forward and reverse primers (table 2.3).

Table 2.3. Primer concentration combinations for optimisation.

Reverse primer concentration (nM)	**Forward primer concentration (nM)**		
	50	**300**	**900**
50	50/50	300/50	900/50
300	50/300	300/300	900/300
900	50/900	300/900	900/900

Efficiency was tested by creating a 10 fold dilution of a known positive DNA/RNA sample and running with the optimised primer/probe set. Efficiency was calculated using the slope of the standard curve produced with the equation:

Reaction Efficiency = $(10^{(-1/slope)}-1) \times 100$

An efficiency of 100% shows each cycle is producing double the amount of PCR product which should occur if all the conditions are optimum. The assay conditions were accepted if the efficiency was greater than 90%. It is particularly important that primer/probe sets being used for relative quantification have a similar efficiency. Primer/probe sequences, working concentrations and efficiency values are summarised in table 2.4.

Table 2.4. Primer/probe details.

Primer/ probe	Working conc.	Efficiency (%)	Viral genome position	Sequence and labels
gB-F	500	104	57499-57519	5' TGT GGA CCT AAA CCT CAC GGT 3'
gB-R	500		57595-57575	5' GTA GTC GAG CAG ACC CGT GTC 3'
gB probe	200		57525-57545	FAM-AGG ACC GCG AGT TCT TGC CGC-TAMRA
Circ F	300	93	811-830	5' CCG CCT CGC CGA TTC 3'
Circ R	300		828-808	5' GCT GCT GTA GTC CGG CAG TAG 3'
Circ probe	200		832-845	FAM-CTG CAC GAC TTT CT-MGB
RR F	200	101	23285-23263	5' CCC AAA AAG ACA TCC TGC ATT AC 3'
RR R	200		23226-23243	5' GCG AGT GCA CCA CTT CGA 3'
RR probe	100		23261-23245	FAM-ATA TCG AGC AGG AGT CC-MGB
actin F	500	97	N/A	5'GAC AGG ATG CAG AAR GAG ATC AC 3'
actin R	500		N/A	5' TCC ACA TCT GCT GGA AGG TG 3'
actin probe	200		N/A	NED-TGA AGA TCA AGA TCA TCG-MGB

2.6.2 Real-time PCR as a diagnostic tool

Real-time PCR can be used as a diagnostic tool for detection of viruses from swabs/tissues or cells. A region of BoHV-1 glycoprotein B was targeted to detect the presence of BoHV-1 in tissue DNA samples prepared as described previously. This assay was previously published for detection of BoHV-1 in semen samples (Wang et al., 2007) and was adapted and optimised for use with cell and tissue DNA samples, for use in this project. The platinum quantitative PCR supermix-UDG (Invitrogen) mastermix containing, 0.1µl ROX reference dye, primer and probes at optimised concentrations (table 2.4) in a final volume of 25 µl was plated out on 96-well reaction plates (Applied Biosystems) before template DNA was added in a separate room to avoid contamination of the PCR setup area. Each plate always included a positive control sample, a DNA extraction negative control and no-template control. All diagnostic samples were run in triplicate. The plates were run on the 7500 ABI Prism system (Applied Biosystems) under the following conditions: 50°C for 2 minutes UDG incubation, 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds.

Results were analysed using the supplied ABI Prism 7500 software (Applied Biosystems). A baseline and threshold level was set according to manufacturer's

guidelines. Samples were considered positive when at least 2/3 wells were positive, or were repeated if only 1/3 wells were positive. If any contamination was suspected in the DNA extraction control by detection late in the reaction a cut-off point below this detection level was made to ensure no false positives were recorded.

2.6.3 Real time PCR as a quantification tool

Absolute quantification

Absolute quantification was used to quantify viral load using standards produced with known copy numbers of the BoHV-1 glycoprotein B fragment in the form of plasmid DNA. Samples were run on 96-well plates on the ABI Prism 7500 system (Applied Biosystems) as described for the diagnostic method, with the exceptions of samples run in duplicate and on each plate the 4 plasmid DNA standards were included containing 10^{10}, 10^{8}, 10^{6}, 10^{4} copies in triplicate. Samples containing known viral copy numbers were calculated as follows:

pCR4-TOPO + gB fragment = 4055 bp
Weight in Daltons = 2676300 g/mol
267300g plasmid DNA = 6.02×10^{23} copies
1µg plasmid DNA = 2.25×10^{11}copies
Samples containing specific copy numbers were made by diluting plasmid DNA accordingly.

The results were analysed by plotting the samples against the standard curve produced by the four standards using the supplied ABI Prism 7500 software. This provided absolute copy number values of virus genome detected in the template DNA. For the swabs this was taken as the final value as there should not have been much variation in the amount of non-viral DNA isolated so different swabs could be directly compared as it was a good representative for the amount of virus being shed in nasal or ocular discharge. For the tissues it was necessary to ensure that the values took into account different DNA quantities in the different samples and tissues. Therefore assuming the viral genome DNA contributed minimally compared to host genome DNA isolated, the number of cells that each sample represented was calculated using the DNA quantification values from the Nanodrop, based on each

bovine diploid cell containing 6.3pg DNA (Gregory 2010). This allowed calculation of viral copy number per million cells to make comparisons between the different samples (Islam *et al.* 2006).

Statistical analysis to compare viral load was done using two methods depending on the sample type. Tissues from BoHV-1 experimental challenge: Minitab was used to conduct a two- way ANOVA to compare viral load between groups infected with the two isolate types, treatment and time post-infection were used as factors, if $p<0.05$ differences were considered significant. Swabs from BoHV-1 experimental challenge were not independent from one day to the next as they were taken from the same animal, therefore a test was needed that would take into account the relationships between the samples. Genstat was used to carry out a REML analysis using covariance structure model power-city block distance. If an interaction of $p<0.05$ was identified the predicted means were studied to identify the days post-infection were most likely to be significantly different between treatments. T-tests were then carried out on these results based on the predicted means table and standard errors of differences calculated by the REML analysis, differences were considered significant if $p<0.05$.

Relative quantification

Relative quantification using real time PCR enables comparison of viral load or gene expression relative to other tissues or samples. DNA was extracted from infected tissue that had tested positive for BoHV-1 using the diagnostic test previously described. For abortion samples the comparative ΔΔCt method (Livak & Schmittgen 2001) was used to compare virus distribution. The platinum quantitative PCR supermix-UDG (Invitrogen) was used to detect a BoHV-1 glycoprotein B fragment and also the host reference gene β-actin, to provide a value for normalisation for the size of tissue sample and yield of DNA extracted. A calibrator sample was used to provide a relative value for viral load in all tissues in comparison with the calibrator sample. For this work the brain was used as the calibrator as it demonstrated a consistent positive but low level of viral load. The equation used to calculate the relative viral load was:

Relative viral load = $2^{-\Delta\Delta Ct}$

$\Delta\Delta Ct$ = (sample gB Ct - sample β-actin Ct) – (calibrator gB Ct- calibrator β-actin Ct)

(Livak & Schmittgen 2001)

Relative quantification was also used to compare viral gene expression during infection. RNA was extracted from infected cells lung slices, or tissue samples from animals used in the experimental challenge. Samples were run on 96 well plates using the Superscript III Platinum One-Step Quantitative RT-PCR System (Invitrogen) according to the manufacturer's instructions. Briefly, 25µl reaction mixes contained Superscript III mastermix with added, forward, reverse primers and probe at optimised concentrations (table 2.3), 0.1µl ROX reference dye and Superscript III RT/Platinum Taq (Invitrogen) in ddH_2O. For each primer/probe set on every plate a reverse transcription control was run to check for DNA contamination by replacing the Superscript III RT/Platinum Taq mix with Platinum Taq DNA polymerase. Plates were run on the ABI Prism 7500 (Applied Biosystems) using the following cycling conditions: 50°C for 15 minutes, 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. On all plates the viral target primer/probes were run alongside the host reference β-actin primers/probes to normalise samples. Further normalisation was achieved by using the same quantity of RNA from each tissue sample as RNA extraction yield varied significantly between samples. The cells produced more consistent RNA yields and therefore the β-actin reference was considered adequate for the normalisation of these samples. The best calibrator would be an uninfected control or 0 time point. However, as no viral gene expression would be present in these samples it was decided to not use a calibrator sample for these samples and use the equation:

Relative viral gene expression = $2^{-\Delta Ct}$

ΔCt = target Ct – reference Ct

(Livak & Schmittgen 2001; Morse *et al.* 2005; Kubota *et al.* 2008)

Statistical analysis of viral load in abortion study tissue was carried out in Minitab using a GLM with tissue and abortion case as factors, a post-hoc Tukey test to

identify which tissues contained significantly different viral loads. Analysis of viral transcript expression differences in tissues from the experimental challenge and infected lung slices were analysed in excel using a two sample t-test to compare the two isolate types and were considered significant if the two-tail p-value< 0.05. In the statistical analysis BT cell and lung slice infection transcription quantification a one way ANOVA and two-sample t test methods were used respectively and a p-value <0.05 was considered significant.

2.7 Histopathology

2.7.1 Handling of samples for histology

All samples for histology were fixed in 10% formal saline for at least 24 hours. Samples were embedded in paraffin wax by trimming and placing into cassette cases. A processing machine was used to dehydrate samples and add paraffin wax to the sample, they were then embedded in paraffin wax blocks using standard protocols. Blocks were stored at room temperature until sections were cut using a microtome. After cutting sections were placed on slides and dried overnight at 37°C. Some tissues required decalcification treatment due to high quantities of cartilage or bone. Blocks were briefly soaked in Decalcifier II (Surgipath) to enable better sections to be cut. Samples for histological analysis were stained with haematoxylin and eosin using standard procedures. Other slides were stored at room temperature until used in immunohistochemical labelling.

2.7.2 Immunohistohemistry

Tissue sections of interest were studied in more detail using the Envision-HRP system (Dako) to stain the glycoprotein C protein of BoHV-1 with a monoclonal mouse antibody to enable localisation of the virus within the tissues. Formal saline fixed sections embedded in paraffin wax were cut and placed on superfrost slides. Samples were then rehydrated by placing the slides consecutively in xylene, 100% ethanol, 70% ethanol and water. Slides were washed with tris buffered saline (TBS) buffer (50mM Tris-HCl, 150mM NaCl in dH_20 adjusted to pH 7.6) and placed in a peroxidase block (3% hydrogen peroxide in methanol) for 30 minutes at room

temperature to bind to any endogenous peroxidises so they do not act on the added substrate. Slides were washed in water and then TBS after placing slides in cover plates (Sequenza). 100µl of 25% normal goat serum in TBS buffer was added to each slide as a further block to prevent non-specific binding of the secondary antibody and incubated at room temperature for 30 minutes. Slides were washed twice with TBS buffer and then 100µl of the primary antibody, anti-gC BoHV-1 (VMRD) (Collins *et al.* 1984), diluted 1:5000 in TBS was added to all positive slides. Each section had a corresponding negative control to which TBS was added instead of the primary antibody. A positive control was also used in the form of a liver section known to be positive for BoHV-1 in each run. The slides were incubated overnight with the antibody at 4°C. After the incubation, slides were washed twice with TBS and 100µl of anti-mouse labelled polymer (Dako) was added to all slides and incubated for 30 minutes at room temperature. Slides were washed twice in TBS buffer before staining with the chromogen DAB (Dako) for 7 minutes, which binds with the anti-mouse polymer to result in brown staining at sites labelled with the primary antibody. Slides were washed with water, counterstained with haematoxylin and dehydrated by placing slides consecutively in 70% ethanol, 100% ethanol and xylene. Finally, coverslips were fixed with slide mountant and slides were studied using a light microscope to look for DAB labelling indicating presence of BoHV-1.

2.7.3 Immunofluorescence

Immunofluorescence was used to image localisation of BoHV-1 in lung slice cultures. Lung slices that had been snap-frozen in liquid nitrogen on gelatine discs and subsequently stored at -70°C were sectioned using a cryostat to cut 10µm sections that could then be placed onto slides. The sections were fixed by placing in acetone for ~30 seconds and allowed to dry at room temperature, before storing at -20°C until they were stained.

Sections were double labelled using a parallel approach with one of two combinations of antibodies (table 2.5). Briefly, slides were put in coverplates (Sequenza) and washed with TBS. 100µl of 10% v/v normal goat serum in TBS was added and slides were incubated for 30 minutes at room temperature to block non-

specific binding of secondary antibodies. After washing twice with TBS 100µl of primary antibodies were added at appropriate concentrations diluted in TBS (table 2.5) and allowed to incubate overnight at 4°C. Slides were washed twice with TBS before adding 100µl of secondary antibodies diluted to the appropriate concentrations in TBS (table 2.5) and incubated for 30 minutes at room temperature. Slides were washed with TBS then PBS before 300mM DAPI in PBS was added for 5 minutes at room temperature to counterstain cell nuclei. Slides were washed in water, any excess water was then removed before Prolong anti-fade mountant (Invitrogen) was used to fix coverslips. This was allowed to cure for 24 hours before sealing coverslips with clear nail varnish.

Control slides were run with no primary antibodies and also with single sets of antibodies to check for cross-reactivity in the parallel approach (e.g cytokeratin primary antibody with BoHV-1 secondary antibody).

Table 2.5. Details of antibodies used for the double immunofluorescence staining.

Targets	**Primary antibody 1**	**Primary antibody 2**	**Secondary antibody 1**	**Secondary antibody 2**
BoHV-1 and endothelial cells (von Williebrand factor)	Monoclonal mouse anti-BoHV-1 gC (IgG2b) 1:1000 dilution (VMRD)	Polyclonal rabbit anti-human von Williebrand factor 1:50 dilution (Dako)	Alexa fluor 488 (green) goat anti-mouse (IgG2b) 1:1000 dilution (Invitrogen)	Alex fluor 647 (far red) goat anti-rabbit 1:100 dilution (Invitrogen)
BoHV-1 and epithelial cells (cytokeratin)	Monoclonal mouse anti-BoHV-1 gC (IgG2b) 1:1000 dilution (VMRD)	Monoclonal mouse anti-human cytokeratin (IgG1) 1:50 dilution (Dako)	Alexa fluor 488 (green) goat anti-mouse (IgG2b) 1:1000 dilution (Invitrogen)	Alex fluor 647 (far red) goat anti-mouse (IgG1) 1:100 dilution (Invitrogen)

Sections were photographed using the Axiovision software (Zeiss Microscopy) at wavelengths of 488nm to detect the green fluorescent antibody and 647nm to detect any red fluorescent antibody at sites where positive virus was identified. Negative controls were checked to ensure labelling was virus specific. Photographs were taken of the negatives using the same exposure as for the positive sections. Images were created by merging photos from 488 and 647 wavelengths in Image J. Areas of co-localisation of red and green secondary antibodies were used to analyse virus association with either epithelial or endothelial cells. To reduce the subjectivity of

interpreting the co-localisation intensity levels were set and co-localisation marked in white at sites where both fluorescent signals reached the specified levels.

2.8 Comparative genomics

2.8.1 Next generation sequencing

Whole genome sequencing of 11 BoHV-1 isolates was carried out by Genepool, University of Edinburgh on one of their next generation sequencing platforms. The DNA was sequenced on the Solexa GAII platform (Illumina) which uses a 'flowcell' system to immobilise, amplify and sequence the DNA to produce millions of sequence reads of ~50 bases at a high coverage to produce reliable base calling.

2.8.2 Sequence read assembly

As a full sequence of BoHV-1 had already been completed this was a re-sequencing project which means the original sequence was used as a scaffold to produce the final consensus sequences from the 50 base reads. This was achieved using the MAQ program which maps the raw Solexa data onto the reference sequence to produce a consensus sequence in a fasta or fastq format (Li, Ruan & Durbin 2009). Sequence data was initially converted from the Sanger text format produced by the Illumina Solexa technology to fastq format that could be used by MAQ for assembly. Next the 'easyrun' assembly method was used in MAQ to map all reads to the scaffold and produce several files for analysis. Coverage and data detailing percentage of the total reads mapped was produced to enable comparison between sequencing runs. It was also possible to view the assembly using Maqview, where any area of low coverage could be easily spotted and further analysed (Trivedi 2008). Further details of this analysis will be discussed in more detail in chapter four.

2.8.3 Sequence comparisons

Comparisons between the isolate consensus sequences were made by looking for SNPs. The MAQ software produced a SNP file alongside the consensus sequence. By comparing the SNPs between each isolate and the reference it was possible to identify the SNP variation across all the isolates. The SNPs were studied for quality

score and a filtered version of SNPs was used to draw the final conclusions. All statistical analysis was carried out in Excel using a one way ANOVA for more than two groups or a two sample t-test for tests, significant differences between samples were identified if $p < 0.05$.

Phylogenetic analysis was also used to look for any patterns in the variation between the isolates. The genome was divided into 3 sections Ul, Us and repeat regions for the purposes of the phylogenetic analysis. Sequences were kept in the nucleotide format to allow non-coding regions to be included in the analysis. A multiple alignment including BoHV-5 and BoHV-1 references alongside all the Solexa sequenced isolates was created using ClustalW (Thompson, Gibson & Higgins 2002). Any aligned columns containing any non-AGTC characters were removed using Base-by-Base (Brodie *et al.* 2004). Using this alignment a neighbour-joining comparison with 1000 bootstraps was made using ClustalX. The alignment was also put into the Phylip software where multiple data files (100 bootstraps) were produced in Seqboot and used as the input for Dnaml to make a bootstrapped maximum likelihood comparison of the sequences. Finally, Consense, also part of the Phylip package was used to produce the consensus tree (Felsenstein 1989).

Chapter Three: Prevalence and pathogenesis of BoHV-1 as an abortifacient agent

3.1 Introduction

BoHV-1 is generally not considered an important abortifacient agent in the UK. The most recent survey on causes of bovine abortions in Scotland found a prevalence of BoHV-1 of less than 2% (SAC VS 2009). Bacterial agents are often more commonly associated with infectious bovine abortion outbreaks as reported in a review from figures in the United States (Anderson 2007). Diagnosis of the cause of abortion can be problematic due to tissue sample autolysis and also the availability of key samples such as the placenta. This means investigations often rely on serology even though this is not the most reliable method of diagnosis (Davison, Otter & Trees 1999). In the case of enveloped viruses such as BoHV-1, virus particles may no longer be infectious by the time tissue has been expelled (up to 7 days post foetal death) meaning a negative result would be found from virus isolation in cell culture. Secondary causes of bacterial infection may also be over-represented, as bacteria can survive without viable cells for longer and are generally easier to grow in culture.

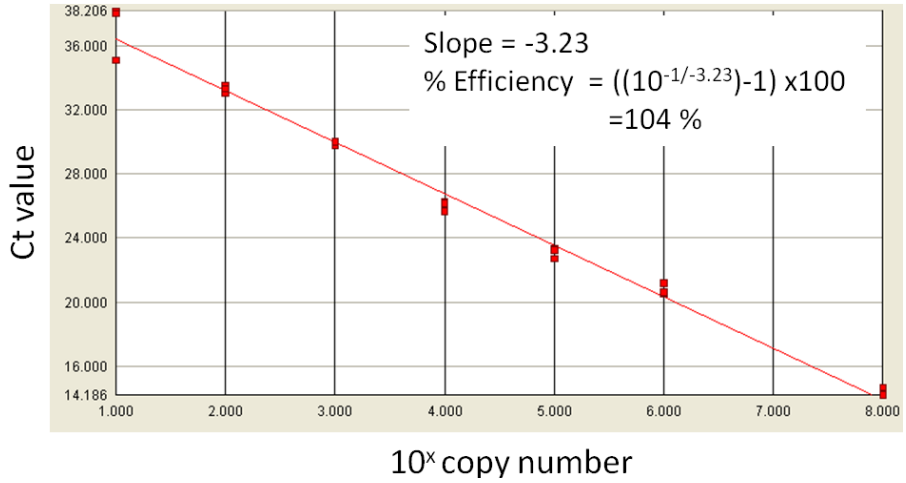
The aim of this study was to evaluate the prevalence of this disease and to compare the effectiveness of different diagnostic techniques for these sample types. Four hundred UK diagnostic abortion cases were tested for the presence of BoHV-1 using a real-time PCR assay, alongside virus isolation and histopathology. The molecular approach of real-time PCR provides the advantage of relying on the detection of a region of the viral genome rather than replicating virus or intact tissue structure. The real-time PCR method used has been validated by an international ring trial for screening bull semen (Wang *et al.* 2007; Wang *et al.* 2008). It was adapted for use with tissue samples for the purposes of this work, the primers and probe were optimised and a standard curve was used to check the efficiency of the assay.

Secondly, this work aimed to investigate the hypothesized but poorly documented transplacental spread of BoHV-1 into the foetus. Since 1963 when BoHV-1 was first recorded as the causal agent of an abortion, little progress has been made in the understanding of how it reaches the uterus from the respiratory tract and crosses the placental barrier to infect the foetus (Ormsbee 1963). In this study relative quantification by real-time PCR and localisation of viral antigen by immunohistochemistry was used to study the distribution of BoHV-1 in positive foetal tissues. Viral load and distribution was compared in foetal tissues, including placenta and foetal fluid, liver, heart, lung and brain, with the hope of learning more about the mechanisms of transplacental spread and foetal infection.

3.2 Real-time PCR as a diagnostic tool in abortion cases

Real-time PCR targeting a glycoprotein B fragment of the BoHV-1 genome (Wang *et al.* 2007; Wang *et al.* 2008), was optimised for use with DNA extracted from virus isolation and fresh tissue samples by testing different primer and probe concentrations (see sections 2.6.1 and 2.6.2 for detailed methods). The results showed the best primer concentrations were 500nM for each of the primers and 250nM for the probe. The assay was further analysed to check the efficiency by running a standard curve with a 10 fold series dilution of plasmid DNA containing the BoHV-1 gB fragment. This enabled calculation of both the efficiency and sensitivity of the assay. The results showed between 1 and 10 viral copies could be reliably detected. The efficiency curve gave a value of 104%, also showing a good exponential amplification of the target DNA fragment (figure 3.1). BoHV-1 DNA was successfully detected in tissue sample DNA and DNA extracted from cells showing the characteristic cytopathic effect typical of BoHV-1 infection.

Figure 3.1. Real-time PCR efficiency curve and calculation for glycoprotein B.



3.3 Prevalence of BoHV-1 in bovine abortions in the UK

Different samples from 400 abortion cases were received for testing. Most originated from Scottish farms with a smaller number from England (between 2007 and 2009). This was a prospective study, and therefore we also had access to the results from previous tests and diagnosis, determined without the use of molecular diagnostic techniques.

All tissues received from each case were initially pooled and extracted DNA was tested in duplicate in the real-time PCR assay. The final results found 10 (2.5%) of the tissue pools tested positive for BoHV-1 by PCR. Further tests were conducted on the individual tissues that were available from these positive samples (table 3.1). Virus isolation used pooled and individual tissue samples to inoculate BT cells. Only one of the placenta samples added to cell culture resulted in any cytopathic effect typical of BoHV-1. Among the 10 PCR positive samples tested using all techniques, histopathological examination and IHC demonstrated pathology consistent with BoHV-1 in 8/10 liver samples, 4/9 lung samples and 3/4 placenta samples (table 3.1 and 3.2). IHC identified a higher proportion of positive results as positive labelling was found in heart, lung, brain and placenta samples where histopathological lesions were not evident (table 3.2). Observations in both the lesions seen from H&E staining and IHC detection of BoHV-1 generally supported the PCR results, (tables

3.1 and 3.2). However, some cases such as 09/462 liver and lung and 07/558 heart and brain demonstrates positive PCR results but no IHC labelling. No IHC, virus isolation or lesions were observed in PCR negative tissue. It should be noted that due to autolysis, lesions in the placenta were only visible after IHC identified the location of BoHV-1 infection. All other lesions reported were visible on histological examination alone. However, cases with lesions did not always correlate with IHC positive cases showing some lesions were insignificant for the investigation of the cause of abortion. For details of lesions see appendix 1.

Independently of this study, the 400 cases were investigated in other laboratories. The previous findings from the 10 BoHV-1 positive cases found here are detailed in table 3.3. Only 2 of the 10 BoHV-1 PCR positive samples (07/128 and 09/334) had been diagnosed previously as potential BoHV-1 abortions. One was confirmed by IHC and the other suggested by a rising BoHV-1 antibody titre in maternal serology results and supported by clinical observations. Testing focussed on BVD and bacterial causes such as *Leptospira hardjo* and *Brucella sp.* Five of the cases had a final diagnosis of a bacterial cause, although at least 3 of these cases showed multifocal necrotic hepatitis, characteristic of a BoHV-1 foetal infection (table 3.3). However, no IHC was conducted to confirm BoHV-1 as the cause of the lesions. Clinical history showed one case which reported clinical signs typical of an IBR infection a month prior to the abortion. Two of the cases confirm no respiratory disease was evident prior to abortion. No clinical history was available for the other cases. Gross pathology revealed lesions of the placenta in two cases (07/327 and 09/334) but no other gross lesions were reported relating to the foetus in any of these cases. Autolysis was reported at varying levels in 8 of the 10 cases. It was also reported that at least one of the cases occurred in a BoHV-1 vaccinated herd.

Table 3.1. Results for each test for the BoHV-1 positive cases. (PCR – real-time PCR, HP- histopathology and IHC- immunohistochemistry, VI- virus isolation, + or - test result testing for BoHV-1 or lesion for histopath., ns – no sample available for this test).

Sample	Pool				Liver				Heart				Lung				Brain				Foetal fluid				Placenta			
Ref No.	PCR	VI	HP	IHC	PCR	VI	HP	IHC	PCR	VI	HP	IHC	PCR	VI	HP	IHC	PCR	VI	HP	IHC	PCR	VI	HP	IHC	PCR	VI	HP	IHC
07/128	+	-	ns	ns	+	ns	+	+	+	ns	-	+	ns	ns	+	+	+	ns	ns	ns	+	ns	ns	ns	ns	ns	-	+
07/232	+	-	ns	ns	+	ns	+	+	+	ns	-	-	+	ns	+	-	+	ns	+	+	+	ns	ns	ns	ns	ns	ns	ns
07/233	+	-	ns	ns	+	ns	+	+	+	ns	-	-	+	ns	-	-	+	ns	-	-	+	ns	ns	ns	ns	ns	ns	ns
07/327	+	-	ns	ns	+	ns	+	+	+	ns	-	+	+	ns	-	+	+	ns	-	+	+	ns	ns	ns	+	ns	+	+
07/558	+	-	ns	ns	+	ns	+	+	+	ns	-	-	ns	ns	-	+	+	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
09/046	+	ns	ns	ns	-	-	-	-	-	ns	-	-	-	-	-	-	-	ns	-	-	-	ns	ns	ns	+	-	ns	ns
09/050	+	ns	ns	ns	+	-	+	+	+	ns	-	+	+	-	-	+	+	ns	-	+	+	ns	ns	ns	ns	ns	ns	ns
09/334	+	ns	ns	ns	+	-	+	+	+	-	-	+	+	-	ns	ns	+	-	ns	ns	+	ns	ns	ns	+	+	+	+
09/461	+	ns	ns	ns	+	-	+	+	+	-	ns	ns	ns	ns	+	+	+	-	ns	ns	+	ns	ns	ns	+	ns	+	+
09/462	+	ns	ns	ns	+	-	-	-	+	-	ns	ns	+	-	+	-	+	-	+	-	-	ns	ns	ns	ns	ns	ns	ns

Table 3.2. Summary of the results from table 3.1 showing comparisons between the diagnostic techniques (no. positive samples/total no. of samples tested)

Tissue	PCR	Virus isolation	Lesion	IHC
Pool	10/400	0/5	-	-
Brain	9/10	0/3	2/6	3/6
Foetal fluid	8/9	-	-	-
Heart	9/10	0/3	0/8	4/8
Liver	9/10	0/5	8/10	8/10
Lung	6/7	0/4	4/9	5/9
Placenta	4/4	1/3	3/4	4/4

Table 3.3. Summary of previous diagnostic details and other available information about abortion cases that have tested positive for BoHV-1 by PCR in this study.

Case	Breed	Gestation (months)	Gross findings	Original testing	Original diagnosis	Other relavent history
07/128	Aberdeen angus cross	~6 months	None	Bacillus cultured. BVD antibody negative. BoHV-1 IHC positive.	BoHV-1 and Bacillus sp.	-
07/232	Simmental cross - Twins	8.5 months	Autolysis - 3/5. .	Salmonella, campylobacter negative. BVD and neospora antibody negative. L. hardjo negative. Multifocal necrotising hepatitis.	Suspected bacterial cause	No sign of respiratory disease
07/233			Autolysis - 3/5	Salmonella, campylobacter negative. BVD and neospora antibody negative. L. hardjo antibody positive. Multifocal necrotising hepatitis.	Suspected bacterial cause	
07/327	Unknown	Unknown	Placenta inflammation. Autolysis.	Salmonella, campylobacter, brucella negative. BVD negative. Seropositive foetal fluid for L. hardjo.	L.hardjo (based on foetal serology)	Abortion in an older unvaccinated cow of same herd
07/558	Aberdeen angus?	~ 7 months	None	Bacillus lichenformis found. Negative by serology for L hardjo, Neospora, IBR and BVD.	Bacillus licheniformis	-
09/046	Aberdeen angus	8.5 months	Moderate autolysis, no lesions.	No bacterial pathogens isolated. No significant changes seen in histopathology. Antibody negative for L. hardjo.	No evidence of infectious cause.	No sign of respiratory disease
09/050	Ayrshire heifer	~ 6 month	Autolysis - 3 /5. No lesions.	Bacteriology - no significant organisms isolated. Foetal fluid negative for BVD and leptospirosis. No histopathology except autolysis. Serology negative for Neospora.	None	Herd IBR vaccinated
09/334	Holstein dairy	7 months	Autolysis - 4/5. Placental vessels slightly injected.	BVD, neospora Ab negative. L. hardjo MAT negative. S. uberis isolated from placenta. Rising maternal Ab titre for IBR. Multifocal necrosis in liver shown by histopath.	BoHV-1	High temp and drop of milk yield 1 month before sample submission
09/461	Unknown	~5 months	Autolysis - 5/5. No lesions	Negative for BVD and L. hardjo, neospora.	None	-
09/462	Unknown	~ 8 months	Autolysis - 3/5. No lesions noted.	Negative for BVD and L. hardjo, neospora. Actinomyces pyogenes. Maternal serology positive for BVD. Histology showed possible viral infection.	None	-

3.4 Molecular investigation of viral distribution in BoHV-1 abortion cases

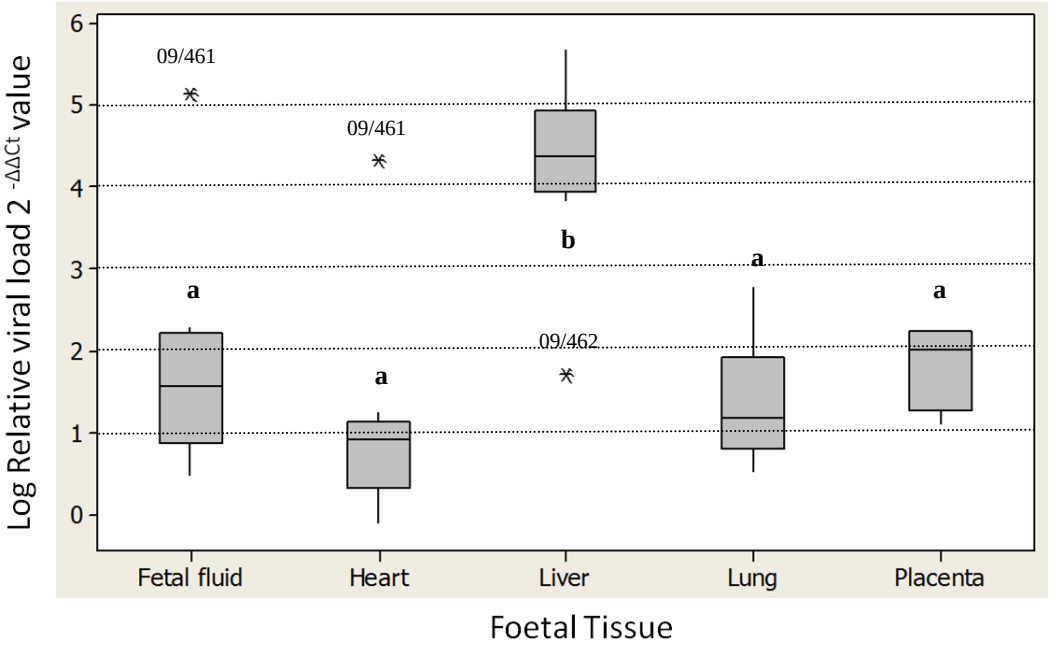
BoHV-1 distribution during an infection of the foetus was investigated by relative quantification of real-time PCR results, to compare BoHV-1 viral load between individual tissues from the 10 positive cases. Individual tissues available from the original pooled samples submitted were homogenized and DNA was extracted. Samples were run in triplicate using the real-time PCR diagnostic method targeting BoHV-1 gB alongside the host β-actin target. The β-actin target was needed as a reference to normalize the amount of DNA present in each sample type, which can vary due to cell density in the tissue and efficiency of DNA extracted from each sample type (see 2.6.3 for detailed methods). The brain was used as the calibrator sample as it was present in all cases and showed a consistent low level of BoHV-1 viral load. These measurements enabled calculation of relative viral load using the comparative ΔΔ Ct method (Livak and Schmittgen, 2001).

BoHV-1 DNA was detected in all individual tissue samples from positive pools, with the exception of one case (09/046- table 3.1) in which BoHV-1 was detected only in the placenta. This set was therefore unsuitable for inclusion in the relative quantification analysis. It may also be relevant that this sample was found to be toxic in virus isolation suggesting other infectious agents were also present.

The relative quantification of individual tissue viral load showed largely consistent differences in each of the cases between the tissues types (figure 3.2). A General Linear Model (GLM) with a Tukey post-hoc test identified a significantly higher viral load in the liver relative to the other tissues ($p<0.01$) with a mean value of 86,000 fold higher viral load than the calibrator brain sample. There was large variation seen between the different cases indicated by the outliers (figure 3.2), but in all cases there was a comparatively higher viral load in the liver than the brain. In particular, it should be noted that the outlier values showing high relative viral load for foetal fluid and heart were from the same abortion case (09/461). The outlier with low relative liver viral load was 09/462. No other tissues showed significantly different viral loads but there is some suggestion that the placenta shows a higher

viral load than the heart, this may have shown statistical relevance if a relative number of placenta samples had been available for study.

Figure 3.2. Boxplot showing BoHV-1 viral load found in fetal tissues relative to viral load in fetal brain calculated by the ΔΔCt method from real-time PCR targeting BoHV-1 glycoprotein B. (Fetal fluid n = 8 ; Heart n = 9 ; Liver n = 9 ; Lung n = 7 ; Placenta n = 3; * represent outlier samples; letters a,b indicate statistical test grouping, GLM; $p < 0.05$).



3.5 Histological and immunohistochemical analysis of the foetal samples

The presence of lesions in foetal organs was investigated in formalin fixed tissue samples embedded in paraffin from those cases that had tested positive for BoHV-1 by PCR (tables 3.1 and 3.2). The distribution of the virus was studied by immunohistochemical labelling of viral antigen (glycoprotein C). In cases where serial sections had been used for H&E and IHC section comparisons could be made between lesion sites and IHC labelling.

H&E stained tissue showed that while lesions were consistently identified in the liver, they were also less frequently observed in the placenta and the lung. Multifocal hepatitis, characterized by numerous necrotic loci, can be clearly demonstrated in the

liver in 8 out of 10 PCR positive cases. These lesions appear to be randomly distributed throughout the parenchyma with no clear relation to the portal spaces or central veins (figure 3.3A). Inflammatory infiltrate was rarely present, but when seen was in the form of mononuclear cells at the periphery of the necrotic foci. IHC on the same 8 samples showed consistent positive detection of BoHV-1 antigen. In all cases the positive labelling was localised to the sites of the multifocal necrotic lesions identified in H&E stained sections (figure 3.3B).

Histological evaluation of the placenta was compromised by the advanced autolysis making it difficult to distinguish between occasional necrosis of endothelial cells and the effects of autolysis. However, immunohistochemical labelling allowed the identification of viral antigen in all cases studied. Lesions were visible in 3 of the 4 samples (figure 3.3C) after the likely lesion sites had been identified by the IHC labelling. BoHV-1 IHC labelling was localised to the vessel endothelium of the microvilli (figure 3.3D).

No lesions were detected in any of the heart samples. However, BoHV-1 was localised in 4 of the 8 cases by IHC labelling. The positive labelling was associated with the vessel walls of the heart in each of these cases (figure 3.3E).

Lesions were seen in 4 out of 9 lung samples in the form of a multifocal pneumonia characterized by a few necrotic foci associated with the alveolar walls with no associated inflammatory inflammation. IHC showed BoHV-1 localisation in alveoli of the lung (figure 3.3F) in two of the cases where lesions were found and another 3 cases, in which no lesions were identified.

In the brain, lesions were seen in 2 out of 6 available fixed samples, one in the form of glial foci, indicating non-purulent encephalitis and in the other case mild haemorrhaging. In the brain BoHV-1 localisation by IHC was found in these two cases and a further case showing no lesions. Positive labelling was found in both neurons and vessel walls (figure 3.3G and 3.3H).

Figure 3.3. Foetal samples from abortions caused by BoHV-1. A) Multifocal necrotic loci (arrows) in the liver (x10 H&E), B) Multifocal necrotic lesions in the liver labelled with BoHV-1 specific antibody (x10 IHC), C) Necrosis of endothelial cells (arrow) in the placenta (x20 H&E), D) Placental necrotic lesion labelled with BoHV-1 specific antibody (x20 IHC).

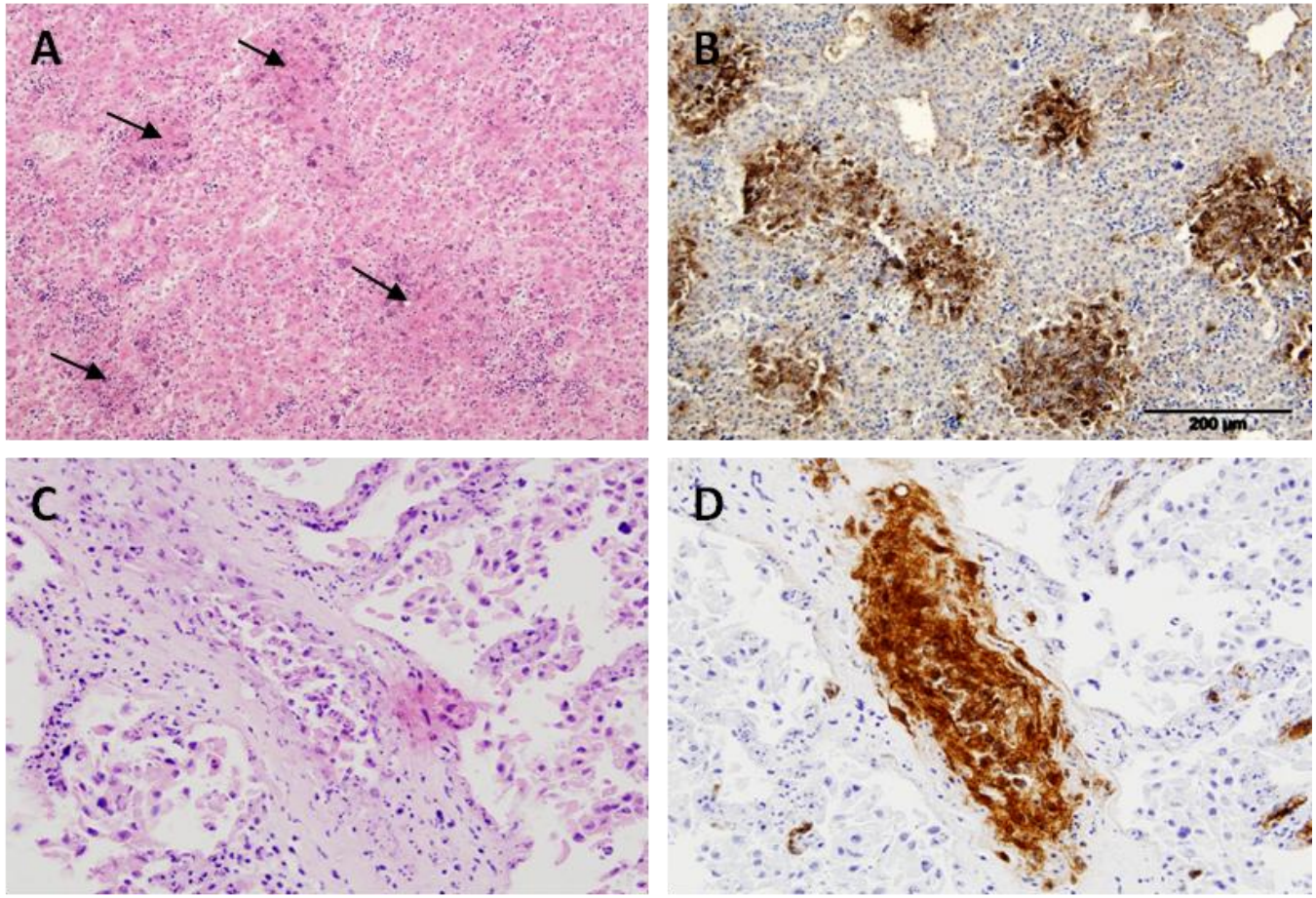
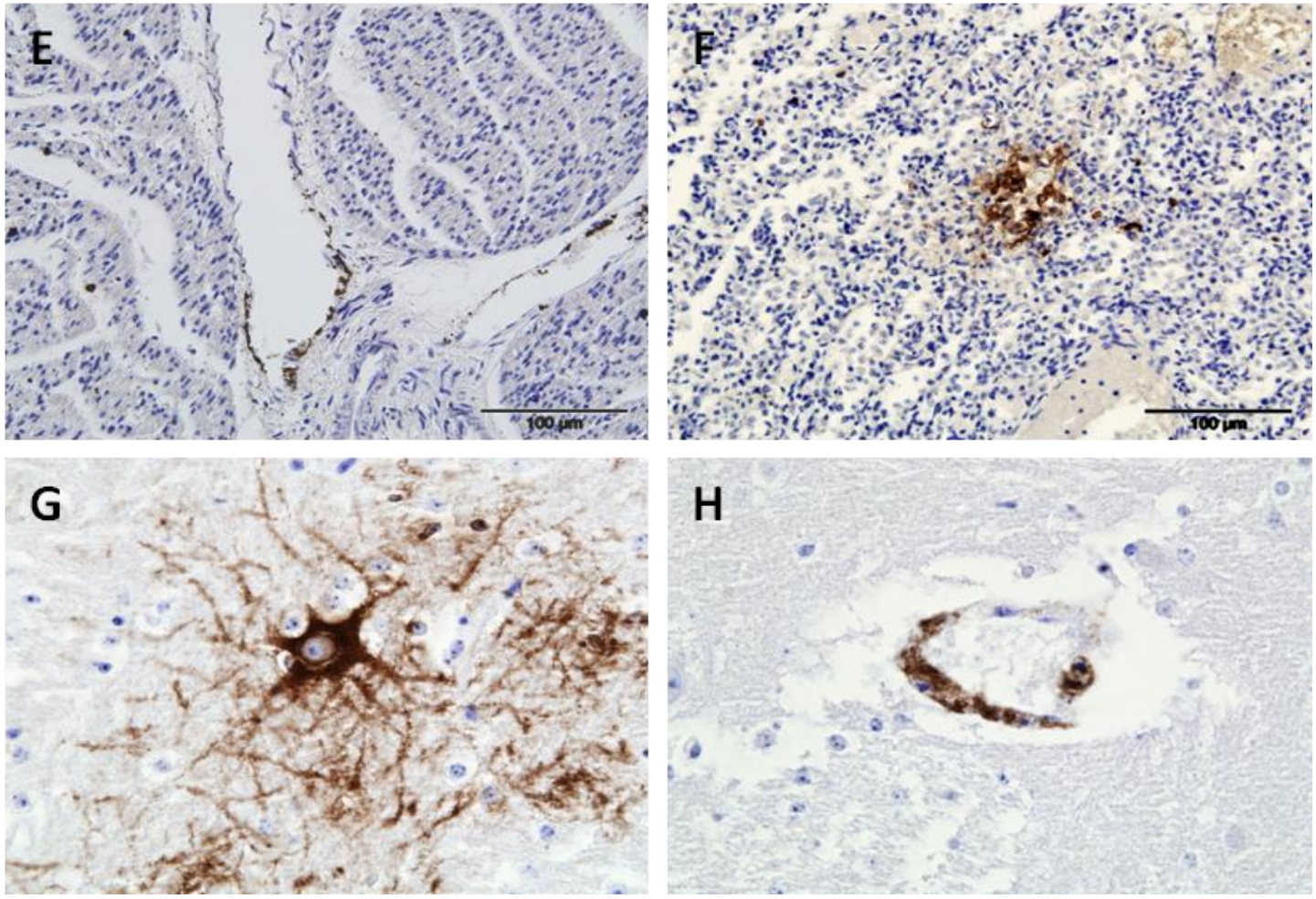


Figure 3.3 cont. E) Heart vessel labelled with BoHV-1 specific antibody (x10 IHC), F) Alveolar cells labelled with BoHV-1 specific antibody (x10 IHC), G) Brain neuron labelled with BoHV-1 specific antibody (x40 IHC), H) Brain vessel labelled with BoHV-1 specific antibody (x40 IHC).



3.5 Discussion

3.5.1 Diagnosis and prevalence of BoHV-1 in bovine abortions

The results here provide a current idea of the prevalence of BoHV-1 as a cause of abortion in Scotland. This has not been previously reported with the use of molecular diagnostic techniques and demonstrates the increased sensitivity PCR can provide over other techniques. The real-time PCR showed a high level of efficiency, both in tests with BoHV-1 positive tissue samples and also for cells in virus isolation. It was therefore considered to be a suitable method to survey abortion cases to identify positive BoHV-1 abortion tissue with a high level of sensitivity. Diagnosis of abortion cases requires tests that do not rely on the presence of infectious virus or good histological structure, as high levels of autolysis have regularly been reported due to the retaining of the foetus for several days after death before it is expelled (Kirkbridge 1992a). This makes real-time PCR a good candidate for accurate diagnosis, but these tests are more expensive and are rarely used in abortion cases. Diagnosis can rarely improve the clinical outcome, which can make these test costs difficult to justify. However, it can highlight the presence of a BoHV-1 infection in a herd and precautionary steps can be taken to prevent spread to other pregnant animals, in which case it becomes a highly economical method to prevent further losses.

The prevalence of BoHV-1 in abortion cases tested was found to be 2.5%. It shows a higher rate of detection than in the most recent UK diagnostic data, which produced a figure of less than 2% BoHV-1 prevalence in bovine abortions in Scotland between 2004 and 2009 (SAC VS 2009). Another bovine abortion study reported no cases of IBR (Caldow *et al.* 1996). However, it is lower than reported in the 1980's in England, which found 13% of bovine abortion cases BoHV-1 positive from samples collected from 54 dairy farms, an estimated 3600 breeding animals. This was based on a relatively low sample number of just 122 abortion cases, with analysis mainly relying on histological and serology studies (Murray 1990). The figure of 2.5% prevalence found here is also a lower than that found in a 10 year study in the USA, which found a prevalence of 5.41% from a much larger sample size of 8,962 bovine

abortions (Kirkbridge 1992b). In other studies in the USA, it could be considered that a general trend of reduced BoHV-1 prevalence in bovine abortions has been seen since the 1960's (Anderson 2007). Based on the data reported here and in other studies the same could also be concluded from the UK data (Murray 1990; SAC VS 2009). However, the method used for diagnosis, sample number and area sampled have varied between these studies, so it is difficult to make any accurate comparisons over time.

Based on the comparisons made between the diagnosis carried out in this study and in other laboratories, without the use of molecular techniques, it can be suggested that BoHV-1 systemic spread is more prevalent than detected by current routine methods, and that as a result BoHV-1 is under-diagnosed as a cause of abortion. This difference is most likely to be due to the use of more sensitive and specific diagnostic tests in the form of real-time PCR and IHC used in this study as was demonstrated by the comparison with findings using virus isolation and histopathology.

Virus isolation detected only one positive result out of the 10 cases, in which BoHV-1 DNA was detected using real-time PCR. This is most likely to be due to the autolysis of a samples compromising viral infectivity. The lipid bilayer envelope is sensitive to desiccation and can therefore only survive for short periods of time outside viable host cells for example in low humidity (Elazhary & Derbyshire 1979). This results in reduced reliability of virus isolation due to loss of viability of the virus in natural cases, before the sample is sent for analysis. Histopathological observations supported the PCR results with the liver consistently demonstrating multifocal necrotic lesions typical of BoHV1 infection. Placental lesions were also evident, but due to autolysis were much more difficult to identify without IHC to localise the lesion sites associated with BoHV-1. Other tissues such as the heart and lung were less reliable in observations of BoHV-1 typical lesions and would therefore not be recommended for use in diagnosis using histopathology without specific labelling. IHC labelling improved the reliability of using histopathology for diagnosis. It confirmed presence of BoHV-1 at sites of necrotic lesions and also identified sites of viral infection in the heart and brain where lesions were not visible

with H&E staining alone. There were also some cases where lesions were found but they were not confirmed by IHC. This shows lesions may not always be reliable indicators of the cause of abortion. In the cases where the sample was found to be positive by real-time PCR but not IHC, Ct values were all >30, which may suggest this represents the limit of detection by IHC labelling.

Real-time PCR clearly showed the highest level of sensitivity and reliability of results. However, it should be noted that only PCR positive samples were tested using other methods. Therefore it cannot be confirmed that none of the other individual samples would have been identified as BoHV-1 positive by other methods, when PCR found them to be negative. This study did not cover all the diagnostic options for BoHV-1. In particular, the immunofluorescent antibody test (IFAT) was not used for comparison, because it is considered to be less sensitive than virus isolation that was used in this experiment (Graham 2007). A study comparing diagnostic techniques for bovine diarrhoea virus in aborted foetuses, reported that IFAT and virus isolation produced frequent false negatives, whilst real-time RT-PCR and antigen ELISA were found to be more reliable with a good correlation between the two methods (Graham *et al.* 2009). In cases when there is more than one potential cause for abortion, consensus PCR methods have been used successfully, for example in the case of equine herpesviruses when EHV-1, EHV-2, EHV-3, EHV-4 and EHV-5 are all thought to cause abortion (Leon *et al.* 2008). Based on these previous findings it seems IFAT is generally considered less sensitive than real-time PCR and would not have altered these findings if it had been included, and overall it correlates well with conclusions drawn from other diagnostic studies.

In this sample set only 2 of the 10 cases had been identified as a potential BoHV-1 infection by the usual diagnostic protocols, one after IHC labelling and another by identifying a rising BoHV-1 antibody titre in serology testing. Several cases showed multifocal necrotic hepatitis typical of BoHV-1 abortion. However this was not followed up with further testing specific for BoHV-1 such as IHC, serology or PCR. This was most likely due to this being a common feature of causes of bovine abortion that enter the foetus via umbilical vessels, such as *Listeria spp.* and *Neospora*

caninum (Anderson 2007). Half of the positive cases here were previously diagnosed as a bacterial cause, based on bacterial culture or serology result, but, this does not necessarily represent a misdiagnosis. These agents may also have been present alongside BoHV-1, with the possibility that secondary bacterial infection contributed to the abortion. Alternatively, the stress of the bacterial infection may have lead to reactivation of latent BoHV-1. This provides another example of the advantage of PCR, when the original cause of infection may have since been overwhelmed by a secondary infection. This makes diagnosis of the original cause, by histopathology for example, difficult, but real-time PCR is still able to detect the presence of primary causes of the infection.

This study also considered the recent history of the aborting dam or of the herd. At least one of the cases came from a vaccinated herd, demonstrating that a BoHV-1 cause of abortion should not be ruled out in vaccinated herds. This is most likely due to infection prior to vaccination that has been reactivated from its latent state during pregnancy. Only one case had recorded clinical signs typical of IBR that had occurred a month prior to abortion. This could indicate that clinical signs are rarely noticed in the dam to support a suspicion of BoHV-1 abortion or that most of these cases are due to the reactivation of the virus from latency which rarely causes any clinical signs of IBR. Testing serology of the dams was not possible in this study and although it may provide good predictions of likely causes of abortion on a herd level it is not possible to make a definitive diagnosis using this method. In the case of BoHV-1, it has been found that levels of herd seroprevelance do not correlate with problems of abortion (Davison *et al.* 1999). There has also been some discrepancy in the change of serum neutralisation titre measured in the dam, from paired samples, that is of significance for BoHV-1 abortion. This is due to the period between BoHV-1 infection and abortion varying from 18-90 days causing wide variation between cases (Murray, 1990).

The results found here add to concern raised about diagnosis of abortion cases in other studies (Takiuchi *et al.* 2005; Rodger *et al.* 2007; Anderson 2007). They have clearly shown the advantage of molecular diagnostic techniques, providing a highly

sensitive method of detecting viral agents such as BoHV-1. It is also reliable in autolysed tissue, as also stated in a study using semi-nested PCR for BoHV-1 abortion (Takiuchi *et al.* 2005). Considering histology, serology and history of the dam can also provide key information but confirmation by real-time PCR provides the quickest method to reach a reliable diagnosis.

3.5.2 Molecular, histological and immunohistochemical analysis of aborted foetal tissue

The positive BoHV-1 foetal tissue collected in this survey provided the opportunity to look at the spread of BoHV-1 in natural cases of bovine abortion. Quantitative real-time PCR and histological techniques were successfully combined to investigate the distribution of the virus throughout the foetal tissues. It complements previous work based on an experimental challenge with findings from natural cases of BoHV-1 abortion, using molecular techniques.

The primary finding was the significantly higher viral load in the liver, compared to other tissues shown by real-time PCR relative quantification calibrated to the viral load found in the brain tissue. The liver also showed consistent severe multifocal necrotic lesions confirmed by IHC to be caused by BoHV-1. Absolute quantification would be needed to confirm this, but relative quantification has been sufficient to identify the liver as an important component in BoHV-1 foetal infection. This was consistent with many published descriptions of the pathology of BoHV-1 abortion tissue (Kennedy & Richards 1964; Owen *et al.* 1964; Molello *et al.* 1966; Kirkbridge 1992b; Rodger *et al.* 2007; Anderson 2007). However, these findings differ from one study based on pathology and serology, which found more lesions in the lung and chorioallantoic membrane of the placenta than the liver (Murray 1990). Real-time PCR data showed a high level of variation between the abortion cases, which is important to consider as it may have reduced the potential impact of these findings, but was limited to high relative viral load in case 09/461 and low viral load in 09/462.

BoHV-1 spread throughout the foetus to the lungs, heart and brain was demonstrated here by viral detection by PCR in all tissues tested and has also been previously reported (Murray 1990; Rodger *et al.* 2007). There was just one exception, where only the placenta tested positive for BoHV-1 in case 09/046. This could have occurred due to a placentitis causing an abortion at an early stage of transplacental spread. This does not support the theory that placental infection is secondary to the foetal infection that occurs after foetal death due to impaired foetal circulation (Molello *et al.* 1966). Other work suggests slow spread through the cotyledons of the placenta before a more rapid infection of the foetus (Kendrick *et al.* 1971). Therefore, it is possible that in this case there may have been another cause for abortion that was coincidental with BoHV-1 infection of the placenta, and occurred before transplacental spread of BoHV-1. The previous investigation of this case found no evidence of an infectious cause for abortion, which would support this theory. However, there was a toxic result reported for virus isolation, which could suggest presence of bacteria. Infection being limited to the placenta has also been observed on occasion in EHV-1 abortions, it has been suggested that placental separation has occurred due to the infection compromising the vascular supply (Smith *et al.* 2004; Kydd *et al.* 2006).

The detection of BoHV-1 DNA was generally supported by the presence of histopathological lesions and antigen detection by IHC, providing further information about virus localisation. BoHV-1 has recently been associated with encephalitis in the foetal brain (Brower et al. 2008). This is supported by the results reported here, showing low levels of viral load, lesions and positive IHC labelling in the brain. This suggests BoHV-1 frequently reaches the foetal brain during an infection. In one of the cases (07/327) positive labelling was associated with vessels in the meninges. Association of BoHV-1 with vascular endothelial cells in the foetal brain was previously reported in an experimental infection study (Rodger *et al.* 2007). This is the first time it has been reported in a natural case in the UK that is not associated with an intra venous viral challenge. It was suggested the virus would have initially infected endothelial cells of the vessels with secondary infection of associated neuronal and glial cells (Rodger *et al.* 2007). The demonstration of

positive labelling of a neuron and vessels in the same case supports this suggestion in natural cases. Secondary infection of neuronal cells would also explain the more prevalent labelling of vessels compared to neurons. Although more cases would be needed to confirm this as only 3 of 6 brain samples collected in this study were IHC positive. It could also be due to better replication occurring in endothelial cells compared to neurons.

There was also positive labelling in the blood vessels of the placenta and heart. Unfortunately, the samples were too autolysed to be able to reliably identify the cell types that were associated with BoHV-1 infection. Further staining using IHC techniques to identify endothelial and epithelial cells was also prevented by the degree of the autolysis. Association of the virus with blood vessels was previously observed in an experimental infection, although as this was an intravenous challenge, association of virus within blood vessels should be interpreted cautiously (Rodger et al. 2007). Pathology of placental lesions were also extensively described in an early experimental infection that used an intramuscular challenge, which noted coagulative necrosis of villus and non-villus chorioallantois membrane, involving both epithelial cells and endothelium of the villi (Molello *et al.* 1966). A similar experimental challenge suggested BoHV-1 does not always cause lesions in the placenta, however virus was detected by fluorescent antibody in the tissue showing virus was still present even though no lesions were observed (Kendrick *et al.* 1971). The work reported here may be the first study to show the association between the BoHV-1 and placental vessels in natural cases of abortion.

In this study, the high viral load observed in the liver by real-time PCR and localisation of virus in the vessels of several tissues, sometimes without association with vessels, provides support for previous suggestions of a haematogenous route of spread (Smith 1997). This can be explained by the virus reaching the foetus via exchange across the chorioallantoic membrane from the maternal blood supply, after spread via replication in the cotyledons the virus enters the foetal blood supply via the umbilical vein resulting in the high viral load in the liver. The virus could then

disseminate via the foetal circulatory system to other foetal organs, where virus was found at lower viral loads.

Placental tissue collected for this study consisted of combined cotyledons and surrounding membranes, further localisation could be useful by testing different parts of the placenta separately. In one experimental challenge study, where intact placentas were available for analysis, the highest number of lesions was found in the chorioallatoic membrane. The placentome also contained several lesions, but no lesions were found in the amnion (Murray 1990). The innermost membrane was not visibly affected by the virus, whereas the membranes that were highly involved in exchange with nutrients and oxygen from blood of the dam were affected, further supporting the haematogenous route of transplacental spread. Similar results have been seen in studies of the transplacental spread of the closely related equine herpesvirus-1 (EHV-1), presence of EHV-1 was demonstrated in the trophoblast and chorionic villi using RT-PCR *in-situ* hybridization to detect viral RNA, thought to demonstrate direct cell to cell spread from endometrial cells to the trophoblasts (Mukaiya *et al.* 2000). This has been supported by *in situ* hybridization detecting localised presence of EHV-1 DNA in the endothelial cells of the endometrial arterioles. Necrosis in the microcotyledon that could allow spread of the virus to the trophoblast epithelium was also demonstrated (Smith & Borchers 2001). Due to the close relation of these viruses it could be suggested that BoHV-1 may behave in a similar way, although a more detailed study of different parts of the placenta would be needed to investigate this.

In EHV-1 abortion, cell associated viraemia has also been clearly demonstrated, which explains the spread to the uterus in this case (Allen & Breathnach 2006). Due to the close relation between BoHV-1 and EHV-1 it seems likely that BoHV-1 acts by similar mechanisms, further study of the placenta would be needed to investigate if this also occurs in BoHV-1. However, to date no cell-associated viraemia has been clearly demonstrated for BoHV-1, so it remains difficult to explain the presence of the virus at the site of transplacental exchange between mother and foetus. There is some evidence BoHV-1 interacts with peripheral blood components *in vitro.* This

suggested BoHV-1 is able to replicate in monocytes and is adsorbed to leukocytes, so *in vivo* this could be how the virus reaches the placenta (Nyaga & McKercher 1979). An *in vivo* study of the pathogenesis of BoHV-1 during pregnancy would be needed to provide more information in this area.

In summary this study shows BoHV-1 does currently play a role in bovine abortion in the UK with an estimated prevalence of 2.5%, although a much larger sample size would be preferable to confirm this. It has highlighted the importance of viral molecular diagnostic techniques, in that they can produce conclusive results identifying a potential causative agent even in autolysed tissue. It has also provided support using natural cases of BoHV-1 abortion and molecular quantification techniques for previous findings, relating to transplacental and foetal spread of BoHV-1 in experimental challenges. Relative quantification and IHC labelling allowed us to identify the liver as the main target organ for BoHV-1 in the foetus. However, more work is still needed to understand more about the initial stages of BoHV-1 infection that allow the spread of the virus from the initial site of infection in the upper respiratory tract to the uterus.

Chapter Four: Comparative genomics of BoHV-1 isolates

4.1 Introduction

This section aims to search for virally encoded genetic variation between different isolates with known clinical outcomes in the field, to attempt to identify a genetic basis, either a specific point mutation or mutations in particular genes associated with virulence characteristics. Previous work in closely related equine herpesvirus-1 found a single nucleotide polymorphism in the viral DNA polymerase (UL30) was apparently related to neurovirulence of the virus (Goodman *et al.* 2007; Nugent *et al.* 2006). Mutation of glycoprotein E has been found to be important in the neurovirulence of BoHV-5, also closely related to BoHV-1 (Al-Mubarak *et al.* 2007). Thymidine kinase has been suggested as a gene that may be involved in BoHV-1 abortion after an experimental challenge with a thymidine kinase-negative deletion mutant virus showed a decrease in abortion occurrence (Miller *et al.* 1991; Miller *et al.* 1991). However, an intravenous route of challenge was used, so that a systemic infection of BoHV-1 was initiated from the start, which therefore does not provide any conclusive evidence of its role in systemic spread. Other alphaherpesviruses have also shown decreased virulence or reactivation in TK mutant viruses (e.g HSV) (Chen *et al.* 2006).

The aim of this section of work was to identify any genetic variation between 'respiratory' and 'systemic' BoHV-1 isolates that may contribute to the systemic spread of the virus. The first approach used here was RFLP which has been extensively used to subtype BoHV-1 isolates since the 1980's (Christensen *et al.* 1996; Mayfield *et al.* 1983; D'Arce *et al.* 2002). Analysis of the viral DNA with restriction enzymes showed similarity was extensive between the isolates, therefore an approach achieving a higher resolution was needed to identify any subtle differences between them. Next-generation sequencing technology has recently

become available and affordable, and was identified as the best approach to enable the analysis of the whole genome rather than trying to predict which genes may be interesting to look at by comparison with other herpesviruses. Therefore 11 isolates (table 4.1) were sequenced using the Illumina Solexa technology (for detailed methods see 2.8.1). From this data consensus sequences were derived using the published reference sequence (NC_001847) (RefSeq 2010), as a scaffold. SNP identification and phylogenetic analysis was conducted to seek differences between the isolate sequences that may contribute to systemic spread.

4.2 RFLP subtyping of isolates

DNA extracted from 19 purified BoHV-1 isolates was digested with *Hind III* (restriction site 5' A/AGCTT 3') and *Eco RI* (restriction site 5' G/AATTC 3') to differentiate between subtypes 1.1, 1.2a and 1.2b (section 2.5.3 and 2.5.7 for detailed methods). Restriction digestion with *Hind III* is known to produce 13 fragments of the BoHV-1 genome, ranging from 21.5 – 0.4 kb (figure 4.1). *Eco RI* digests the BoHV-1 genome into 7 fragments in BoHV-1.1 ranging from 58.2kb–3.1kb, and 6 fragments in subtype 1.2b, with fragments G and D combining to produce a fragment of 18.4 kb (figure 4.1).

RE digests were analysed by agarose gel electrophoresis and visualised with SYBR Gold staining and UV transillumination. Eighteen isolates were identified as subtype 1.1 and one, 06/1093, was identified as subtype 1.2b (figure 4.2 and table 4.1). The subtypes were differentiated by the 3 bands produced by DNA fragments J, K and L ranging from 7-10 kb (figure 4.1). Subtype 1.2b has an extra band (fragment I) at approximately 10.4 kb and two slightly smaller bands of 7.7 and 7.2 kb, but has the same 9kb fragment as subtype 1.1 (figure 4.2). *Eco RI* (figure 4.3) was also used to attempt to identify any genetic variation between the isolates. Differences found in 06/1093 by the *Hind III* digest were confirmed with some evidence of a different *Eco RI* pattern (figure 4.3). There is a band at approximately 18kb representing fragment C of the 1.2b subtype pattern in the 06/1093 lane 18 that is not visible in the other

lanes which contain 2 smaller fragments, G and D in the subtype 1.1 pattern (figure 4.3b).

This method was successful in subtyping the isolates and showing that type 1.1 appears to be the predominant subtype in this random selection of BoHV-1 diagnostic cases from around Scotland and Northern England from 2004-2007. However, as can be seen the DNA was not always fully digested producing faint bands that were difficult to type. It also clearly shows the relative crudity of relying on RE analysis, targeting only 6 base sequences to type BoHV-1 isolates. A method providing better coverage of the whole genome was required to search for genetic differences between these isolates was needed.

Figure 4.1 Restriction enzyme maps of BoHV-1.1 and BoHV-1.2b for *Hind III* and *EcoRI*. Letters indicate fragment and numbers indicate size of fragment (kb). Adapted from (Mayfield *et al.* 1983).

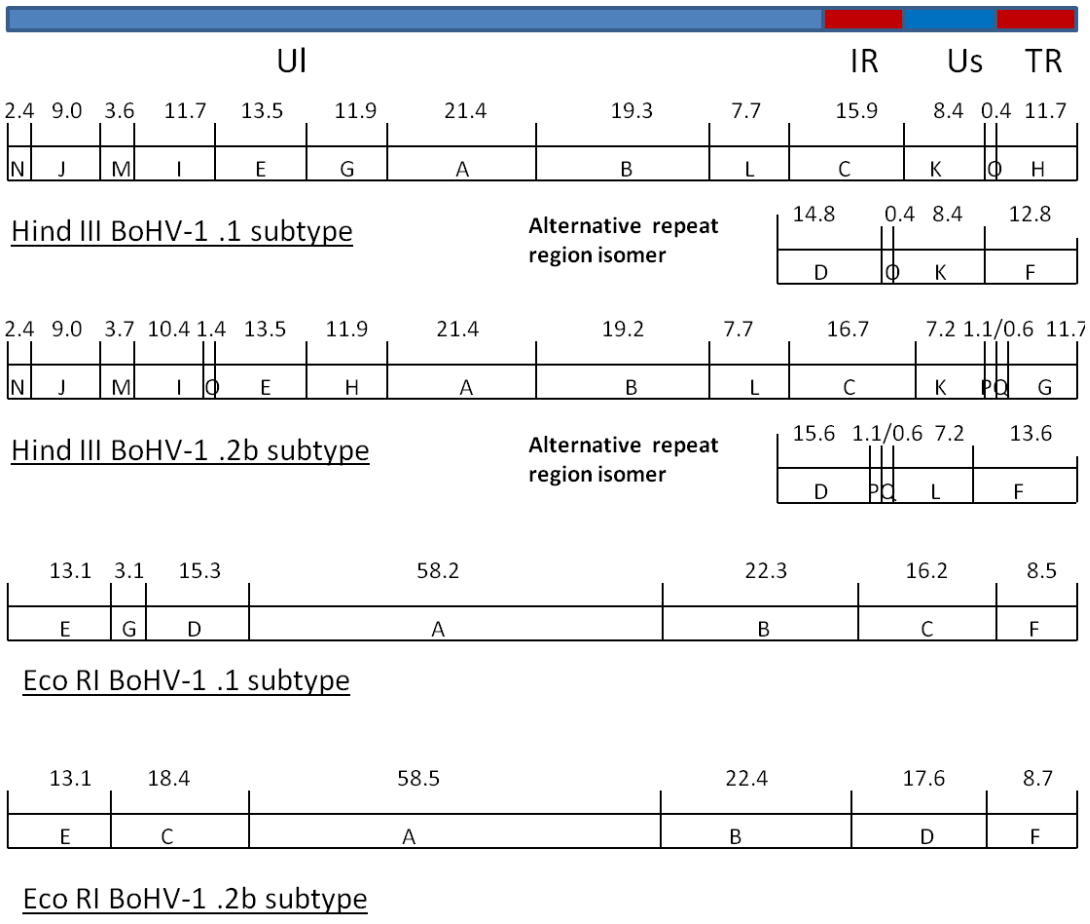


Figure 4.2. RFLP analysis of BoHV-1 isolates cut with restriction enzyme *Hind III* to classify BoHV-1 subtypes on an agarose gel stained with SYBRGold

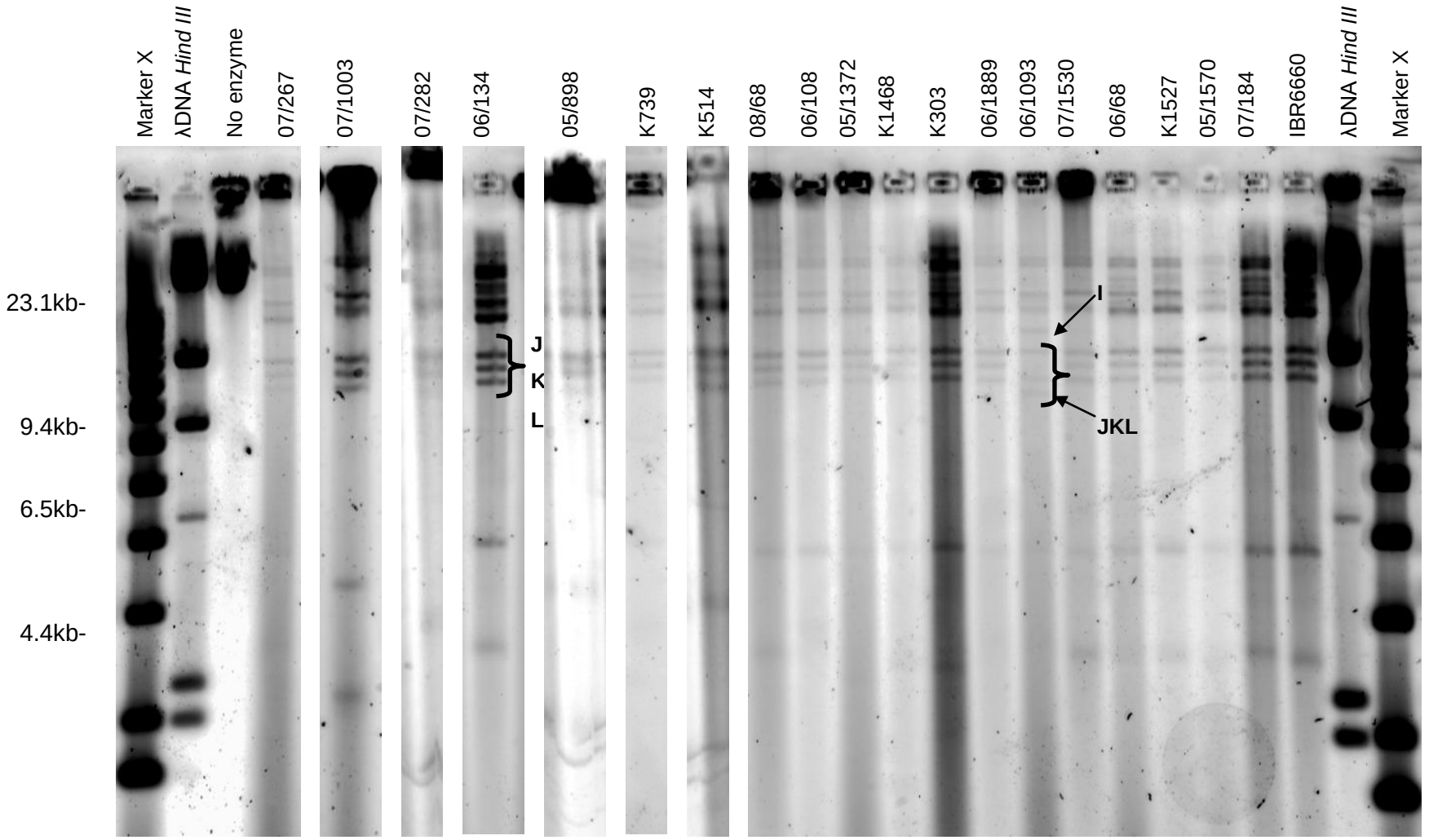
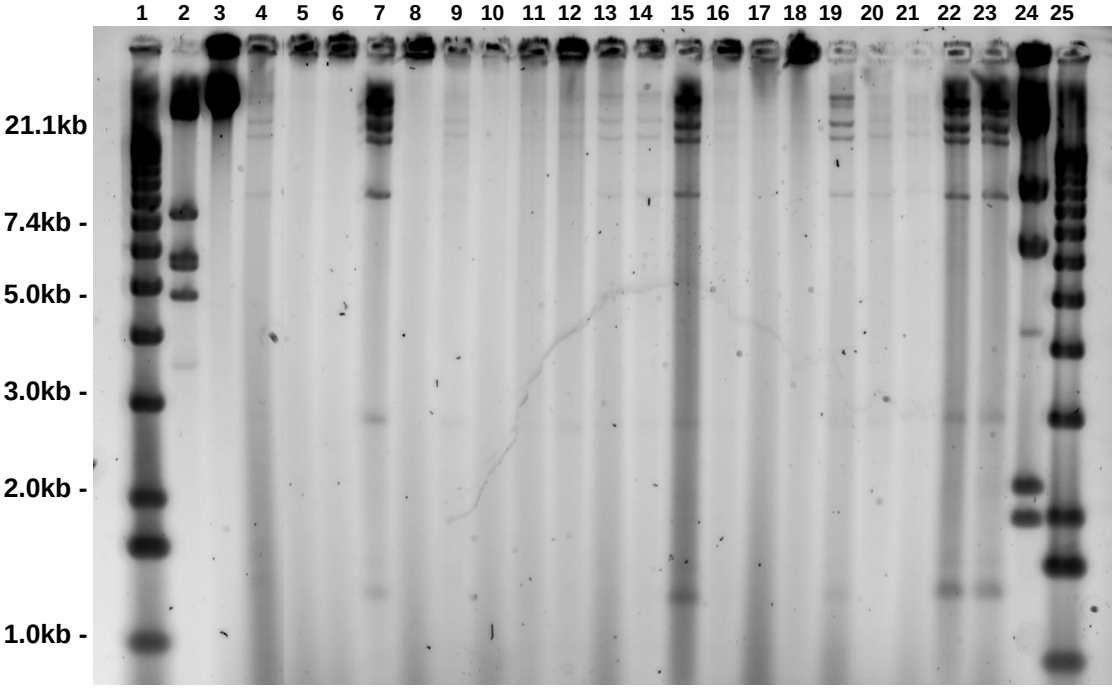
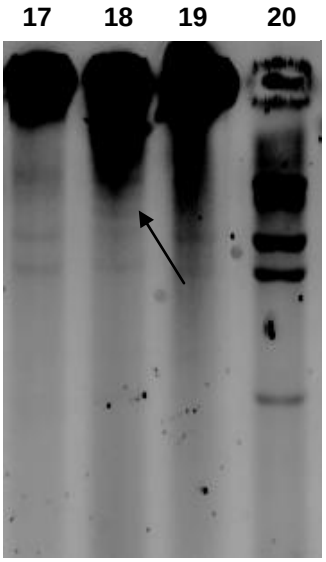


Figure 4.3 RFLP analysis of BoHV-1 isolates cut with restriction enzyme *EcoRI*.





Gel lanes

1) Marker X
2) λDNA
3) Enzyme control
4) 07/267
5) 07/1003
6) 06/005
7) 07/282
8) 06/134
9) 05/898
10) K739
11) K514
12) 08/68
13) 06/108
14) 05/1372
15) K1468
16) K303
17) 06/1889
18) 06/1093
19) 07/1530
20) 06/68
21) K1527
22) 05/1570
23) 07/184
24) λ DNA Hind III digest
25) Marker X

Table 4.1. Summary of RE analysis results determining subtype and isolates chosen for further investigation using whole genome sequencing methods.

Isolate	Respiratory or systemic source	Subtype	Used for Solexa Sequencing
07/267	Respiratory	1.1	No
07/1003	Respiratory	1.1	No
07/282	Systemic	1.1	Yes
06/134	Systemic	1.1	Yes
05/898	Systemic	1.1	Yes
K739	Abortion	1.1	Yes
K514	Systemic	1.1	Yes
08/68	Systemic	1.1	Yes
06/108	Respiratory	1.1	No
05/1372	Respiratory	1.1	No
K1468	Respiratory	1.1	No
K303	Respiratory	1.1	No
06/1889	Respiratory	1.1	No
06/1093	Respiratory	1.2b	Yes
07/1530	Abortion	1.1	Yes
06/068	Respiratory	1.1	No
K1527	Respiratory	1.1	Yes
05/1570	Respiratory	1.1	Yes
07/184	Respiratory	1.1	Yes

4.3 Solexa sequencing

Eleven virus isolates from the twenty that were subtyped were chosen for sequencing (table 4.1) including isolates that had caused both respiratory and systemic clinical presentations of BoHV-1 and the one 1.2b subtype, ensuring there was a range of isolates from a range of locations in Scotland and Northern England and isolates collected at different time points from 2004-2008. Viral DNA was extracted from isolates that had undergone just one passage in cell culture minimising the amount of mutations acquired in comparison with the original isolates. Isolates were sequenced using Illumina Solexa technology at The Gene Pool, University of Edinburgh (see section 2.8 for detailed methods). DNA from 10 purified BoHV-1 isolates was sequenced in 50bp reads and with one as a trial run in 36bp reads. These were then successfully mapped to the reference BoHV-1.1 genome (NC_001847) to produce whole genome sequences for all isolates, allowing specific genome regions to be studied in more detail for identification of single nucleotide polymorphisms (SNP) compared to the reference sequence and phylogenetic analysis of the whole genome.

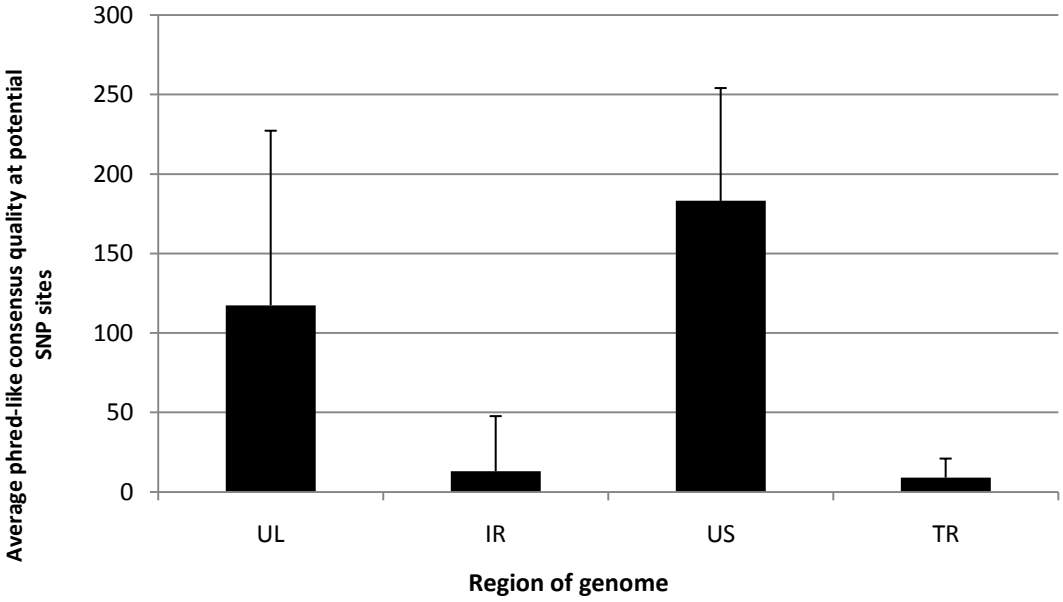
The number of 50bp reads produced from the solexa sequencing ranged from 2.4 million to 7.8 million reads per isolate, with a range of 1.94-58.87% of these reads being mapped onto the reference genome of BoHV-1 with average depth of reads ranging from 19-1140 (table 4.2). The reads that were not mapped to the reference sequence were considered most likely host DNA (table 4.2). K514 was considered to have a too low percentage of mapped reads along with a low overall total no. of reads mapped and poor depth of coverage. and therefore was excluded from further analysis. To improve the mapping percentage and reduce the number of gaps the alignments were studied. At 15 gap sites where all the sequenced isolates differed from the reference sequence, the reference scaffold sequence was edited to match the sequenced isolates (Davison 2009). When the raw sequence data was re-mapped, the gaps were filled with the exception of gaps of less than 50 bp found in UL47 and both internal and terminal BICP4 ORFs.

Not unexpectedly, repeat regions showed the lowest percentage of mapped reads and were investigated further to ensure the mapped data was as reliable as those in the unique regions of the genome (figure 4.4). Phred scores measure the accuracy and probability of errors in the base calling of high-throughput sequencing methods (Ewing *et al.* 1998; Ewing & Green 1998). A similar method is used by the MAQ software for mapping of short read sequences and was used in the SNP analysis (Li *et al.* 2009). The phred-like consensus quality scores provided by the mapping and SNP identifying software, MAQ reflected the lower percentage of mapped reads in the repeat regions (figure 4.4). The repeat regions were re-mapped separately from the rest of the genome. Much higher phred-like quality scores were produced throughout the repeat regions using this method. However, when these separately mapped repeat regions were aligned to the original consensus repeat regions mapped with the rest of the genome, the sequences were identical. Therefore, despite the suggestions of poor reliability in the repeat regions demonstrated in figure 4.4, it has been shown this does not impact on the findings.

Table 4.2 Summary of sequenced isolates including the total number of reads produced by Solexa sequencing ,the percentage of those reads which successfully mapped to the reference sequence and the average depth of the reads mapped across the length of each consensus sequence (Refseq: NC_001847) using MAQ software.

Virus isolate	Isolate type	Total no. of reads	% reads mapped to reference	Average depth of coverage
07/184	Respiratory 1.1	2417100	18.8	420.73
K1527	Respiratory 1.1	4398945	15.15	171.31
05/1570	Respiratory 1.1	7646052	19.89	530.13
07/1530	Abortion 1.1	7329816	35.9	538.30
K739	Abortion 1.1	4791757	36.52	601.17
05/898	Systemic 1.1	6803731	10.85	225.349
08/68	Systemic 1.1	4936619	24.93	413.87
07/282	Systemic 1.1	7886584	58.87	1139.34
06/134	Systemic 1.1	4747578	17.45	611.80
06/1093	Respiratory 1.2b	5612960	2.97	58.47
K514	Systemic 1.1	2813960	1.94	19.18

Figure 4.4 Average phred-like consensus quality score at potential SNP sites across the BoHV-1 isolate genomes compared to the reference genome produced by MAQ mapping software.



4.4 SNP identification

SNPs were identified and filtered using MAQ software and studied for conservation with other isolates in the same group. All SNPs were identified as compared to the reference genome; all isolates, with the exception of 06/1093 (the 1.2b type) showed

between 206 and 254 SNPs compared to the reference sequence (table 4.3). 139 of these SNP sites were identical in all isolates showing SNPs were conserved between all isolates with the exception of the reference sequence. 06/1093 (the 1.2b subtype) showed more variation compared to all the other isolates with 871 SNP sites identified when compared to the reference isolate and can therefore be considered to show variation when compared to the other isolates sequenced.

SNPs in all isolates were fairly evenly spread across the genome with the exception of less frequent SNPs being identified in UL42 (DNA polymerase processivity protein), UL41 (host-shut-off protein), UL28 (DNA packaging terminase) and BICP4 (transcriptional regulator) in the terminal region (figure 4.5). The SNPs were also fairly evenly distributed between genes classified by different functions, although as the figure indicates there was a large amount of variation between the different genes in each category (figure 4.6). BoHV-1 genes have been classified as non-essential and essential genes in tissue culture (Robinson et al. 2008). A comparison of these two gene groups showed non-essential genes had significantly more frequent SNPs averaging every 350 bp, compared to essential genes, averaging a SNP every 611 bp (t-test; p-value =0.03) (figure 4.7).

After a filtering process step, removal of SNPs present in all isolates compared to the reference sequence and removal of any SNPs related to ambiguous bases that were unlikely to be true SNPs, 204 SNPs were found with at least one isolate demonstrating a different base to the others (table 4.4). As is shown in table 4.4 most sites have SNPs in just one of the 10 isolates; i.e. none of the SNPs observed showed any conservation between isolates of respiratory/systemic derived strains. Table 4.5 demonstrates this for SNPs found between two isolates, one respiratory and one systemic isolate, as a representative example. 40 SNPs were identified between these two isolates, 33 of which were in coding regions. 13 were non-synonymous (resulting in amino acid changes) in several genes including glycoprotein C, thymidine kinase, tegument and capsid proteins, but there was no specific association with either isolate type. When these SNPs were cross-referenced with other isolates

of the same type there was no conservation of SNP sites in other respiratory or systemic isolates.

Table 4.3. Total no. of SNPs between sequenced isolates and reference sequence.

Sample	Total SNP no.
K1527	213
07_184	245
05_1570	219
K739	222
07_1530	233
07_282	254
05_898	206
06_134	245
08_68	228
06_1093	871

Recent research identified 10 microRNA sites on the bovine herpesvirus-1 genomes that are considered important in gene expression (Glazov *et al.* 2009). As they have previously been associated with control of virulence in other herpesviruses the isolates were checked for variations in these regions. No variation was found at any of the 10 microRNA sites compared to each other or the published sequence (NC_001847) at the genomic level from the data collected in this study.

Figure4.5. Graph showing average distribution of SNPs across genome length (blue = Ul, red = repeat regions, green = Us). SNP frequency was calculated from the average number of SNPs in each coding regions compared to the no. of bases in that coding region to provide a SNP frequency shown in the y–axis of the graph. x-axis, shaded areas – repeat regions.

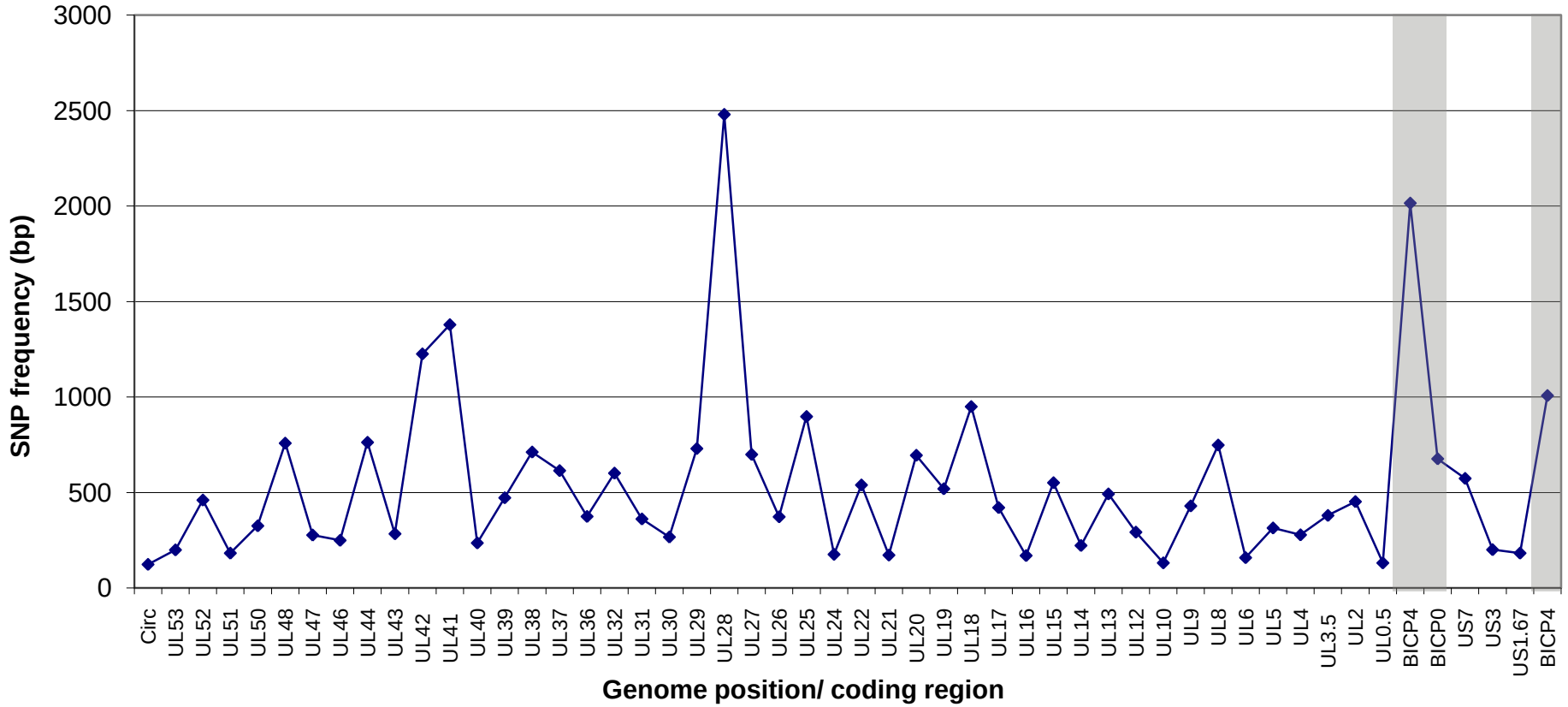


Figure 4.6. Distribution of SNPs in different genes assigned to different functional groups.

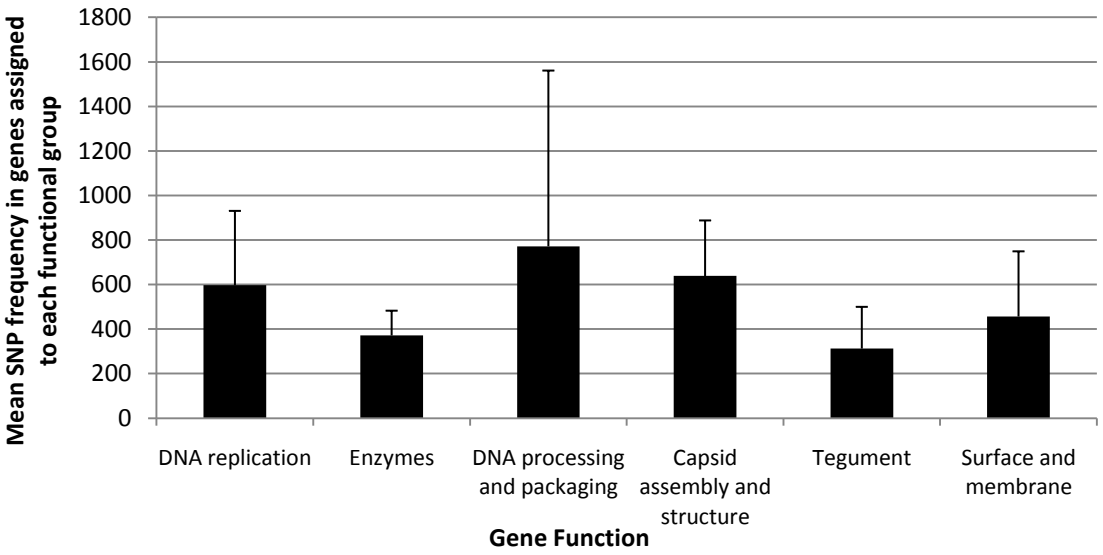


Figure 4.7. A comparison of SNP frequencies between essential and non-essential genes.

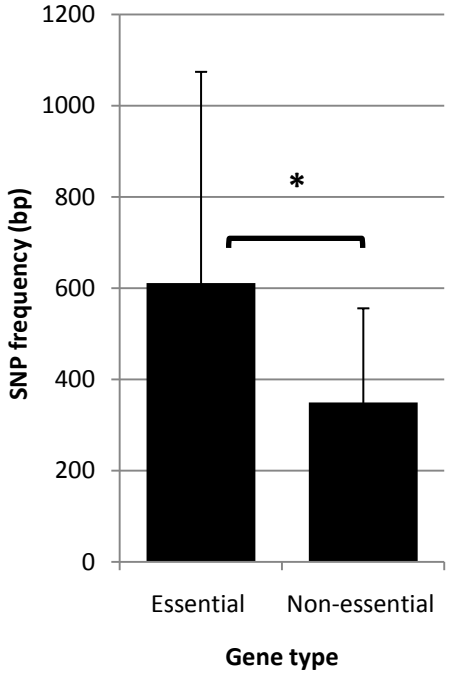


Table 4.4. Filtered SNPs between all isolates and the reference sequences, excluding ambiguous bases that may represent base calling uncertainty.

			Respiratory			Systemic					
Gene	Position	Ref	K1527	05/1570	07/184	K739	07/1530	06/134	08/68	07/282	05/898
circ	1010	C	C	C	C	C	C	T	C	C	C
	1021	T	W	W	W	W	W	A	W	W	T
	1108	G	G	G	G	A	G	G	G	G	G
UL53	3431	G	C	G	C	G	C	G	G	C	C
	3685	G	G	G	C	G	G	G	G	G	G
	3686	C	C	C	G	C	C	C	C	C	C
	3881	C	T	T	T	T	T	T	T	C	T
	3882	T	C	C	C	C	C	C	C	T	C
UL52	5318	G	G	G	G	G	G	A	G	G	G
	5781	C	C	C	C	C	C	C	C	C	G
	5782	G	G	G	G	G	G	G	G	G	C
	6267	C	T	C	C	C	C	C	C	C	C
UL51	7268	C	C	C	C	C	T	C	C	C	C
	7595	C	C	C	C	C	C	C	C	C	T
UL50	8291	T	T	T	T	T	T	T	T	T	G
	8299	C	T	C	C	C	C	C	C	C	C
	8752	C	C	C	C	C	C	C	C	A	C
UL47	12005	G	G	G	G	G	C	G	G	G	G
	12006	T	T	T	T	T	G	T	T	T	T
	12313	T	T	T	C	T	T	T	C	T	T
	13020	T	T	T	K	C	T	T	T	K	T
	13157	C	C	G	C	G	C	C	C	C	C
	13169	C	G	C	G	C	G	C	C	C	C
	13252	T	C	C	C	T	C	C	C	C	C
	13364	C	G	G	G	C	G	G	G	G	G
UL46	14346	C	C	C	C	C	T	C	C	C	C
	14697	G	G	A	G	G	G	G	G	G	G
	15804	G	A	G	G	G	G	G	G	G	G
	15921	T	C	C	T	T	T	T	T	T	T
	15957	T	T	T	T	C	T	T	T	C	T
	15990	T	T	T	T	T	T	T	T	C	T
	16287	G	A	G	G	G	G	G	G	G	G
	16521	C	T	C	C	C	C	C	C	C	C
UL44	16705	C	C	C	C	C	C	C	C	T	C
	18152	A	A	A	A	A	G	A	A	A	A
UL43	18523	G	G	G	A	G	G	G	G	G	G
	18916	C	C	C	C	C	C	T	C	C	C
	19377	C	C	C	C	C	C	T	C	C	C
	19392	C	C	C	C	C	C	T	C	C	C
UL42	20107	C	C	C	C	T	C	C	C	C	C
UL41	21983	C	T	C	C	C	C	C	C	C	C
UL40	22786	A	G	G	G	G	G	G	G	A	A
	22791	T	T	A	T	T	T	T	A	T	T
	22786	A	A	T	A	A	A	A	A	G	A
	22874	C	T	C	T	T	T	T	T	T	T
UL39	23628	C	C	C	C	C	C	C	C	C	T
	23761	C	C	C	T	C	C	C	T	C	C
	24075	C	T	C	C	C	C	C	C	C	C
	25332	G	G	G	G	G	A	G	G	G	G
UL38	27562	G	G	G	G	G	A	G	G	G	G
UL37	28312	G	G	A	G	G	G	G	G	G	G
	28892	G	G	C	G	C	C	C	C	C	G
	29583	C	C	C	C	C	C	T	C	C	C
UL36	33035	C	C	C	C	C	C	C	T	C	C
	33291	C	C	C	C	C	C	C	C	C	T
	34033	G	A	G	G	G	G	G	G	G	G
	34714	G	G	G	G	G	G	G	G	A	G
	35319	G	G	G	G	A	G	G	G	G	G
	35348	C	C	G	C	G	C	G	C	G	G

Table 4.4 (cont.)

			Respiratory			Systemic					
Gene	Position	Ref	K1527	05/1570	07/184	06/134	08/68	K739	07/1530	07/282	05/898
UL36	35349	G	G	C	C	C	G	C	G	C	C
cont	35875	C	C	C	C	C	C	C	T	C	C
	36133	G	G	A	A	A	A	A	A	A	A
	38532	A	R	A	R	G	R	R	R	R	A
	38559	A	A	G	R	G	R	G	A	G	A
	38580	C	C	C	T	C	T	C	C	C	C
	38734	C	C	C	C	C	T	C	C	C	C
	38791	G	G	G	A	G	G	G	G	G	G
	38976	C	C	T	Y	T	Y	Y	Y	T	T
	38988	C	Y	C	Y	T	Y	C	C	T	C
	39047	G	G	G	G	G	G	A	G	G	G
	39171	C	C	C	C	C	T	C	C	C	C
	39219	G	G	G	G	T	G	G	G	G	G
	39222	A	A	A	A	C	A	A	A	A	A
UL32	43135	T	T	T	T	T	T	C	T	T	T
	43137	G	G	G	G	G	G	C	G	G	G
	43142	C	C	C	C	G	C	C	C	C	C
UL31	44350	C	C	C	T	C	T	C	C	C	C
UL30	46147	C	T	C	C	C	C	C	C	C	C
	46232	A	A	A	A	A	A	A	A	A	G
	46249	G	C	C	G	C	C	G	C	G	G
	46250	C	C	C	C	G	G	C	C	C	C
	46261	C	C	T	C	C	G	G	C	C	C
	46249	G	G	G	G	G	G	G	G	C	G
	46945	C	C	C	C	C	G	G	G	S	C
	46962	C	C	C	C	C	C	C	C	T	C
	47908	C	C	C	C	T	C	C	C	C	C
	48048	C	C	C	C	C	C	C	C	C	T
	48698	G	G	G	G	G	G	A	G	G	G
	48719	T	C	T	T	T	T	T	T	T	T
UL29	50109	G	G	G	G	G	A	G	G	G	G
	50716	C	C	T	C	C	C	C	C	C	C
	51450	G	G	G	G	G	G	G	A	G	G
	52747	G	G	G	G	G	G	G	G	C	G
	52748	G	G	G	G	G	G	G	G	C	A
UL28	55067	C	T	C	C	C	C	C	C	C	C
UL27	56878	C	T	C	C	C	C	C	C	C	C
	58180	C	C	C	C	T	C	C	C	C	C
UL26	58736	G	G	G	G	G	G	G	A	G	G
	58859	A	A	A	A	A	A	A	A	G	A
	59533	C	C	C	C	T	C	C	C	C	C
	59878	C	C	T	C	C	C	C	C	C	C
	60238	G	G	A	G	G	G	G	G	G	G
UL25	61978	C	C	C	C	C	C	C	C	A	C
	62026	G	G	G	G	G	G	G	A	G	G
UL24	62989	G	G	G	G	G	G	G	A	G	G
	63166	C	C	C	C	T	C	C	C	C	C
	63412	C	C	C	C	A	C	C	C	C	C
UL21	67370	G	T	G	G	G	C	T	G	G	G
	67970	G	G	T	T	T	T	G	T	T	T
	68017	A	A	R	A	R	A	G	G	G	A
	68052	G	G	C	G	G	G	G	G	G	G
	68152	G	G	G	C	G	G	G	G	G	G
	68246	G	G	G	C	G	G	G	G	G	G
	68296	C	G	C	G	C	C	G	G	G	C
	68297	G	C	G	C	G	G	C	C	C	G
	68307	C	C	G	C	G	G	G	S	S	G
	68701	A	A	A	A	A	A	A	A	G	A
UL20	69176	G	G	G	G	A	G	G	G	G	G
UL19	70373	C	C	C	C	G	C	C	G	G	C
	70374	G	G	G	G	C	G	G	G	C	C
	71158	C	G	C	G	C	C	G	G	G	G
	71159	G	G	G	G	G	G	G	C	C	G
	71313	C	C	C	C	C	C	C	C	T	C
	72000	C	G	C	G	G	G	G	C	G	G
UL18	74356	G	G	G	G	A	G	G	G	G	G
UL15	75944	G	G	G	A	G	G	G	G	G	G
	76317	T	T	T	T	T	T	T	T	C	T

Table 4.4 (cont.)

			Respiratory			Systemic					
Gene	Position	Ref	K1527	05/1570	07/184	06/134	08/68	K739	07/1530	07/282	05/898
UL17	76432	G	G	G	G	G	A	G	G	G	G
	77084	G	G	G	G	A	G	G	G	G	G
	77152	T	T	C	T	T	T	T	T	T	T
	77827	G	G	A	G	G	G	G	G	G	G
	78207	G	G	G	G	G	G	G	A	G	G
UL16	79094	C	C	C	C	C	G	C	G	C	G
	79096	G	G	G	A	G	G	G	G	G	G
	79097	A	A	A	A	A	G	A	G	A	G
	79367	G	C	C	C	C	G	C	C	C	C
	79367	G	G	G	G	G	C	G	G	G	G
UL15	80389	C	C	C	C	C	C	C	T	C	C
UL14	80941	C	C	C	C	C	C	C	T	C	C
	81268	G	G	G	G	G	A	G	G	G	G
UL13	81409	C	C	G	C	C	C	C	C	C	C
	81414	G	C	C	G	G	G	G	G	G	G
	82444	G	G	G	G	G	G	G	G	A	G
UL12	82830	C	G	C	C	C	C	C	C	C	G
	82832	C	C	C	C	C	C	C	C	S	C
	82984	A	A	M	A	C	A	A	C	A	A
	83579	C	C	C	C	C	C	C	C	G	C
	83581	G	C	G	G	G	G	G	G	C	G
UL10	84575	C	G	G	G	G	G	G	G	G	C
	84577	G	T	T	T	T	T	T	T	T	G
	84615	G	G	G	G	G	G	G	G	G	A
	84643	G	C	C	G	C	C	C	C	C	C
	84910	G	C	G	C	G	C	G	G	C	C
	84906	G	G	A	G	G	G	G	G	G	G
	84910	G	G	C	G	G	G	G	G	G	G
	85331	C	C	C	G	C	C	C	C	G	C
UL9	86994	A	A	A	A	A	A	G	A	A	A
	87322	C	C	G	C	G	G	G	G	G	C
	87384	C	C	C	C	G	C	C	C	C	C
	87390	G	G	G	S	G	G	G	G	G	G
	87686	C	C	C	C	C	C	T	C	C	C
	88424	G	G	G	A	G	G	G	G	G	G
UL8	89264	C	C	C	C	T	C	C	C	C	C
	90116	C	C	C	T	C	C	C	C	C	C
	90747	A	A	A	A	G	A	A	A	A	G
UL6	92247	C	C	G	C	G	G	G	G	C	C
	92250	G	G	C	G	C	C	C	G	G	G
	92508	C	C	G	G	G	G	G	G	G	G
	92511	C	C	G	C	G	G	G	G	G	G
	92518	G	G	G	G	G	G	G	C	G	G
	92747	T	G	T	T	T	T	T	T	T	T
	92748	T	T	C	T	T	T	T	T	T	T
	92879	C	T	C	C	C	C	C	C	C	C
	93099	G	A	G	G	G	G	G	G	G	G
	93124	C	G	G	G	G	G	G	C	G	G
	93125	G	C	C	C	C	C	C	G	C	C
	93367	G	G	G	G	G	G	G	G	G	C
	93693	T	T	T	T	T	G	T	T	T	T
UL5	94653	C	T	C	C	C	C	C	C	C	C
	94907	C	C	C	C	C	C	C	T	C	C
	94913	C	C	C	C	C	C	C	T	C	C
	95092	A	A	A	A	A	A	A	A	A	G
	95094	T	T	T	T	T	T	T	T	T	C
	95362	C	C	C	C	T	C	C	C	C	C
	95645	T	T	T	C	C	T	C	C	C	T
	95645	T	C	C	T	T	C	T	T	T	C
UL4	96564	C	C	C	C	T	C	C	C	C	T
	96566	T	T	T	T	C	T	T	T	T	C
UL2	98384	G	G	G	A	G	G	G	G	G	G
UL0.5	99959	C	C	C	C	T	C	C	C	C	C
	100653	G	G	G	G	G	G	A	G	G	G
BICP0	101075	C	T	C	C	C	C	C	C	C	C
	101282	C	C	C	C	C	T	C	C	C	C
BICP4	105282	C	C	C	C	C	G	C	C	C	C
	105304	C	C	C	C	T	C	T	T	T	C
US1.67	114594	C	C	C	C	C	C	T	C	C	C
US3	116803	C	C	C	C	T	C	C	T	T	C
	116914	G	G	G	A	G	A	G	G	G	G
	116998	C	C	C	C	C	C	T	C	C	C
US7	121021	G	G	G	G	G	G	A	G	G	G
BICP4	132851	C	C	C	C	G	G	C	G	G	C
	132855	C	C	C	C	G	G	C	G	G	C
	132865	G	G	G	G	G	G	G	C	G	G
	132871	C	C	C	C	G	C	C	G	G	C

Table 4.5 SNPs between two isolates representing the respiratory and systemic groups

Gene						
	Genome position	05/1570	07/1530		Non-Synonymous?	Gene function for non-synonymous SNP sites
NC	52	A	G			
UL52	7268	C	T		Syn	
UL46	14346	C	T		Syn	
	14697	A	G		Syn	
	15921	C	T		Syn	
UL44	18152	A	G		S-P	gC type 1 memebrane protein, contains a single peptide, binds cell surface heparan sulphate, binds complement C3b to block neuralization
UL39	25332	G	A		Syn	
UL37	28891	S	C		Syn	
	35875	C	T		Syn	
	38559	G	A		Y-C	Tegument protein complexed with large tegument protein, function - virion morphogenesis
	38988	T	C		P-L	
UL30	46261	T	C		Syn	
UL29	50716	T	C		T-M	Single stranded DNA-binding protein for DNA replication and possibly gene regulation (contains zinc ring finger)
	51450	G	A		V-I	
NC	58649	G	K			
UL26.5	58736	G	A		P-L	Capsid scaffold protein for capsid morphogenesis (clipped near C terminus)
NC	59878	T	C			
	60238	A	G			
UL25	62026	G	A		R-C	DNA packaging tegument protein for DNA encapsidation, located on capsid near vertices, possibly stabilizes capsid and retains the genome
UL24	62989	G	A		Syn	
UL23	64094	C	A		T-P	Thymidine kinase for nucleotide metabolism
NC	67006	T	Y			
UL21	68017	R	G		?	
	68307	G	S		Syn	
NC	69671	C	A			
UL17	77152	C	T		L-P	DNA packaging tegument protein for capsid transport (capsid associated)
	77827	A	G		R-H	
	78207	G	A		A-T	
NC	80389	C	T			
	80690	C	T			
UL14	80941	C	T		A-V	Tegument protein involved in virion morphogenesis
UL12	82984	M	C		Syn	
UL6	92518	G	C		R-G	Capsid portal protein involved in DNA encapsidation, dodecamer located at one capsid vertex in place of a penton.
	92750	C	T		Syn	
UL5	94907	C	T		Syn	
	94913	C	T		Syn	
US1.67	114428	G	K		Syn	
	114939	G	K		Syn	
US3	116169	R	A		Syn	
	116803	C	T		Syn	

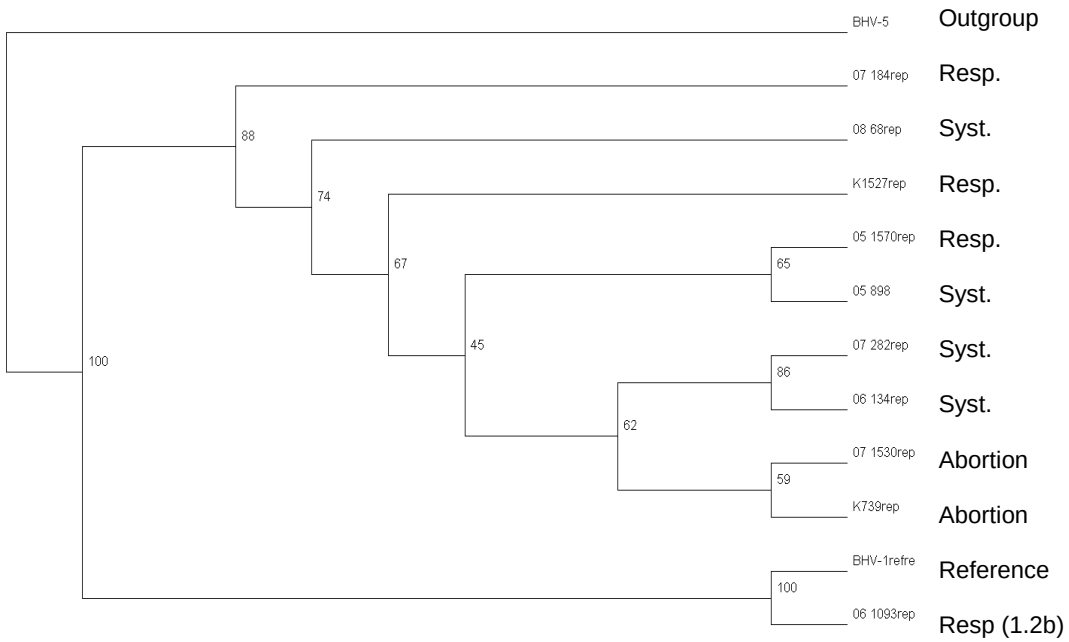
4.5 Phylogenetic analysis

Phylogenetic analysis was carried out to confirm that data extracted from the SNP identification, suggesting minimal genetic variation between the isolates, and did not indicate a viral genetic basis in terms of coding sequences for systemic spread. Both the neighbour-joining (a distance based algorithm) and maximum-likelihood methods were employed to analyse the evolutionary differences between isolates (section 2.8.2 for detailed methods). The isolate genomes were split into 3 regions for the alignment process: unique long, unique short and repeat regions. Alignments were kept at the nucleotide level to enable analysis of non-coding regions as well as coding regions. Both sets of analysis were bootstrapped to find a consensus tree with 100 bootstraps used for maximum likelihood and 1000 bootstraps for neighbour joining, with the exception of the unique long region which could only be calculated with 500 bootstraps due to the length of the sequence alignment.

The phylogenetic trees showed no grouping of the systemic or respiratory isolates in any of the genome regions to suggest there was any conservation of genetic variation among those isolates (figure 4.8, 4.9, 4.10). This observation is supported by low bootstraps consistently found at the nodes separating the sequenced isolates. However, in the repeat region analysis, the two abortion isolates K739 and 07/1530 are grouped together with a bootstrap value of 62 using the maximum likelihood method, separating them from their nearest other isolates of 07/282 and 06/134, both from the systemic group (figure 4.8A). In the neighbour joining tree they do not form a separate branch and are associated more closely with the 07/282 and 06/134 isolates (figure 4.8B). The BoHV-1 reference sequence was found to be separated from the isolates in the unique short region analysis. However, in the repeat regions it is associated with 06/1093, indicating similarities in the repeat regions of these sequences (figure 4.8). In the unique short region it is associated with the K1527 sequence (figure 4.10).

Figure 4.8.A) Maximum-likelihood phylogenetic tree of clustalW aligned repeat (rep) regions created in Phylip (x100 bootstraps). B) Neighbour joining tree formed in clustalX (x1000 bootstraps).

A)



B)

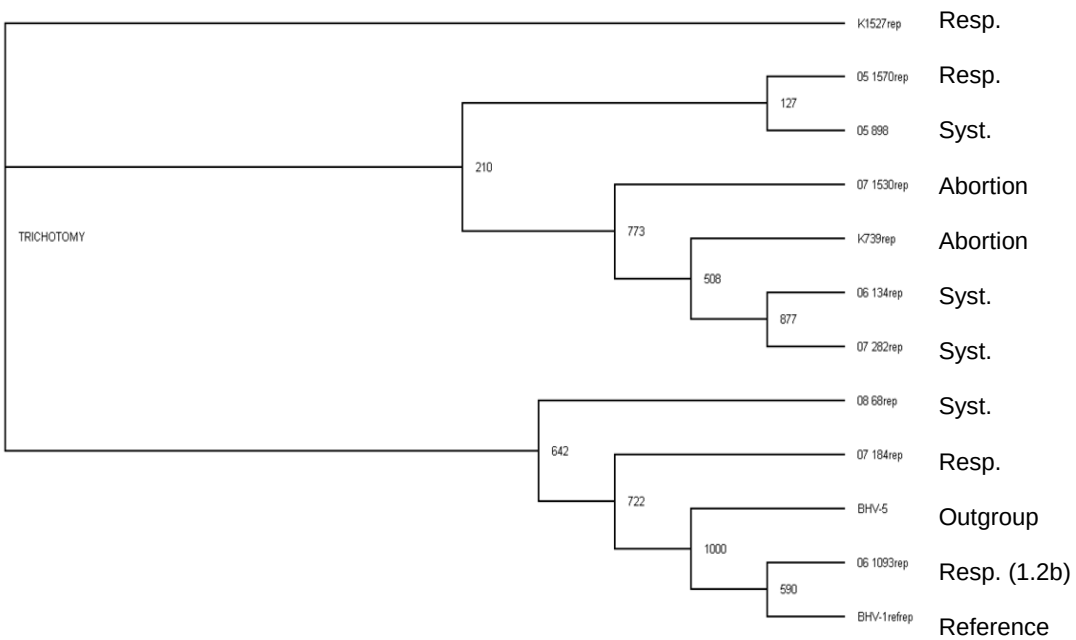
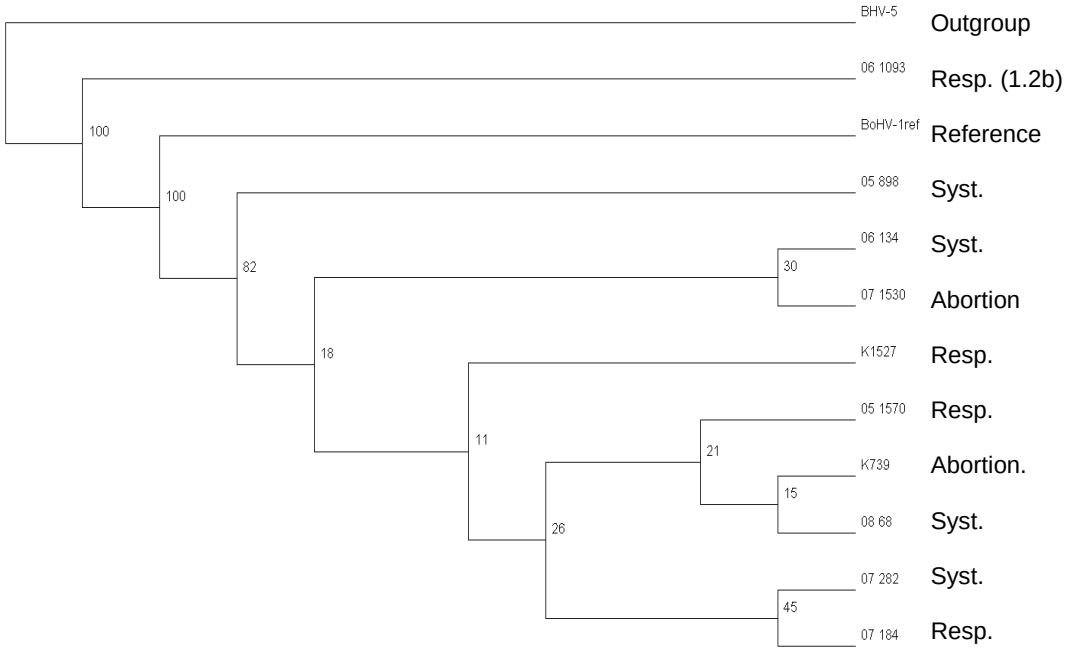


Figure 4.9. A) Maximum-likelihood phylogenetic tree of clustalW aligned unique long regions created in Phylip (x100 bootstraps). B) Neighbour joining tree formed in clustalX (x500 bootstraps).

A)



B)

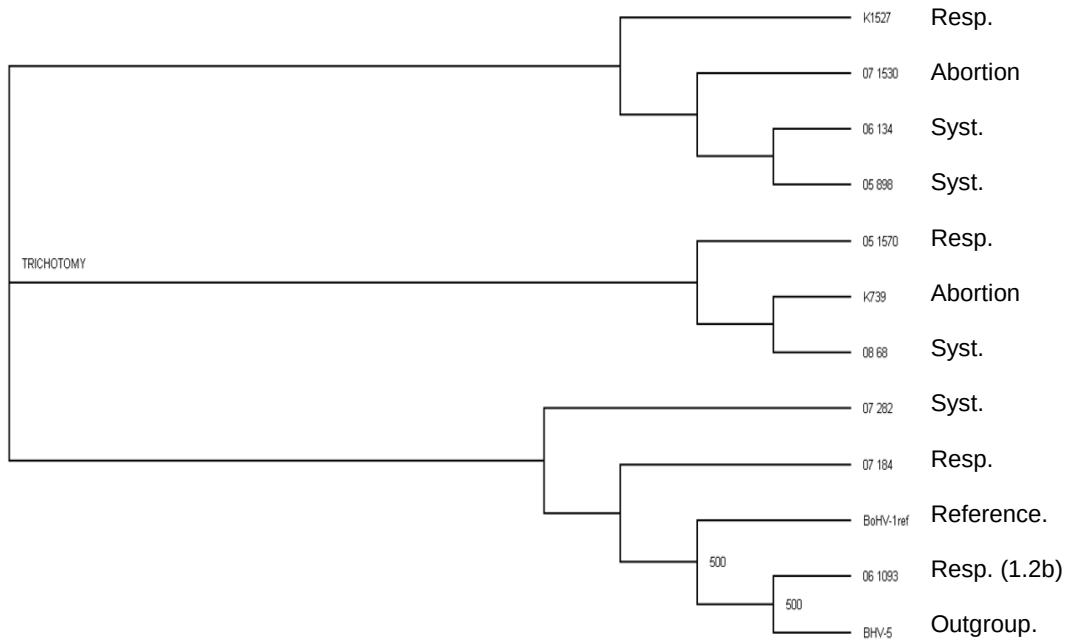
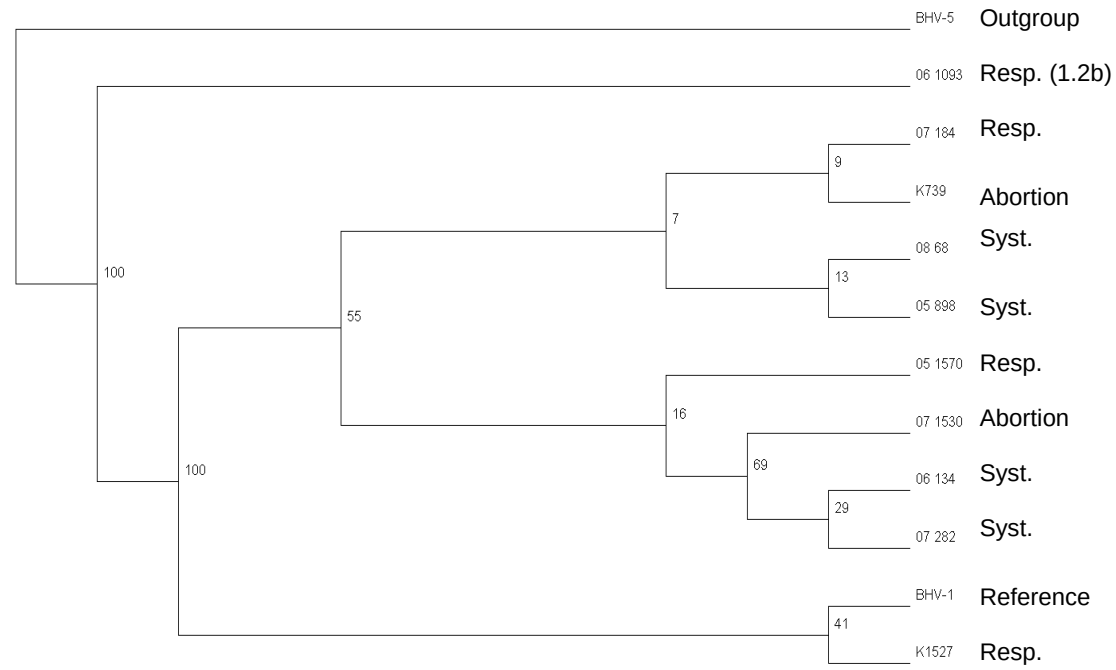
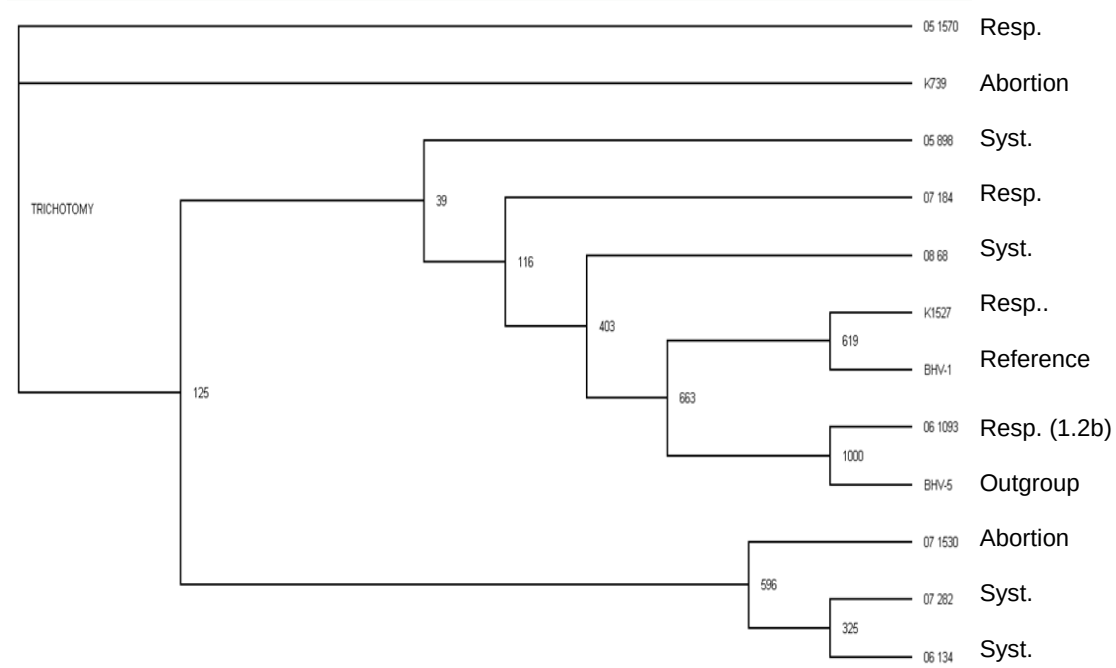


Figure 4.10. A) Maximum-likelihood phylogenetic tree of clustalW aligned unique short regions created in Phylip (x100 bootstraps). B) Neighbour joining tree created in ClustalX (x1000 bootstraps).

A)



B)



4.6 Discussion

The RFLP analysis was used to define the conventional subtyping of the isolates. Isolates could easily be subtyped using the *Hind III* digestion, with the differences in fragment sizes determined by the subtype used to classify the isolates (Mayfield *et al.* 1983). It showed the majority of isolates collected were subtype 1.1, most commonly associated with the respiratory form of the infection (IBR). Only one was identified a subtype 1.2b, most commonly associated with the genital form of infection (IPV). However, as none of these isolates originated from genital cases of disease this indicates that these subtypes are not strict definitions of the clinical signs that should be expected (Christensen *et al.* 1996). This finding was in contrast to a similar study in New Zealand when 24 of 28 isolates were identified as subtype BoHV-1.2b (Wang, Horner & O'Keefe 2006), indicating a geographical difference in predominant subtypes. The main finding from the *Hind III* digest and the use of other restriction enzymes such as *EcoRI* quickly revealed the difficulty of discrimination between isolates using RE analysis due to the poor resolution of this technique and reinforced the homogeneity between the isolates. This was supported by a previous RFLP study which sequenced a 438bp fragment of the Us region and found 99% identity with the published reference strain in Genbank (Wang *et al.* 2006). Therefore it was clear that a more detailed, global analysis was going to be necessary to identify any differences, which were likely to be individual SNPs rather than large recombination or rearrangement events that could potentially be picked up using RFLP analysis.

High-throughput sequencing on the Illumina Solexa sequencing platform provided an opportunity to collect a large amount of data on 11 BoHV-1 isolates, which were used to analyse and compare the isolates using a range of bioinformatic techniques. Just one reference sequence was available for BoHV-1 from a sequencing project in the early 90's (Schwyzer & Ackermann 1996). Therefore, this project provided a dramatic increase in the available sequencing data for BoHV-1, with 11 fully sequenced isolates that can be annotated and published on Genbank.

One of the main challenges with sequencing viral DNA is separating the viral DNA from host DNA. An important step in the isolation of viral DNA was the purification of virus from the BT cell DNA. This was successfully carried out by ultracentrifugation of the viral supernatant through a sucrose cushion to filter out the larger cell debris. The DNA extracted from the virus pellet was successfully subjected to Solexa platform sequencing. A good percentage of viral reads that mapped with significant depth across the reference genome, with the exception of isolate K514 that was subsequently removed from further analysis. This may have been due to poor quality DNA being produced from the sample. 06/1093 also had a relatively low mapping percentage but the overall number of reads sequenced was similar to the other isolates so it was included in the later analysis. This was considered to be due to 06/1093 being a different subtype to the reference sequence, so it would be expected to have a lower mapping percentage. *De novo* assembly may have provided a more reliable sequence, but was not attempted in this study.

The repeat regions also caused some concern with lower depth of reads after mapping and lower phred-like consensus scores; most of the small gaps were found in these regions. This was an anticipated problem that commonly arises in the sequencing of herpesviruses. Although the gaps could not be filled, the data was considered reliable after remapping the repeat regions alone, which dramatically increased the phred-like consensus scores, while the consensus sequences themselves did not change. This leads to the conclusion that the repeat region mapping already completed was reliable. This is a known problem for mapping Solexa data, solutions include specific mapping programs to address the problem such as GNUMAP (Clement *et al.* 2010). The use of this software should be considered in future projects but a brief trial with the BoHV-1 data did not significantly improve the amount of repeat sequence data that was mapped. This is likely to be due to the data already being reliable, as the further analysis of the data using the MAQ software showed.

Minor editing of the reference scaffold sequence was needed to maximise the mapped reads (Davison 2009). This was a successful method of getting the most

from the data and was most likely to be necessary due to the reference sequence being put together from isolates of several strains (K22, Cooper, P8-2, 34, and Jura) (Genbank 2006). After minor editing of the reference scaffold sequence and subsequent mapping using MAQ, consensus sequences were formed for all isolates. A gap was observed in all sequenced isolates in the repeat regions in the BICP4 ORF. This could be due to several reasons. As the repeat regions are repeats of the same bases, regions could be mapping more frequently to either the internal or terminal repeat regions rather than equally between the two. This seems unlikely, as gaps were seen in both repeat regions. Alternatively, the observed gaps could represent true deletions compared to the reference isolate. There was also a conserved gap region of 15-30bp (depending on isolate) that were called as N's. This could possibly represent a gap seen in all isolates within the UL47 ORF, which codes for a tegument protein VP13/14. However, a protein blast on this region with the gap region removed shifts the ORF and produces stop codons mid-gene. Therefore, it appears likely that it may just represent a region of poor sequence where base calling was difficult due to the characteristics of this region. A previous study has shown BoHV-1.1 subtypes have fewer GC-repeats than subtype 1.2b downstream from US7 coding region for gI (Nadin-Davis 1996). The findings here may suggest the same could occur in other regions of the genome, if the gaps represent true deletions.

The search for SNPs between the isolate groups provided several important results; SNPs appeared to be random and not conserved between isolate types, the reference sequence may not have been a reliable scaffold, and 06/1093 had a very different SNP pattern to all the other isolates. Phylogenetic analysis generally provided support for these findings, although the bootstrap values were consistently low between the sequenced isolates in the maximum likelihood analysis indicating a poor level of consensus in the tree structure in terms of the 10 sequenced isolates. The close-relationship between the isolates that is indicated makes it difficult to distinguish the phylogeny reliably.

A general overview of the SNP distribution was gained by measuring SNP frequency in each coding region. This showed a widespread distribution across the genome with

just a few sites showing fewer SNPs than others including UL42 (DNA polymerase processivity protein), UL41 (host-shut-off protein), UL28 (DNA packaging terminase) and BICP4 (transcriptional regulator), showing these genes are very well conserved between isolates. There was no significant variation in the frequency of SNPs associated with particular gene functions. However, a significantly more frequent number of SNPs were identified in genes identified as non-essential compared to essential genes in tissue culture. This could represent the ability of these genes to evolve at a faster rate due to their non-essential contribution to virus replication; therefore random mutations are less likely to be lost as they are less likely to affect the successful replication of the progeny virus. However, it should also be considered that any passage through cell culture is likely to result in mutations of these genes, this seems unlikely to be the cause in this case as only one passage in cell culture had occurred. During an *in vivo* infection these genes are likely to be most important for the spread of the virus, not necessary in cell culture. A comparison between isolates that had undergone different numbers of cell culture would be needed to find out whether this is the cause for the higher number of SNPs observed here, or whether this may represent the greater variation in genes involved in the *in vivo* spread of the virus, showing more variation than those involved in replication.

SNPs were found to be more prevalent between all the isolates sequenced and the reference rather than between the sequenced isolates. This was most likely due to the reference sequence consisting of sequence data from several different sequencing projects using 5 different strains to produce the reference sequence as discussed previously (Genbank 2006). This could explain the poor mapping at the repeat regions as most of these regions were sequenced using the K22 1.2b strain and may also explain the grouping of 06/1093 and the reference BoHV-1 sequence in the repeat region phylogenetic analysis, as in this region they would have shown a higher level of similarity. The high bootstrap values seen on the nodes separating the reference isolates and 06/1093 from the other isolates, demonstrated this was specific to the newly sequenced isolates, and also supported the significantly higher number of SNPs found between the reference and 06/1093. This could possibly represent

temporal or geographical factors, as all sequenced isolates originated from Scotland or northern England over a five year period, compared to the reference sequence which was mostly represented by a strain, originally isolated in California in 1956 (Madin *et al.* 1956). A geographical cause seems most likely as this was found to be a powerful influence in a genetic analysis of varicella-zoster isolates from around the world (Muir, Nichols & Breuer 2002). There was one exception in the unique short region maximum likelihood analysis which found K1527 associated with the BoHV-1 reference sequence, but the bootstrap value of 41 is not high enough to consider this a significant finding. It is more likely to be a reflection of the short length of this region, and therefore fewer SNPs identified.

Very few SNPs were conserved between any of the isolates, just 40 between 05/1570 and 07/1530; this number was typical of all isolates shown fully in table 4.3. It was not considered necessary to make a complete set of pair-wise comparisons as no conservation between isolate types was observed. Only 13 SNPs of the 05/1570-07/1530 pairing were non-synonymous. When cross-referenced with other isolates they were not found to be conserved between the groups associated with respiratory or systemic clinical signs. This extensive homology was not unexpected and reflects finding from a previous study of phylogenetic comparison of the glycoprotein C region which found genomic similarity of 98.7-99.8% between BoHV-1 isolates (Esteves *et al.* 2008). A study of varicella-zoster genotypes from different geographical regions identified just 16 non-synonymous SNPs, 10 x fewer than the genetic diversity observed in herpes simplex virus (Muir *et al.* 2002). A whole genome study of 18 varicella-zoster isolates identified homology at a nucleotide level of >99.8 between isolates and the Dumas reference strain and demonstrated the occurrence of recombination (Peters *et al.* 2006). In Varicella-Zoster most variation was observed within the reiteration patterns of the repeat regions, origins of replication and intergenic homopolymer patterns (repetition of a single base) of the repeat regions (Tyler *et al.* 2007). HSV type 2 isolates collected from Tanzania, Norway and Sweden demonstrated 99.6% similarity based on sequencing of 3.5% of the genome (all in the unique short region), which also identified evidence of recombination (Norberg *et al.* 2007).

Overall, this sequencing data provides information on the natural variation found between BoHV-1 isolates collected between 2002 and 2007 in Scotland and northern England. It confirms the slow evolution that is expected from the stable genomes of alphaherpesviruses, although recent SNP analysis on the varicella-zoster virus genome shows a constantly evolving genome in which discovery of new genotypes that may contribute to new phenotypes may occur (Faga *et al.* 2001). As there also seemed to be no grouping of SNPs according to gene function or genome position it seems likely that the variation observed between isolates is representative of natural variation rather than positive selection for virulence. As there is only one reference genome, these further sequences are useful information that will be of interest in terms of the evolution of BoHV-1. Phylogenetic analysis also supported these findings with no grouping of isolates assigned to the same group except the two abortion isolates in the repeat regions. The bootstrap value of 62 separating them from two systemic isolates on this branch is not entirely convincing as it was not conclusively supported by the neighbour joining method. Sequencing of this region of further isolates with higher bootstraps would be needed to confirm any significance of this, as it was not reflected in the SNP analysis so there is not enough evidence to conclude anything from this finding here. However, it does provide some evidence that these two isolates may show more similarity with each other compared to the other isolates and this should therefore be considered in further investigations. In particular it raises the question of whether the abortion isolates should be grouped with the isolates from systemic infection in neonates as this result indicates they may be different.

Recently discovered microRNAs encoded by BoHV-1 were potential areas of interest as they have been associated with virulence in other herpesviruses (Boss *et al.* 2009). However, there is no evidence of variation at these sites at the genomic level, although this does not necessarily mean they are all being expressed and able to bind with mRNA to influence translation. Further experiments would be required to monitor expression of these microRNAs.

Recombination is thought to play a significant role in alphaherpesvirus evolution. An *in vitro* co-infection of two BoHV-1 strains showed this recombination involves negative genetic interference, which means one incidence of exchange is likely to result in another recombination event in the close vicinity of the first event (Muylkens *et al.* 2009). More single isolate whole genomes are needed to establish whether recombination of BoHV-1 has ever occurred in the natural environment, but there was no evidence of recombination in the isolates examined here. Specialist software was not used to look for potential recombination sites. It may also have been hidden by using a reference sequence to map short reads to the predetermined scaffold. 454 sequencing or de novo assembly may be more appropriate to identify any potential sites of recombination.

In conclusion, unlike the neurovirulence of EHV-1, the systemic spread of BoHV-1 does not appear to be related to a SNP or other variation such as insertions or deletions in the viral genome, as demonstrated by viral genome sequencing. There could also be transcriptional factors causing the gene expression to vary between isolates. Transcriptomic analysis may be a useful technique to investigate this further as was used in the study of pseudorabiesvirus and porcine epithelial cells during infection (Flori *et al.* 2008). The next chapters will use another approach to consider this and investigate the pathogenesis of different sequenced isolates in both the natural host and model systems, considering both the dissemination of the virus and viral gene expression throughout an infection.

Chapter Five: Experimental infection to compare pathogenesis of respiratory and systemic isolates

5.1 Introduction

The previous chapter compared BoHV-1 genomes obtained from animals showing different clinical presentations in the field. No genetic differences were found to differentiate between these different clinical outcomes. However, to rule out any virally encoded control of the systemic spread of BoHV-1 it was necessary to observe how these isolates behaved in a natural experimental challenge in the definitive host, and establish whether there were *in vivo* effects. No other studies have directly compared two BoHV-1 isolates in this way, using a natural route of infection. Most other studies which have aimed to provide information on the systemic route of spread have used an intravenous route of infection, which obviously influences dissemination of the virus. Studying viral pathogenesis in the natural host also adds the important factor of the host immune response to the experiment.

Two of the fully sequenced isolates were chosen, one recovered from a calf with respiratory disease (05/1570) and the other representing a systemic infection that was isolated from aborted material (07/1530). They will be referred to as 05/1570 and 07/1530 throughout this chapter. These were used to infect two groups of nine calves, by an intranasal route of infection with a dose of 10^7 $TCID_{50,}$ a third group of calves remained uninfected. Daily clinical scores were recorded and blood, nasal and ocular swab samples taken for the first 11 days, then twice weekly, thereafter. Three calves from each infection group were euthanased 4, 10 and 24 days post infection. A post-mortem examination allowed collection of tissues for molecular and histopathological analysis. All calves used were BoHV-1 antibody negative to ensure

they had no previous exposure to the virus or adaptive immune response. See 2.4 for detailed methods.

5.2 Clinical observations

Clinical signs typical of IBR were observed in infected groups and not in the control group from day 1 post infection (pi). The clinical signs calculated from the mean of each group, increased on day 4 pi which represented the peak for the 05/1570 infected group. This increase in clinical signs lasted until 8-10 days pi with a peak in the 07/1530 infected group on day 8 pi (figure 5.1A). The primary observations were a fever with an average body temperature of 40°C recorded in both groups, compared to 38.5°C in the uninfected control group. However, this peak quickly subsided over the following few days (figure 5.1B). Very little coughing was observed but, when observed it was seen most frequently to be associated with the respiratory isolate, 05/1570 (figure 5.1C). Nasal discharge was observed in both infected groups from day 4 pi, peaking on day 5 for the 07/1530 infected group and day 6 in the 05/1570 infected groups. The score for nasal discharge remained higher than the control group until day 11 pi in both groups (figure 5.1D). Signs of rhinitis, including reddening and lesions associated with the nasal passage peaked later in both groups between days 7-10 pi, and lasted longer for 07/1530 infected animals, although this was not statistically significant (figure 5.1E). Conjunctivitis and ocular discharge were observed in both groups, but were visibly more severe and lasted longer in the systemic isolate infected group. These differences between infected groups did not reach statistical significance (figure 5.1F). No significant clinical signs were observed in any of the animals in the uninfected control group. No observations of depression, loss of appetite, dehydration and body condition were recorded in any of the groups and therefore are not shown, but data was included in the average clinical score results. One animal suffered from an increased respiratory rate that was found to be unrelated to the viral infection and is therefore also not shown in detail.

Figure 5.1. Clinical scores throughout the experimental infection with BoHV-1 isolates. A) Average clinical score from all clinical scores measured (table 2.4). B) Average body temperature. C) Average score for coughing. D) Average score for nasal discharge. E) Average rhinitis score. F) Average conjunctivitis/ocular discharge score.

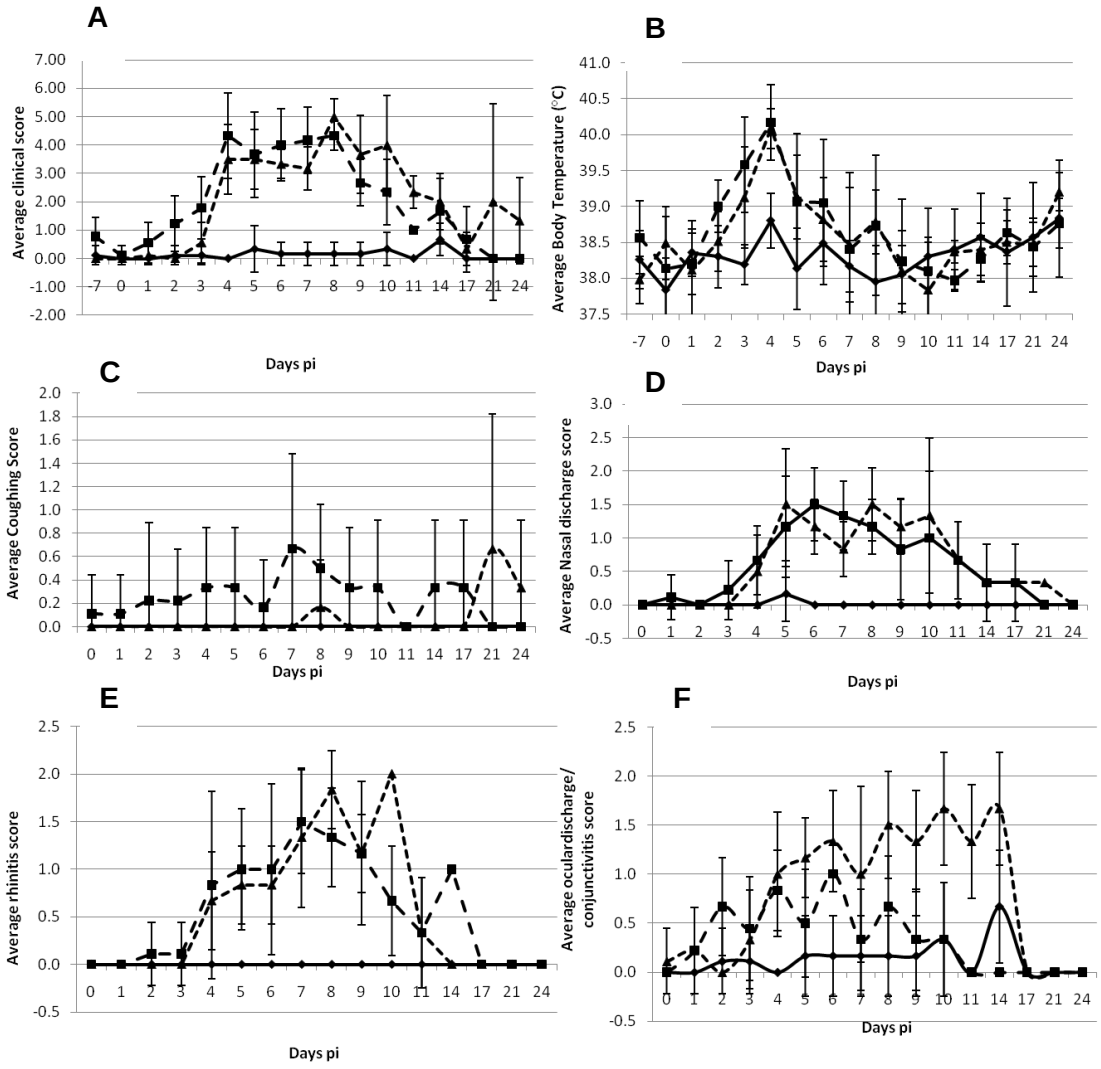
Control uninfected group;

Respiratory 05/1570 isolate infected group;

Systemic 07/1530 isolate infected group.

Days 0-3 n=9, days 4-9 n=6, days 10-24 n=3.

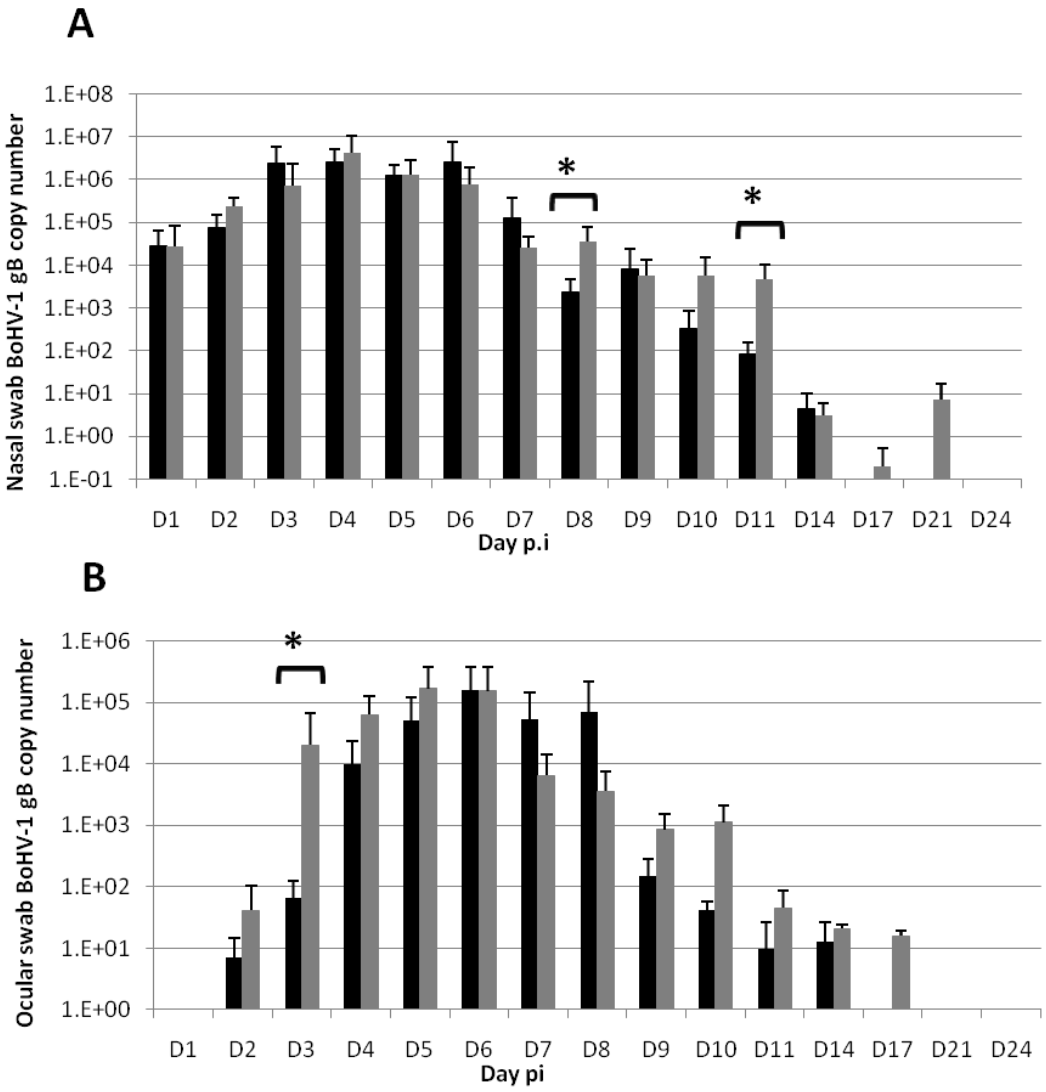
Fisher's exact test showed significant differences between infected groups and uninfected control groups but not between the two infected groups.



5.3 Viral shedding

Ocular and nasal swabs were collected daily for 11 days, then twice weekly until day 24 pi. Real-time PCR with absolute quantification of BoHV-1 gB was used to determine BoHV-1 viral load in the swabs (see 2.6.3 for detailed methods). As anticipated, using an intranasal infection, virus was detected in nasal swabs from day 1 in both groups and virus load peaked on day 4 (figure 5.2A). In the ocular swabs viral DNA was not detected until day 2 pi and virus load peaked slightly later than in the nasal swabs, on days 5 and 6 (figure 5.2B). Virus load on nasal swabs was consistently detected at similar levels in both groups early in infection, but from day 8 pi a decrease was detected in the 05/1570 infected group, whilst the viral load in the systemic isolate infected animals remained high. This was found to be statistically significant on days 8 and 11 ($p<0.05$). The viral loads detected in the ocular swabs demonstrated more differences between the two groups. Early in the infection (from day 2 until day 5 pi) the systemic isolate infected group showed higher viral loads, which was found to be significantly different on day 3 ($p<0.01$). On day 7 and 8 the 05/1570 infected group showed a higher viral load, but this rapidly decreased from this time point onwards, whilst the 07/1530 infected group continued to show high viral loads from the eyes until day 10 pi and was still detectable on day 17. No virus was detected in any of the swabs collected from the uninfected control group.

Figure 5.2. Viral loads collected from nasal (A) and ocular (B) swabs after experimental infection with BoHV-1 isolates. Black – swabs from respiratory 05/1570 isolate infected animals, Grey – swabs from systemic 07/1530 isolate infected animals. D1-3 n=9, D4-9 n=6, D10-D24 n=3. A REML covariance mode with subsequent t tests was used to compare viral load between infected groups and differences were considered significant if $p<0.05$ indicated with a (*).



5.4 Viral distribution and lesion characterization throughout infection

5.4.1 Comparison of viral load in tissues measured by RT-PCR

The viral load of BoHV-1 in tissues including the respiratory tract, brain, lymph nodes, viscera and buffy coat was detected and measured using absolute quantification by real-time PCR (see 2.4 and 2.6.3 for detailed methods). The aim was to look for evidence of viraemia and identify the extent of viral spread over the time period of infection. Whole heparinised blood was collected daily for the first 11 days pi and then twice weekly until day 24 pi. Buffy coat was recovered each day and stored at -20°C prior to testing. Calves were euthanased and tissue samples collected at 3 time points; day 4 pi representing the expected peak of clinical signs, day 10 pi representing expected recovery from clinical signs and day 24 pi when the virus would be expected to have entered the latency phase.

No BoHV-1 DNA was detected at any of the time points in the buffy coat samples. Serum from clotted blood samples was also tested at the peak of infection, on day 4 to check for cell-free viraemia, but no BoHV-1 DNA was detected.

BoHV-1 DNA was detected in tissues at each of the three time points sampled. Neither of the BoHV-1 isolates were detected outside of the respiratory tract or the CNS. No BoHV-1 DNA was detected in the control group, with the exception of one trachea sample, which is likely to have been due to contamination at some point in the testing process, as it was not seen in any other control samples and was histopathology and IHC negative. Due to the high variation between individual animals there were not many statistically significant differences in viral load between the isolate types, however several trends can be observed (figure 5.3).

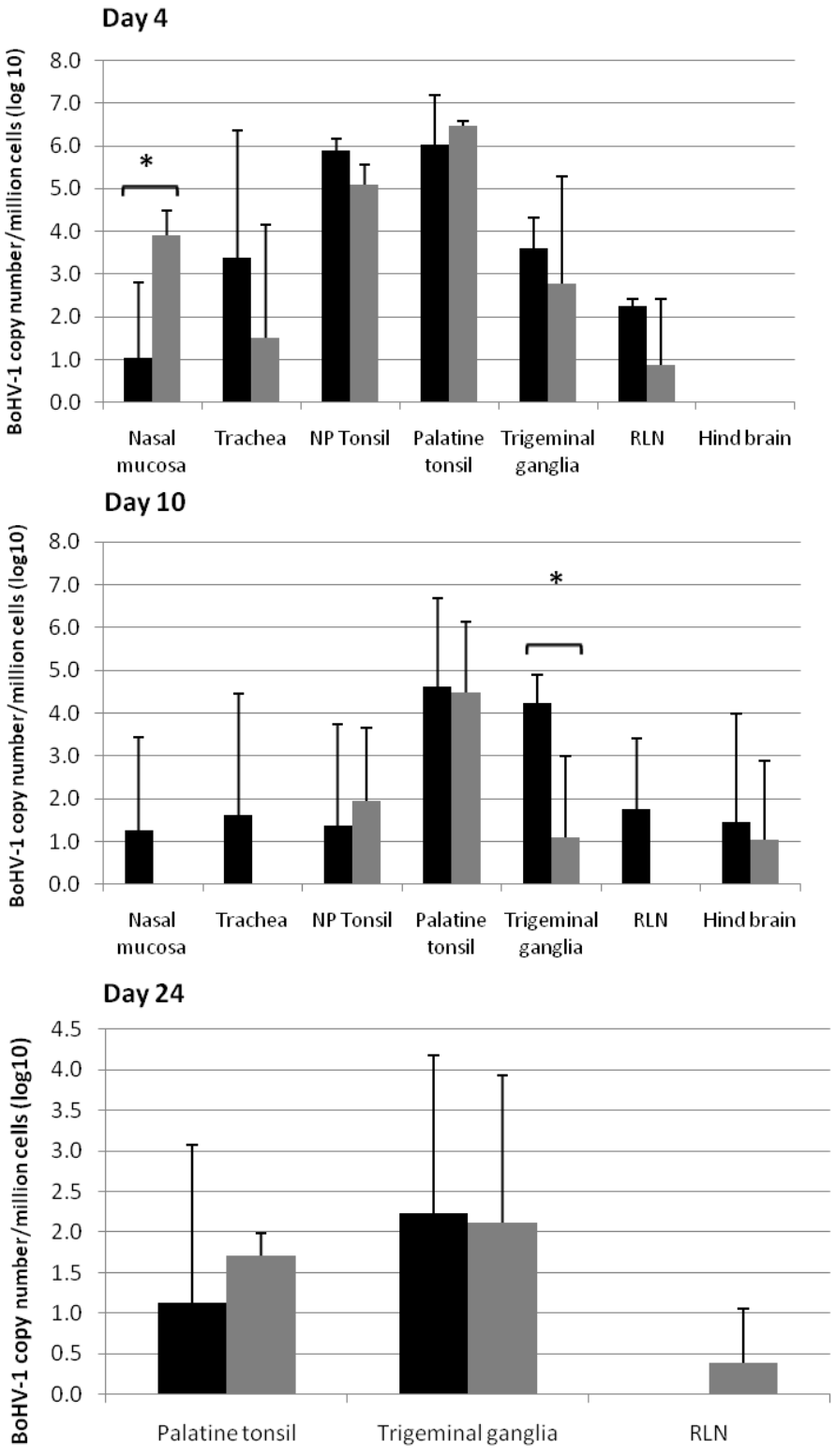
On day 4 virus DNA was detected in the nasal mucosa, trachea, tonsils, trigeminal ganglia and the retropharyngeal lymph node in both isolate infected groups. There was a significantly higher viral load in the nasal mucosa of the 07/1530 infected group on day 4, whilst the 05/1570 infected group showed some evidence of a higher

viral load in the trachea. In other tissues viral loads detected were similar in both groups (figure 5.3).

On day 10 post infection virus DNA was detected in animals infected with isolate 05/1570 in the same tissue where it was present on day 4 and was also detected in the hindbrain. However, those animals infected with isolate 07/1530 no longer had detectable levels of virus in the nasal mucosa or trachea. There was also significantly higher viral load observed in the trigeminal ganglia of the 05/1570 isolate infected group (figure 5.3).

In both infected groups on day 24 virus was detected in the palatine tonsil and trigeminal ganglia. Similar levels of viral loads were found in both infected groups in both of these tissues. There was also evidence of viral DNA in the retropharyngeal lymph node of the 07/1530 isolate group (figure 5.3).

Figure 5.3. BoHV-1 viral load found in tissues collected at day 4, 10 and 24 of the experimental infection. Viral load was calculated to give a copy number per million cells (see 2.6.3). Black – Respiratory isolate infected animals, Grey – Systemic isolate infected animals (n=3). T-test – * - $p<0.05$)



5.4.2 Gross and histopathological examination and immunohistochemical localisation of virus

Gross pathological examination of tissues during post mortem demonstrated lesions in the nasal mucosa (figure 5.4). Mild rhinitis characterized by reddening of the nasal mucosa, was observed in all infected animals on day 4, and occasionally associated with mucopurulent exudate (figure 5.4B). By day 10 local and superficial ulceration of the mucosa with an overlay of fibrinous exudation was present in all 3 of isolate 05/1570 infected calves and one of the 07/1530 infected calves (figure 5.4C) There was evidence of mild mucopurulent tracheobronchitis of all 3 07/1530 infected calves on day 24 pi (figure 5.5), but not in the 05/1570 infected calves.

Tissues collected on day 4, 10 and 24 pi were also examined histopathologically using H&E staining to identify lesions and immunohistochemistry (IHC) to demonstrate the localisation of BoHV-1 antigen in the tissues in which at least one sample had tested positive for PCR (see 2.7 for detailed methods). Comparisons of the results of PCR, histological examination and immunohistochemical labelling are summarised in table 5.1.

H&E staining allowed identification of lesions that were consistent with BoHV-1 infection. There were also some lesions identified in control group animals, mainly in the trachea where a non-purulent inflammatory infiltrate was observed in 7 of the 9 control group animals. Lesions were also seen in the nasal mucosa, palatine tonsil and retropharyngeal lymph nodes of 1-3 control animals, although these were considered to be caused by other agents than BoHV-1 as they tested negative by PCR and IHC specific for BoHV-1. These lesions were considered as background and taken into account when looking at lesions in the infected groups. IHC was used to label BoHV-1 antigen in sections, from the same paraffin blocks used for H&E staining. Viral antigen was only found in the nasal mucosa, tonsils and trigeminal ganglia.

Figure 5.4. Saggital section of the nasal cavity. A) Control uninfected calf. B) Mild rhinitis on day 4 pi. C) Focal ulcerative fibrinous rhinitis on day 10 pi.

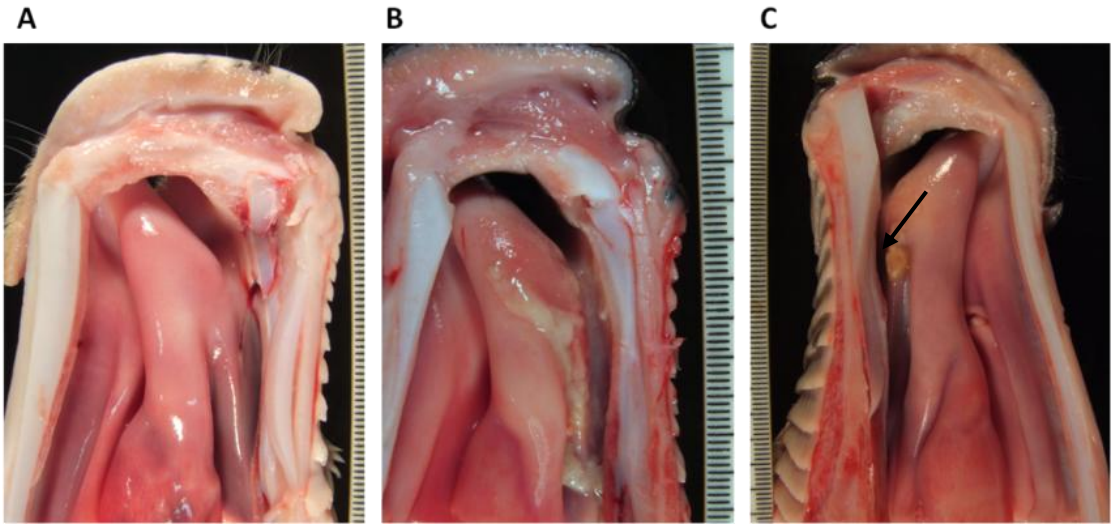
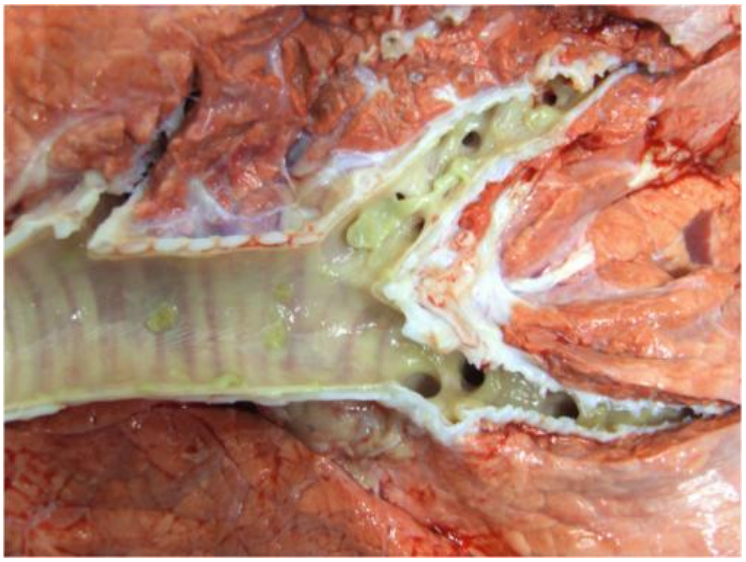


Figure 5.5. Mucopurulent exudates in trachea and bronchi on day 24 pi in systemic isolate (07/1530) infected calf.



On day 4 pi the nasal mucosa showed evidence of an inflammatory infiltrate, consisting of macrophages, lymphocytes and plasma cells in all 3 07/1530 infected animals. The nasal mucosa showed BoHV-1 antigen in a necrotic area of the epithelium in one systemic isolate case on day 4 pi (figure 5.7D), compared to no evidence of necrosis or IHC labelling in the respiratory isolate infected samples (figure 5.7C). The trachea also demonstrated differences between the infected groups on day 4 pi. The 05/1570 infected group showed a non-purulent inflammation of the

epithelium in all 3 trachea samples that was not considered to be more severe than observed in the 07/1530 infected group and control group. The nasopharyngeal and palatine tonsil demonstrated evident lesions on day 4 in all infected animals, however the characteristics of these lesions differed between the groups. Tonsils from the 05/1570 infected group showed multifocal necrosis in the lymphoid tissue (figure 5.6A), whereas in the 07/1530 infected calves a multifocal to coalescent neutrophilic infiltrate was seen in the lymphoid tissue and epithelium (figure 5.6B), with only one sample showing necrotic foci. Both the palatine and nasopharyngeal tonsils were IHC positive in all calves of both infected groups at 4 days pi. Multifocal labelling was observed, associated with both epithelial and follicle regions of the tissue, with no obvious differences in the localisation of the antigen between the two infected groups (figure 5.7A and 5.7B). The retropharyngeal lymph node was reactive and showed some focal infiltration in the 07/1530 infected group and control group. However, no viral antigen was detected by IHC. The trigeminal ganglia showed mild focal inflammation on day 4 pi in 2 out of 3 of the 05/1570 infected animals (figure 5.6E), which was not seen in any samples from the 07/1530 infected group. In the trigeminal ganglia only two samples were IHC positive, both from the 05/1570 infected group on day 4 pi. Labelling was localised to single neurons (figure 5.7 E). The brain showed no sign of lesions on day 4 in either group.

On day 10 pi, inflammatory infiltrate was observed in the nasal mucosa in both infected groups, although it was considered to be more diffuse in the 05/1570 infected group. In the trachea there was a mixed, thick and diffuse infiltrate in the samples infected with isolate 05/1570. More inflammatory infiltrate was observed in the trachea of isolate 07/1530 infected animals, compared to day 4, although it remained non-purulent, with the exception of one case (figure 5.6C and 5.6D). No significant lesions were observed in the tonsils at 10 days pi. However, viral antigen was detected in both groups in the palatine tonsil and in one 05/1570 infected animal in the nasopharyngeal tonsil. In the trigeminal ganglia, a more severe response formed by multifocal non-purulent aggregates of inflammatory cells was observed in the 07/1530 infected group samples. Lesions were also observed in the brain samples at 10 days pi, with evidence of a non-purulent encephalitis characterized by glial foci

and perivascular cuffs. This was observed in both infected groups, but was considered to be more severe in the systemic isolate infected samples (figure 5.6F).

On day 24 pi, inflammatory infiltrate in the nasal mucosa was present in both infected groups with no significant differences noted between the groups (table 5.1). Mild focal non-purulent aggregates of inflammatory cells were observed in trigeminal ganglia from 07/1530 infected animals, as seen in day 10 samples. Only one trigeminal ganglion sample from the respiratory isolate infected group demonstrated a non-purulent inflammatory infiltrate at this time point. On day 24 pi in the brain, non-purulent encephalitis, as described above, was observed in 1 of 3 respiratory isolate infected samples and in 2 of 3 systemic isolate infected samples. Tonsils showed lesions in 1 of 3 05/1570 infected samples from the nasopharyngeal tonsil and 1 of 3 07/1530 infected samples from the palatine tonsil. This palatine tonsil sample was also positive for viral antigen by IHC. The retropharyngeal lymph node remained reactive, but was not considered to be significantly different to the response seen in control samples.

Other tissues demonstrated very few or no lesions throughout the infection period. Regarding the lungs, where seen, lesions were more frequent in the systemic isolate infected group with one or two animals affected at each time point. However, the lesion, a non-purulent pneumonia, was very mild and was also observed in a control animal at day 10. Lesions in the mesenteric lymph node, liver, kidney, spleen and testicle were sporadic and very mild and considered to be incidental.

Table 5.1. Summary of presence of lesion, viral antigen and viral DNA in all groups. C1-C9 are uninfected control calves, R1- R9 are respiratory isolate 05/1570 infected calves, S1-S9 are systemic isolate 07/1530 infected calves. A positive result is indicated with a (+) and a negative result with (-). NS indicates sample was not suitable or not tested.

Day	Animal	Nasal mucosa			Trachea			Nasopharyngeal tonsil			Palatine tonsil			RLN			Lung- caudal			Lung - cranial			Trigeminal ganglia			Brain-cortex			Hindbrain			Liver			Kidney			Spleen			Mesenteric lymph node			Testicle		
		H&E	IHC	PCR	H&E	IHC	PCR	H&E	IHC	PCR	H&E	IHC	PCR	H&E	IHC	PCR	H&E	IHC	PCR	H&E	IHC	PCR	H&E	IHC	PCR	H&E	IHC	PCR	H&E	IHC	PCR	H&E	IHC	PCR	H&E	IHC	PCR	H&E	IHC	PCR	H&E	IHC	PCR
4	C1	-	-	-	+	-	-	-	-	-	NS	-	-	-	-	-	-	NS	-	-	NS	-	-	-	-	-	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	C2	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	NS	-	-	NS	-	-	-	-	-	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	C3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NS	-	-	NS	-	-	-	-	-	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	R1	-	-	-	+	-	+	+	+	+	+	+	+	-	-	+	-	NS	-	-	NS	-	-	-	+	-	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	R2	-	-	+	+	-	+	+	+	+	+	+	+	-	-	+	-	NS	-	-	NS	-	+	+	+	-	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	R3	-	-	-	+	-	-	+	+	+	+	+	+	-	-	+	-	NS	-	-	NS	-	+	+	+	-	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	S1	+	+	+	NS	-	+	+	+	+	+	+	+	+	-	-	-	NS	-	+	NS	-	-	-	+	-	NS	-	-	-	-	-	NS	-	+	NS	-	-	NS	-	-	NS	-
	S2	+	-	+	+	-	-	+	+	+	+	+	+	+	-	+	+	NS	-	-	NS	-	-	-	+	-	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	S3	+	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	NS	-	-	NS	-	-	-	+	-	NS	-	-	-	-	+	NS	-	+	NS	-	-	NS	-	-	NS	-
10	C4	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	NS	-	-	NS	-	-	-	-	+	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	C5	-	-	-	+	-	-	-	-	-	NS	-	-	+	-	-	-	NS	-	+	NS	-	-	-	-	-	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	C6	+	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-	NS	-	-	NS	-	-	-	-	-	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	R4	+	-	+	+	-	+	+	+	+	+	+	+	-	-	-	-	NS	-	-	NS	-	+	-	+	-	NS	-	-	-	+	+	NS	-	-	NS	-	-	NS	-	-	NS	-
	R5	+	-	-	+	-	-	-	-	-	+	+	+	-	-	+	-	NS	-	-	NS	-	NS	-	+	-	NS	-	+	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	R6	+	-	-	+	-	-	-	-	-	+	-	+	-	-	+	-	NS	-	+	NS	-	+	-	+	-	NS	-	+	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	S4	+	-	-	+	-	+	-	-	-	+	+	+	-	-	-	-	NS	-	+	NS	-	+	-	-	-	NS	-	+	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	S5	+	-	-	+	-	-	-	-	+	-	+	+	-	-	-	-	NS	-	-	NS	-	+	-	-	-	NS	-	+	-	+	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	S6	+	-	-	+	-	-	-	-	+	-	+	+	-	-	-	-	NS	-	-	NS	-	+	-	+	+	NS	-	+	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
24	C7	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	NS	-	-	NS	-	-	-	-	-	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	C8	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	NS	-	-	NS	-	-	-	-	-	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	C9	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	NS	-	-	NS	-	-	-	-	-	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	R7	+	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	NS	-	-	NS	-	+	-	-	-	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
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	S7	+	-	-	+	-	-	-	-	-	+	+	+	-	-	-	+	NS	-	-	NS	-	+	-	+	-	NS	-	+	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	S8	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	NS	-	-	NS	-	+	-	+	-	NS	-	+	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-
	S9	+	-	-	+	-	-	-	-	-	-	-	+	+	-	+	+	NS	-	-	NS	-	+	-	-	-	NS	-	-	-	-	-	NS	-	-	NS	-	-	NS	-	-	NS	-

Figure 5.6. Histological lesions at different locations in both infected groups.. A) Respiratory isolate infected calf; nasopharyngeal tonsil necrosis, day 4, pi. B) Systemic isolate infected calf; nasopharyngeal tonsil neutrophilic infiltrate, day 4 pi. C) Respiratory isolate infected calf; trachea inflammatory infiltrate, day 10 pi. D) Systemic isolate infected calf; trachea with no inflammatory infiltrate, day 10 pi. E) Respiratory isolate infected calf; trigeminal ganglia inflammatory infiltrate, day 4 pi. F) Systemic isolate infected calf; brain showing inflammatory infiltrate, day 10 pi. (H&E x10).

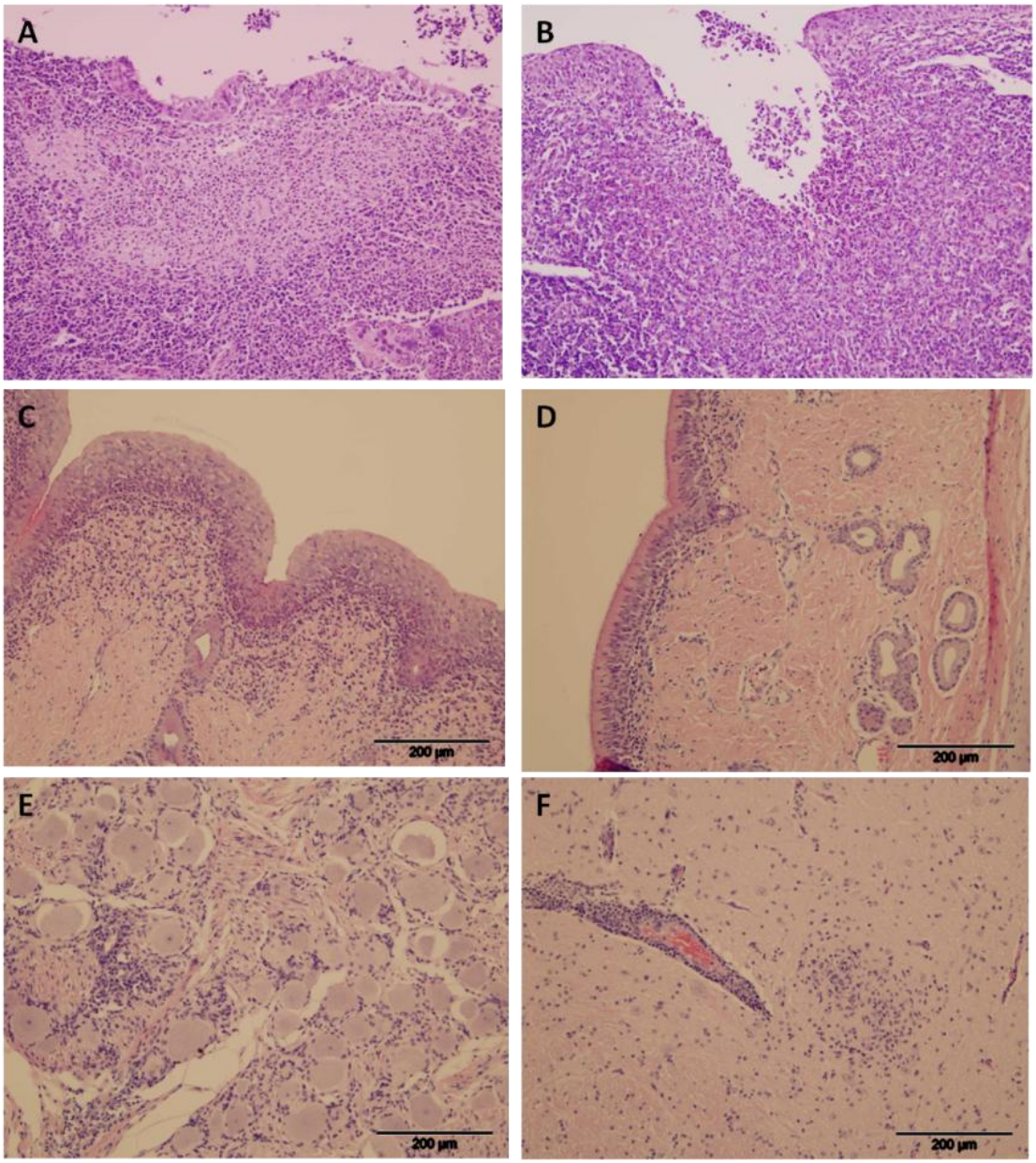
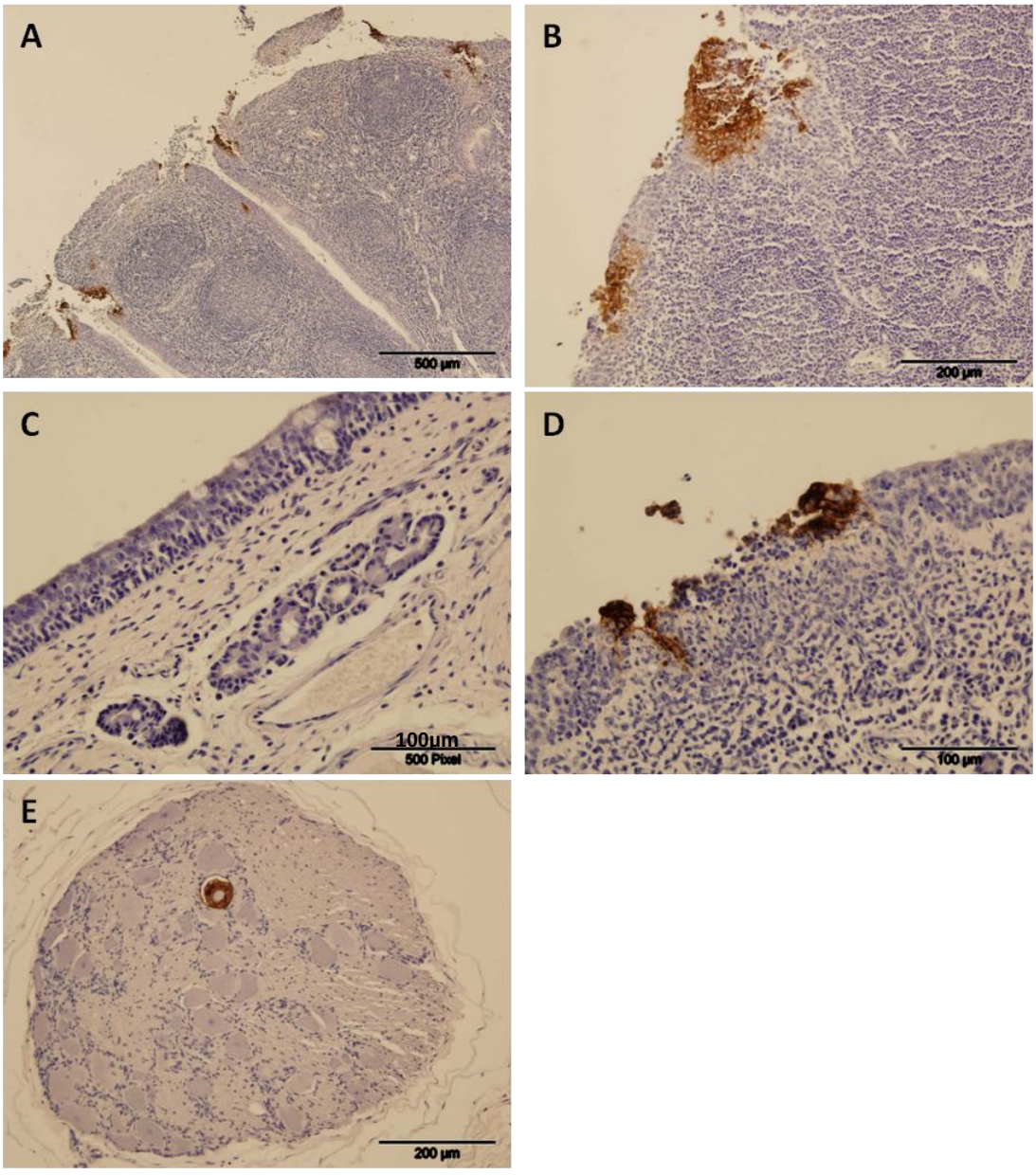


Figure 5.7. Immunolabelling of viral antigen at different locations in both groups. A) Respiratory isolate infected calf; nasopharyngeal tonsil showing scattered foci of positive labelling, day 4 pi (x4). B) Systemic isolate infected calf; nasopharyngeal tonsil showing scattered foci of positive labelling, day 4 pi (x10). C) Respiratory isolate infected calf; nasal mucosa without inflammatory infiltrate or viral antigen, day 4 pi (x20). D) Systemic isolate infected calf; nasal mucosa showing positive labelling for BoHV-1 in the epithelial cells at the periphery of a necrotic lesion (x20). E) Respiratory isolate infected calf; trigeminal ganglia on day 4 pi showing a positive labelled neuron (x10).



5.5 Transcript expression in different tissues throughout infection

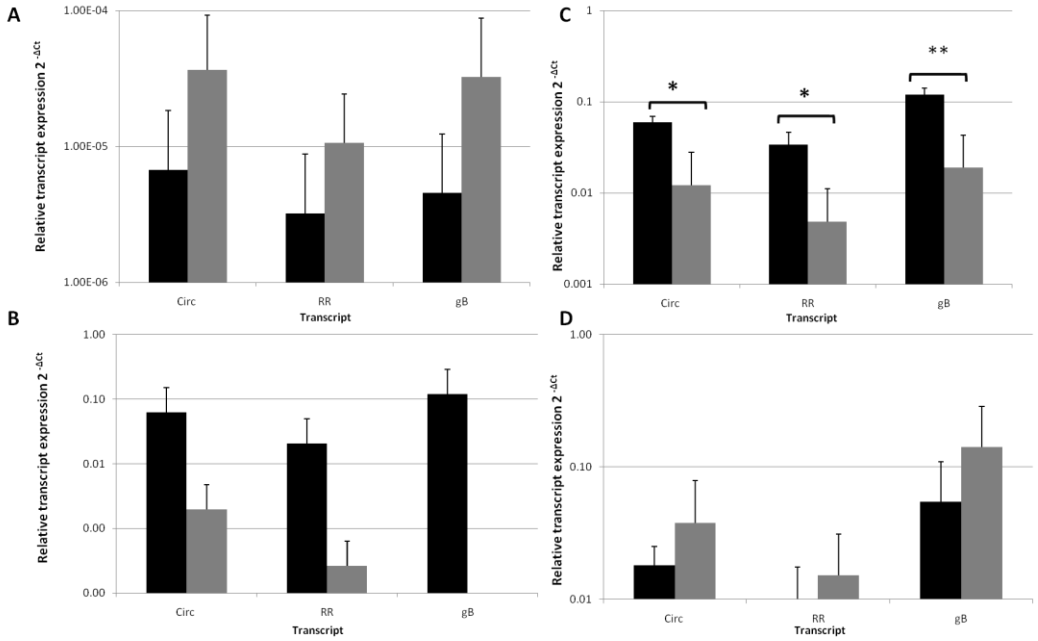
The expression of three transcripts, circ, ribonucleotide reductase (RR) and glycoprotein B (gB), representing each stage of the temporal cascade of transcript expression was measured using reverse transcriptase RT-PCR (see 2.6.2 for detailed methods). The tissue samples tested (nasal mucosa, trachea, tonsils, trigeminal ganglia and hindbrain and retropharyngeal lymph node) had previously tested positive for BoHV-1 DNA in one or more animals during the experimental infection. The samples were collected at three time points during infection, day 4, 10 and 24.

Positive detection of viral RNA was only consistently detected on day 4 pi, in four tissues (nasal mucosa, trachea, palatine tonsil and nasopharyngeal tonsil). On day 10 viral RNA was detected in one or two animals in the tonsils, trigeminal ganglia and retropharyngeal lymph node. On day 24 viral RNA was detected only in the trigeminal ganglia. No viral RNA was detected in the hindbrain. The information collected from day 10 and 24 samples was not sufficient to make any comparisons. For the day 4 samples relative quantification of BoHV-1 transcript expression was calculated by comparison to the expression of the β-actin host transcript calculated using the $2^{-\Delta Ct}$ method (Kubota *et al.* 2008; Livak & Schmittgen 2001; Morse *et al.* 2005).

These results showed differences in viral transcript expression between tissues from calves infected with the two BoHV-1 isolate types. In the nasal mucosa a trend of higher level of transcript expression was observed for all transcripts in the 07/1530 infected tissue, although due to the level of variation observed this was not found to be a statistically significant (figure 5.8A). In the trachea, only 2 of 3 05/1570 infected samples were positive for *circ* and 1 of 3 for RR and gB. No expression of gB was observed in any of the 07/1530 infected group samples. This made these results difficult to analyse, with only two samples from each group included in the analysis and therefore were not suitable for any statistical analysis. However, the results did show a trend for a higher level of transcript expression in the 05/1570 infected samples (figure 5.8B). The findings in the nasopharyngeal tonsil provided more

conclusive results; all 3 transcripts were expressed at significantly higher levels in the 05/1570 infected samples (figure 5.8C). In the palatine tonsils the opposite was seen with higher BoHV-1 transcript expression in the 07/1530 infected samples, although this was not found to be statistically significant.

Figure 5.8. Relative quantification real-time PCR targeting circ, ribonucleotide reductase (RR) and glycoprotein B (gB) fragments of BoHV-1 and normalised targeting β- actin of the host genome on day 4 pi (n=9) Black = respiratory 05/1570 isolate infected calves, Grey = systemic 07/1530 isolate infected calves. A) Nasal mucosa (n=3). B) Trachea (n=2). C) Nasopharyngeal tonsil (* = $p<0.05$; ** = $p<0.01$ Two sample t-test, n=3). D) Palatine tonsil (n=3).



5.6 Discussion

An experimental challenge of BoHV-1 was administered to calves by intranasal nebulisation, a natural route of infection. Clinical signs typical of IBR were observed, including a fever, ocular and nasal discharge, coughing, conjunctivitis and rhinitis as described in other experimental infections (Edwards, Newman & White 1991; Meyer *et al.* 2001). Viral shedding was detected via both nasal and ocular routes and there was evidence of virus within the respiratory tract and anticipated latency sites. Therefore it can be concluded that the BoHV-1 challenge successfully resulted in IBR disease and samples collected can be used to study both the virus and host response when infected with the two BoHV-1 isolates (respiratory 05/1570 and systemic 07/1530).

Clinical signs and tissue viral load demonstrated differences between the two BoHV-1 isolate infected groups, but there was a large amount of individual variation observed within each group, which compromised the statistical analysis and limited the conclusions that could be drawn. For example, despite clear visible differences in the severity of clinical signs, such as ocular discharge/conjunctivitis, they were not found to be significantly different between infected groups. However, they were significantly different from the control group, demonstrating a clear effect of the BoHV-1 infection. The difficulty in comparing the infected groups is likely to be due to the scoring system of 0, 1 and 2 which provided very little difference in the average score between groups. The small number of animals in each group is likely also to have played a part.

The monitoring of clinical signs showed trends that suggested the different isolates may be causing different presentations of the disease, although there were no significant differences in the extent of spread between the two isolates. Signs of conjunctivitis and ocular discharge were more evident in calves infected with the systemic (07/1530) isolate. This is also reflected in the viral load from the ocular swabs which was significantly higher on day 3 and lasted longer post infection compared to the respiratory infected group.

Real-time PCR was used to quantify virus collected from nasal and ocular swab sampling to represent viral shedding. Although it should be considered that quantitative PCR of swabs does not confirm that infectious virus particles were being shed. This provided data on the spread of the virus from the nasal cavity to ocular areas. It demonstrated that 48 hours was needed for the virus to spread from the nasal passage and being shed via the ocular route, which was also found in another study comparing BoHV-1 strains (Kaashoek *et al.* 1996). This data also showed that viral DNA could be detected for at least 21 days pi, which shows a long time period when calves would have been a risk factor for the rest of the herd, if they had been infected in their natural environment. This contrasted with other studies which showed no shedding after day 17 pi by virus isolation (Kaashoek *et al.* 1996) and day 19 pi by immuno-PCR (Mweene *et al.* 1996). Neither of these studies took measurements beyond these time points, but this may also represent the increased sensitivity of the quantitative PCR method used in this study compared to virus isolation and immuno-PCR. The peaks of viral shedding coincide with the initial peak of clinical signs, as would be expected, also representing the peak of viral replication.

No cell-associated or cell free virus was detected in the blood buffy coat or tissues outside the respiratory tract and CNS. This leads to the conclusion that there was no systemic dissemination of the virus, as has been found in natural cases (Higgins & Edwards 1986). Systemic spread of the virus has been shown in other intranasal experimental infections, although no histopathological or virus isolation evidence is provided to support the viral detection by immuno-PCR in the ovaries, spleen and peripheral blood leukocytes (Mweene *et al.* 1996). Another study demonstrated presence of BoHV-1 in serum, adrenal glands, kidneys and liver by virus isolation with supporting pathological evidence in the tissues after an intranasal BoHV-1 infection (Kaashoek *et al.* 1996). Systemic spread has also been demonstrated in cases of vaccination with modified-live vaccine for IBR and parainfluenza-3 in neonatal calves (Bryan *et al.* 1994). However, the vaccine had been administered intra-muscularly so the natural route of virus dissemination does not apply to this case. Serum was also tested from day 4 samples at the peak of clinical signs to check for a cell-free viraemia but this also gave negative results. In conclusion, the results

show that the isolates tested do not result in the same clinical presentation on infection and therefore some host factors must also be involved in control of systemic spread.

Despite the lack of evidence of systemic spread there were several observations made from the analysis of tissue for the three time points sampled. Comparisons between tissues from calves infected with the two isolates demonstrated potential differences in their infection of the host. On day 4 pi, at the peak of clinical signs and viral shedding, evidence of virus was detected by PCR in the nasal mucosa and trachea of the respiratory tract, associated tissues including palatine and nasopharyngeal tonsils and retropharyngeal lymph node in both infected groups. Other studies have recorded presence of BoHV-1 in lung tissue in experimental BoHV-1 infections of 3-5 month year old calves with $10^{7.8}$ PFU/ml of the LA strain (Mweene *et al.* 1996). However, no evidence of virus in the lungs was demonstrated in our study, the virus remained confined to the upper respiratory tract. Another study using endobronchial administration of virus has shown that BoHV-1 increases the severity of histopathological lesions observed in pneumonia when compared to a *Pasturella haemolytica* only infection and also demonstrates a BoHV-1 only infection causing pneumonia (Narita *et al.* 2000b; Narita *et al.* 2000c). Lung disorders are usually associated with secondary bacterial infections, rather than the virus itself due to reduced defences caused by BoHV-1 (Jones & Chowdhury 2007; Ellis 2009). Overall in a natural intranasal infection it seems unlikely BoHV-1 would reach the lower respiratory tract based on findings in this experimental challenge and previous data.

There was also some indication of differences in tissue tropism between the two isolate types at day 4 pi. A significantly higher viral load was found in the nasal mucosa in the 07/1530 infected group compared to the trend of higher viral load in the trachea of the 05/1570 infected group. This was also supported by histopathological findings with more severe inflammation observed in the nasal mucosa and positive IHC labelling of antigen within a lesion in the 07/1530 infected group. The infection of epithelial cells is characteristic of a BoHV-1 infection

(Muylkens *et al.* 2007). Studies *in vitro* have shown the infection of these cells results in cytokine expression and allows secondary infection of bacteria such as *Mannheimia haemolytica*, known to lead to pneumonia (Rivera-Rivas, Kisiela & Czuprynski 2009). There was also a higher level of transcript expression detected in the nasal mucosa of the 07/1530 infected animals on day 4, suggesting this was a viral replication site. In the trachea, more inflammation was observed at this time point in the 05/1570 infected tissue, although there was no positive antigen labelling by IHC to confirm presence of replicating BoHV-1. There was also evidence of inflammation in some of the control cases so this may not have been related to the BoHV-1 infection. However, real-time PCR measurement of transcript expression in the trachea, confirmed presence of replicating virus in at least some of the samples. In positive cases it was found to be higher in the respiratory isolate infected tissue.

The tonsils showed differences in their response to the virus. Similar viral loads were detected in both infected groups, however the characteristics of the lesions were different depending on the isolate type. Animals infected with the respiratory isolate showed multifocal necrosis, whilst the systemic group showed no sign of necrosis, but extensive multifocal neutrophil infiltration. However, the IHC labelling did not confirm these differences. A multifocal pattern of labelling was observed in the tonsil tissues, which was similar in tissues from both isolate types. This suggested it was the host response that differed rather than the extent of viral infection. Significant differences in the transcript expression detected between the isolates were also found. The 05/1570 infected group demonstrated a significantly higher level of transcript expression in the nasopharyngeal tonsil for all 3 transcripts. This may indicate this isolate was able to replicate more readily at this site than the systemic isolate, or that the neutrophil infiltrate response was potentially limiting the replication potential of the systemic isolate. A previous study specifically looking at BoHV-1 infection of the nasopharyngeal tonsil described the progression of infection resulting in histopathological lesions. After an intranasal infection, multifocal epithelial erosions and increased infiltration of neutrophils, with erosions then becoming ulcerated and necrosis extending into adjacent lymphoid tissue (Schuh *et al.* 1992). This could suggest we observed two of these stages, increased neutrophilic

infiltration in 07/1530 associated tissue and the latter stages of necrosis in the 05/1570 associated tissues. However, we did not observe any necrosis occurring alongside the neutrophilic infiltration. A study focussing on the response of lymphoid tissue after infection found BoHV-1 infected CD4+ T cells, causing apoptosis, at 7 days pi, which was thought to contribute to the immunosuppression caused by the virus (Winkler, Doster & Jones 1999), also suggesting cell death at this site, is secondary to the peak of clinical signs on days 4-5 pi. Therefore the observations made in our study could represent a difference in host response or differences in the speed of infection progression as it was not possible to confirm the same response was not observed in either isolate before or after the 4 day pi time point.

Finally, with reference to observations on day 4 post infection was the detection of virus DNA in the trigeminal ganglia. This showed BoHV-1 was able to spread to the trigeminal ganglia in just 4 days, with similar viral loads to those found in the upper respiratory tract. Histopathology demonstrated a mild focus of inflammation in the 05/1570 infected group, which was also positive by IHC, suggesting the presence of replicating virus in neurons. No lesions or positive IHC labelling was observed in the 07/1530 infected tissue, suggesting less spread of this isolate at this stage of infection. The positive labelling of neurons in the trigeminal ganglia of 05/1570 infected animals supports previous studies that have shown gene expression in the trigeminal ganglion mainly occurs within the neurons (Schang & Jones 1997). Previous studies have also identified an interstitial mononuclear cell infiltration of the trigeminal ganglia during acute infection (Winkler *et al.* 2002), consistent with our observations in the 05/1570 infected animals. Another study demonstrated a brief period of viral transcript expression at the peak of acute infection (Schang & Jones 1997), which may explain the positive IHC at this time point.

On day 10 the clinical signs were resolving and viral shedding decreasing. Studies of the tissues at this time point also reflected this. Fewer tissues were found to be IHC positive or PCR positive for transcript expression, suggesting little active viral replication was occurring for either isolate. However, viral DNA was still found by

real-time PCR in the tonsils, trigeminal ganglia, retropharyngeal lymph node and hindbrain of both infected groups. Viral DNA was also detected in the nasal mucosa and trachea of the 05/1570 infected group only, which suggests the virus had cleared from the upper respiratory tract tissues in the 07/1530 infected calves. This may have been related to the more severe inflammatory infiltrate provoked by the 05/1570 isolate, compared to milder inflammatory infiltrate observed in the 07/1530 infected tissue. A neutrophilic infiltrate as observed in the 05/1570 isolate has also been demonstrated in an *in vitro* study. Activation of neutrophils was shown during BoHV-1 infection of epithelial cells (Rivera-Rivas *et al.* 2009).

The palatine tonsil showed IHC labelling in animals infected by both isolates and PCR indicated it was also the tissue containing the highest viral load. This may be related to the tonsils being considered a secondary site of latency (Winkler *et al.* 2000). As the tonsils consistently showed high viral load, supported by labelling of viral antigen by IHC it may also suggest a tropism for lymphoid tissue. However, as this was not supported by detection of transcript expression, it could also be related to their immune surveillance role earlier in infection. Further work would be needed to differentiate between these possibilities, specifically to establish whether virus was replicating, latent or if virus debris from an earlier infection was being detected. The trigeminal ganglia demonstrated an inflammatory response in all cases of the 07/1530 infected samples, which was not seen in the 05/1570 infected samples. However, the respiratory isolate demonstrated a significantly higher viral load by PCR. This could be considered to be evidence of the respiratory isolate entering the latent phase, whilst the systemic isolate was still causing an initial inflammatory response, although detection of LAT gene expression would be needed to confirm this. Previous findings demonstrated inflammation of the trigeminal ganglion on day 7 pi after intranasal and ocular infection with the BoHV-1 (Winkler *et al.* 2002). As inflammation was present on day 4 in the respiratory group but not at day 10, it may therefore have occurred later in the 07/1530 infected group. However, this does not explain the lack of any inflammation in 05/1570 infected group, as in previous studies inflammation was observed until day 15 pi (Winkler *et al.* 2002). More frequent sampling of the tissues would be needed to look into this further.

On day 10 there was also presence of BoHV-1 DNA in the hindbrain, although only one sample from each infected group was found to be positive. There was also evidence of inflammation in 5 of the 6 hindbrain samples at this time-point which was more severe in the 07/1530 infected group. No lesions were observed in the control group so this can be attributed to the BoHV-1 infection, but there was no IHC labelling to confirm that this was related to BoHV-1 infection. Spread to the brain has also been seen in natural cases, particularly in cases of young calves with lesions similar to those observed in our study. Non-suppurative encephalitis, characterized by perivascular mononuclear cell cuffing and gliosis was demonstrated during histological examination in a calf from a natural systemic BoHV-1 outbreak (Higgins & Edwards 1986). Another study has shown it can sometimes be accompanied with neurological signs (Penny *et al.* 2002). It is unknown whether spread to the brain occurs by axonal transport in neurons or via a haematogenous route. A study in mice infected intraperitonealy with either BoHV-5 or BoHV-1 showed a viraemia was needed to cause neuronal infection by BoHV-1, in contrast to BoHV-5 that was transported to the CNS by an axonal pathway, based on detection of viral DNA and antigen in different tissues sampled on days 3 and 6 days post infection (Abril *et al.* 2004). This would suggest a haematogenous route may also be more likely for the systemic spread of BoHV-1, although a mouse model with an intraperitoneal route of infection may not be a good representation for natural infection with BoHV-1. It also raises the question of whether we should consider spread to the brain as evidence of systemic spread in this study.

By day 24, when almost all clinical signs had subsided, there was little evidence of the presence of virus. Only the palatine tonsil and trigeminal ganglia tested positive by real-time PCR for both infected groups, which are both known latency sites so it can be suggested that both isolates had reached the latency phase at this time point. A potential exception to this is S9, as viral DNA was detected in the retropharyngeal lymph node in this animal. All other cases were only positive at latency sites, supporting the findings of a previous study. Calves were infected intranasally with the Cooper strain and latency related (LR) transcript was detected in the tonsils by

nested RT-PCR at 60 days post infection. They also showed successful reactivation of BoHV-1 from the tonsils using intravenous dexamethasone, a corticosteroid treatment to trigger reactivation from latency providing conclusive evidence of the tonsils as a latency site (Winkler *et al.* 2000). However, these results contrast with findings from an intranasal BoHV-1 infection that detected BoHV-1 DNA in ovaries, lungs, nasal mucosa, tracheal mucosa, spleen, lymph nodes and peripheral blood leukocytes on day 22 pi (Mweene *et al.* 1996). There was some evidence that the systemic isolate was not entirely latent, as there was a positive IHC result in the palatine tonsil suggesting the virus was still replicating. In the trigeminal ganglia inflammation was detected in all 3 07/1530 infected animals. This was also observed in a study that compared lymphocyte infiltration between an LR transcript mutant and a wild-type strain of BoHV-1, which found an increased presence of inflammatory infiltrate in the LR transcript mutant infected trigeminal ganglia. It was suggested that infiltration of lymphocytes in LR transcript mutant strains results in increased apoptosis at the end of the acute infection, and therefore fewer neurons available for the establishment of latency (Perez *et al.* 2006). This was also shown in a rabbit model that showed apoptosis of neurons was preceded by viral gene expression (Delhon, Gonzalez & Murcia 2002). This could indicate that the systemic isolate was not expressing the LR transcript at this time point, resulting in increased lymphocyte infiltration. Study of the LR transcript would be needed to confirm the latency strategy of the virus.

Overall, this experimental infection has provided an important insight into how molecular and histopathological observations of two sequenced BoHV-1 isolates may relate to the clinical presentation of disease. Neither of the isolates demonstrated systemic spread beyond the respiratory tract or brain, and generally showed similar patterns of infection. This leads to the conclusion that previous systemic spread by an isolate does not mean it will behave in the same way in the next infection, and therefore host factors must also play a role. Differences in host responses were potentially demonstrated in the tonsils of infected calves. Further work is needed to confirm that this was not simply a difference in the speed of infection between the isolates, as it was not possible to confirm from this work that these effects weren't

seen a day later or earlier in animals infected with the other isolate. There was also some indication of differences in virus tropism in the upper respiratory tract. Unfortunately, these results were difficult to interpret due to a high level of variation between samples from the different animals, as was also reflected in the clinical signs. More isolates of each type need to be tested to determine whether any of the observations made here were significant. In the next chapter the possibility of model systems will be investigated, as for a study involving more isolates an experimental infection in the natural host is not suitable. This aims to provide data with less variation, more repetitions and more isolates of each type which should establish whether any of the differences observed here are true differences between 'respiratory' and 'systemic' isolates.

Chapter Six: Comparison of respiratory and systemic isolates in model systems

6.1 Introduction

The previous chapter demonstrated differences in virus distribution and transcript expression between two BoHV-1 isolates, in calves challenged intra-nasally. In this chapter *in vitro* model systems were used to study these isolates in a more controlled and reproducible environment. It also provided an opportunity to compare a greater number of isolates. Respiratory and systemic isolates were compared during an infection of BT cells in terms of replication patterns, by measuring production of virus and gene expression from the three temporal phases of replication. It also provided a comparison of different infection models. BoHV-1 is one of the most studied herpesviruses because it grows well in cell culture, making this an easy way to study the virus (Peterson & Goyal 1988). This cell culture model was used as the first model to compare the isolates used in the *in vivo* challenge, through the analysis of transcript expression over the infection period.

The second part of this work used bovine lung slices kept in culture for four days and infected with BoHV-1 on the second day. It tested whether the lung slices remained viable and were able to support the replication of virus. Organ culture was first used in studies in the late 1970's (Bouffard & Derbyshire 1978), and is now re-emerging as a more commonly used infection model (Booth *et al.* 2004; McNeilly *et al.* 2007; Niesalla *et al.* 2009). An air-liquid interface system was previously used for the culture of respiratory epithelium, which was then infected with BoHV-1 (Niesalla *et al.* 2009). Similar systems involving submerged tissue, more similar to the original techniques have been used to culture sheep tracheal and lung tissue for the infection of maedi-visna virus (McNeilly *et al.* 2007) and also ovine pulmonary adenocarcinoma infection in sheep lung (Cousens 2010). Previous studies in our

laboratory showed that infection of lung slices was also a suitable method for the study of BoHV-1 and demonstrated successful replication of virus.

This method has several advantages in comparison to cell culture infection. A large number of lung slices taken from the same organ can be infected with several different isolates. It also preserves the range of cell types and structure present in the original organ. However, it does have some limitations, for example the influx of inflammatory cells or the route of infection cannot be studied. Innate immune responses such as the inflammatory response are thought to be maintained and a recent study has successfully measured TNF-α in an air-liquid interface infection of respiratory epithelium with BoHV-1 (Niesalla *et al.* 2009). This makes it a good approach to compare different virus isolates, ultimately to determine whether viral or host factors play the most important roles in systemic infection. If successful this system may provide a good alternative to experimental infection in the natural host, or other animal models such as rabbits (Lupton *et al.* 1980).

6.2 *In vitro* time course infection in bovine turbinate cell culture

Two BoHV-1 isolates from natural cases, one associated with respiratory presentation (05/1570), and one associated with systemic spread (07/1530) (estimated M.O.I of 2) were used to infect BT cells. These were also the same isolates used for the *in vivo* infection described in chapter 5. Both isolates had been previously titrated and diluted as necessary to ensure minimal differences in the infection titre between isolates. When harvesting infected cells for transcript expression analysis cytopathic effect (CPE) was recorded and cell monolayer samples were taken at 3 hourly intervals over a 48 hour period post infection to study transcripts from each phase of viral gene expression using real-time PCR, to provide an insight into the viral infection at a molecular level. RNA for viral transcript expression studies was extracted from the infected cells. Transcripts that are known to be expressed in each of the temporal phases of gene expression were measured, *circ* in the immediate-early phase, RR in the early phase and gB in the late phase

(Muylkens *et al.* 2007). These genes were also measured in the *in vivo* infection allowing comparison between the two experimental models (section 5.5). The detailed methods are described in section 2.2, 2.5.4 and 2.6.3. Relative quantification of the transcripts was calculated using the ΔCt method, using the host transcript β-actin as the reference transcript.

At each time point the percentage CPE was estimated before harvesting the cells. This provided a comparison of the viral replication and speed of virus cell to cell spread between isolates. Cultured cells infected with the respiratory (05/1570) isolate showed a faster development of CPE. Cell rounding and development of plaques was observed at 6 hours pi, compared to 9 hours pi in the systemic isolate infected cells (figure 6.1). Later in the time course, 100% CPE was observed 6 hours earlier in the respiratory isolate infected cells than the systemic isolate (07/1530) infected cells.

Figure 6.1. Development of CPE in BT cells during a time-course infection of either a respiratory, (05/1570) and systemic (07/1530) BoHV-1 isolates.

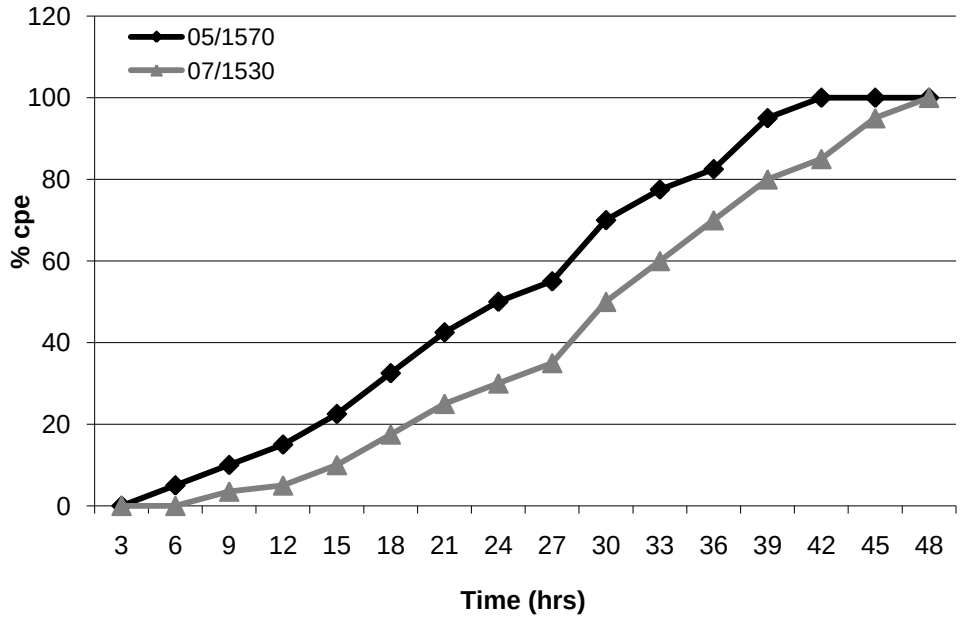
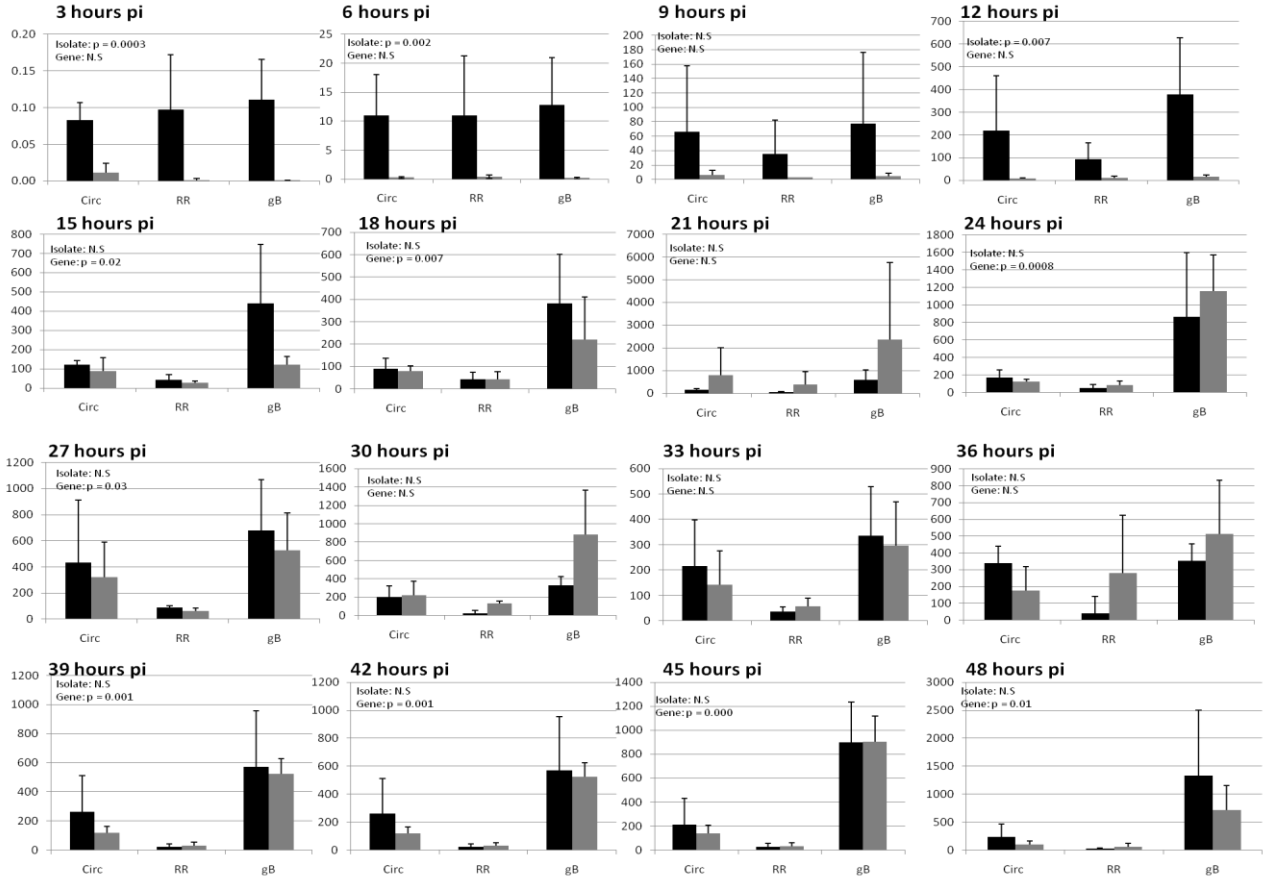


Figure 6.2. Relative viral transcript expression compared to host reference gene β-actin over a 48 hour period of two BoHV-1 isolates. Respiratory (05/1570) – Black; Systemic (07/1530) – Grey. y-axis = relative viral gene expression $2^{-\Delta Ct}$, x-axis = transcript expressed. Error bars represent standard deviation of the 3 biological replicates. P-values calculated using a 2-way ANOVA (Factors: BoHV-1 isolate v gene, n=3).



The results from the real-time PCR study demonstrated several aspects of viral transcript expression during this BoHV-1 infection. The first activity of viral transcription was detected 3 hours after infection for both isolates (figure 6.2). During the first 12 hours of the infection, in all 3 phases of transcript expression, the respiratory isolate (05/1570) showed comparatively higher transcript abundance than the systemic isolate (07/1530). A two-way ANOVA demonstrated a significantly higher number of respiratory isolate transcripts at 3, 6 and 12 hours pi (figure 6.2). From 15 hours pi, the two isolates showed no significant differences, which may be due to high levels of variation between the biological replicates. However, there were differences observed between the transcript levels of the immediate early, early and late transcripts, for both isolates. Expression of the late transcript (gB) was higher than RR and *circ* at 15, 18, 24, 27 and 39-48 hours, although at 27 hours pi *circ* was also highly expressed. It should also be noted that there was a decrease in the host beta actin expression which influenced the ΔCt calculations, meaning the results could not be compared over time.

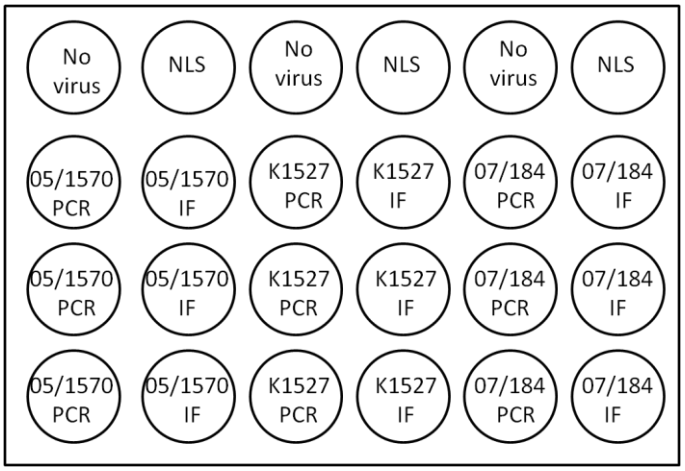
6.3 *In vitro* BoHV-1 time course infection in bovine lung slices

A 3 day time course infection of lung slice cultures with 6 different BoHV-1 isolates (table 6.1) was designed to study viral replication, viral gene expression and cellular localisation of virus in tissue. This provided an intact organ structure that would be targeted in a natural infection. For detailed methods see 2.3, 2.6.3 and 2.7.3. Briefly, lung slices were set up in 24 well plates and infected with BoHV-1 isolates (figure 6.3). Viral replication was measured in the lung slice medium that was replaced each day, using absolute quantification real-time PCR to calculate viral load produced each day. Viral transcript expression of *circ*, RR and gB was measured by RT-real-time PCR and subsequent relative quantification calculation, using host beta actin as a reference in RNA samples extracted from whole lung slices. Cellular localisation of the virus was studied using immunohistofluorescence double labelling of BoHV-1 and either epithelial or endothelial cells.

Table 6.1. Isolates used in lung slice infection.

Isolate	Classification
05/1570	Respiratory
K1527	Respiratory
07/184	Respiratory
07/1530	Systemic
07/282	Systemic
K739	Systemic

Figure 6.3 Example of lung slice plate set up for the respiratory isolates. Three of each of the respiratory and systemic plates were set up, one to be harvested each day. (NLS = no lung slice control; No virus = lung slice with no virus added control; PCR = lung slice and media to be used in PCR analysis; IF = lung slice to be used for immunohistofluorescence.)



6.3.1 Measuring viral load in lung slice cultures

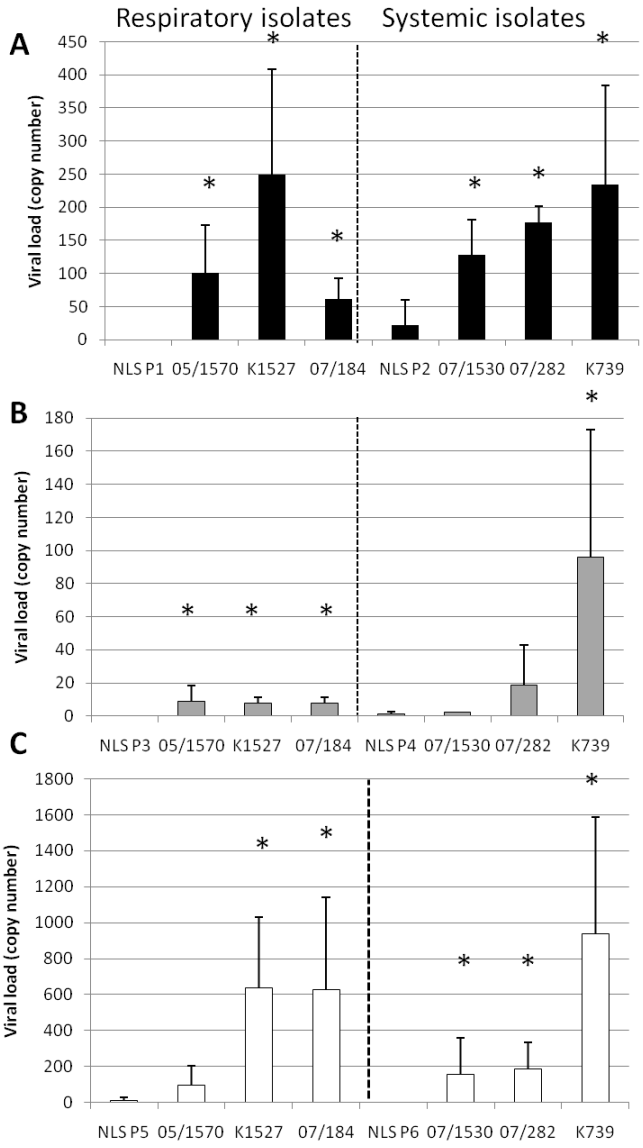
Viral load was measured in culture media containing the lung slices using real-time PCR absolute quantification to detect BoHV-1 DNA. Plasmid standards of known copy number were included on each run to create a standard curve that the tested samples could be plotted against. This indirectly checked the lung slice was viable, as it was supporting viral replication.

Media tested from the wells containing no lung slices tested positive for BoHV-1 DNA in one of 3 wells on each of the plates on each day indicating there was some virus adherence to the plate surface. In most cases the mean virus copy number detected in the wells containing lung slices was still significantly higher than the average of the no lung slice control wells (figure 6.4). On the occasions when it was

not significantly different, this was due to high variation between samples infected with the same isolate type. Overall, there was sufficient evidence of virus replication (and therefore viable lung slices) to continue the study of gene expression and localisation of virus within the lung tissue.

This study also showed that less virus was produced on day 2 in all isolates than on days 1 and 3. There were no significant differences observed between isolates or isolate groups in the amount of virus that was produced on each day (figure 6.4).

Figure 6.4. Viral load produced each day in lung culture and comparison with no lung slice (NLS) controls on each plate from each day (P1-6). A) Day 1, B) Day 2, C) Day 3; Two sample t-test, * indicates statistical differences between NLS control and each isolate of $p<0.05$ (n=3), error bars show s.d.

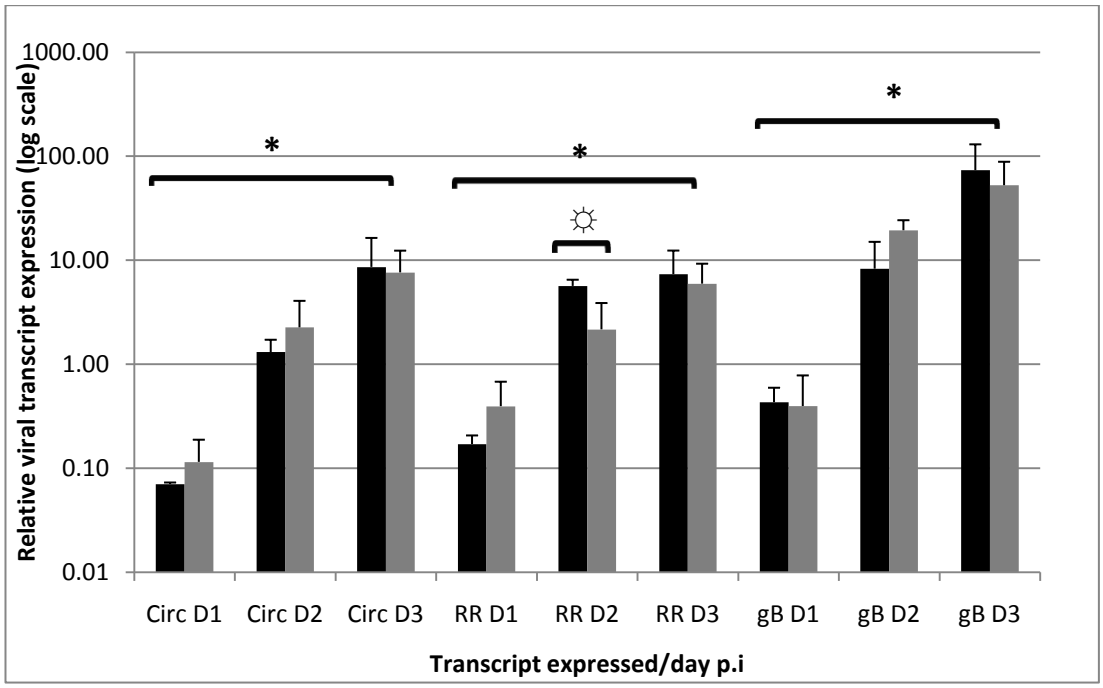


6.3.2 BoHV-1 gene transcription in lung slice culture

Viral gene transcription was measured using real-time RT-PCR relative quantification with the transcript of interest compared to host gene transcription of β-actin (Livak & Schmittgen 2001; Kubota *et al.* 2008). This data can be used to compare between isolates and between infection models (as this was the same method used in the cell culture in section 6.2 and the *in vivo* challenge in chapter 5).

Viral transcript abundance of all three transcipts measured generally increased over the three day infection period (figure 6.5). Both isolate groups showed a similar pattern of viral gene transcription, as seen in the BT cell culture, when only one of each isolate type was used (figure 6.2), with the exception of RR transcription on day 2, when a significantly higher relative transcript level was observed in the respiratory isolates, compared to the systemic isolates (figure 6.5). There was also a significant increase in each transcript with time over the 3 day infection period (one- way ANOVA; $p < 0.01$).

Figure 6.5 Viral transcript expression in lung slice culture. Black – average of three respiratory isolates (05/1570, K1527, 07/184), Grey – average of three systemic isolates (07/1530, 07/282, K739). A two sample t-test identified any significant differences in transcript expression between isolate types (☼ $p < 0.01$) (n=3), error bars represent s.d). A one-way ANOVA identified significant differences of transcript accumulation between days 1 and 3 (* $p<0.01$).



6.3.3 Immunohistofluoresence labelling to localise BoHV-1 in lung slice culture

As the lung slices contained a population of different cell types, it was possible to determine which cell type was infected by the virus, in order to study differences in cell tropism between the isolate types. Immunofluorescence labelling was used as a pilot technique for future investigation, to identify virus-infected, epithelial and endothelial cells represented by labelling of BoHV-1 gC (a late expressed gene indicative of virus production), cytokeratin and von Williebrand Factor (vWF) respectively. Co-localization of virus with either vWF or cytokeratin was used to indicate the presence of the virus in endothelial or epithelial cells respectively (table 6.3). See 2.7.3 for detailed methods.

Negative controls (table 6.2), that tested background fluorescence and antibody specificity showed no significant labelling (figure 6.6 A, C, E). These control images also show the positive labelling of the individual targets when individual labelling was used. Cyt and vWF labelling was localised to cellular areas and BoHV-1 was shown to be localised within some of these cells (figure 6.6 B, D, F). Specifically, cytokeratin labelling was identified by red fluorescence signal within the epithelial lining of a bronchiole (figure 6.6B). vWF labelling was identified also by red fluorescence signal on the internal lining of a vessel within the lung structure (figure 6.6F). BoHV-1 positive signal appeared in individual cells or groups of cells as green fluorescence (figure 6.6D). Maintenance of lung structure can be most clearly seen in figures 6.6E and F. It should be noted that it seems the antibodies were not working at an optimum level of sensitivity under the conditions used in this experiment as more cells would be expected to be positively labelled using the cell targeted labelling.

Virus antigen was not detected in several sections, including all the samples infected with isolates 07/1530 and K1527, therefore these samples could not be evaluated for any co-localisation (table 6.3). Where virus was detected, co-localization was found with both cell types for both isolate types. On day 1 pi virus was only found in epithelial cells (figure 6.7 A and B), whilst on days 2 and 3 pi the virus was found to be localised in both epithelial and endothelial cell types in two of the BoHV-1

infected samples (figure 6.7 C and D). Both viral antigen and areas of co-localization were greater on day 3 post infection than on days 1 and 2 (figure 6.7).

Table 6.2. Controls used in immunofluorescence double labelling to validate positive labelling and co-localisation.

	Primary Antibody	**Secondary Antibody**	**Testing**
Control 1	None	BoHV-1 and Cyt	Specificity of fluorescent secondary antibody for primary antibodies
Control 2	None	BoHV-1 and vWF	Specificity of fluorescent secondary antibody for primary antibodies
Control 3	BoHV-1 only	Cyt only	Cross reaction between BoHV-1 primary and Cyt secondary antibodies
Control 4	BoHV-1 only	vWF only	Cross reaction between BoHV-1 primary and vWF secondary
Control 5	Cyt only	BoHV-1 only	Cross reaction between Cyt primary and BoHV-1 secondary
Control 6	vWF only	BoHV-1 only	Cross reaction between vWF primary and BoHV-1 secondary
Control 7	None	None	Background fluorescence of tissue

Table 6.3. Co-localization of virus antigen and markers for different cell types. ns indicates that no virus was visible and therefore co-localisation could not be evaluated. + or – indicates the extent of co-localisation between the virus and the indicated cell-type marker.

	D1		**D2**		**D3**	
	Cyt	vWF	Cyt	vWF	Cyt	vWF
05/1570 (R)	+	-	+	-	+	ns
K1527 (R)	ns	ns	ns	ns	ns	ns
07/184 (R)	ns	ns	++	-	++	++
07/1530 (S)	ns	ns	ns	ns	ns	ns
07/282 (S)	ns	ns	ns	+	++	+
K739 (S)	+	-	ns	ns	ns	ns

Figure 6.6. Control and individual labelling of the targets using immunofluorescence. A) Negative control for Cyt – secondary antibody only. B) Positive labelling of bronchiolar epithelium for cytokeratin in red. C) Negative control for BoHV-1 – secondary antibody only. D) Positive BoHV-1 labelling in green. E) Negative control for vWF – secondary antibody only. F) Positive vWF labelling in red. Blue represents DAPI which labels all cells.

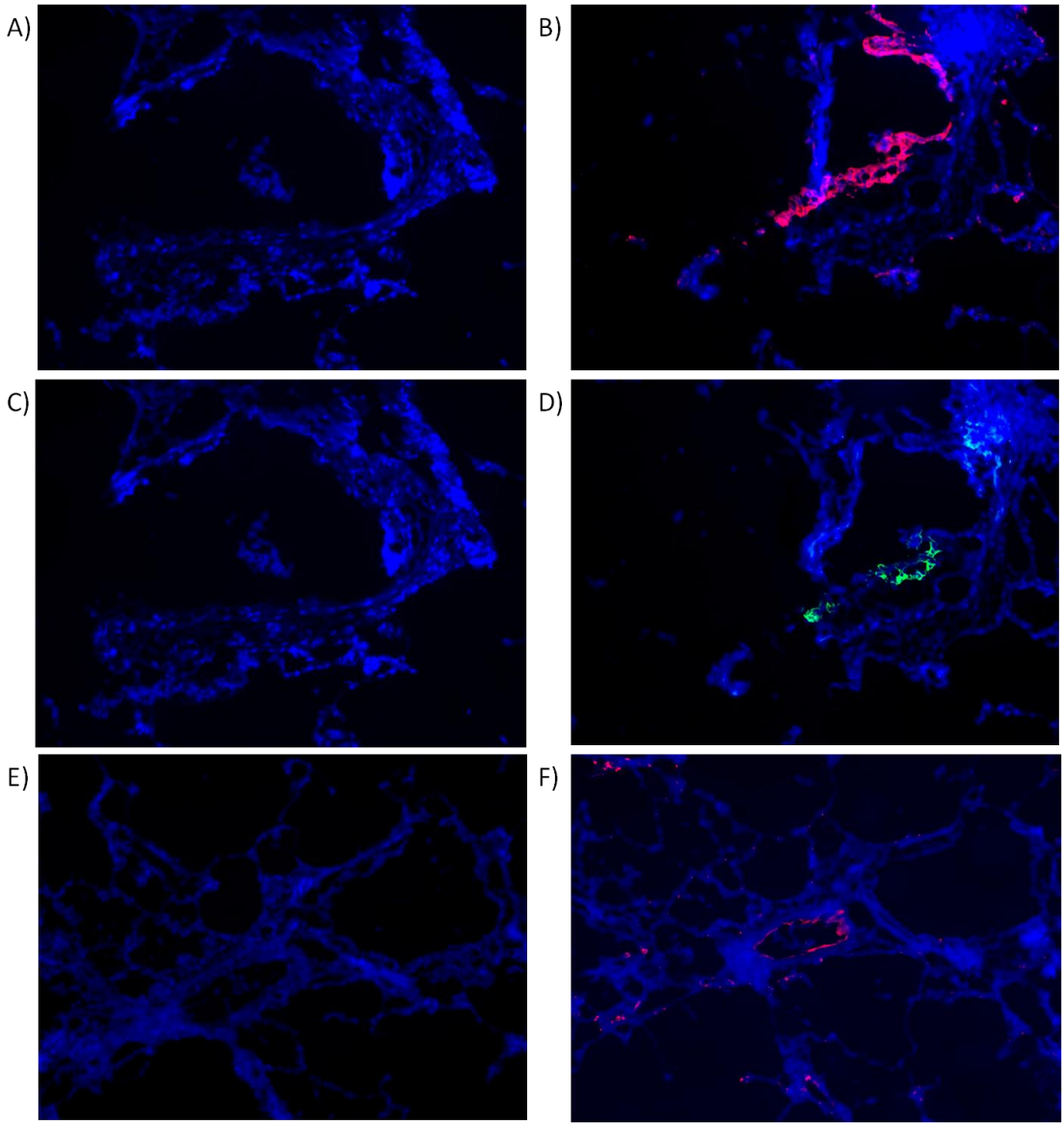
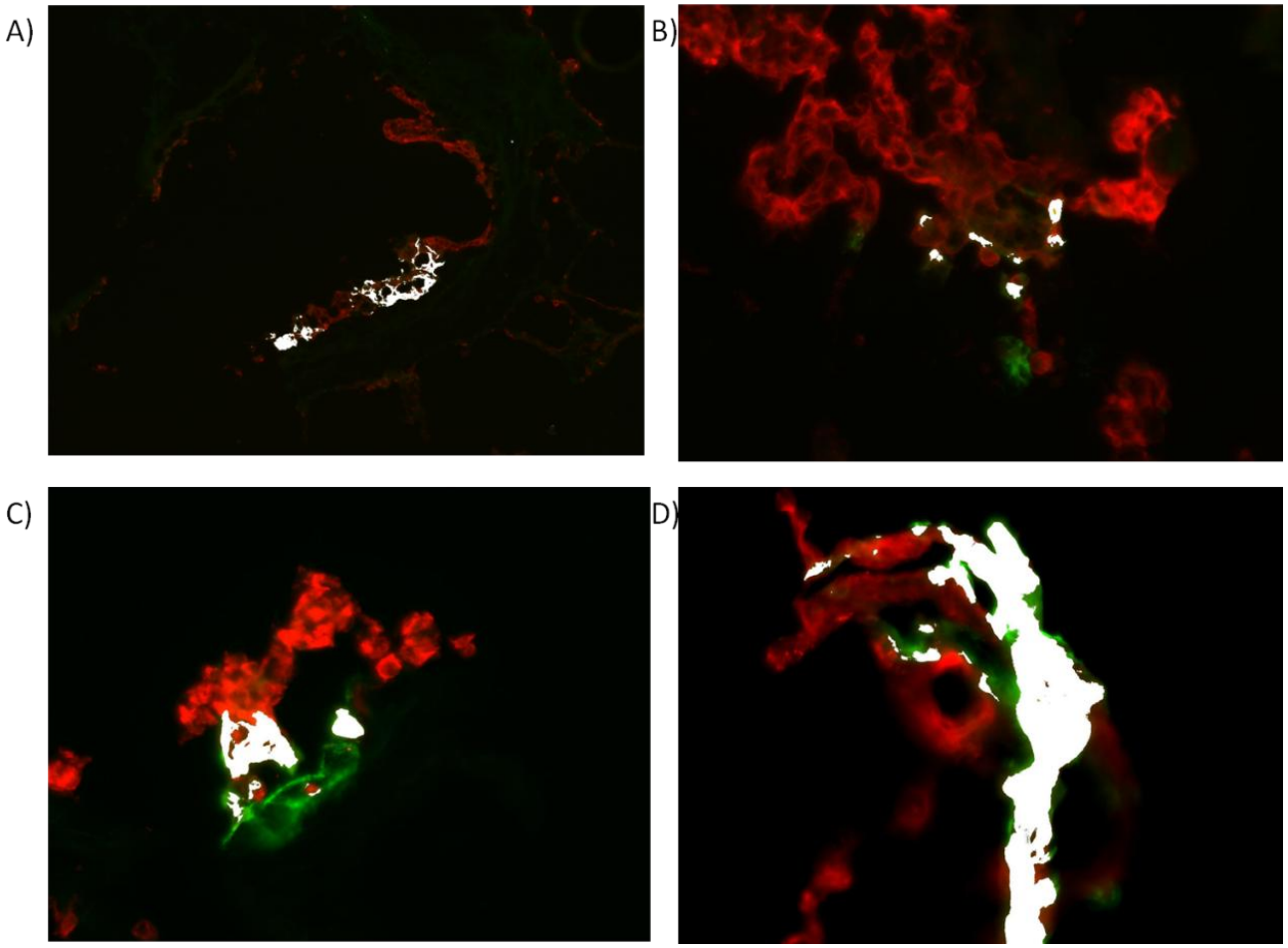


Figure 6.7. Co-localization images after immunofluorescence labelling. Red – cytokeratin (epithelial) or vWF (endothelial), green – BoHV-1, white – co-localisation of green and red determined using imageJ software (Rasband 1997). A) K739 BoHV-1 isolate day 1 pi localized to epithelial cells. B) 07/184 BoHV-1 isolate day 2 pi localized to epithelial cells. C) 07/184 BoHV-1 isolate day 3 pi localized to endothelial cells. D) 07/282 BoHV-1 isolate day 3 pi localized to epithelial cells.



6.4 Discussion

These experiments have used *in vitro* model systems to investigate BoHV-1 isolates studied in the *in vivo* experimental challenge and further respiratory or systemic isolates that had also been sequenced.

The infection of BT cells is a common method of studying BoHV-1 due to its rapid replication and clearly visible CPE (Peterson & Goyal 1988). Infection of BT cells with two isolates found the respiratory (05/1570) isolate resulted in a faster development of CPE than the systemic (07/1530) isolate. This could be an indication of faster viral replication to produce progeny viruses that can spread, resulting in further cell death, creating visible plaques in the cell monolayer. However, this is a subjective method of comparing the viral replication. It would have also been useful to produce growth curves from the cell cultures by titrating the supernatant to further quantify the differences of infection between the isolates. However, as time was limited, to provide quantitative measure of viral replication viral transcript abundance was measured over the infection period for both isolates using quantitative real-time PCR which could be compared to the method used in the experimental challenge.

The results of this transcript analysis provided evidence of viral RNA just 3 hours after infection, most evident in the respiratory (05/1570) isolate, but also seen in the systemic isolate (07/1530). This is similar to previous experiments monitoring transcript expression *in vitro* using the Cooper, Jura and K22 strains, which also demonstrated transcript accumulation at 2-3 hours pi (Wirth *et al.* 1989; Seal *et al.* 1991; Schang & Jones 1997). Two of these studies report evidence of immediate early transcript expression earlier in the infection compared to the other transcript phases (Schang & Jones 1997; Seal *et al.* 1991). In our study, all three phases of transcript expression are represented at the 3 hour time-point, which was also observed in a previous BoHV-1 BT cell infection (Seal *et al.* 1991). More frequent measurements of the first 3 hours of infection, are needed to identify the transcript phases more clearly, as distinctions between the phases in other studies have been

made between as early as 2 hours pi (Wirth *et al.* 1989). In the first 12 hours pi, higher levels of all 3 transcripts tested were detected in the respiratory isolate (05/1570) infection than the systemic isolate (07/1530) infection. This correlates with the faster CPE development observed in the respiratory isolate infection. Throughout the rest of the cell culture infection period studied (12-48 hours), both isolates showed similar patterns of transcription for each of the three transcripts detected. gB transcription predominated between 15 and 48 hours post infection.

Based on the current knowledge of BoHV-1 replication (Muylkens *et al.* 2007); earlier viral transcription would ultimately be expected to allow faster production of progeny virus and spread to neighbouring cells, resulting in plaque formation. However, production of progeny virus is dependent on the subsequent increase in viral gene translation, leading to faster protein expression, which was not determined in this experiment. Previous studies have shown that differences in viral transcription timing does not always relate to differences in viral replication in culture (Aguilar *et al.* 2006). Therefore, protein expression needs to be determined to investigate viral replication in culture. It would also be useful to study the transcription of more viral genes, in pseudorabies virus this has been achieved with the use of transcriptomic analysis (Aguilar *et al.* 2006; Flori *et al.* 2008). The timing of transcription is largely dependent on the expression of tegument protein α-TIF and its subsequent binding to promoter regions, which stimulates immediate early transcription (Muylkens *et al.* 2007). This may make the monitoring of α-TIF expression an interesting starting point for future studies.

This study in BT cells found that, in comparison with the other transcripts studied, gB transcription was higher 15-48 hours post infection. Transcripts of *in vitro* cell culture systems have been studied previously only up to 16 hours post infection (Wirth *et al.* 1989; Seal *et al.* 1991) and, with the exception of recent transcriptome studies in HSV and pseudorabiesvirus, have not been quantitative (Aguilar *et al.* 2006; Flori *et al.* 2008). Therefore comparisons with previous work are not possible. Further work, looking at transcript accumulation of other glycoproteins and subsequent protein expression is needed to establish whether this is an important

finding. Investigating BoHV-1 protein expression in infected cells was trialled in a recent project, and indicated this may be a good starting point for future work in this area (Dryburgh 2009). Increased expression of gB could potentially relate to the final packaging and enveloping of progeny virions, as glycoproteins surround the virion envelope. It could also influence spread to surrounding cells, as many glycoproteins are essential for cell entry (Muylkens *et al.* 2007). *Circ* was also expressed later in the infection. Although it is known as an immediate-early transcript due to the timing of its initial transcription, it is known to be expressed throughout all phases of infection but its exact function is not fully understood (Fraefel *et al.* 1993).

This is not the first time the speed of transcript accumulation has been compared between two herpesvirus isolates representing different clinical signs. Previous analysis using northern blot RNA analysis of BoHV-1 isolate transcripts also demonstrated differences between two isolates (Jura and K22), representing different subtypes (1.1 and 1.2b respectively). Evidence of transcription in the Jura strain appeared earlier, at 3 hours pi and in higher abundance than K22, though a similar pattern of transcription was found later in the infection period (Wirth *et al.* 1989). A slower transcript accumulation has also been shown in HSV-2 during a transcriptomic study comparing it to HSV-1. In addition, four transcripts were identified, UL4, UL29, UL30 and UL31, all involved in nuclear organization and viral DNA localisation, showing a low correlation between the two strains throughout the transcription cascade. The clinical significance of these differences, in terms of the pathology demonstrated by the two HSVs could not be concluded from this data (Aguilar *et al.* 2006).

There was also variation between the three replicate time courses that were run, which compromised statistical comparison between isolates. There are several possible reasons for this. The ΔCt calculations result in small differences in Ct values between samples, becoming much larger when made relative to the host reference gene, β-actin. Alternatively, it may represent biological variation between different infections of the same isolate that could be caused by variation in the host cells, for example in the stage of the cell cycle or degree of confluence. This could either

indicate that this model does not provide the reproducibility that would be expected from an *in vitro* system, or it could be caused by rapidly changing expression patterns in BoHV-1, which would mean the three hour snapshot would provide variation between repeat infections.

This experiment has also highlighted the problem of relative quantification, as variation was seen in the host reference gene over time, most likely due to the decrease in viable cells due to viral infection. Finding appropriate reference genes or calibrator samples is a particular problem for viral infections. This is due to the possible effect of virus infection on host cell transcription making comparison with host genes problematic. Other reference genes are available such as ubiquitin and glyceraldehyde-3-phosphate dehydrogenase. No studies have been conducted to identify the most suitable reference to use for BoHV-1 transcripts. Future relative quantification of viral transcription would be improved by a survey of possible reference genes that are unaffected during BoHV-1 infection, in order to find the most suitable host reference gene for time course transcript studies such as this. Another possible approach would be to measure viral gene expression using an absolute quantification method, but this experiment has still provided a good comparison at different time points of infection between two isolates. As some differences were observed between isolates using this method, it highlighted the importance of testing more than one of each isolate type, this could have been done using this system had time allowed but it was decided that the lung slice model had the potential to provide more information if successful.

The infection of lung slices was a more complex system of infection than BT cells, with maintenance of tissue structure and multiple cell types. As it was not possible to observe CPE, as in BT cells, it was important to establish the viability of the lung slices and their ability to support BoHV-1 replication. This was assessed by measuring the release of virus into the culture medium over time. The virus genome copy number produced on each day of the infection was measured using real-time PCR absolute quantification. Infected lung slice viral load was also compared to control wells containing no lung slices, but were still infected with virus to measure

any residual virus that stuck to the wells between washes. These control wells should contain significantly less virus than infected lung slices if the lung slices were supporting viral replication.

The results showed that most lung slices produced significantly more virus than the no lung slice controls. On 3 of the 6 plates no virus was detected in the no-lung-slice control wells. On the other 3 plates where a small amount of virus was detected in the control wells, values were much higher than in the control wells, with the exception of one plate. However, the extent of variation between lung slices meant that at some time points this did not achieve levels of statistical significance. This was due to the high variability of virus copy number detected, perhaps due to differences in viability of individual lung slices. The viability of the lung slices may have been compromised by the method of euthanasia used for the donor animal, which was killed by barbiturate overdose, which could cause toxicity and interfere with cell culture. Overall, it was considered that as 13 of the 16 triplicate wells demonstrated significantly higher viral load compared to the no-lung-slice control wells that this provided enough evidence to continue with the analysis of transcript expression and localisation of virus.

There was no difference in the amount of virus produced each day between the two isolate groups, based on mean values from the 3 respiratory isolate and 3 systemic isolate lung slice infections. In all cases, there was less virus produced on day 2 of the infection than days 1 and 3. This is in contrast to a BoHV-1 infection of upper respiratory tract in an air-liquid interface, which shows a consistent increase in viral production over 6 days (Niesalla *et al.* 2009). There is also a possibility that the higher viral load on day 1 could represent residual virus left from the infection, compared to day 2 sampled after another wash stage. As lower transcript levels were not observed in the lung slices on day 2, this provides further support that it may be due to residual virus in the media on day 1 or could alternatively indicate the viral DNA extraction from the media was not as efficient for the day 2 samples.

Transcription of 3 genes was measured using relative quantification by real-time RT-PCR. The results showed an accumulation of gene transcripts over the three days. This provided further confirmation of the replication of the virus in the lung slices and therefore of the suitability of this *in vitro* model. The two isolate types, respiratory (05/1570), and systemic (07/1530) showed a similar pattern of gene transcription throughout the 3 days of infection, with the exception of RR on day 2, when the 05/1570 infections showed a higher gene transcription than the 07/1530 infections.

Comparing these *in vitro* studies with the *in vivo* experiment described in chapter 5, a higher level of transcript expression was also apparent in the respiratory isolate in the nasopharyngeal tonsil and possibly the trachea. In the nasal mucosa (which could be considered most similar to the BT cells) no clear difference was demonstrated *in vivo* between the isolates, though a trend was observed of higher transcript accumulation by the systemic (07/1530) BoHV-1 isolate. The differences between *in vitr*o and *in vivo* models could be due to the limitations of *in vitro* systems to mimic all the mechanisms involved in the pathogenesis of disease, for example the involvement of the immune system, which also stimulates immune evasion responses from the virus (Babiuk, van Drunen Littel-van den Hurk & Tikoo 1996). These results could demonstrate that the immune response, not present in this model system, can influence viral transcription and subsequent viral replication when comparing these isolates. A higher RR transcript abundance was observed on day 2 in respiratory isolate infected lung slices. This could be considered to correlate with the observations in the *in vivo* study on day 4 pi, in the trachea and the nasopharyngeal tonsil, which also showed higher transcript expression of all 3 transcripts after infection with the respiratory isolate (05/1570). The consistency of this finding, both in the *in vivo* and *in vitro* infection with 3 ‘respiratory’ isolates suggests this may be of significance in the replication of this isolate type. Increased expression of RR could be related to an increase of viral DNA replication at this time point as RR is important in the synthesis of dNTPs (Schang & Jones 1997). However, further experiments to measure RR protein expression and other early genes or proteins involved in DNA replication would be needed to support this suggestion.

The use of lung slices provided a further opportunity to study the cell tropism of the different virus isolates. Immunofluorescent double labelling was used to determine whether BoHV-1 was present in epithelial, endothelial cells or both cell types, over the three days of infection. Co-localization of virus in both epithelial and endothelial cells was found in a number of cases. Unfortunately, many of the slices did not contain enough viral antigen to be detected by the antibodies used, which limited the analysis of the results. In the limited samples available for analysis, it was found that both respiratory and systemic isolates of BoHV-1 localised to epithelial cells on day 1 of infection, and in both epithelial and endothelial cells by day 2 pi. Further optimisation of the antibodies and conditions should improve the labelling reliability, but the initial results are encouraging and this technique could be used to provide an insight into viral tropism and the progress of infection.

More extensive examination of this system is needed to establish whether these observations are of any significance. If this was demonstrated in more cases there are several explanations. Initial infection of epithelial cells would be expected as this is the first site of entry in the upper respiratory tract (Muylkens *et al.* 2007). Later, infection of endothelial cells could represent a potential mechanism for systemic spread via the circulatory system as seen in closely related viruses such as pseudorabies virus and EHV-1 (Nauwynck & Pensaert 1992; Allen & Breathnach 2006). Tropism for endothelial cells has been previously associated with virulence in EHV-1. An *in vivo* study found tropism for endothelial cells may be an important factor in the increased virulence of EHV-1 strains (Smith *et al.* 2000). Alternatively, this could occur due to both epithelial and endothelial cells being equally exposed to virus, and reflects slower replication in endothelial cells. No evidence of differences between the isolate types was detected, although the data set available was too small to confirm this. Both quantitative PCR and immunohistofluorescence techniques showed an increase, of either viral transcripts or antigen on day 3, compared to days 1 and 2. The increased presence of viral antigen on day 3 provides more evidence of the suitability of lung slices to support viral replication, and also suggests that the increase in transcript production is related to an increase in protein expression.

This technique has advantages over classical *in vitro* cell culture, and with further optimisation it may also provide an initial approach when targeting a specific aspect of pathogen behaviour such as cell tropism in an environment when it is possible to test many variables. This should enable better design and refinement of any subsequent *in vivo* studies. It provides information on cell tropism that an infection of BT cells cannot provide, and did not present the same problems with relative quantification of real-time PCR data, as no decrease in beta actin expression was observed over the infection period. It also uses multiple replicate samples from the same organ and animal, which adds to the power of any statistical analysis. The system could be optimised for more tissue types, and could make a good model for tissue tropism by measuring viral replication in the different tissue types and demonstration of cell type specific localisation of virus. Bovine tracheal and nasal epithelium has been used in the study of BoHV-1 using an air-liquid interface system. This system provided the possibility of measuring early host responses at the molecular level, as well as demonstrating a viable BoHV-1 infection, showing there are many potential opportunities in the use of these organ culture systems (Niesalla *et al.* 2009). In the lung slice system used in this study, the tissue was submerged in medium, similar to the trachea culture system developed earlier (Bouffard & Derbyshire 1978), more recently in Maedi-Visna infection of the trachea and lung (McNeilly *et al.* 2007), and also in the study of the herpesvirus that causes infectious laryngotracheitis (Williams *et al.* 1992). However, our study shows that like the air-liquid interface system, it can be used to investigate molecular aspects of viral expression whilst the structural integrity of the organ can be maintained, to allow imaging of the viral infection to investigate factors such as cell tropism.

Overall, the use of *in vitro* model infections has not clearly confirmed the differences in transcript expression between the two isolate types that were observed *in vivo*, but it has provided further evidence of subtle differences between the isolates. Further experiments such as growth curve analysis and comparison of more isolates in BT cells would be useful to clarify these observations. Further information has been provided on the transcript expression of BoHV-1 using two infection models, complemented by imaging of infection, which showed potential for cell tropism

studies. A system that provides a relatively simple way of studying BoHV-1 has been developed, which with further optimisation could be transferred to upper respiratory tract tissue. This could provide a good model for further investigation of BoHV-1 tropism with limited need for experimental animals.

Chapter Seven: General Discussion

This project has studied BoHV-1 from the molecular level of the viral genome and to its pathogenesis in the natural host. It has focussed on the comparison of isolates that have been associated with different pathology in the field, isolated from either a respiratory IBR infection, or tissue from a BoHV-1 abortion case or systemically infected calf.

Before any direct comparisons between the isolates were started a survey of the prevalence of BoHV-1 in abortion cases in Scotland and Northern England was conducted. This showed BoHV-1 may be more prevalent as a cause of abortion than recent reports suggest (SAC VS 2009) (Murray 1990). Molecular diagnostic methods such as real-time PCR appear to play an important role in accurate diagnosis. It also highlighted that specific tests are needed to identify BoHV-1 in abortion cases, histopathology provided little reliable evidence without specific IHC labelling. Specific tests can be expensive but in the case of real-time PCR this could be made more economical several causes could be targeted in a multiplex real-time PCR assay that includes likely viral causes such as BoHV-1 and BVD alongside bacterial causes.

The molecular mechanisms of BoHV-1 systemic spread from the upper respiratory tract to other organs or across the placental barrier have not been clearly demonstrated. This study quantified BoHV-1 in the foetal tissue from BoHV-1 cases. It found the highest abundance of BoHV-1 in the foetal liver by real-time PCR, and localisation of virus to vessels in the placenta and also foetal tissues such as the heart. This provided support for previous studies that have suggested a haematogenous route infection from the placenta to the foetus (Smith 1997; Rodger *et al.* 2007). However, the route from the respiratory tract to the placenta still remains undetermined and needs further investigation.

The sequencing of 10 BoHV-1 whole genomes provided an in-depth insight into the variation between the isolates and added extensively to the BoHV-1 genome data available. No conserved differences were found between the isolate types confirmed by SNP and phylogenetic analysis. This suggests coding differences are not related to virulence or tropism as was found in EHV-1(Nugent *et al.* 2006; Allen & Breathnach 2006). However, it does not rule out differences at a transcriptional level and also did not distinguish between circulating isolates and reactivated isolates which both may be key to understanding systemic infection in terms of viral factors.

Despite no differences found between the whole viral genomes, this did not rule out differences at the transcriptional level contributing to the different pathology produced by the isolates. It was also not known whether isolates would reproduce the same pathology or if it was entirely dependent on the host. An *in vivo* infection allowed this to be tested. Differences were observed in the clinical outcome of the infection with more respiratory clinical signs caused by the respiratory (IBR) isolate and more ocular signs caused by the systemic (abortion) isolate. There were also differences observed in tissue tropism at day 4 post infection and the host immune responses observed in the nasopharynegeal tonsil. A study of transcript expression representing the three phases of temporal BoHV-1 gene expression showed differences between the isolates. This was most clear in the nasopharyngeal tonsil where gene expression was significantly higher in all genes tested in the respiratory (IBR) isolate on day 4 pi. As the clinical signs suggested that the systemic isolate was affecting the hosts at a slower rate, it is not clear whether this is simply representative of the stage of infection rather than an actual difference in transcript levels associated with the isolate type. There was also evidence of spread to the brain in a couple of cases that could be considered as evidence of systemic spread. However, with the exception of the brain no evidence of BoHV-1 was found in either the buffy coats or any tissue outside of the respiratory tract or associated tissues. This contrasted to previous intranasal challenges that had found BoHV-1 in the spleen, kidneys and liver (Mweene *et al.* 1996; Kaashoek *et al.* 1996). Overall, the results from this study lead to the conclusion that an isolate previously causing a systemic infection does not reproduce this in the infection of another host. This adds further

weight to the sequencing data showing no virally encoded evidence for systemic spread.

Model systems were used to provide more replicative conditions to look at BoHV-1 transcript expression in an *in vitro* infection of BT cells and lung slices from a donor animal. The lung slices allowed for imaging of the viral infection at different stages using histopathology and immunofluorescence techniques, to potentially investigate virus tropism at the cellular level. The differences observed in the cells between the isolates showed a more rapid development of CPE and higher levels of transcript abundance the respiratory (IBR) isolate infected cells. To confirm the observations made that have suggested differences between the isolates, both in BT cell infection and the *in vivo* challenge several isolates from each classification needed to be studied in the same system. The lung slice infection used 3 isolates of each type to provide biological replicate data. This aimed to establish whether the differences observed were just showing natural isolate variation as the sequencing results suggest or something specific to the previous pathology they were known to have demonstrated in the field. No differences were observed except for higher RR transcript expression on day 2 in the respiratory group of isolates. It would have been useful to gain further information from this using growth curves and more isolates but as time was limited the lung slices were focussed on to provide a more complex model and the potential to provide more detailed results. For example the use of lung slices demonstrated the potential to investigate cellular localisation of the virus which would not be possible from basic cell culture or growth curve experiments. With further optimisation the cellular tropism of different isolates or strains could be established.

In both cases when more than one of each isolate type was used (the sequencing and the lung slice model) few differences were observed. This indicates that virally encoded involvement in the systemic spread of BoHV-1 is likely to be minimal. However, there were some clear differences identified in the *in vivo* infections between the isolates. Overall, it is likely that the host immune response and environmental conditions are the major factors determining systemic spread. The

immune response has also been noted of importance in equine abortion caused by EHV-1 when presence of cytotoxic lymphocytes have been found to protect against abortion (Kydd *et al.* 2003).

If this project were to continue several directions of further work could be followed. As some evidence was observed in differences in transcript expression between the isolates, further work is needed to establish how the differences in transcript expression translates if at all, into variation in protein expression. A more thorough study including a wider representation of BoHV-1 transcripts would also be needed to rule out involvement at a transcriptional level, for example transcriptome sequencing during an infection. There is also much work that could be done on the host immune response, as this research suggests it is likely to be a major factor in the systemic spread of BoHV-1 as viral control appears to be limited. To investigate any of these areas further it is also important to try to establish the mechanisms involved in the initial dissemination of the virus from the respiratory tract, which is the initial cause of any systemic infection and remains unclear. Comparisons could then be made between bovine immune responses during respiratory and systemic infections.

This project has provided several areas of novel research that can be summarised below:

- The extent of bovine abortions related to BoHV-1 in Scotland and Northern England were established suing molecular diagnostic techniques.
- Next-generation sequencing technology was used or the comparison of multiple BoHV-1 genomes significantly adding to the BoHV-1 sequencing data available.
- Two field isolates have been tested in an *in vivo* challenge of the natural host to provide the information that an isolate that has previously demonstrated systemic spread does not necessarily cause systemic spread in a new infection. It has also used lung slice culture for molecular and imaging studies to simultaneously compare 6 BoHV-1 isolates cellular tropism and gene expression

The main findings from this research are:

- BoHV-1 abortion is potentially under diagnosed and molecular diagnostics such as real-time PCR could play an important role in producing more accurate data.
- Comparison of BoHV-1 genome sequences demonstrated no significant difference between islates limited to the respiratory tract infections and those spreading systemically to cause systemic disease or abortion.
- An *in vivo* study comparing two of the sequenced isolates demonstrated clinical differences, potentially different routes and speed of spread through the respiratory tract and differences in transcript expression which could relate to viral replication. However, this could not be attributed with certainty to the respiratory/systemic classification of the isolates.
- *In vitro* work using more of the sequenced isolates supported the sequencing work with no clear differences in the transcript expression or tropism at a cellular level of the respiratory/systemic classed isolates.

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Appendices

Appendix 1. Table detailing findings from histopathological and immunohistochemical labelling of BoHV-1 positive foetal tissues.

Case	Tissue	Lesion	IHC	Labelling localisation
07/128	Placenta	None	Positive	Vessel endothelial cells/white blood cells
	Heart	None	Positive	Vessel endothelial cells/white blood cells
	Lung	Multifocal necrosis	Positive	Alveoli
	Liver	Multifocal necrotic hepatitis	Positive	Necrotic foci and endothelial cells of vessels
	Brain	-	-	-
	Fetal fluid	-	-	-
07/232	Brain	Glial foci	Positive?	2 sites within neuropile
	Liver	Multifocal necrotic hepatitis	Positive	Necrotic foci and endothelial cells of vessels
	Heart	No lesion	Negative	-
	Lung	Haemmorage	Negative	-
	Fetal fluid	-	-	-
07/233	Brain	No lesion	Negative	-
	Heart	No lesion	Negative	-
	Liver	Multifocal necrotic hepatitis	Positive	Necrotic foci and endothelial cells of vessels
	Lung	No lesion	Negative	-
	Fetal fluid	-	-	-
07/327	Lung	No lesion	Positive	Vessels and scattered cells in alveoli
	Brain	No lesion	Positive	Endothelial cells/white blood cells in vessels of meninges
	Heart	No lesion	Positive	Endothelial cells of vessels
	Liver	Multifocal necrotic hepatitis	Positive	Necrotic foci and endothelial cells of vessels
	Placenta	Focal necrosis	Positive	Endothelial cells/white blood cells in vessels of the villi
	Fetal fluid	-	-	-
07/558	Lung	No lesion	Positive	One area of focal necrosis positively stained in alveoli
	Liver	Multifocal necrotic hepatitis	Positive	Staining associated with sites of multifocal necrosis
	Heart	No lesion	Negative	-
	Brain	-	-	
09/046	Liver	No lesion	Negative	-
	Heart	No lesion	Negative	-
	Lung	No lesion	Negative	-
	Brain	No lesion	Negative	-
	Fetal fluid	-	-	-
	Placenta	-	-	-
09/050	Lung	No lesions	Positive	Vessels and scattered cells in alveoli
	Liver	Multifocal necrosis	Positive	Vessel associated
	Heart	No lesions	Positive	Vessel associated
	Brain	No lesions	Positive	Scattered small foci
	Fetal fluid	-		
09/334	Liver	Multifocal necrosis	Positive	Necrotic foci and endothelial cells of vessels
	Heart	No lesions	Positive	Scattered cells and some association with vessel walls
	Placenta	Multifocal necrosis	Positive	Vessels and necrotic foci
	Lung	-	-	-
	Fetal fluid	-	-	-
	Brain	-	-	-
09/461	Placenta	Multifocal necrosis	Positive	Necrotic foci and endothelial cells of vessels
	Liver	Multifocal necrosis or could be autolysis	Positive	Vessels and scattered cells in alveoli
	Lung	Multifocal necrosis	Positive	Mainly in villi endothelial cells?
	Heart	-	-	-
	Brain	-	-	-
	Fetal fluid	-	-	-
09/462	Liver	No lesions	Negative	-
	Lung	Small focis of inflammatory	Negative	-
	Brain	Haemmoraging	Negative	-
	Heart	-	-	-
	Fetal fluid	-	-	-