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IMPACT OF MAEDI-VISNA ON SHEEP BREEDING FLOCKS

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Abstract

Maedi-visna (MV), is a chronic wasting disease of sheep and goats caused by the small ruminant lentivirus (SRLV), maedi-visna virus (MVV). With no known cure or treatment, an asymptomatic period of sometimes several years and an infection that ultimately results in death, the finding that prevalence is on the rise within the UK is of great concern.

In this study, a diagnostic was developed for detection and quantification of as a yet unidentified SRLV strain circulating within the UK in 2014. Identification of the viral strain was attempted to characterise this current circulating strain. Tissues and blood samples were collected from 28 seropositive rams over a period 28 months as part of a longitudinal case study after which semen harvested from 13 of these rams was used in an artificial insemination (AI) trial to estimate the risk of MVV transmission within a natural mating model.

The viral strain was partially characterised at the molecular level and found to show similarities with previously reported UK strain (EV1). A qPCR assay was developed and showed successful detection of virus within both blood and tissue samples of seropositive animals but failed to detect any viral sequences with inseminated naïve ewes 7 weeks post insemination. In addition, proviral loads within blood were shown to be higher than previous reported findings.

Finally, regression modelling of milk production data collected from a UK dairy flock suggested an outbreak of MVV of an unknown strain to cause a reduction in milk yield within seropositive ewes. Overall, this study demonstrates the impact of disease of a newly identified circulating strain of MVV within the UK.

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ABBREVIATION	MEANING
AI	Artificial Insemination
AGID	Agar Gel Immunodiffusion
AMV	Avian Myeloblastosis Virus
APOBEC3	Apolipoprotein B mRNA-editing Catalytic Polypeptide-like 3 Protein
BIV	Bovine Immunodeficiency Virus
CA	Capsid Protein
CAE	Caprine Arthritis and Encephalitis
CAEAS	Caprine Arthritis and Encephalitis Accreditation Scheme
CAEV	Caprine Arthritis and Encephalitis Virus
CBFB	Core-Binding Factor Beta
CCR5	C-C Chemokine Receptor Type 5
CD4	Cluster of Differentiation 4
CNS	Central Nervous System
CUL5	Cullin 5
CYPA	Cyclophilin A
dNTP	Deoxyribonucleotide Triphosphates
dUTPase	Deoxyuridine 5'-Triphosphate Nucleotidohydrolyase
EIAV	Equine Infectious Anemia Virus
ELISA	Enzyme-Linked Immunosorbent Assays
ENTV	Enzootic Nasal Tumor Virus
FIV	Feline Immunodeficiency Virus
GPCR	G Protein-Coupled Receptor
GWAS	Genome Wide Association Study
HIV	Human Immunodeficiency Virus
IELISA	Indirect Enzyme-Linked Immunosorbent Assay
IFN-β	Interferon-β
IN	Integrase
JSRV	Jaagsiekte Retrovirus
LC3	Microtubule-Associated Protein 1A/1B-Light Chain 3
LTR	Long Terminal Repeat
M-MLV	Moloney Murine Leukemia Virus
MA	Matrix Protein
MHC-II	Major Histocompatibility Complex II
MR	Mannose Receptor
MV	Maedi-Visna
MVAS	Maedi-Visna Accreditation Scheme
MVV	Maedi-Visna Virus
NGS	Next Generation Sequencing

NC	Nucleocapsid Protein
OaA3Z2-Z3	Ovine APOBEC3-Z2-Z3
OaA3Z3	Ovine APOBEC3-Z3
OvLv	Ovine Lentivirus
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reation
PM	Post-Mortem
PPT	Polypurine Tract
PR	Protease
QPCR	Quantitative Polymerase Chain Reaction
RRE	Rev Responsive Element
RT	Reverse Transcriptase
SCC	Somatic Cell Count
SIV	Simian Immunodeficiency Virus
SNP	Single Nucleotide Polymorphism
SRLV	Small Ruminant Lentivirus
SU	Surface Glycoprotein
TM	Transmembrane Glycoprotein
TMEM154	Transmembrane Protein 154

Chapter 1: Literature Review

Maedi-Visna (MV), also known as Ovine Progressive Pneumonia, Zwoergersiekte or Graaff-Reinet disease and caprine arthritis and encephalitis (CAE) are chronic wasting diseases affecting sheep and goats worldwide (Rovid Spickler 2015). They are the result of infection by the lentiviruses maedi-visna virus (MVV) and caprine arthritis encephalitis virus (CAEV), respectively. Affected animals are asymptomatic during the majority of infection (>2 years). The appearance of clinical signs occurs with a gradual onset which progressively worsens with time ultimately leading to a 100% fatality rate in infected individuals. This observation of prolonged infection was first described for MVV and resulted in the concept of 'slow viruses' (Bennet and Kimberlin 1976).

1.1 Small Ruminant Lentiviruses

1.1.1 Viral Taxonomy

MVV and CAEV were originally regarded as two completely separate viral species. But recently, due to shared host species (sheep and goats) and genetic and phenotypical similarities these viruses are now widely regarded as a viral continuum under the grouping of small ruminant lentiviruses (SRLVs). These viruses are of the *Retroviridae* family and *Orthoretrovirinae* subfamily and the genus *Lentivirus* which also includes human, simian, bovine and feline immunodeficiency viruses (HIV-1, SIV, BIV and FIV) and equine infectious anaemia virus (EIAV) (Figure 1.1.1.1) (ICTV 2015).

Zanoni (1998) suggested a classification system consisting of 6 clusters (I, II, III, IV, V and VI) based on the phylogenetic

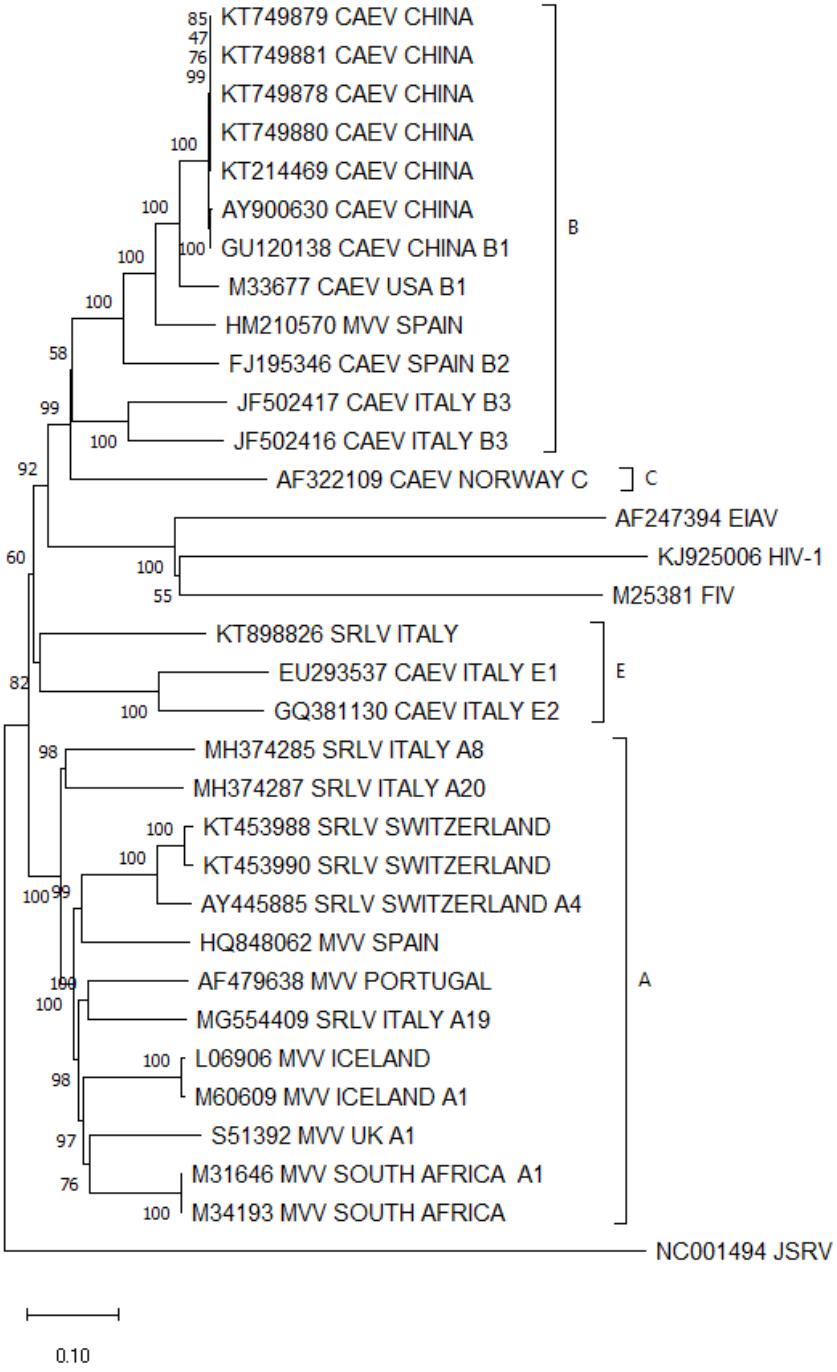


Figure 1.1.1.1 Phylogenetic tree of 33 full retrovirus genome sequences. 29 small ruminant lentiviral (maedi-visna virus (MVV) and caprine arthritis encephalitis (CAEV)), 3 lentiviral (human immunodeficiency virus type 1 (HIV-1), feline immunodeficiency virus (FIV) and equine infectious anaemia virus (EIAV)) and 1 retroviral (jaagsiekte retrovirus (JSRV)) sequences were aligned using MUSCLE software after which a phylogenetic tree was constructed by neighbour-joining.

analysis of 64 SRLV sequences ranging in size from 129 bp to 3146 bp from various regions of the viral genome in addition to one full genome CAEV and three full genome MVV sequences. In 2004, a second study analysing 104 SRLV isolates made up of 284 bp gag, 1.2 kb pol and/or 1.8kb gag-pol fragments isolated from 91 seropositive goats and 13 seropositive sheep sourced from 115 swiss herds was completed (Table 1.1.1.2) (Shah et al. 2004b). From this, SRLVs were reclassified into four principal sequence groups: A-D. Between groups, a sequence variability of 25-37% was observed when comparing *gag* and *pol* sequences. In addition to these groups, A and B were divided into seven and two further subgroups, respectively, with a sequence variability of 15-27% seen between subgroups. Group A represents a clustering around MVV isolates kv1772, EV1 and SA-OMVV while group B sequences share high similarity to the CAEV Cork isolate (Sonigo et al. 1985; Braun et al. 1987; Querat et al. 1990; Saltarelli et al. 1990). Groups C and D represent more diverse sequences isolated during the Shah study that did not cluster with group A and B. Since the Shah reclassification, groups A and B have been further expanded upon with a current total of twenty-two and five subgroups, respectively. In addition, Ramírez et al. (2013) suggested group D to be in fact a part of group A exhibiting divergence within the *pol* gene. This was after phylogenetic analysis of the *gag* gene of group D sequences classified them as group A. This in tandem with the fact that said group has only been identified within one study may suggest an error in classification. Finally, a fifth group was added in 2007, E, which was later expanded to two subgroups in 2011 (Grego et al. 2007; Reina et al. 2009a; Giammarioli et al. 2011).

Table 1.1.1.2 SRLV classification systems. Table illustrates the two recognised classification systems for SRLVs, stating the currently known locations of circulation and host species with source references. Adapted from (Shah et al. 2004b).

Classification System				
Shah et al (2004)		**Zanoni (1998)**		
Group	Sub Group	Cluster	Source of Isolation	Reference
A	A1	I	MVV-like from sheep and goats worldwide	[1]
	A2	II	Sheep from North America, Spain and Turkey	[2]
	A3	-	Sheep and Goats from Switzerland, Spain and Turkey	(Shah et al. 2004b; Glaria et al. 2012; Muz et al. 2013)
	A4	-	Sheep and Goats from Switzerland and Germany	[3]
	A5	-	Sheep and Goats from Switzerland, Germany, Turkey and Slovenia	[4]
	A6	VI	Sheep and Goats from France	(Leroux et al. 1995)
	A7	-	Goats from Switzerland	(Shah et al. 2004b)
	A8	-	Goats from Italy	(Grego et al. 2007)
	A9	-	Sheep and Goats from Italy and Turkey	(Grego et al. 2007; Giammarioli et al. 2011; Muz et al. 2013)
	A10	-	Goats from Italy	(Pisoni et al. 2010; Molaee et al. 2020)
	A11	-	Sheep and goats from Italy, Germany and Turkey	(Giammarioli et al. 2011; Muz et al. 2013)
	A12	-	Sheep from Poland	(Olech et al. 2012)(Kuhar et al. 2013a)
	A13	-	Sheep from Poland	(Kuhar et al. 2013a)
	A14	-	Goats from Slovenia	(Kuhar et al. 2013a)
	A15	-	Sheep from Slovenia	(Kuhar et al. 2013a)
	A16	-	Goats from Poland and Germany	(Olech et al. 2018)
	A17	-	Goats from Poland	(Olech et al. 2018)
	A18	-	Sheep from Poland	(Olech et al. 2019)
	A19	-	Goats from Italy	(Colitti et al. 2019)
	A20	-	Sheep from Italy	(Colitti et al. 2019)
	A21	-	Sheep in Germany	(Molaee et al. 2020)
	A22	-	Sheep in Iran, Lebanon and Jordan	(Molaee et al. 2020)
B	B1	V	CAEV-like from goats worldwide	[5]
	B2	IV	Sheep and Goats from France, Spain, Poland and Switzerland	[6]
	B3	-	Sheep and Goats from Italy	(Bertolotti et al. 2011; Giammarioli et al. 2011)
	B4	-	Goats from Canada	(Santry et al. 2013)
	B5	-	Goats from Belgium	(Michiels et al. 2020)
C		III	Sheep and goats from Norway	(Gjerset et al. 2006; Gjerset et al. 2007; Gjerset et al. 2009)
D		-	Goat from Switzerland and Spain	(Shah et al. 2004b)
E	E1	-	Goats from Italy	(Grego et al. 2007; Reina et al. 2009a)
	E2	-	Goats from Italy	(Giammarioli et al. 2011)

[1] (Sonigo et al. 1985; Querat et al. 1990; Sargan et al. 1991; Leroux et al. 1995; Gjerset et al. 2007; Grego et al. 2007; Olech et al. 2012)
[2] (Woodward et al. 1995; Karr et al. 1996; Glaria et al. 2012; Fras et al. 2013; Muz et al. 2013; Santry et al. 2013)
[3] (Shah et al. 2004a; Shah et al. 2004b; Cardinaux et al. 2013; Deubelbeiss et al. 2014; Blatti-Cardinaux et al. 2016; Molaee et al. 2020)
[4] (Shah et al. 2004b; Kuhar et al. 2013a; Muz et al. 2013; Molaee et al. 2020)
[5] (Chiu et al. 1985; Saltarelli et al. 1990; Zanoni et al. 1992; Leroux et al. 1995; Chebloune et al. 1996; Germain and Valas 2006; Grego et al. 2007; Giammarioli et al. 2011; Olech et al. 2012; Fras et al. 2013; Kuhar et al. 2013a)
[6] (Leroux et al. 1995; Shah et al. 2004b; Germain and Valas 2006; Grego et al. 2007; Glaria et al. 2009; Giammarioli et al. 2011; Crespo et al. 2012; Olech et al. 2012; Perez et al. 2015; Pérez et al. 2015)

1.1.2 Viral Structure

The SRLV genome consists of two single-stranded positive-sense RNA strands 9.2 kb in size. Each strand contains the full complement of genetic information and are often identical. The information present on these strands, codes for three structural genes (*gag*, *pol* and *env*) and three auxiliary genes (*vif*, *vpr* and *rev*) (Pépin et al. 1998). The organisation of these genes within the RNA strands is illustrated in Figure 1.1.2.1a. When comparing the genetic structure and organisation of SRLVs to other viruses of the lentiviral group several differences can be observed. Although the three structural proteins, *gag*, *pol* and *env*, are maintained throughout all lentiviruses, the number and composition of accessory genes varies greatly (Gifford et al. 2012). An example of this, HIV-1 possesses six accessory genes; *tat*, *rev*, *vpu*, *nef*, *vif* and *vpr*, while EIAV only has four; *ttm*, *tat*, *rev*, and *S2* (Beisel et al. 1993; Cullen 1998; Li et al. 2000). These variations can be categorised according to clade of host species with lesser variations seen within these groups.

Of the three structural genes, the *gag* (group-specific antigen) gene encodes for precursor $Pr55^{gag}$ which is cleaved into three proteins: capsid protein (CA), nucleocapsid protein (NC) and matrix protein (MA) (Figure 1.1.2.1b) which are responsible for the formation of the hydrophobic virion core, coating viral RNA and association of capsid with the viral membrane, respectively (Cheevers et al. 1988). In contrast to *gag,* which codes for the internal structural proteins of the virion, *env* (envelope) codes for two external glycoproteins scattered throughout the host cell derived lipid bilayer which forms the viral envelope. These glycoproteins: transmembrane (TM) and surface (SU) (Figure 1.1.1.2b) are formed upon cleavage of the Env precursor

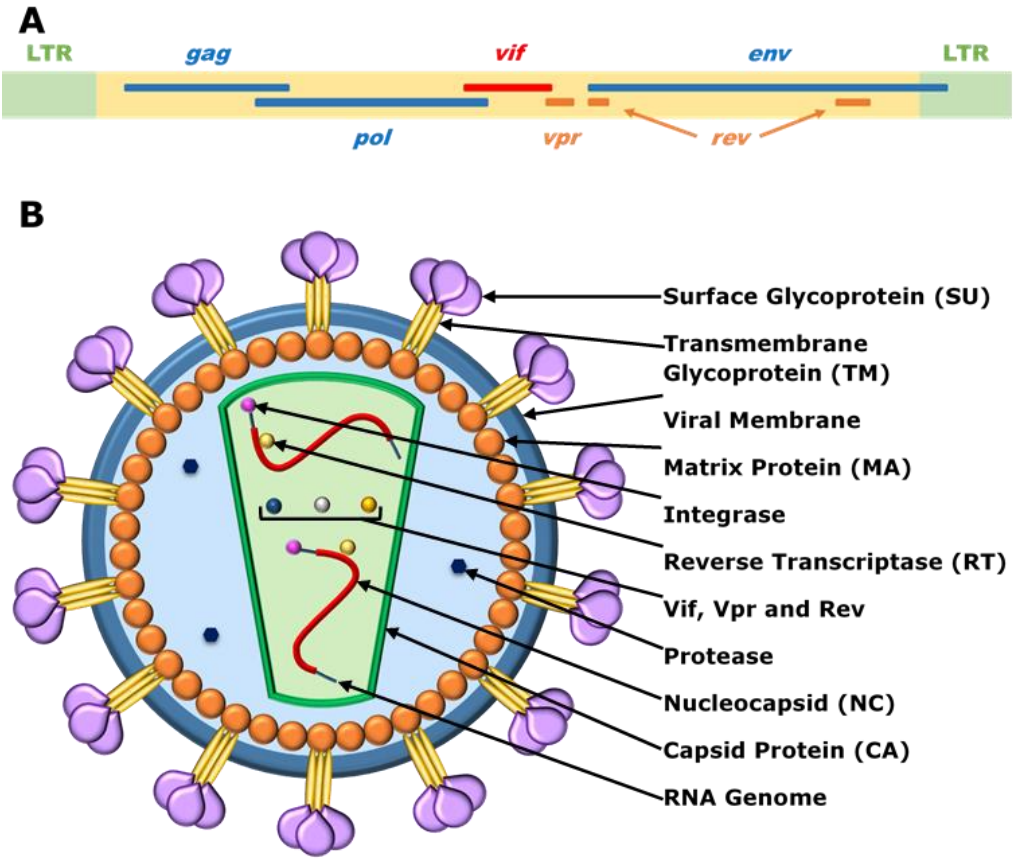


Figure 1.1.2.1 Genomic and viral structure of small ruminant lentiviruses. (A) The structure of a small ruminant lentivirus (SRLV) RNA genome. The genome is comprised of two long terminal repeat (LTR) regions found either end of the RNA strand (green region), 3 structural genes (blue; gag, pol and env) and 3 accessory genes (red/orange; vif, vpr and rev). Adapted from (Minardi da Cruz et al. 2013). (B) The structure of a SRLV viral particle illustrating the individual protein components and enzymes present. Virus comprises an icosahedral nucleocapsid core containing two identical ssRNA viral genomes and multiple vital proteins surrounded by a capsid protein shell within a host derived viral membrane from which virus derived glycoproteins protrude.

coded for by the *env* gene and provide the epitopes required for interactions between the virus and the host receptors whilst also inducing neutralising antibodies. The final structural gene, *pol* (polymerase), codes for important enzymes critical for successful viral replication within a host cell. In total, five enzymes are produced from the *pol* portion of the initial gag-pol polyprotein precursor; reverse transcriptase (RT), integrase (IN), protease (PR), RNase H and deoxyuridine 5'-triphosphate nucleotidohydrolyase (dUTPase) (Pépin et al. 1998).

The *vif* (virion infectivity factor) gene, also known as *Q* or *sor*, is essential for infectivity of SRLVs in host target cells and present in all lentiviruses with the exception of EIAV (Kristbjörnsdóttir et al. 2004). One function of the Vif protein, a small basic protein rich in tryptophans (28 kDa), is the neutralisation of host-specific Apolipoprotein B mRNA-editing catalytic polypeptide-like 3 (APOBEC3) proteins. These polynucleotide cytosine deaminases attenuate virus through the production of G to A hypermutations in the viral plus strand, although this is not their only means of antiviral activity (Depboylu et al. 2007; Franzdóttir et al. 2016). In the case of MVV in sheep, ovine APOBEC3-Z3 (OaA3Z3) and ovine APOBEC-Z2-Z3 (OaA3Z2-Z3) are the targets of Vif, with Vif deficient MVV being shown to be restricted by these proteins (Simon et al. 1995). This neutralising ability of MVV and CAEV Vif is produced by utilisation of multiple host cellular proteins including cyclophilin A (CYPA), Cullin5 (CUL5) and Elongin B/C for the formation of the Vif-mediated E3 ubiquitin ligase complex which allows for degradation of APOBEC3 proteins via a ubiquitin/proteasome-dependent pathway (Zhang et al. 2014; Zhao et al. 2019). These co-factors have been found to vary between lentiviruses such as the primate lentivirus co-factor

core-binding factor beta (CBFB). This difference in co-factors has been associated with the high variability seen in *vif* genes between viruses (Yoshikawa et al. 2016). Secondary to this function, Fu et al. (2020) reported that Vif protein can produce a down regulation of interferon-β (IFN-β) production, thereby providing the virus a means of evasion from the host immune system. Finally, it has also been suggested that Vif can modulate autophagy within host cells, which could be linked to the ability of Vif to bind microtubule-associated protein 1A/1B-light chain 3 (LC3), a central protein of the autophagy system, although further research is required to expand upon this (Aðalbjörnsdóttir 2016).

In SRLVs, the *vpr* (viral protein R) gene was initially thought to code for a Tat-like protein and was correspondingly named as such until 2003 (Villet et al. 2003). Tat proteins, as seen during HIV-1 infection, have an important role in stimulating transcription from the LTR promotor, a function that did not align with that seen from the SRLV protein (Das et al. 2011). Following this, similarities were identified between this protein functionally and structurally to the HIV-1 Vpr protein (Villet et al. 2003). This, in addition to differences seen in localisation of protein during and after replication in host cells compared to HIV Tat during infection suggested the protein product of this gene to be Vpr-like. Since then, SRLV Vpr (10 kDa) has been shown to induce G_2/M cell cycle arrest in transfected cells, also seen in HIV infection in humans which has been linked to interaction of Vpr with CRL4A (DCAF1), E3 ubiquitin ligase and SLX4 (Romani and Cohen 2012; Berger et al. 2015). This may also hold true for SRLV Vpr in sheep and goats but has yet to be confirmed.

The final accessory gene present in SRLVs, *rev* (regulation of virion protein expression), encodes a 19 kDa protein which ensures replication competency of virus in permissive cell lines (Toohey and Haase 1994; Pépin et al. 1998). This can be linked to the Rev protein's function in expression of viral proteins. This is accomplished by it binding to the RRE (*rev* responsive element) present within the *env* gene in SRLVs close to the SU/TM cleavage site (Lesnik et al. 2002). This initiates a cascade that facilitates the movement of viral transcripts into the cytoplasm.

1.1.3 Viral Lifecycle

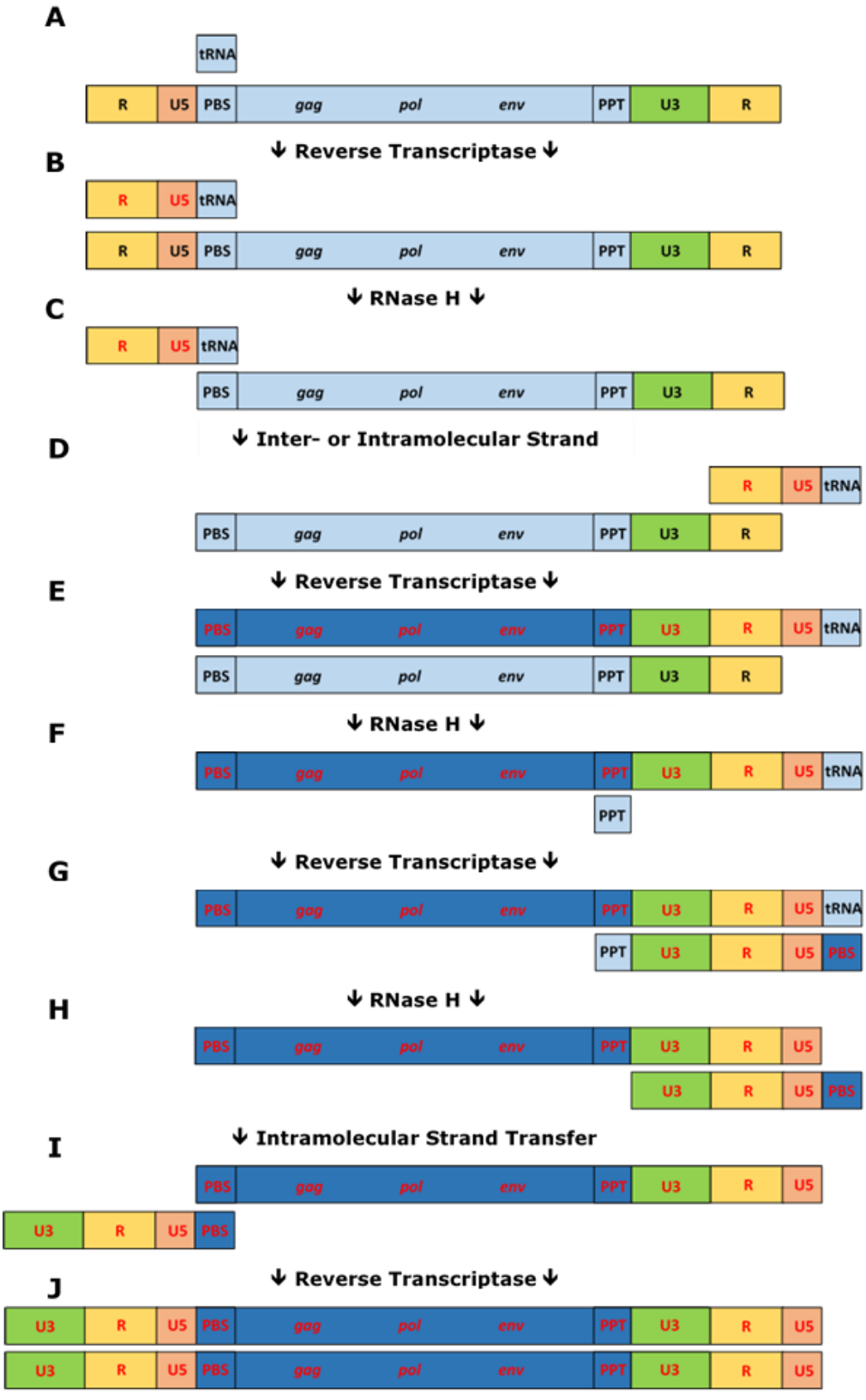
As seen with the majority of viruses, the lifecycle of SRLVs can be summarised in 5 key steps: cellular entry, genome replication, transcription and translation, maturation of virion and exit from the cell.

To allow for entrance into cells, SRLV must first bind to specific receptors present on target cells. With regards to other retroviruses, all have been shown to bind membrane bound glycoproteins (Weiss and Tailor 1995), with lentiviruses showing a requirement for the presence of 2 molecules (a receptor and co-receptor) to initiate entry (Broder et al. 1999). Currently no specific receptor for MVV or CAEV has been identified although based on their ability to infect a variety of cell lines in addition to target cells, it is suggested to be a commonly occurring cell membrane molecule (Brodie et al. 1995). A number of candidates have been identified, such as major histocompatibility complex II (MHC-II), the mannose receptor (MR) and a variety of membrane proteins in sheep and goats ranging in size from 15 kDa to 50 kDa (Crane et al. 1991;

Dalziel et al. 1991; Barber et al. 2000; Bruett et al. 2000; Crespo et al. 2011; Crespo et al. 2012).

Following receptor binding, viral fusion, entry and uncoating occur resulting in the release of the two identical strands of genomic RNA into the host cell cytoplasm. Currently, little is known about these processes during SRLV infection. Reverse transcription of ssRNA to dsDNA occurs next. Thought to occur via a similar mechanism seen in other retroviruses, it is initiated by tRNA lysine, an amino acid bound to the approximately 16 bp primer binding site flanking the 5' LTR of the ssRNA genome (Figure 1.1.3.1a) (Sonigo et al. 1985; Carey and Dalziel 1993; Burmeister 2001). RT then begins synthesis of the negative DNA strand from the primer binding site to the 5' end (Figure 1.1.3.1b). The RNA region of the resulting RNA-DNA hybrid is then removed from the RNA genome through RNase H activity (Figure 1.1.3.1c).

➔ **Figure 1.1.3.1 Reverse transcription of retroviral ssRNA genome to dsDNA.** Depicts stages of retroviral reverse transcription of genomic RNA to dsDNA for integration into host genome. Initial binding of tRNA lysine to primer binding site for - DNA synthesis (a), elongation via RT of - strand to 5' end (b), degradation of 5' end of RNA strand by RNase H (c), annealing of - strand to 3' R sequences of RNA strand (d), elongation of - DNA strand to 5' end (e), degradation of RNA strand by RNase H and binding of PPT RNA to DNA strand to initiate synthesis of + strand (f), elongation of + strand by RT to 5'end (g), removal of RNA primer elements via RNase H degradation (h), + strand transfer and annealing to - strand via PBS region (i), final elongation of both DNA strands via RT activity resulting in formation of full viral genomic copy of dsDNA (j). Black font and light blue represent RNA while red font and dark blue represents DNA. Adapted from (Heaton et al. 2012).


A
tRNA
R U5 PBS gag pol env PPT U3 R
Reverse Transcriptase
B
R U5 tRNA
R U5 PBS gag pol env PPT U3 R
RNase H
C
R U5 tRNA
PBS gag pol env PPT U3 R
Inter- or Intramolecular Strand
D
R U5 tRNA
PBS gag pol env PPT U3 R
Reverse Transcriptase
E
PBS gag pol env PPT U3 R U5 tRNA
PBS gag pol env PPT U3 R
RNase H
F
PBS gag pol env PPT U3 R U5 tRNA
PPT
Reverse Transcriptase
G
PBS gag pol env PPT U3 R U5 tRNA
PPT U3 R U5 PBS
RNase H
H
PBS gag pol env PPT U3 R U5
U3 R U5 PBS
Intramolecular Strand Transfer
I
PBS gag pol env PPT U3 R U5
U3 R U5 PBS
Reverse Transcriptase
J
U3 R U5 PBS gag pol env PPT U3 R U5
U3 R U5 PBS gag pol env PPT U3 R U5

The negative sense DNA strand next anneals to the 3’ end of either viral genome strand via the repeated (R) sequences present within the LTRs found at either end of the genome (Figure 1.1.3.1d). From here, RT elongates the DNA to form a complete DNA-RNA hybrid spanning from the PBS to the 3’ end (Figure 1.1.3.1e). RNase H mediated degradation of the RNA strand produces a single DNA strand to which the polypurine tract (PPT) binds to initiate synthesis of the positive DNA strand (Figure 1.1.3.1f). Following elongation, RNase H removes the initial RNA primers from both strands and the positive strand anneals to the 5’ end of the negative strand via the primer binding site, where the final elongation of each strand occurs producing a double stranded DNA copy of the viral genome (Figure 1.1.3.1g-j).

Upon producing a dsDNA copy of the viral genome, the next step is integration into the host genome. The process for SRLVs has yet to be confirmed but in other retroviruses integration occurs in two steps: end processing and joining (Hindmarsh and Leis 1999). During end processing, 2 nucleotides are removed from the 3’ end of each strand within the U3 and U5 regions of the LTRs exposing 3’ hydroxyl groups through a reaction involving a nucleophile, commonly water. This reaction in HIV infection is mediated by the IN protein and has been found to be a site-specific hydrolysis of a phosphodiester bond which results in the release of 2 nucleotides (Vink et al. 1991). During joining, IN mediates a nucleophilic attack via the exposed hydroxyl groups of target DNA, resulting in a simultaneous cleavage of target DNA and joining of 3’ ends of viral DNA to the 5’ end of cleaved target DNA (Engelman et al. 1991). This process results in 2 nucleotide overhangs at the 5’ ends of viral

DNA and single stranded regions of the target DNA and upon removal and repair of these regions, integration is complete.

Upon successful integration of viral DNA into the host genome, the next step in the viral life cycle is transcription, both for the production of viral proteins and to generate full length genomic RNA for packaging into virions. At this stage, the differences seen between *in vitro* and *in vivo* infection become more pronounced. *In vivo*, SRLV infection results in persistent infection suggesting a life cycle with minimal or no cell lysis. In comparison, *in vitro*, SRLV infection normally undergoes a lytic cycle which resolves within days of inoculation resulting in complete cell death. One reason for these apparent differences can be attributed to restrictions in expression of viral genes *in vivo*. This has been observed within immature monocytes harvested from the ventricles of experimentally infected sheep (Peluso et al. 1985). An increase in expression within these cells was observed following maturation of monocytes into macrophages (Gendelman et al. 1986). This change in expression level was partially linked to the LTR region of MVV, which can act to enhance expression. In another study, transgenic mice were used and showed that transcription directed by the LTR region was initiated following macrophage activation (Small et al. 1989). This therefore demonstrated a means by which MVV may be restricted during an infection which helps in maintaining persistent infection. The lytic cycle observed *in vitro*, suggests a lack of restrictive elements which can likely be attributed to the use of a single cell line. From these cells it has been shown that MVV has a temporally regulated pattern of transcription in which low levels of smaller mRNAs attributed to *vpr* and *rev* are produced early in infection (approximately 24 h.p.i.) with larger mRNA's being produced

later in infection (approximately 72 h.p.i.) (Gourdou et al. 1989). These larger species are believed to represent the structural genes (*gag, pol* and *env*) and *vif*. In addition, it has been reported that lytic infection results in high levels of amplification of viral genomes (Brahic et al. 1977). Crespo et al. (2013) looked at SRLV infection of two alternatively differentiated small ruminant macrophages, M1 and M2 cells, responsible for antimicrobial and pro-inflammatory responses short term or anti-inflammatory and immune suppressive responses long term, respectively. They found that SRLVs had reduced replicative ability in M1 cells and enhanced ability in M2 cells. This block, found to occur post-entry, may be associated with the presence of APOBEC proteins which have been shown to be expressed by M1 cell stimuli in small ruminants, similar to that seen in HIV-1 infection in humans (Cassol et al. 2009).

Finally, two copies of genomic RNA, structural proteins and essential proteins are packaged and leave the cell via budding either from the cell surface or into macrophage vacuoles (Georgsson et al. 1990).

1.1.4 Cellular Tropism

During natural infection *in vivo*, the monocyte/macrophage cell lineage and dendritic cells have been shown to be the main target of SRLVs (Ramírez et al. 2013). The monocyte/macrophage lineage appears to be a common target cell for the lentivirus genus, with some species also targeting lymphocytes (not a target of SRLVs). Initial infection by SRLVs occurs in monocytes where the virus becomes latent until cell differentiation into macrophages occurs. At this time, the expression of two cellular proto-oncogenic transcription factors, c-Fos and c-Jun, is enhanced. Previously, it has been shown

that these transcription factors bind to the AP-1 and AP-4 promotor binding sites present within the LTR of the viral genome triggering expression of proviral DNA resulting in replication and productive infection (Narayan et al. 1983; Shih et al. 1992). Therefore, the replication of SRLVs is dependent on the maturation of monocytes into macrophages.

The cellular receptors of SRLVs have been suggested to be a receptor (e.g. MHC-II or MR) present throughout the body, therefore the cell tropism of SRLVs is thought not to be solely determined by the presence of target receptors (Dalziel et al. 1991; Crespo et al. 2011). In support of this, Agnarsdóttir et al. (2000) found that a 53 bp region of the LTR cloned into chimeric virus in either single or duplicate copies presented varying ability to replicate within permissive cell lines (e.g. sheep choroid plexus cells and sheep fibroblasts) with viruses containing a single copy showing reduced productive capabilities compared to viruses containing duplicates. This finding suggests that the LTRs of SRLVs provide at least one determinant of cell tropism during infection.

1.1.5 Variability

With the steady increase in number of SRLV sequences over the years, the large variability between SRLV strains, as seen in other lentiviruses, has become more apparent. This variation can be mainly attributed to three mechanisms: mutation, recombination and selective pressure.

Mutations, whether they be missense, insertion, deletion etc. are critical for the persistence of lentiviruses within their host species as they give rise to the ability to evade the host's immune system. Of these mutations, most occur during the reverse transcription phase of the viral life cycle. This is due to

the RT possessing no proof-reading function which results in a high error rate quantified as being 0.2-2 mutations per genome per cycle (Ramírez et al. 2013). One potential factor influencing this rate of mutation is dUTPase. In both CAEV and FIV it has been shown that inactivation of dUTPase results in an increase in the mutation rate with an accumulation in guanine to adenine mutations which can ultimately result in production of non-viable viruses (Lerner et al. 1995; Turelli et al. 1997). In contrast to this, two SRLV isolates belonging to genotypes E1 and E2, naturally lack the dUTPase coding region within their genome but showed no increased rate of mutations or accumulation of guanine to adenine mutations compared to other natural strains (Reina et al. 2009a; Reina et al. 2010).

Recombination is the act of combining fragments of two different parental viruses into a new unique virus. This can occur both between two different strains of the same virus (e.g. MVV-MVV) and between two different viruses (e.g. MVV-CAEV). Within lentiviruses, recombination occurs in varying frequencies such as that seen between primate lentiviruses. Previously, recombination between HIV and SIV strains (HIV-1-SIV and HIV-2-SIV) has been shown to occur at high frequencies (Chen et al. 2006). While this recombination between viruses of different host species occurred at high frequencies, Motomura et al. (2008) reported recombination between HIV-1 and HIV-2, two viruses afflicting humans, to occur at low frequency. The causes of these discrepancies have yet to be assessed. With regards to SRLVs, recombination was demonstrated within naturally MVV and CAEV co-infected dairy goats (Pisoni et al. 2007b). Envelope sequence analysis clearly showed SRLV variants possessing sequence fragments belonging to both MVV and CAEV, shown by alignment of MVV and CAEV sequences

obtained from the same goat. In addition, Andrésdóttir (2003) has shown MVV strain 1514 to undergo frequent recombination within the envelope gene which has been suggested to contribute to antigenic variation of MVV.

Selective pressure refers to that applied upon an infecting virus by the host immune system. This can be attributed to the presence of quasispecies. First proposed by Manfred Eigen, quasispecies are defined as a set of viruses found in an infected individual (Eigen 1971; Ojosnegros et al. 2011). With mutation and recombination occurring at a constant pace, new quasispecies are being constantly produced and dominating the 'unevolved' previous species. But, these earlier forms are 'archived' as integrated DNA in the host genome and therefore can re-emerge resulting in a further increase in diversity of quasispecies. Pasick (1998) proposed the idea of treating MVV and CAEV as quasispecies instead of distinct viruses. Arnarson et al. (2017) demonstrated selective pressure. They found that during natural transmission there was the occurrence of positive selection of quasispecies possessing mutations within the neutralising epitopes therefore providing antigenic variance allowing for persistence of infection by immune evasion.

Taking the principal of quasispecies further, lentiviruses have been shown to compartmentalise within a single host with genetically different viruses present within different organs and systems of the body (Becquart et al. 2002). It has even been seen that quasispecies within these compartments to possess divergent cell tropisms and pathogenicity (Smit et al. 2004). Compartmentalisation of SRLVs has been suggested to occur within the peripheral blood and colostrum of goats and central nervous system (CNS), lungs and mammary glands of sheep (Pisoni et al. 2007a; Ramírez et al. 2012).

Despite these actions causing constant changes within the viral genomes of SRLVs there are regions highly conserved between strains present. These regions include the PBS, PPT and the RRE (Ramírez et al. 2013).

1.2 Maedi-Visna and Caprine Arthritis and Encephalitis

1.2.1 Natural History

The first identified cases of MV were during the Icelandic epidemic, 1933-1965, following the importation of 20 sub-clinically infected karakul sheep from Halle, Germany. These sheep sourced from a university farm were certified as being free from the known diseases of the time. Following a brief isolation period of 2 months, sheep were distributed across Iceland resulting in the spread of three untreatable, progressively fatal diseases: Jaagsiekte retrovirus (a beta retrovirus causing lung tumours), *Mycobacterium paratuberculosis* (Johnes disease, the cause of chronic gastrointestinal inflammation, diarrhoea and wasting) and MV. During the following years, jaagsiekte and paratuberculosis were detected in 1934 and 1938, respectively, while MV was not detected until 1939. Before this time, MV had never before been described and was found to present as two differing disorders: maedi and visna ('dyspnea' and 'wasting' in Icelandic, respectively) (Sigurdsson et al. 1952; Sigurdsson et al. 1957). In order to control and eradicate these diseases and therefore decrease the impact on its main source of agricultural trade; Iceland implemented quarantine zones and began an extensive depopulation and repopulation programme. These programmes resulted in the eventual eradication of jaagsiekte and MV from Icelandic sheep in 1952 and 1965, respectively,

which has since been maintained. In contrast, paratuberculosis has remained an issue to agriculture in Iceland.

Prior to the Icelandic outbreak, clinical signs consistent with MV were described in South Africa, 1915 and USA, 1923 and termed Graaff-Reinet disease and Montana progressive pneumonia, respectively. In both cases symptoms described coincided with those associated with the respiratory form of the disease (maedi). During the Icelandic outbreak, cases similar to MV were described as 'la bouhite' in France, 1940 and 'zwoegersiekte' in Holland, 1943.

It was however not until 1960 that the actual virus (MVV) was isolated from affected sheep (Sigurdsson et al. 1960).

With regards to CAEV, the disorder was initially observed in a herd of Toggenberg goats with adults suffering from an arthritic disorder and young kids with leukoencephalomyelitis (Cork et al. 1974). Initially, disorders were considered separate with further work into the disease in kids suggesting viral infection due to the transmissibility by inoculation with filtrate sourced from infected animals with the addition of the inability to isolate bacteria. Crawford et al. (1980a) later determined this virus to be a retrovirus and designated it CAEV with successful isolation of virus.

1.2.2 Clinical Signs

High genetic variability seen between SRLVs has contributed to the range of clinical signs seen in infected individuals. In sheep the clinical signs of MV are strain dependent while the presentation of caprine arthritis and encephalitis (CAE) in goats has been associated with the age of host animals. Despite this variation in clinical signs, it remains common that most infected

animals present as asymptomatic, especially in recent infections and upon appearance of clinical signs, disease progressively worsens to eventual death (Straub 2004).

Two main presentations of MV have been characterised: maedi and visna ('dyspnea' and 'wasting' in Icelandic, respectively) depending on the infecting strain of MVV (Narayan and Cork 1985). The most common form of MV, maedi, typically shows as wasting and progressive dyspnea with a possible dry cough. Less common signs associated with this form of disease include fever, bronchial exudates and depression (Sigurdsson 1954). Death of animals with this disease usually results from anoxia or secondary bacterial infections. In comparison, visna is less frequent, especially in the UK (although higher incidences seen in countries such as the USA). Onset normally begins insidiously with subtle neurological signs such as hind limb weakness, a trembling of lips or a head tilt (Sigurdsson et al. 1962). This is accompanied by a gradual loss in condition of the infected animal. Following this, disease progresses to ataxia, incoordination, muscle tremors, paresis and paraplegia. In rare instances, other neurological signs such as blindness are also seen. The clinical course of this form of MV from onset to severe neurological signs is approximately a year with animals, if unattended, usually dying of inanition. In addition to the clinical signs stated above, progressive arthritis with severe lameness and chronic indurative mastitis have been seen in animals affected by either form of MV (Sigurdsson et al. 1957).

CAE, as with MV, normally presents as one of two forms: an arthritic form mainly seen in adults and a neurological form seen mostly in kids (Narayan and Cork 1985). In kids, the neurological form of disease primarily occurs in kids aged between 2-6 months old. Initial presentation can include

lameness, ataxia, hind limb placing deficit, hypertonia and hyperreflexia, despite which kids appear bright and alert with no changes in eating or drinking (Cork et al. 1974). Following this the disease worsens to paraparesis, tetraparesis or paralysis. Other signs observed in affected kids include depression, a head tilt, blindness and nystagmus. Infection will normally result in either death by secondary cause (e.g. pneumonia or exposure) or euthanasia due to economic or welfare concerns. The arthritic form of CAE, the main clinical form of disease seen in adults, is characterised by a chronic polyarthritis normally accompanied by synovitis and bursitis (Crawford et al. 1980b). During early infection, lameness and distension of the joint capsule is seen, especially but not exclusively in the carpal joint, with progressive worsening over time. A loss in condition and dull coats have also been associated with diseased animals. As with MV, chronic indurative mastitis has also been seen in affected does with some cases resulting in agalactia at parturition (Lara et al. 2005). In addition, in cases in which goats have serological evidence of infection without any typical clinical signs, chronic interstitial pneumonia and progressive dyspnea have been observed, although, these cases may be associated with cross species transmission of MVV.

1.2.3 Pathology

Following successful infection, SRLVs locate to monocytes from where they are disseminated throughout the body and from which onset of disease begins. Unlike other viruses of the lentivirus group, including HIV, BIV and FIV, MVV and CAEV do not cause immunodeficiency. Instead, lesions caused by these viruses can be characterised by persistent inflammation and infiltration and proliferation of mononuclear cells in target

organs (Nathanson et al. 1976). In addition, high proviral loads have been found to correspond to lesions of higher severity.

Animals suffering from the respiratory form of disease show pathology of firm, dense, enlarged lungs that fail to collapse following opening of the thoracic cavity (Spickler 2015). In addition, lungs are typically discoloured with areas of consolidation or small white foci, although, this discolouring may not be obvious during early infection. Enlargement of the neighbouring lymph nodes, which may become edematous, is also common in both infections. Histologically, some variation between viral diseases is observed. In MVV infection, thickening of the alveolar septa due to infiltration and hyperplasia of the smooth musculature of the septa and of the epithelium within the bronchi and bronchioles following increased proliferation occurs. Fibrosis can also occur, although, is more commonly observed in severe cases (Georgsson and Palsson 1971). For animals suffering CAEV infection, chronic interstitial pneumonia can occur (higher prevalence in younger animals) as can enlargement of the alveolar septa. In addition, bronchopneumonia, perivascular cuffing and pulmonary alveolar proteinosis have also been known to develop.

The neurological forms of both diseases, with the exception of wasting of the carcass, typically will only show gross pathology within the brain and spinal cord. Macroscopically, focal brown/pink regions may be found within the white matter of the CNS and on the ventricular surfaces, although this is not always visible. In addition, the spinal cord may appear swollen and the meninges may show a cloudy discolouration. Histologically, inflammation and demyelination are common (Georgsson et al. 1982). It was found that early in infection, intensive inflammation is expected with aggregation of leukocytes within

the perivascular spaces (perivascular cuffing) and evidence of glial cell proliferation (gliosis). As a result of this inflammation, demyelination can occur within the white matter which in severe cases can result in extensive destruction of white matter within the cerebrum, cerebellum and other parts of the brain.

The arthritic form of disease is most common within adult goats suffering CAEV infection although MVV has also been shown to cause arthritis. Adams et al. (1980) studied the early stages of development of joint lesions following CAEV infection. One day post infection an increase in synovial fluid cell count was seen followed by the development of morphological changes in synovial membrane. Lesions then worsened from mild synovial cell hyperplasia and perivascular mononuclear cell infiltration to severe synovial cell hyperplasia and mononuclear cell infiltration with villous hypertrophy. In addition, CAEV has been shown to mainly target the carpal joints within goats with the tarsal joints being targeted less frequently. Ultimately, infection can lead to severe destruction of cartilage, ruptured ligaments and tendons and the formation of periarticular osteophytes (bone spurs). In comparison, SRLV associated arthritis in infected sheep has been shown to be much milder.

Indurative mastitis in infected animals has been characterised by mononuclear infiltration of the periductular stroma which results in the destruction of normal mammary tissue. Interestingly SRLVs have been shown to be associated with mammary epithelial cells within tissue with the permissive nature of these cells being confirmed in primary culture and immortalised cell lines (Lerondelle et al. 1999; Mselli-Lakhal et al. 2001; Bolea et al. 2006).

Lesions have also been noticed to be present within the kidneys of infected animals with evidence of vasculitis upon microscopic analysis (Angelopoulou et al. 2006). Finally, Pálfi et al. (1989) characterised lesions within testicles of MVV infected rams. In this study infiltration of the interstitium of the testicles by lymphocytes, histiocytes and plasma cells, fibrosis and atrophy of seminiferous tubules with resultant impacts on spermatogenesis were suggested to be associated with MVV infection. Interestingly, despite disturbances in spermatogenesis, semen was still demonstrated to be capable of transmitting MVV (Cutlip et al. 1981).

1.2.4 Treatment

To date, no successful treatment option has been recognised for MV or CAE. It is possible to ease the clinical signs associated with disease by supportive therapy, but this would not be able to treat the causal agent, SRLV infection and cannot prevent disease progression. With HIV in humans, the use of antiretroviral therapies (ARTs) maintains viral suppression, preventing propagation of virus and progression of disease and has been largely successful in controlling clinical signs and prolonging life expectancy to near normal (Raffi et al. 2016). The expense and difficulty of using these drug therapies has however largely precluded their use in domestic animals.

1.2.5 Vaccines

The inability to treat SRLV infection within small ruminants highlights the importance of preventative measures. One of the most commonly used examples of these are vaccines, used to prime the host immune system, providing protection against future infection. Unfortunately for SRLV, successful vaccines have proved elusive. There have been many studies attempting

to identify functional vaccines that provide reliable protection against SRLV infection. To date, multiple vaccine production systems have been tested including inactivated or attenuated virus, viral clones and recombinant plasmids (Reina et al. 2009b).

Two of the first vaccines tested for both MV and CAE were inactivated virus vaccines (McGuire et al. 1986; Cutlip et al. 1987). In both studies the vaccines did not provide protection following challenge with virus, with data from the CAEV vaccine trial suggesting that vaccination facilitated infection with animals developing arthritis more rapidly. This ability to aid viral infection was also seen in a MVV vaccine containing the MVV *gag* gene (Torsteinsdóttir et al. 2007). Animals were vaccinated a total of 8 times over 30 months after which they were challenged with MVV intratracheally. All animals showed a strong rise in antibody titres indicative of infection 2-3 weeks post challenge, much sooner than expected suggesting that vaccination may have again facilitated infection in host animals. Another vaccine trialled in goat was a recombinant vaccinia virus expressing CAEV surface and transmembrane envelope glycoproteins but provided no protection from intravenous challenge (Cheevers et al. 1994). Partial protection has been demonstrated for an attenuated MVV clone mucosal vaccine (Pétursson et al. 2005). Partial protection was characterised by superinfection following challenge after which a reduced frequency in isolation of virus from the blood and lungs was seen in vaccinated animals compared to unvaccinated. This inability to provide complete protection from SRLV infection further limits the preventative tactics available in reducing prevalence.

1.2.6 Genetic Susceptibility

Absence of a curative treatment or available vaccines requires other means of controlling SRLV infection. The identification of genetic traits that provide improved resistance against infection could provide one such avenue. It has long been recognised that the rate of transmission of MVV is lower in particular breeds of sheep. Studies in Iceland identified that specific bloodlines within the Icelandic breed possessed resistance to disease expression, this resistance was more pronounced following cross breeding of the Icelandic breed with Border Leicester breeds (Pálsson 1976). This resistance was not against infection but to the onset of disease with these breeds showing slower disease progression. Following this, Cutlip et al. (1986) compared the susceptibility of two breeds, Border Leicester and Columbia. By comparison of the frequency and severity of clinical signs and lesions attributed to infection it was found that Border Leicester were more susceptible to infection when compared to Columbia sheep. The underlying reasons for these apparent differences are still yet to be fully understood, however modern molecular methods are shedding some light on this.

Following a genome wide association study (GWAS) of naturally infected ewes with the intent of identifying any genetic associations with SRLV infection, the transmembrane protein gene (TMEM154) was identified (Heaton et al. 2012). The role of TMEM154 has yet to be ascertained, although a GWAS in humans looking at asthma severity identified a SNP within the TMEM154 gene that was associated with an increase severity (Li et al. 2010). This link to asthma may suggest a link between TMEM154 and airway immunity in humans. As the human protein shares only 67% amino acid identity with the ovine

protein it is also possible that this link may not be present within sheep. Analysis at the nucleotide level within 40 breeds of sheep revealed ten missense and two frameshift mutations that occur within the TMEM154 gene. In combination these mutations result in 12 different haplotypes (1-4, 6, 9-15) which have currently been identified (Table 1.2.6.1). Since then, Yaman et al. (2019) has recently identified a 13th haplotype within german flocks (will be referred as 16). Of these, the impact on susceptibility is only known for three (1, 2 and 3) (Heaton et al. 2013). Haplotype 3 was reported as being the ancestral allele, this is apparent upon comparison of haplotype sequences in which all variations deviate from the ancestral sequence by a maximum of two mutations (Table 1.2.6.1). Leymaster et al. (2015) compared the incidence of MVV infection between ewes with TMEM154 diplotypes "1 1", "1 3" and "3 3" in a natural exposure setting. It was found that after a period of 39 months approximately 10% of animals with diplotype "1 1" were infected compared to the approximate 90% prevalence seen in the remaining two groups. TMEM154 diplotype "1 1" therefore seems to provide resistance to infection. At the amino acid level, haplotype 1 differs from haplotype 3 by a missense mutation causing a change from glutamate (E) to lysine (K) at position 35. As this is the only difference between haplotypes this suggests that this change from E to K is key for resistance to MVV infection. In addition to haplotype 1, this missense mutation is also present within haplotype 10 which might suggest that this haplotype also provides resistance, but this is yet to be tested. Heaton et al. (2012) also sequenced TMEM154 genes from domestic and mountain goats. These sequences showed no variation from the ancestral sheep haplotype at the locations previously mentioned. This in combination with the high similarity of CAEV and MVV suggests

Table 1.2.6.1 Amino acid sequence mutations in TMEM154 haplotypes against ancestral sequence. Missense and frameshift mutations present in 12 TMEM154 amino acid sequences (1, 2, 4, 6 and 9-16) when compared against the ancestral haplotype (3). All haplotype variation deviates from the ancestral sequence by a maximum of 2 mutations. Blue region denotes the amino acid position associated with resistance to SRLV infection as seen in haplotype 1. Adapted from (Heaton et al. 2012).

	TMEM154 aa Position												
	Exon 1				Exon 2								
Haplotype	4	13	14	25	31	33	35	38	44	70	74	82	102
3	R	A	L	T	E	D	E	G	T	N	I	E	I
1	-	-	-	-	-	-	K	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	I	-	-	-
4	AΔ	-	-	-	-	-	-	-	M	-	-	-	-
6	-	-	-	I	-	-	-	-	-	-	-	YΔ	-
9	-	-	-	-	-	N	-	-	-	-	-	-	-
10	-	-	H	-	-	-	K	-	-	-	-	-	-
11	-	-	-	I	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	F	-	-
13	-	V	-	-	-	N	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	T
15	-	-	-	-	Q	-	-	-	-	-	F	-	-
16	-	-	-	-	-	-	-	R	-	-	-	-	-

that TMEM154 does not provide resistance in goats although this might not be representative of the global population.

The frequency of TMEM154 genotypes was determined for 2759 sheep representing 74 breeds worldwide (Heaton et al. 2013). From this data, a predicted value for the susceptibility to MVV infection was determined for these breeds. Those with the highest predicted susceptibility included Deccani, Chios and Scottish Texel Breeds while breeds with the lowest predicted susceptibility included animals of Valley Red Sheep, Rambouillet and Dorset Horned breeds. Multiple studies have attempted to quantify the relative risk of infection when comparing diplotypes (Heaton et al. 2012; Molaee et al. 2018; Yaman et al. 2019). These studies suggested on average an infection risk 2-3 fold lower in animals with resistant diplotypes when compared to those possessing 1 or more susceptible haplotypes. Greater variation within these studies has been suggested to be due to experimental factors such as sheep breed and circulating virus strain.

C-C chemokine receptor type 5 (CCR5), is a G protein-coupled receptor (GPCR) with roles in chemotaxis and immunity. In humans, it has been identified as a co-receptor for HIV-1 infection alongside the cluster of differentiation 4 (CD4) glycoprotein (Deng et al. 1996; Dragic et al. 1996). Interestingly, a 32bp deletion was identified that gave resistance to HIV-1 infection in those homozygous for this mutation (Liu et al. 1996). This deletion occurs within the transmembrane domain of CCR5 causing a frameshift at amino acid 185 which produces a premature stop codon that results in the formation of a non-functional protein. Initially thought to provide almost complete protection against protection, cases of

HIV infection within homozygous individuals have since been reported (Balotta et al. 1997; O'Brien et al. 1997).

White et al. (2009) determined the sequence for ovine CCR5 using primers derived from the bovine CCR5 sequence and found it to share 83.5% identity with human CCR5. They also identified a 4bp deletion within the octamer protein binding site within the promotor region which they found in homozygous individuals and resulted in reduced proviral loads during infection. This interaction requires further investigation, especially for the reason that CCR5 has been previously ruled out as a potential receptor for MVV infection (Lyall et al. 2000; Hötzel and Cheevers 2002). Later work has thus far failed to confirm a link between this ovine CCR5 deletion and reduced proviral loads, in fact the opposite was seen with increased infection risk in certain breeds (Alshanbari et al. 2014; Molaee et al. 2018). Research has since continued into the relationship of CCR5 and SRLV infection due to lack of a definitive answer, resulting in the recent discovery of a single nucleotide substitution within caprine CCR5 associated with an increased proviral load within goats infected by CAEV (Colussi et al. 2019). The caprine CCR5 amino acid sequence showed 98% identity with ovine CCR5. This in conjunction with the high similarity between SRLVs as a group suggests this variant nucleotide sequence influencing CAEV infection may be present within sheep and MVV infection.

1.2.7 Transmission

Asymptomatic persistent infection associated with SRLVs highlights the importance of preventative measures to limit the risks of infection of healthy animals. In order to develop such strategies, the dynamics by which viruses are transmitted must

be understood. For SRLVs, the routes of transmission from infected individuals to naïve animals currently identified include inhalation of respiratory secretions, ingestion of colostrum or milk and faecal contamination of drinking water (Brodie et al. 1998; Blacklaws et al. 2004).

The respiratory route has traditionally been regarded as the main route by which horizontal SRLV transmission occurs within sheep and goats. Close contact between individuals is required to allow for successful transmission, with confined unventilated spaces as seen in indoor housing providing optimal conditions (Leginagoikoa et al. 2010). To reduce this, it has been shown that for outdoor enclosures a gap of two metres between infected and non-infected animals is sufficient in preventing transmission. McNeilly et al. (2007) demonstrated that cell-free MVV was sufficient in transmitting infection especially when instilled into the lower lung. Following this it was also found that MVV associated with alveolar macrophages was able to transmit infection but only following instillation into the lower lung (McNeilly et al. 2008). This same study also looked at the role of alveolar macrophages in transmission of the virus from the lung to the rest of the body. From this it was found that alveolar macrophage migration after infection did not play a role in transferring virus to the rest of the body suggesting that this occurred via an intermediate route.

The next major mode of transmission of SRLVs is the ingestion of colostrum or milk from infected ewes/does. As previously described SRLV infection can target mammary tissue causing chronic mastitis in both sheep and goats (Cutlip et al. 1985; Gregory et al. 2009). In addition, multiple studies have also demonstrated the presence of SRLVs within colostrum and milk within both cell-free milk and milk derived cells (Adams et al.

1983; Leroux et al. 1997; Álvarez et al. 2006). It is suggested that this route of transmission is of more significance in SRLV infections when compared to other lentiviruses such as SIV and HIV due to the increased permeability of the kid/lamb's digestive tract following birth (Preziuso et al. 2004; Pisoni et al. 2010). The cells responsible for harbouring virus within milk have been suggested as being macrophages and epithelial cells, two cells previously described as being permissive to SRLV infection. Transmission via colostrum or milk has been demonstrated for both MVV and CAEV (Adams et al. 1983; Pépin et al. 1998). Interestingly, when comparing SRLV strains A10 and B1, Pisoni et al. (2010) found differing efficiencies between strains for transmission by milk or colostrum. Álvarez et al. (2005) quantified the significance of colostrum in transmission of MVV, comparing lambs suckling from seropositive dam or bottle-fed colostrum from seropositive ewes. When comparing percentage of seropositive animals to a control group fed bovine colostrum, a 16% increase in the number of seropositive animals was seen when lambs were suckled by dams whilst those bottle fed showed an increase of 29-61%. It was suggested that this greater risk in bottle fed animals may be due to bottle fed animals having a larger colostrum intake compared to those suckling or due to bottle feeding increasing the risk of inhalation of colostrum leading to respiratory transmission of MVV (Houwers 1990). Cutlip et al. (1981) have also proposed transplacental transmission to occur within animals following isolation of virus from ovine fetuses and newborn lambs which may also play a part in the previous study's findings.

Following the identification of pathological lesions within the testes of rams infected with MVV, the possibility of sexual

transmission was raised (Pálfi et al. 1989). Sexual transmission is not a new concept in lentiviruses and is a common occurrence in primates infected with SIV or HIV-1 (Zhang et al. 1999; Haase 2011). In addition, there is evidence suggesting occurrences within feline immunodeficiency virus (FIV) infected cats (Jordan et al. 1995). Sexual transmission of SRLV in sheep and goats was further supported by the finding of proviral DNA within semen and the reproductive tract of infected rams and bucks (Ali Al Ahmad et al. 2008; Peterson et al. 2008). Further, Ali Al Ahmad et al. (2012) artificially inseminated seronegative does intrauterine with semen containing CAEV with virus being subsequently detected in uterine smears, flushing media and uterine swabs suggesting again the potential for sexual transmission. This finding was later confirmed in a study that saw 60% of intrauterine inseminated does seroconverting thirty days post insemination (Souza et al. 2013). It is important to note that intrauterine insemination bypasses many innate defences present within the reproductive tract that may prevent transmission. Therefore, further study is required to confirm the potential risks of sexual transmission both with artificial insemination (AI) and with natural mating. In addition, the possibility of sexual transmission alongside the observed seroconversion of artificially inseminated animals, the potential transmission of virus when using reproductive biotechnologies such as embryo transfer and IVF is a concern (Cortez-Romero et al. 2013).

1.2.8 Diagnostic Tests

Accurate diagnostic tools are critical for the efficient identification of infected animals and are essential for an effective control strategy. The two currently prescribed tests for international trade are the agar gel immunodiffusion (AGID)

and a variety of enzyme-linked immunosorbent assays (ELISA) for serological diagnosis (OIE 2016). While the AGID is specific, reproducible and easy to perform, interpretation of results can be difficult without experience. In contrast, ELISA's are more economical and quantitative with an ability to be automated providing an advantage when testing large quantities of sera. Both tests detect the presence of specific anti-viral antibodies with their specificity and sensitivity directly linked to the viral strain used for the assay, the viral antigens used and the standard of the comparison assay used (e.g. western blot or radio-immunoprecipitation). Other laboratory techniques employed for diagnosis include polymerase chain reaction (PCR), western blot and radioimmunoprecipitation (RIPA) with the latter two being implemented mostly as confirmatory tests (Herrmann-Hoesing 2010).

In the UK, the current commercially used testing kits are the CAEV/MVV p28 Antibody Screening Kit (IDEXX), an indirect ELISA (iELISA) based on the immunogenic peptide, TM, and the recombinant protein, CA. The CAEV/MVV Total Antibody Test (IDEXX) and the ID Screen® MVV/CAEV Indirect ELISA Test (ID.VET) which targets a panel of TM peptides, the envelope glycoprotein gp135 and major core protein, p25. In addition, there have been over 30 different ELISAs reported for SRLV diagnosis. Of these the majority are iELISAs with a few examples of competitive ELISAs (cELISAs) using monoclonal antibodies being reported (Houwers and Schaake 1987; Schalie et al. 1994; DeMartini et al. 1999; Fevereiro et al. 1999). The ability of ELISAs to detect CAEV and MVV in milk also potentially enables a less invasive sampling strategy than the blood tests currently used in most programmes (Plaza et al. 2009; Brinkhof et al. 2010).

PCR can also be a reliable tool for diagnosing SRLVs from a variety of sources including blood, tissue, milk and semen (Ali Al Ahmad et al. 2008; Brinkhof et al. 2010). The limitations with PCR relate to the specificity of primers required for successful amplification. These require an up to date sequence of the infective strain to maximise probability of detecting virus. In addition, the constant mutations occurring within the SRLV genome can prevent primer binding and therefore prevent any amplification and subsequent diagnosis. Currently PCR is mainly used as a confirmatory test following ELISA or AGID (de Andrés et al. 2005). Carrozza et al. (2010) have designed two probe based real time PCR assays targeting the *gag* and *pol* genes of the EV1 strain of MVV.

These diagnostic tools are critical for the control of SRLV spread as they allow identification of infected animals which enables quick actions to be put in place to limit the impact of infection on a flock/herd.

1.2.9 Control Programmes

Due to the lack of vaccine or treatment available for SRLV infection, governments and producer bodies worldwide have introduced control schemes to limit transmission and reduce national prevalence. Countries included in this number are the UK, Germany, Norway, Switzerland, Canada and the USA.

The MVV and CAEV accreditation schemes (MVAS and CAEAS), currently in place within the UK, are completely voluntary schemes which aim to help reduce the prevalence of disease (SRUC 2020). However, Ritchie et al. (2010) when comparing seroprevalence within the country from 2010 to those recorded in 1995 found that individual animal prevalence had quadrupled, suggesting the current accreditation scheme to be

ineffective. It is worth noting that the farms sampled during these surveys were mostly a part of the accreditation scheme therefore producing bias towards virus free flocks which may have resulted in an under estimation of the actual UK prevalence. In addition, the voluntary nature of the schemes results in the majority of participating farms holding high value flocks e.g. rare breeds or breeding stocks. This is due to higher degree of economic impact on these farms with smaller holdings declining testing due to the monetary requirements necessary to take part in the schemes. This therefore means that viral presence is unchecked within these animals resulting in persistence within the national flock/herd.

This is similar to the situation previously seen in Switzerland who implemented a CAEV eradication programme in 1984 on a voluntary basis (Peterhans et al. 2004). The scheme was made mandatory as of 1998 and involved annual serological testing for all goats. Seropositive animals were culled, and the source farms quarantined until three consecutive negative results from all adult animals. The scheme resulted in the initial flock prevalence of 83% dropping to 1% in 2002. This therefore suggests that a mandatory scheme is necessary in the UK to truly combat the prevalence of SRLV infection.

For MV, flocks wishing to join the MVAS must be situated in Great Britain and must ensure that the flock meets and abides by the strict requirements and rules of the MVAS. For a flock to be accredited they must initially undergo two qualifying ELISA tests carried out 6-12 months apart with all animals older than 12 months being tested. Upon accreditation, flocks must be tested within 12 months, after which, providing results are negative, the next periodic testing must occur within 2 years. Following this the time interval between testing is dependent on

the presence of non-accredited animals on holdings. The number of animals tested following accreditation is dependent on flock size (Table 1.2.9.1). In situations where contact occurs with non-accredited animals, the MVAS requires isolation of any such individuals for a period of 6 months and subsequent testing to ensure negative status. In cases in which an animal shows seroconversion, removal and a further 6 months of isolation is required for those remaining. Should any animals be returned to flock without adhering to these terms then accredited status is removed. Further to these terms, the MVAS requires strict animal handling conditions, especially in holdings possessing both accredited and non-accredited animals. These include boundaries to prevent any stray animals from coming into contact with the flock, no shared unventilated spaces (e.g. barns), a 2 metre divide between accredited and non-accredited animals, no use of shared equipment between groups and only embryos/semen from accredited animals can be used for impregnation/insemination. Transport of animals is also highly regulated; accredited and non-accredited animals cannot be transported in the same vehicle, with vehicles previously used to transport animals having to be cleaned and disinfected before use by accredited flock. For goats, the CAEAS is identical to the MVAS except in that it refers to goats, accredited and non-accredited animals must be separated by a distance of 3 metres and at temporary locations a 3 metre tall solid barrier is required to separate animals.

1.2.10 Geographical Distribution

Following initial discovery in 1939 and 1974, SRLV infection has since been shown to be present worldwide, affecting all

Table 1.2.9.1 Sample sizes based on flock size for regular testing as part of the MVAS.

Total Number of ewes and rams aged 18 months or older	Sample Size
1-55	All
56-60	55
61-65	60
66-70	65
71-80	70
81-90	75
91-100	80
101-120	85
121-140	90
141-160	95
161-180	100
181-200	105
201-250	110
251-300	115
301-350	120
351-400	125
401-500	130
501-700	135
701-900	140
901-1000	145
1000+	150

continents apart from Antarctica. In addition, the distribution demonstrated by available reports appears random with countries showing varied presences of virus (Figure 1.2.10.1). Excluding Iceland, which has reported eradication of SRLVs 93 countries have reported cases of SRLV infection (16 MVV, 32 CAEV and 45 both). It is important to note that no reports do not mean that SRLVs are not present within these countries, as many do not test. Testing might not be carried out for varying reasons such as low economic importance, absence of clinical signs or lack of awareness of the disease.

1.2.11 Economic Impact

Several studies have characterised the impacts of SRLV infection on small ruminant production systems. Within milk production systems research has shown contradicting impacts on milk yield with studies reporting decreased, unchanged or increased milk yield within differing populations of sheep and goats during SRLV infection (Nord and Dnøy 1997; Leitner et al. 2010; Lipecka et al. 2010). Therefore, the true impact of SRLV on milk yield during SRLV infection is unclear and requires further investigation.

SRLV infection in young animals is also an important aspect of disease impact. Kids suffering from CAE can develop neurological disease which, depending on severity can lead to the culling of kids on the grounds of welfare. This therefore leads to a loss in future profit associated to these animals. In MV, disease in lambs is less pronounced with infected animals presenting with a reduced growth rate (Keen et al. 1997).

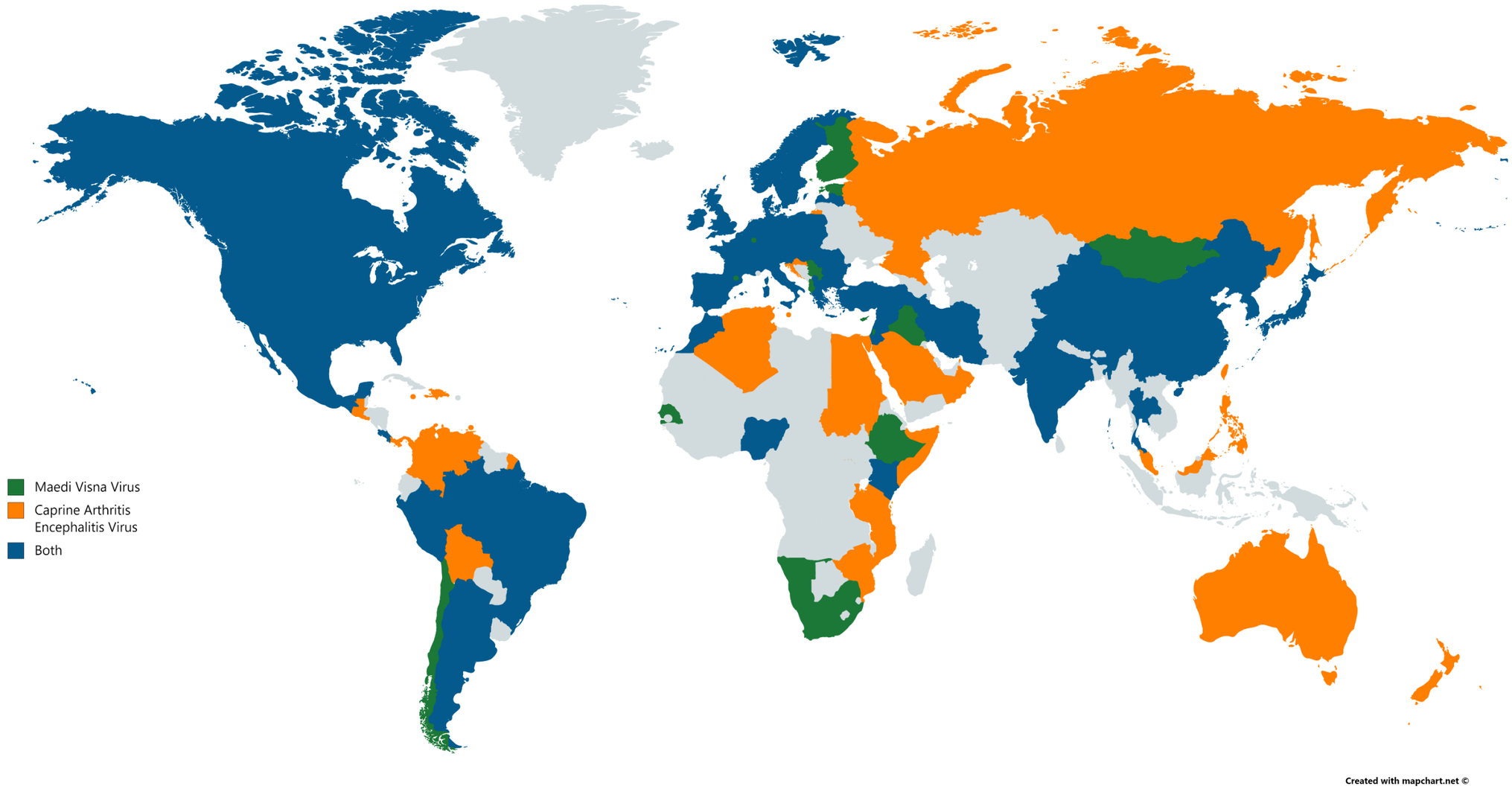


Figure 1.2.10.1 Worldwide distribution of small ruminant lentiviruses. World map illustrating countries with reported evidence of small ruminant lentiviruses (SRLV) presence. Colours denote viral species reported; maedi-visna virus (green), caprine arthritis encephalitis virus (orange) or both (blue). Evidence sourced from (Straub 1970; Wandera 1970; Süveges et al. 1973; Hugoson 1978; Crawford and Adams 1981; Eguiluz and Aluja 1981; Oliver et al. 1982; Adams et al. 1983; Caporale et al. 1983; Snyder et al. 1983; Adams et al. 1984; Belino and Ezeifeka 1984; Gonzalez Angulo et al. 1984; Mahin et al. 1984; Dawson and Wilesmith 1985; Adair 1986; Payne et al. 1986; Agrimi et al. 1987; Gonzalez et al. 1987; Houwers et al. 1987; Houwers and van der Molen 1987; Surman et al. 1987; Grant et al. 1988; Mogollon Galvis et al. 1989; Pereira et al. 1989; Alluwaimi et al. 1990; Baumgartner et al. 1990; Kita et al. 1990; Krieg and Peterhans 1990a; Sargan et al. 1991; Giangaspero et al. 1992; Giangaspero et al. 1993; Hötzel et al. 1993; Burgu et al. 1994; H. 1994; Leroux et al. 1995; Celer and Ni~mcova 1997; Sung and Chol 1997; Valas et al. 1997; Nord et al. 1998; Daltabuit Test et al. 1999; Masalski et al. 1999; Sihvonen et al. 1999; Masalski 2000; Schaller et al. 2000; Ayelet et al. 2001; Ravazzolo et al. 2001; Fevereiro et al. 2002; Robles and Layana 2003; Barros et al. 2004; Konishi et al. 2004; Sz. Kusza 2004; Karanikolaou et al. 2005; Al-Qudah et al. 2006; Christodoulopoulos 2006; Shuljak 2006; Vidic et al. 2008; Fallas et al. 2009; Ghanem et al. 2009; Hananeh and Barhoom 2009; Kaba et al. 2009; Mitrov et al. 2009; Sidelnikov et al. 2009; Elfahal et al. 2010; Noordin et al. 2010; Lin et al. 2011; Sakhaee et al. 2011; Huang et al. 2012; Oem et al. 2012; Paethaisong et al. 2012; Tageldin et al. 2012; Gudnadóttir et al. 2013; Kuhar et al. 2013b; Max et al. 2013; Muz et al. 2013; Santry et al. 2013; Tolari et al. 2013; Zhang et al. 2013; Oguma et al. 2014; Norouzi et al. 2015; Padiernos et al. 2015; Tabet et al. 2015; Tariba et al. 2015; Villagra-Blanco et al. 2015; Waseem et al. 2015; Enache et al. 2016; Gumusova and Memıs 2016; Linderot de Cardona et al. 2016; Hamza and Özkan 2017; Tabet et al. 2017; YANG et al. 2017; Barták et al. 2018; Michiels et al. 2018; Davaasuren et al. 2019; Enache et al. 2019; Idres et al. 2019; Cana et al. 2020; Itzcoatl Martínez-Herrera et al. 2020)

In addition, SRLV infection has been shown to reduce fertility within infected does and ewes, impacting both dairy and meat production markets (Burmeister 2001). In cases of successful pregnancy and birth SRLV infection has then been shown to impact upon birth weight and subsequent growth rate of the offspring, by reducing both (Dohoo et al. 1982; Arsenault et al. 2003; Peterhans et al. 2004).

Gibson et al. (2018) investigated further the monetary losses that could be attributed to an outbreak of SRLV infection in sheep. The study followed an outbreak in a flock of 825 breeding stock in size. Over a four-year period following initial outbreak, the study reported a cumulative loss of £131,953. It is important to note that these costs may be further enhanced depending on purpose of flock (i.e. dairy, meat or breeding).

1.3 Aims and Objectives

Over the course of this project, four aims were put forward for investigation:

1. Development of a more reliable and cost-effective diagnostic for detection of MVV strains within the UK.
2. Quantification of the risk of MVV transmission following intravaginal insemination using semen collected from naturally MVV infected rams.
3. Longitudinal case study of morbidity and mortality due to MVV infection in naturally infected rams.
4. Estimation of the impact of MVV infection on milk production and SCC within a UK dairy flock.

Development of a more reliable and cost-effective diagnostic for detection of MVV strains within the UK

Despite current schemes in place to control the spread of MV and CAE in the UK, Ritchie et al. (2010) calculated the prevalence of MV to have nearly quadrupled over the course of 15 years (1995-2010). Although this can in part be associated to the voluntary nature of the scheme it can also be attributed to the inability of current diagnostic tests to identify all strains of MVV due to the high variability, characteristic of lentiviruses. To try and combat this the first aim of this project was to develop a more reliable and cost-effective PCR based diagnostic test for the detection of MVV infection.

Quantification of the risk of MVV transmission following intravaginal insemination using semen collected from naturally MVV infected rams.

Ali Al Ahmad et al. (2012) previously demonstrated transmission of SRLV via intrauterine insemination with semen proven to contain virus. Although this highlights the risk of sexual transmission, intrauterine insemination bypasses the natural innate defences of the reproductive tract and therefore cannot be used as a reliable model for natural mating. Therefore, the second aim of this study was to quantify the risk of MVV transmission following intravaginal insemination of a group of naïve ewes using semen from naturally infected rams.

Longitudinal case study of morbidity and mortality due to MVV infection in naturally infected rams.

In 2015, the University of Nottingham acquired 28 naturally MVV infected rams. Over the course of 2 years, blood samples and tissue samples following sudden death/euthanasia were collected. Using these samples, the third aim of this study was to quantify the long-term impact of MV by a longitudinal case study of morbidity and mortality due to disease within these individuals.

Estimation of the impact of MVV infection on milk production and SCC within a UK dairy flock.

Finally, an opportunistic data set was received from a flock of 319 dairy ewes identified as MVV infected during routine serological screening. Data provided included milking history, somatic cell counts (SCCs) and individual ewe characteristics. To date, conflicting reports have been made regarding the impact of SRLV infection on milk production (Nord and Dnøy

1997; Leitner et al. 2010; Martínez-Navalón et al. 2013). In addition, SRLV have been shown to cause variation in SCC between seronegative and seropositive individuals with further differences between breeds (Lipecka et al. 2010). Therefore, the final aim of this study was to estimate the impact of SRLV infection on milk production and SCC within this flock by way of multivariable regression modelling.

Chapter 2: Development of qPCR Protocol for Quantification of Maedi-Visna Virus

2.1 Introduction

A previous study into the prevalence of SRLV infection within the UK has suggested that the number of infected individuals to be on the rise (Ritchie et al. 2010). Between 1995 and 2010, seroprevalence of MVV was reported to have nearly quadrupled (0.19% -> 0.74%), a rate of increase that if sustained would result in a seroprevalence of 1.11% in 2020. Despite this dramatic increase in prevalence rates no further studies of current seroprevalence have been published within recent years.

Introduced in 1982, the current MVV/CAEV accreditation scheme within the UK is not efficient enough in preventing this increase in prevalence (SRUC 2020). The degree to which the scheme reduces the rate of spread is unknown but the current rise in seroprevalence suggests a need for enhancement (Ritchie et al. 2010). Several factors can be identified that may contribute to this inability to reduce prevalence such as the voluntary nature of the current scheme, lack of reports on quantification of cost benefits, or viral strain variability (Ramírez et al. 2013; Ogden et al. 2019).

As of 2017, 6056 sheep flocks and goat herds are listed as participating in the scheme within the UK (SRUC 2017). In comparison, the total number of sheep holdings in the UK in 2015 was 72,272 (NFU 2017). This difference, likely due to the scheme being voluntary is likely a large contributor to persistence of SRLV presence within the UK, with unaccredited

flocks and herds acting as viral reservoirs. Farmer perspective is of great importance in this regard. One such factor of importance to individuals is the costs associated with acquiring accreditation as opposed to the benefits of being free of virus. One recent study quantified the losses associated with MVV breakdown and found losses of £132,000 over a 5-year period in a flock originally consisting of 800 individual breeding ewes (Gibson et al. 2018).

Accreditation provides several benefits including entrance to accredited only shows and sales, advertisement of accredited status to purchasers, increased value of accredited stock and allowance for export to certain MV/CAE free countries (SRUC 2020). These benefits are likely to be of more advantage to larger, high value flocks such as pedigrees flocks with limited interest for small holdings such as hobbyists. These small isolated flocks can act as viral reservoirs which will not be detected using a voluntary accreditation scheme and therefore can contribute to viral persistence on a national level.

To date, only a single full genome MVV sequence has been reported within the UK (EV1 strain) (Sargan et al. 1991). Initial identification occurred in 1991, approximately 30 years ago. Given the rate of mutation and ability of virus to recombine, it is possible that this strain as sequenced in 1991 is no longer circulating naturally within the UK population (Ramírez et al. 2013). Therefore, diagnostics designed targeting this strain of virus may prove ineffective and provide false results highlighting the importance of identifying viral strain within outbreaks for such variable viruses as SRLVs. Further to this, the most commonly used diagnostic tests, ELISA, AGID and PCR each require specific components designed to work at peak efficiency with a specific strain of virus (Fevereiro et al. 1999;

Carrozza et al. 2010). This may be combated through production of multiple testing kits for detection of differing strains, testing of larger sample sizes per flock (to account for strains that are detected sub-optimally by the current tests) or constant adaptation of current diagnostic tests to match circulating strains at specific times. Although it is important to note the costs associated with such changes which in many cases render this economically unfeasible.

In this chapter, the objective was to design and develop a qPCR based diagnostic assay for detection and quantification of SRLV viral loads within a naturally infected group of breeding rams within the UK. The virus strain was unknown and therefore deep sequencing technologies were implemented to allow for identification and classification of circulating virus within these animals. This work also allowed for the assessment of feasibility for design and creation of a broad-spectrum qPCR for efficient and reliable detection of all circulating SRLV strains.

2.2 Materials and Methods

2.2.1 DNA Extraction

DNA was extracted from blood using the Nucleospin® Tissue Kit (Macherey-Nagel) for detection of MVV using PCR based methods.

Blood clots were processed by following the supplementary protocol for extraction of genomic and viral DNA from blood samples. Before the protocol was carried out, a small amount (approximately 25 mg) of blood clot was added to 200 µl of PBS. A sterile 5mm steel bead was then added and mixture homogenised by a Retsch MM300 bead mill (Qiagen) at a frequency of 25/s for 2 minutes. The protocol was then followed substituting homogenised blood clot and PBS for 200 µl of fresh blood. DNA was eluted from the column in a final volume of 60 µl. DNA was stored at -20℃ until use.

Successful DNA extraction was confirmed by quantification of DNA content using the NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific, UK).

2.2.2 Primer Design - 1

Primers were designed for PCR and qPCR and sequencing using primerBLAST software (NCBI). Reference viral sequences were attained from the GenBank genetic sequence database (NCBI). Twenty-two full length SRLV sequences and 681 partial sequences were aligned as whole virus and by gene segment by MUSCLE using CLC sequence viewer software (Appendix 1). From this, alignment primers were designed based on regions conserved between sequences. Degenerate primers were designed where necessary due to the high variability between

viral strains. Primers were produced by Sigma Aldrich or Eurofins.

2.2.3 PCR

Amplification of desired DNA sequences for the determination of the ability of specific primers to amplify these sequences was carried out by PCR. For template, 1 µl of DNA extracted from the blood of MVV seropositive rams was used in a reaction mixture of 25 µl. Each reaction contained 5 units of *Taq* DNA Polymerase, 1x standard *Taq* (Mg-free) reaction buffer (NEB), 3mM magnesium chloride ($MgCl_2$) (NEB), 0.04 pmol of forward and reverse primers (Table 2.2.3.1) and 0.4mM deoxynucleotide (dNTP) solution mix (Thermo Scientific). Standard PCR cycling conditions consisted of an initial denaturation phase of 95^{o}C for 5 minutes followed by 45 cycles of 95^{o}C, 45-68^{o}C and 68^{o}C, each for 15-60 seconds depending on expected product size. Reactions were carried out within a Thermal cycler Life ECO (Bioer Technology). Primers were tested over a gradient of annealing temperatures determined by primer melting temperature to allow for optimum conditions for amplification. Successful amplification was determined by gel electrophoresis of PCR products. Primers used are listed within Table 2.2.3.1 stating target gene, sequence and source.

2.2.4 Gel Electrophoresis

To allow identification of products produced by PCR, gel electrophoresis was utilised. Expected PCR products were smaller than 1000 bp, therefore, a 2% Agarose gel was prepared to which 1 µl of Nancy-520 (Sigma Aldrich) per 20 ml Tris/acetate/EDTA solution (TAE) had been added. Products were loaded on the gel alongside a 100bp DNA ladder (NEB)

Table 2.2.3.1 PCR Primers tested for virus detection

Primer	Target Gene	Sequence	Reference
EV1 POL F	MVV *pol*	AGATTGGGGAAATAAAGCAATAGAAT	
EV1 POL R		TTATTACCTCTTGTGTAAGCTTTTGT	(Carrozza et
EV1 POL Probe		*6-FAM*-CGCTTTAATGCTCTGCTGTGCTTGAC-*BHQ1a*	al. 2010)
MVV pol q F1	MVV *pol*	RGARGATGCDGGVTATGA	*
MVV pol q R1		CYTGATAYCCHGARTCTA	
MVV pol q F2	MVV *pol*	BAARTGGCATCARGATGC	*
MVV pol q R2		TCYACYTGCCARTGRTCTA	
MVV pol q F3	MVV *pol*	GTVTGGRTAGAAACAAATTC	*
MVV pol q R3		GCTTGHGAYTGNGGRTTCCA	
MVV pol q F4a	MVV *pol*	TGGTCTGGGTAGAAACAAATTC	*
MVV pol q F4b		ATACTATTAGTGTGGGTAGA	
MVV pol q F4c		TGGRTAGAAACAAATTCAGG	
MVV pol q F4d		TTRGCWGAVGCGCARTTAGG	
MVV pol q R4a		TTTGTTTCTACCCAGACCAATA	
MVV pol q R4b		TTGTTTCTACCCACACTAATAG	
MVV pol q R4c		GAATTTGTTTCTACCCATACTA	
MVV pol q R4d		CCTGAATTTGTTTCTACCCATAC	
MVV pol q R4e		CCTGAATTTGTTTCTATCCA	
MVV pol q R4f		GGTAACACCTTCCAATAATATC	
MVV gag q F1	MVV *gag*	TTGACDGAAGGRAAYTGT	*
MVV gag q R1		GTYTCDGGYTTCATNCCCAT	
MVV gag q F2	MVV *gag*	MWGTDGCWATGCCARCAT	*
MVV gag q R2		DATATCYTTRCTWGTCCA	
MVV gag q F3	MVV *gag*	NCARGCHAAYATGGATCA	*
MVV gag q R3		TGACARTCTGYRCTDGCAT	
MVV env q F1	MVV *env*	TGTGARGARTGGTGYTGGTA	*
MVV env q R1a		TTTTCCCAATATACCCGCTG	
MVV env q R1b		TTYTCCCAATATACYCTTTG	
MVV env q F2	MVV *env*	RGAYTCNYTRTAYATAGC	*
MVV env q R2a		YTGRTGCATCATYCCATC	
MVV env q R2b		YTGRTGCATCATACTATC	
CAEV pol q F1a	CAEV *pol*	GCAGRRGCNCCAGAGGAWTGGAC	*
CAEV pol q F1b		GGMARRGCNCCCCCACAWTGGAC	
CAEV pol q R1a		GTRAAATATCCATCYCCTATATC	
CAEV pol q R1b		GTRAAATATCCATCYCCTATGTC	

*Primers designed in this study

and run at 100 V and for 45 minutes. Gels were viewed and photographed by ImageQuant LAS 400 (GE Healthcare Life Science, UK) under ultraviolet (UV) light. Confirmed products for which the nucleotide sequence was required were prepared for sequencing using the Nucleospin® Gel and PCR Clean-up kit (Macherey-Negal) following the recommended protocol for PCR clean-up.

2.2.5 Sanger Sequencing and Analysis

Nucleotide sequences were acquired by Sanger sequencing carried out by Source BioScience. 5 µl of product to be sequenced was prepared at 10 ng/µl per 5 µl of primer, at a concentration of 3.2 pmol/µl. Sequence analysis was completed using BioEdit v7.2 and CLC Sequence Viewer software v8.0 (Qiagen).

2.2.6 RNA Extraction

RNA for next generation sequencing (NGS) was extracted from 6 seropositive ram (3 alive at project commencement and 3 which died at differing time points before) lung and mediastinal lymph node tissues. The RNeasy Mini Kit (Qiagen) following the recommended protocol for extraction of total RNA from animal tissue was used. Final RNA was eluted in a total volume of 100 µl. Successful extraction was confirmed using the NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific). The quantity and quality of RNA extracted was determined using the 2100 Bioanalyzer system (Agilent) following the manufacturer's recommended protocol. The ram's (Ram 8, Ram 13 and Ram 26) RNA which showed best quality and yield from both tissue sources were selected for NGS.

2.2.7 Next Generation Sequencing

NGS was carried out by the Imperial BRC Genomics Facility of Imperial College, London. An initial quality check of samples was carried out by the facility and library preps completed targeting total RNA with additional ribosomal RNA depletion. Library preps were then quantified before sequencing. Utilising the Illumina NextSeq 500 system (Illumina), the 6 pooled samples were run over two lanes at MID output. Paired end reads of 150bp in length were sequenced with an estimated 36-42 million fragments per sample produced. Data files were returned for analysis.

2.2.8 Next Generation Sequencing Analysis

NGS data was analysed for acquisition of maedi-visna strains. Analysis was carried out using the Cloud Infrastructure for Microbial Bioinformatics (CLIMB) a cloud-based cyber-infrastructure developed as a collaboration between Warwick, Birmingham, Cardiff, Swansea, Bath and Leicester universities (Connor et al. 2016). The process of analysis is illustrated in Figure 2.2.8.1.

Initial raw data obtained from Imperial BRC Genomics Facility was compiled by ram and tissue before being checked for sufficient quality and removal of labelling barcodes through skewer software. Sequences were then aligned against the sheep genome (v3.1, accession number GCA_000298735.1) at which point sequences which successfully aligned were removed from the sequence pool using HISAT2 software (Archibald et al. 2010; Kim et al. 2015). Next, non-sheep sequences were classified against a reference database using Kraken2 software (Wood and Salzberg 2014). Reference database of Archaea, bacteria and virus sequences was

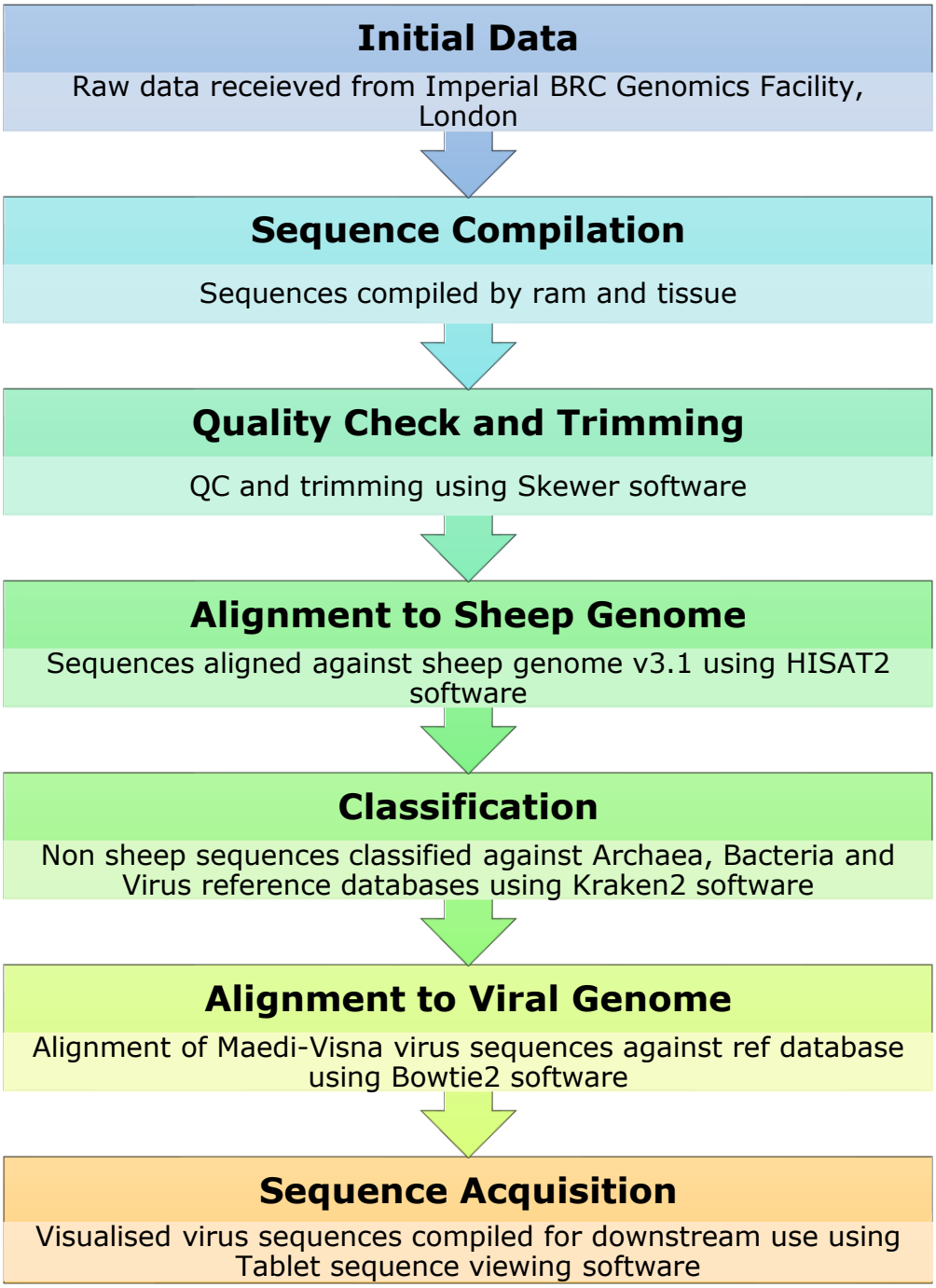


Figure 2.3.8.1 Next generation sequencing analysis flowchart. Chart illustrating the step by step analysis of data for acquisition of maedi-visna strain sequence.

compiled using free to use kraken database. To improve detectability for SRLV sequences, a further 22 full genome and 681 partial sequences (Appendix 1) were added to the reference database. Sequences that were successfully classified as SRLV were removed from the sequence pool and aligned against a custom reference database exclusively consisting of the SRLV sequences using Bowtie2. Finally using Tablet sequence viewing software, alignments were viewed for read depth and localisation within the viral genome (Milne et al. 2013).

2.2.9 Primer Design – 2

Compiled virus sequences acquired from NGS were used for the design of qPCR primers using primerBLAST software (NCBI), targeting the three structural proteins of SRLVs; Env, Gag and Pol (Appendix 2). Regions targeted were determined by a read depth of >2 with a target product size approximately 100bp (Table 2.2.9.1). Ability to amplify viral sequences was confirmed by end point PCR. Amplification of correct product was confirmed by Sanger sequencing.

Table 2.2.9.1 PCR primers designed from sequences acquired by NGS

Primer	Target Gene	Sequence	Product Size
NGS Env1 F	SRLV env	GACTAGGCATTGTGCTTGCT	84 bp
NGS Env1 R		ATGACTGCTGCACGGCATTA	
NGS Gag1 F	SRLV *gag*	CAAGCCACATTGGCATGCTT	76 bp
NGS Gag1 R		TTATTCCCCTTGCTGCCTGC	
NGS Pol1 F	SRLV *pol*	AGGGGATGCATACTTTACTATACCA	97 bp
NGS Pol1 R		TCTTGTGCATGGCCCTAAAT	

2.2.10 qPCR Design

A Sybr green based qPCR procedure was designed for detection of the SRLV strain in this study. Each of the 3 primer sets previously shown to successfully amplify viral sequences were

tested for potential use in this diagnostic assay. For initial testing, standards were created using large overlapping primers approximately 50bp in length covering the target viral sequence (Table 2.2.10.1).

Table 2.2.10.1 PCR primers for synthesis of standard products

Primer	Sequence
NGS Env Std F	GACTAGGCATTGTGCTTGCTATCATGGCAATAATAGCTGCTGCAGGAGCTGG
NGS Env Std R	ATGACTGCTGCACGGCATTAGCAACCCCGAGTCCAGCTCCTGCAGCAGCTATT
NGS Gag Std F	CAAGCCACATTGGCATGCTTAATGTGTAGTCAAATGGGAATGAAGC
NGS Gag Std R	TTATTCCCCTTGCTGCCTGCACTGTCTCGGGCTTCATTCCCATTTGA
NGS Pol Std F	AGGGGATGCATACTTTACTATACCATTATATGAACCCTATAGACAATATACATGC
NGS Pol Std R	TCTTGTGCATGGCCCTAAATTATTCGGACTTAGCAGAGTGAAGCATGTATATTGTC

In addition to the standard acting as a positive control, the primers were tested against ram blood DNA from a seropositive animal, ewe blood DNA from a naïve animal and water, which acted as a negative control. From these tests, the most suitable primer pair was selected for downstream testing.

Reaction mixtures for initial tests consisted of 1x qPCRBIO SyGreen Mix Lo-ROX master mix (PCR Biosystems), 0.04µM forward and reverse primers and 1µl of test DNA or standard in a total volume of 20 µl. Reaction conditions consisted of a starting incubation of 95^{o}C for 15 minutes followed by 45 cycles of 95^{o}C for 5 seconds, 60^{o}C for 30 seconds and 72^{o}C for 10 seconds. After cycle completion a melt curve was carried out ranging from 65^{o}C to 95^{o}C. All reactions were carried out within a CFX Connect Real-Time PCR Detection System (Biorad Laboratories).

Following primer selection, optimization of primer concentration and annealing temperature were carried out to determine optimal conditions. Primer concentrations tested were 0.02µM, 0.04µM, 0.08µM and 0.4µM. Annealing temperatures tested ranged from 55^{o}C to 65^{o}C.

For analysis, a standard curve was produced for each reaction. A 1:10 standard dilution series of PCR product derived standards was carried out on each plate ranging in concentration from 2.41×10^{11} copies/µl to 2.41×10^{4} copies/µl. All analysis of results was carried out using Bio-Rad CFX Maestro software.

2.2.11 Sequence Analysis

To characterise sequences obtained through next generation sequencing and to identify viral strain present within seropositive rams, sequences were analysed using MEGA X software (Kumar et al. 2018). Phylogenetic trees were constructed using the Maximum likelihood method and Tamura-Nei model (Tamura and Nei 1993). The percentage of bootstrap values was based on 500 repititions.

2.3 Results

2.3.1 Primer Testing

Primers listed in Table 2.2.3.1 were tested for ability to amplify SRLV present within samples collected from known seropositive rams, with focus on potential use for a diagnostic test. The results from these PCR reactions were collated within Table 2.3.1.1.

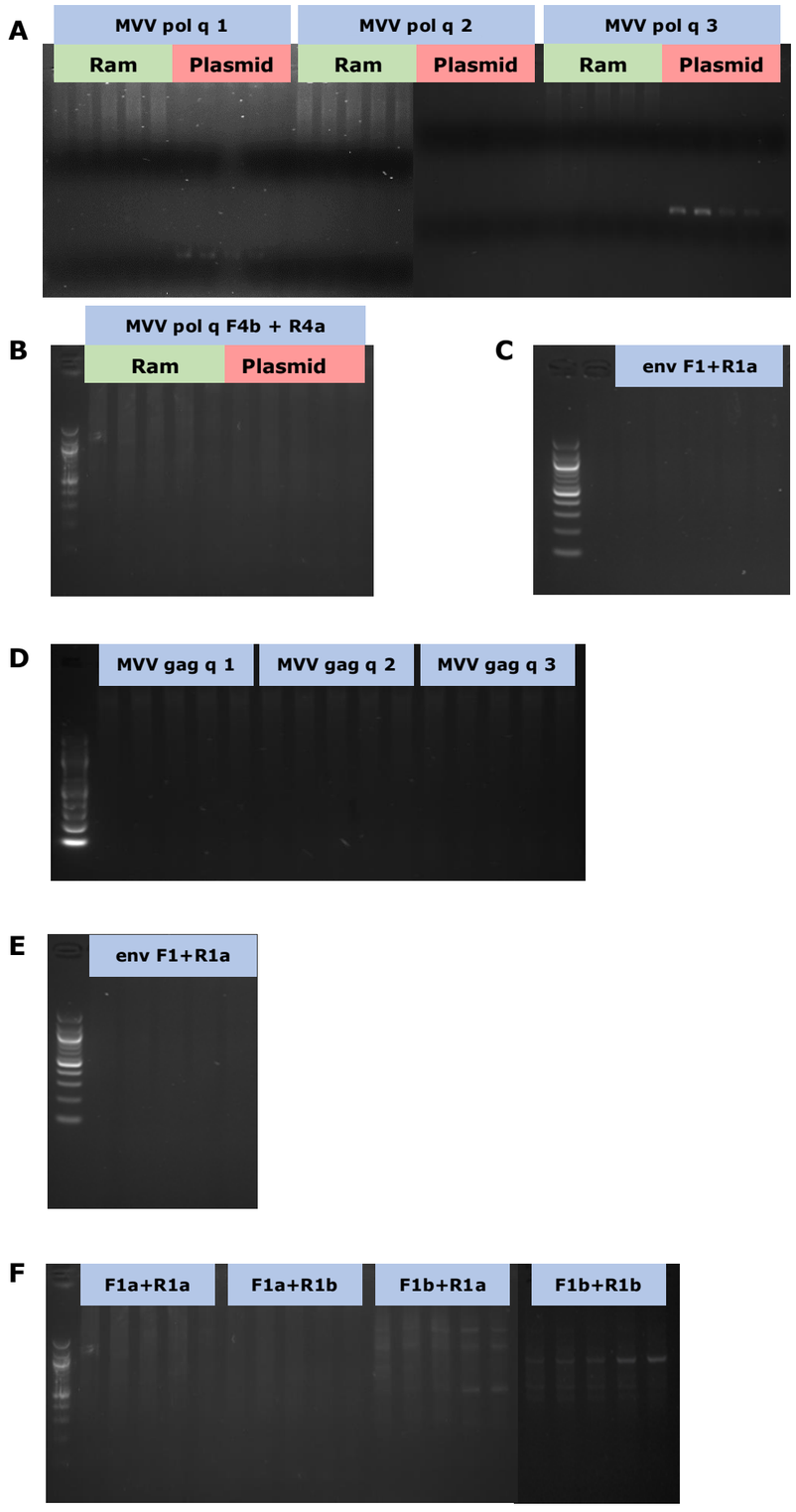
Of the primers targeting the *pol* gene, 3 of 5 primer sets showed successful amplification of plasmid DNA constructed from the *pol* gene of the EV1 strain (UK strain) of MVV. Despite this, no primer set targeting the *pol* gene showed amplification of any product when run using DNA extracted from seropositive ram lung and lymph tissue. Primers targeting the *env* and *gag* genes in addition showed no successful amplification.

Primers designed to target the *pol* gene of CAEV were also tested. PCR reactions resulted in the amplification of multiple bands which upon sequencing were confirmed not to be SRLV sequence.

Table 2.3.1.1 SRLV PCR results following initial primer design for viral detection.

Primer	Target Gene	Sequence	PCR – EV1 Plasmid	PCR – Ram DNA	Gel*
EV1 POL F	MVV *pol*	AGATTGGGGAAATAAAGCAATAGAAT	Positive	Negative	**-**
EV1 POL R		TTATTACCTCTTGTGTAAGCTTTTGT			
EV1 POL Probe		*6-FAM*-CGCTTTAATGCTCTGCTGTGCTTGAC-*BHQ1a*			
MVV pol q F1	MVV *pol*	RGARGATGCDGGVTATGA	Positive	Negative	**A**
MVV pol q R1		CYTGATAYCCHGARTCTA			
MVV pol q F2	MVV *pol*	BAARTGGCATCARGATGC	Negative	Negative	**A**
MVV pol q R2		TCYACYTGCCARTGRTCTA			
MVV pol q F3	MVV *pol*	GTVTGGRTAGAAACAAATTC	Positive	Negative	**A**
MVV pol q R3		GCTTGHGAYTGNGGRTTCCA			
MVV pol q F4a	MVV *pol*	TGGTCTGGGTAGAAACAAATTC	Negative	Negative	**B**
MVV pol q F4b		ATACTATTAGTGTGGGTAGA			
MVV pol q F4c		TGGRTAGAAACAAATTCAGG			
MVV pol q F4d		TTRGCWGAVGCGCARTTAGG			
MVV pol q R4a		TTTGTTTCTACCCAGACCAATA			
MVV pol q R4b		TTGTTTCTACCCACACTAATAG			
MVV pol q R4c		GAATTTGTTTCTACCCATACTA			
MVV pol q R4d		CCTGAATTTGTTTCTACCCATAC			
MVV pol q R4e		CCTGAATTTGTTTCTATCCA			
MVV pol q R4f		GGTAACACCTTCCAATAATATC			
MVV gag q F1	MVV *gag*	TTGACDGAAGGRAAYTGT	-	Negative	**D**
MVV gag q R1		GTYTCDGGYTTCATNCCCAT			
MVV gag q F2	MVV *gag*	MWGTDGCWATGCCARCAT	-	Negative	**D**
MVV gag q R2		DATATCYTTRCTWGTCCA			
MVV gag q F3	MVV *gag*	NCARGCHAAYATGGATCA	-	Negative	**D**
MVV gag q R3		TGACARTCTGYRCTDGCAT			
MVV env q F1	MVV *env*	TGTGARGARTGGTGYTGGTA	-	Negative	**C**
MVV env q R1a		TTTTCCCAATATACCCGCTG			
MVV env q R1b		TTYTCCCAATATACYCTTTG			
MVV env q F2	MVV *env*	RGAYTCNYTRTAYATAGC	-	Negative	**E**
MVV env q R2a		YTGRTGCATCATYCCATC			
MVV env q R2b		YTGRTGCATCATACTATC			
CAEV pol q F1a	CAEV *pol*	GCAGRRGCNCCAGAGGAWTGGAC	-	Negative –	**F**
CAEV pol q F1b		GGMARRGCNCCCCCACAWTGGAC		confirmed by	
CAEV pol q R1a		GTRAAATATCCATCYCCTATATC		sequencing	
CAEV pol q R1b		GTRAAATATCCATCYCCTATGTC			

* Single example gel image shown where multiple negatives indicated



➔ **Figure 2.3.1.2 Gel electrophoresis images of SRLV primer results.** Images of gels ran using PCR products obtained from testing of SRLV primers targeting the (A+B) MVV pol gene, (C+E) MVV env gene, (D) MVV gag gene or (F) CAEV pol gene. Primers were run against DNA extracted from lung tissue of seropositive rams. MVV pol primers were additionally ran against plasmid DNA encoding the pol gene of a previously identified UK strain of MVV (EV1).

2.3.2 Bioinformatic Analysis

RNA isolated from both lung and lymph tissue from 3 seropositive rams was sequenced by NGS to allow for identification of viral sequence present in rams. Bioinformatic analysis carried out is outlined in Figure 2.3.2.1.

Alignment of compiled sequences to the sheep genome (v3.1) identified 74106108 'non sheep' sequences. Kraken2 classified 115114 of these sequences successfully to reference database of which 4052 were classified as SRLV. Of this number, 3242 (85%) were assigned to CAEV sequences with the remaining 810 (15%) sequences assigned to MVV and ovine lentiviral sequences. Of this number, 312 (7.7%) were successful aligned to SRLV sequence database which provided 2046bp of sequence supported by a read depth >2 (Appendix 2). Acquired sequence consisted of 10 fragments spanning over three genes (*Gag* = 4, *Pol* = 2 and *Env* =4). Most sequences obtained lay within the *Gag* and *Env* genes (862 bp and 859 bp, respectively), double that obtained from the *Pol* gene (325 bp). Alignment of obtained sequences to EV1 strain (Accession No. S51392) showed 84.5% nucleotide identity. Nucleotide identity within individual genes *Gag, Pol* and *Env* when compared to EV1 was 86.33%, 83.79% and 83.20%, respectively.

Raw Sequencing Data

Data Handling

Sequences compiled from RNA extracted from lung and mediastinal lymph node of 3 MVV seropositive rams. Quality check and trimming completed using skewer

Alignment to Sheep Genome

Sequence data aligned to sheep genome v3.1 using HiSat2. Sheep and non-sheep sequences were separated for classification.

74106108 non-sheep sequences were isolated

Sheep

Non-Sheep

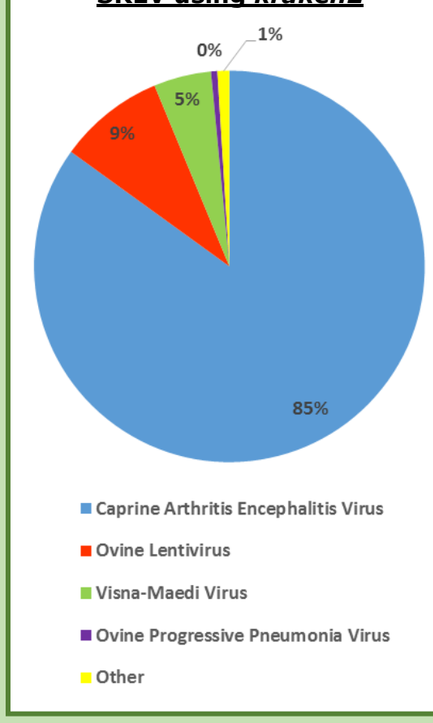
Classification

74106108 sequences classified by kraken2 against reference database of archaeal, bacterial and viral sequences. 115114 (0.16%) of sequences successfully classified.

Table of classified sequences

	Seq. Count	%
Archaea	659	0.0009
Bacteria	97063	0.1
Virus	17392	0.02
SRLV	4052	0.005
Unclassified	73990994	99.8

Pie chart of composition of 4052 sequences classified as SRLV using *kraken2*



Alignment to Viral Genome

4052 sequences aligned against database of SRLV full genome and partial sequences using Bowtie2.

Acquired reads were included in final sequence following a read depth >2. 312 sequences were successfully aligned against SRLV sequences.

Sequence Acquisition

Sequence reads were compiled and ordered by alignment against EV1 strain of MVV (Accession No. S51392).

2046 bp of the unknown strain was obtained, which shared 1736 bp (84.5%) identity with EV1 strain. Sequence amounts to approximately 21.5% of viral genome (Appendix 2).

Differences in unknown strain consist 318 SNPs, 8 deletions and 5 insertions.

Table of gene composition

Gene	Bases	Loci
Gag	862 bp	493-872, 899-1122, 1193-1341, 1391-1503
Pol	325 bp	2103-2242, 2457-2644
Env	859 bp	6315-6580, 7162-7382, 7980-8195, 8612-8780

Figure 2.3.2.1 Outline of bioinformatic analysis for identification of SRLV strain. Description and results of bioinformatic analysis of NGS data set obtained from 3 SRLV seropositive rams for identification of infecting viral strain.

2.3.3 qPCR Design

Three primer sets were tested for suitability for use in a qPCR assay for detection of the partially sequenced strain of SRLV within seropositive rams. Primer sets were designed targeting three structural genes (*Gag, Pol* and *Env*). Testing of primers against synthesised positive controls gave successful amplification of a single product in Gag and Pol primers (Figure 2.3.3.1a). Negative control showed no amplification with any primers (Figure 2.3.3.1b). All primer sets showed amplification of DNA extracted from seropositive ram blood, but only Pol showed amplification of a single product of expected melting temperature (Figure 2.3.3.1c). Finally, qPCR testing of DNA extracted from the blood of seronegative ewes showed marginal amplification using Gag and Env primers, whilst no amplification was seen with Pol primers (Figure 2.3.3.1d).

Amplification of a single product of equal melting temperature in positive control and seropositive ram DNA whilst showing no amplification in negative controls and seronegative ewe DNA led to selection of Pol primers for use in qPCR assay. The reaction efficiency when using Pol primers was calculated as 85.5%. During DNA/RNA testing, samples were determined to be positive when fulfilling three criteria: a Ct value <40, a correct melt curve peak and consistent results between replicates.

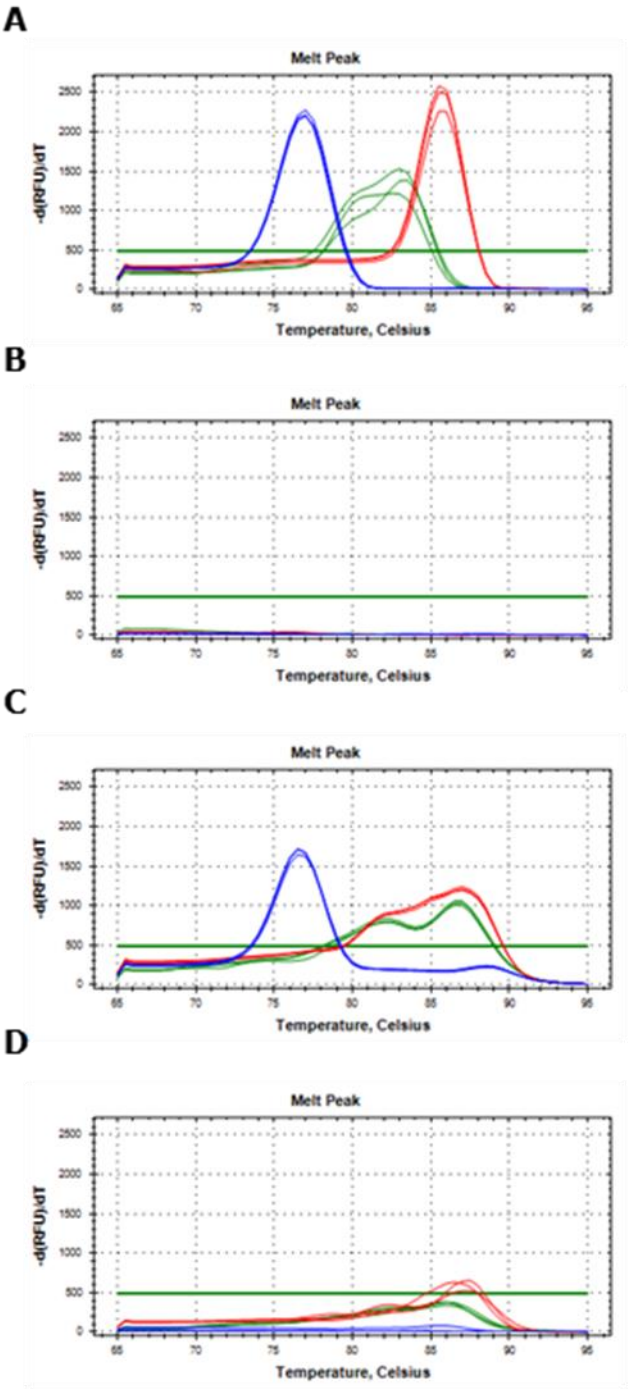


Figure 2.3.3.1 Melt Curves for testing of qPCR primers. 3 primer sets were designed targeting the 3 structural genes of SRLVs: *Gag* (green), *Pol* (blue) and *Env* (red). Primers were tested against synthetic oligo positive control (a), negative water control (b) and DNA extracted from the blood of seropositive rams (c) and seronegative ewes (d).

2.3.4 Sequence Analysis

Fragmented sequences of an unknown circulating strain of SRLV were analysed in an effort to characterise viral strain with regard to previously reported sequences. Phylogenetic trees were generated based on comparison to 2046 bp genome sequences (Figure 2.3.4.1), 862 bp of *Gag* gene (Figure 2.3.4.2), 251 bp of *Gag* gene (Figure 2.3.4.3), 325 bp of *Pol* gene (Figure 2.3.4.4) or 859 bp of *Env* gene (Figure 2.3.4.5).

Obtained sequences were found to be strongly related with genotype A sequences in all trees. Trees comparing the full 2046 bp of obtained sequence to available full genome sequences showed affiliation with subtype A19, although, multiple subtypes (A: 2, 3, 5-7, 9-18, 20-22; B: 4, 5) were not represented in this tree due to lack of full genome sequences (Figure 2.3.4.1). Phylogenetic trees constructed with 862 bp of the *Gag* gene and 325 bp of *Pol* gene showed closest association with sequences of the A1 subtype (Figure 2.3.4.2+4). Again many genotype A sequences were not included due lack of classified sequences spanning these regions of the viral genomes (*Gag:* A3, 5-7, 9-11, 14-15, 18, 21, 22; *Pol:* A6, 9-13, 15-19, 21, 22). The phylogenetic tree of 251 bp region of *Gag* spanned sequences of all genotype subtypes with the exception of A6 (Figure 2.3.4.3). The sequences obtained in this study were found to be affiliated with a cluster of sequences consisting of 5 subtypes (A1, 2, 13, 18 and 21). *Env* sequences closest match was found to be the UK strain of MVV (EV1).

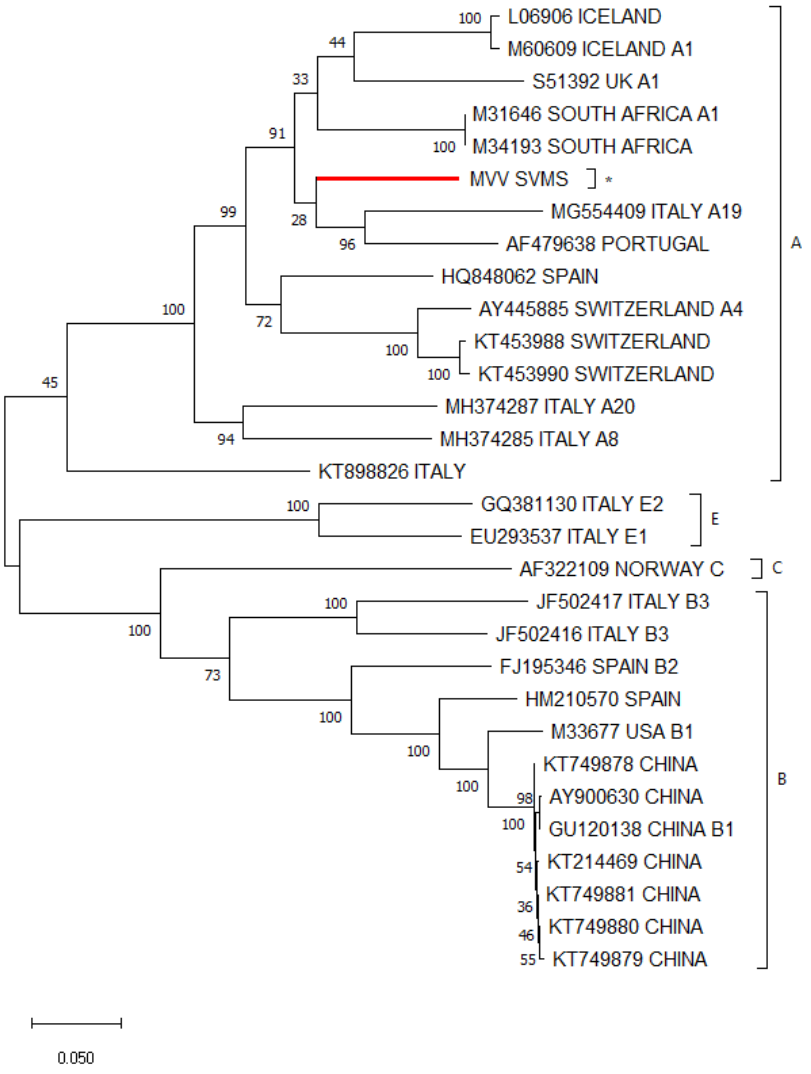


Figure 2.3.4.1 Phylogenetic tree of 2046 bp spanning across SRLV genome. Phylogenetic tree was generated using the Maximum Likelihood method and Tamura-Nei model and assessed by bootstrap method. Tree comprises unknown strain and 29 publicly available SRLV genomes listed in Appendix 1. Analysis was carried out using MEGA X.

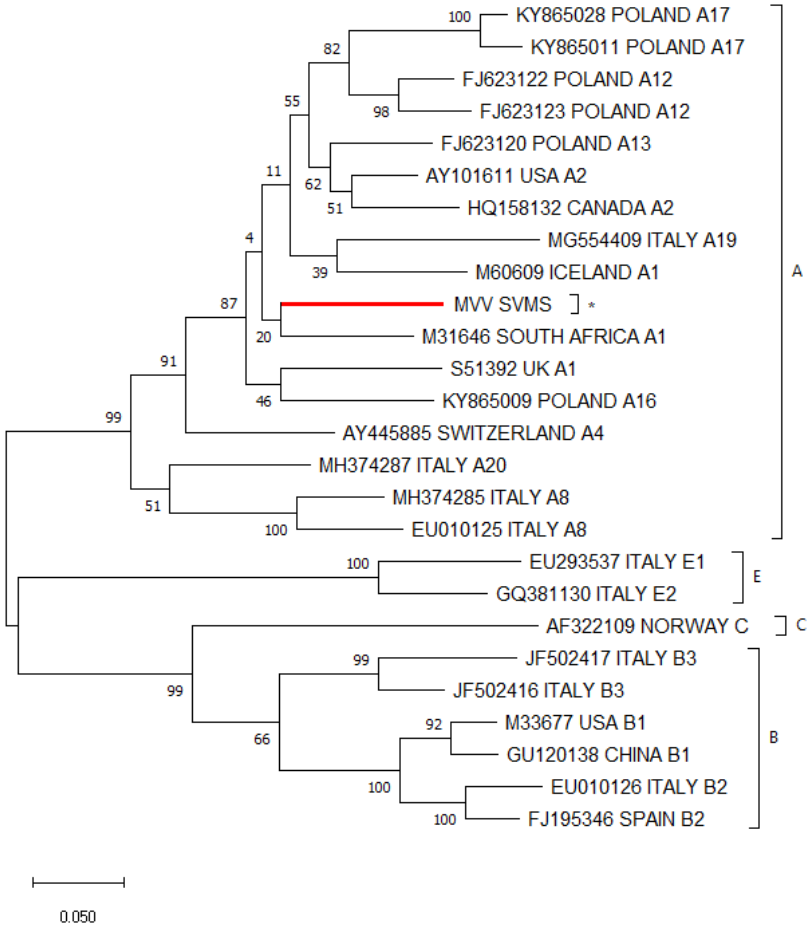


Figure 2.3.4.2 Phylogenetic tree of 862 bp spanning across SRLV *Gag* gene. Phylogenetic tree was generated using the Maximum Likelihood method and Tamura-Nei model and assessed by bootstrap method. Tree comprises unknown strain and 25 publicly available SRLV genomes listed in Appendix 1. Analysis was carried out using MEGA X.

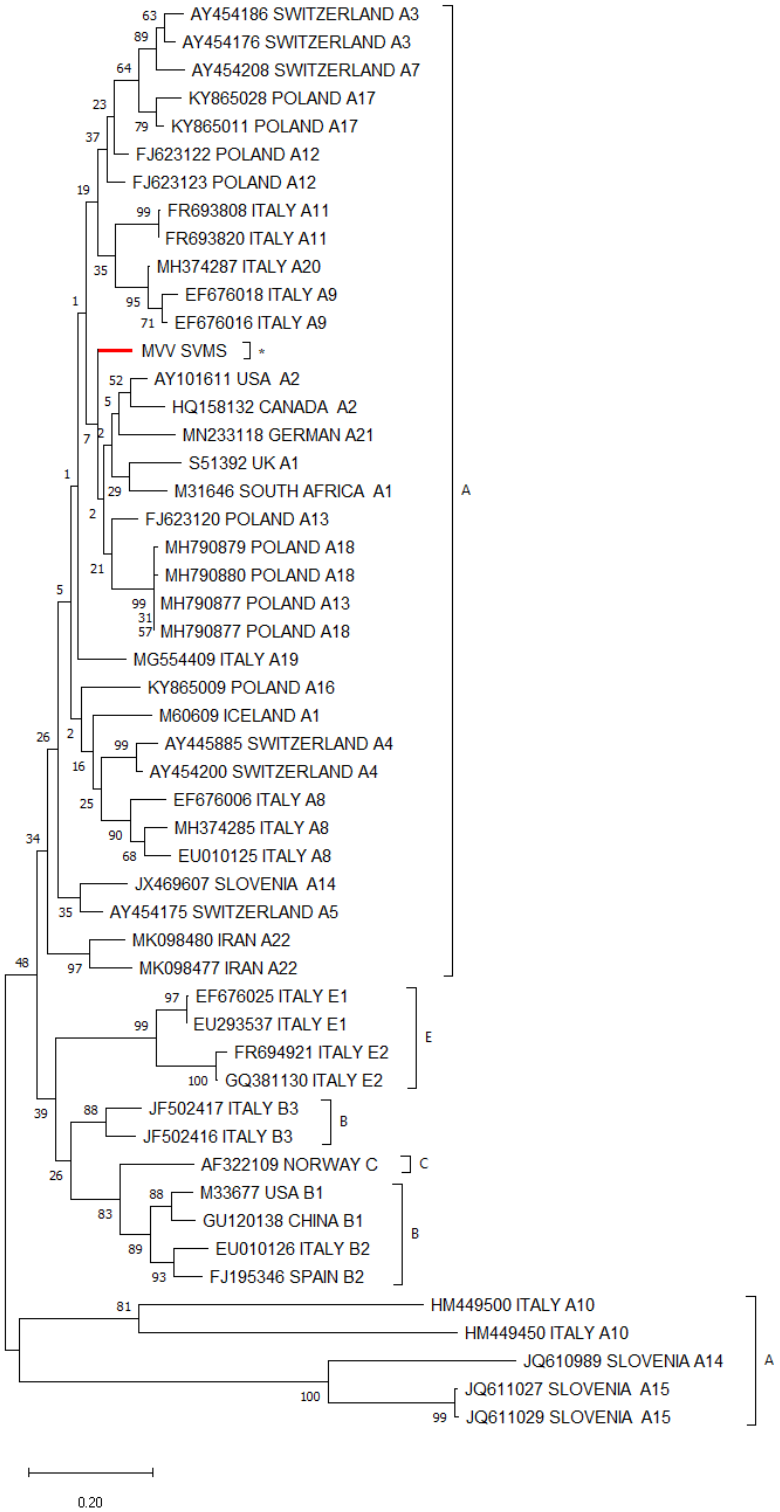


Figure 2.3.4.3 Phylogenetic tree of 251 bp spanning across SRLV *Gag* gene. Phylogenetic tree was generated using the Maximum Likelihood method and Tamura-Nei model and assessed by bootstrap method. Tree comprises unknown strain and 50 publicly available SRLV genomes listed in Appendix 1. Analysis was carried out using MEGA X.

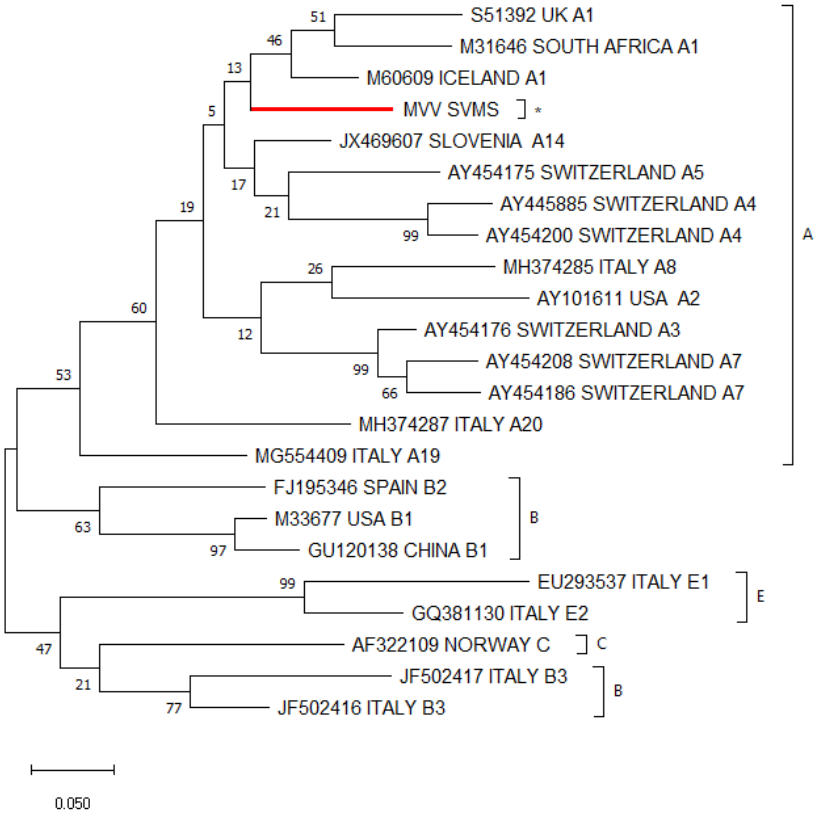


Figure 2.3.4.2 Phylogenetic tree of 325 bp spanning across SRLV *Pol* gene. Phylogenetic tree was generated using the Maximum Likelihood method and Tamura-Nei model and assessed by bootstrap method. Tree comprises unknown strain and 22 publicly available SRLV genomes listed in Appendix 1. Analysis was carried out using MEGA X.

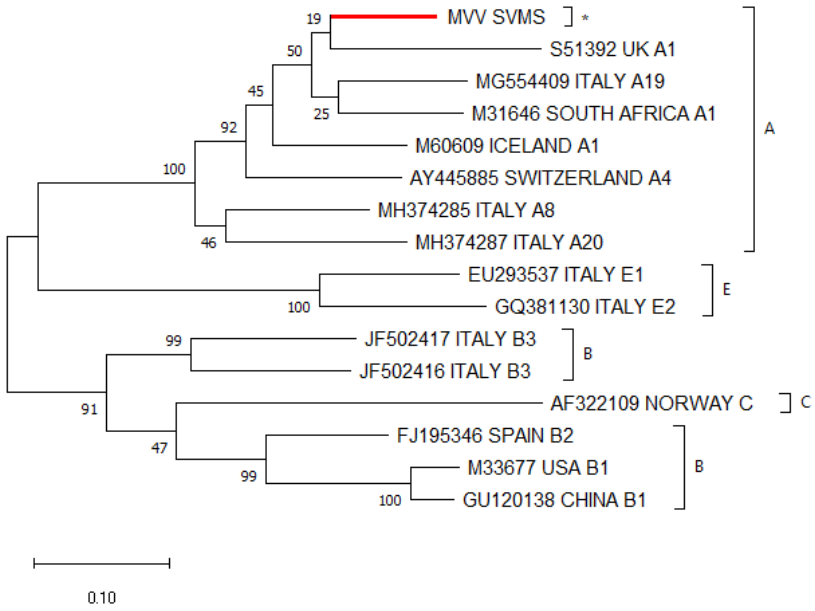


Figure 2.3.4.2 Phylogenetic tree of 859 bp spanning across SRLV *Env* gene. Phylogenetic tree was generated using the Maximum Likelihood method and Tamura-Nei model and assessed by bootstrap method. Tree comprises unknown strain and 16 publicly available SRLV genomes listed in Appendix 1. Analysis was carried out using MEGA X.

2.4 Discussion

In this chapter a qPCR assay for detection of an unknown strain of MVV within a group of seropositive rams was designed. A Sybr green based assay targeting the *Pol* gene was designed and shown to effectively target viral sequences present within DNA of infected individuals.

Blood was selected during testing phase due to high use in commercial diagnostics (e.g. PCR, qPCR, ELISA and AGID), and was found to support quantification of viral loads within tested samples. Previous studies evaluating SRLV PCR based diagnostics have investigated the efficiency of viral detection when sampling milk (Extramiana et al. 2002a; Brinkhof et al. 2010; Barquero et al. 2011). Although findings from such studies has failed to produce a conclusive answer, with varying degrees of agreement reported between blood and milk testing. The ability to collect samples in a less invasive manner than blood sampling whilst not requiring a qualified practitioner does provide advantages for future testing strategies. Even if just as an indicator of circulating virus within a flock. Unfortunately, due to seropositive animals in this study being rams, milk samples could not be tested with the devised assay to determine efficiency.

Acquisition of NGS sequencing data allowed for identification of 21.5% of the unknown strain of virus. This allowed for comparison of sequence to previously reported and characterised strains of SRLV. Phylogenetic trees designed based on all or a selection of segments of obtained sequence suggest strongly that the strain belongs to genotype A. The subgroups with which the sequence from this project clustered

consisted of A1 and A19. A1 sequences have been identified worldwide with infection seen in both goats and sheep, in comparison, A19 sequences have only been reported in goats of Italy (Shah et al. 2004b; Colitti et al. 2019). True classification is not currently possible with the limited sequence data available, as was seen with the previously suggested genotype D which was designated on solely the sequence of the *Pol* gene. Ramírez et al. (2013), later suggested genotype A as a classification following sequencing of the *Gag* gene showed clustering within genotype A.

Despite inability to fully characterise the sequence identified in this study, clustering within phylogenetic trees with subgroup A1 is interesting. The previously reported full genome SRLV sequence for the UK (EV1) is also classified as A1 sequence (Sargan et al. 1991). During initial testing of primers for detection of virus, the previously reported EV1 primers and primers designed for broad spectrum detection of SRLVs with a particular focus on EV1 consistently failed to amplify our virus isolate, despite detecting sequence of the the original EV1 isolate reliably (Figure 2.3.1.1) (Carrozza et al. 2010).

The differences observed here in detectability of virus when comparing strains of the same genotype and potentially of the same subgroup, illustrates one of the key difficulties in diagnosing SRLV infections. Variation in SRLVs is high, which can in part be attributed to the high frequency of mutations occurring (Ramírez et al. 2013). Inclusion of an additional ability to produce recombinant virus in cases of co-infecting strains means that SRLV sequences are 'fluid' and constantly changing with time (Pisoni et al. 2007b).

Initially, one of the objectives for this study included the design of a universal diagnostic assay for UK strains of SRLVs. Inability to design an assay for detection of merely 2 of these circulating strains suggests that such the development of such a diagnostic test may not be feasible. Such a qPCR-based assay would likely be required to be multiplex to ensure amplification of all possible strains. In addition, monitoring of circulating viral strains would be vital to ensure introduction of a strain undetectable by existing qPCR assays does not occur.

Following on from this study, it would be useful to fully identify the strain of the currently circulating SRLVs within the UK, especially as the only UK strain reported prior to this study was sequenced nearly 30 years ago (Sargan et al. 1991). Identification of 21.5% of the genome over 10 fragments spanning the whole genome provides a helpful scaffold to build upon. Methods that could be utilised include the use of PCR based assay to 'bridge' the gaps between fragments to acquire the whole genome. In addition, sequences obtained within this study could be fed back into the NGS pipeline to attempt to fish out further fragments of sequence. Finally, a de novo assembly could be attempted using sequence data following removal of sheep genomic sequence. Time constraints however precluded the completion of this work within this study.

The qPCR assay designed in this study allows for the detection of a newly identified circulating strain of SRLV within the UK. Although identification is currently limited to one group of animals, identification of this new strain highlights the need for further investigation into the current state of SRLV prevalence within the UK.

Chapter 3: AI Model to Estimate Risk of Sexual Transmission During Natural Mating

3.1 Introduction

SRLV outbreaks within sheep flocks and goat herds can be a costly situation for many farmers, especially those accredited under the UK MVV/CAEV accreditation scheme. The current response stipulated within the regulations for participation within the scheme states that following identification of seropositive animals, accredited status is suspended (SRUC 2020). In addition, all confirmed positive sheep and lambs suckling from seropositive ewes are to be removed (preferably slaughtered to remove risk of further transmission) from the flock. Accreditation status cannot be restored until diagnostic testing has been carried out twice with clear results with a period of 6-12 months between each test, with the first being carried out at the earliest of 6 months post-outbreak. Therefore, a minimum period of 1 year is required to restore accredited status (SRUC 2020).

Following this scenario, financial losses to farmers can be attributed to veterinary fees, replacement of infected animals, loss of at-risk offspring (when applicable), loss of sales and loss in value of infected animals (Anderson et al. 1985; Keen et al. 1997; Peterhans et al. 2004). This in addition to the production losses induced by the actual disease. The value attached to individual animals can greatly vary with both breed and function of a flock. In addition to monetary losses, outbreaks within breeding ram flocks and other high value breed flocks result in the loss of valuable genetics. Theoretically, these genetics could be rescued through harvesting of semen before removal of an

animal from a flock. A difficulty arises then however, as there exists a risk of transmission of virus through the use of semen from infected rams for insemination within naïve ewes (Travassos et al. 1999; Peterson et al. 2008).

The main routes of transmission of SRLVs have long been identified as being via the ingestion of infected milk/colostrum and inhalation of respiratory secretions in conjunction with close proximity (Brodie et al. 1998; Blacklaws et al. 2004). Sexual transmission has been clearly demonstrated within the Lentivirus genus of viruses, but it's role in SRLV transmission in sheep and goats has yet to be fully investigated (Marks et al. 2006; Haase 2011). As natural mating would require exposure of naïve ewes to seropositive rams, therefore putting animals at risk of horizontal transmission via droplet transmission, sexual transmission was investigated in this study in relation to AI techniques.

Within sheep, two insemination techniques that can be used are vaginal insemination and laparoscopic intrauterine insemination, with the latter preferred due to increased success rates (Gourley and Riese 1990; Paulenz et al. 2003; Anel et al. 2005). Transcervical insemination is another technique carried out in other ruminants such as cows. This technique has been shown to have difficulties when attempted in sheep and regularly results in cervical trauma, reduced fertility and failed pregnancy (Wulster-Radcliffe and Lewis 2002; Moutou et al. 2004). Studies into the occurrence of sexual transmission during SRLV following AI have been carried out in goats (Ali Al Ahmad et al. 2012; Souza et al. 2013). During these studies naïve does were inseminated by laparoscopic intrauterine insemination and transcervical insemination depositing semen directly into the upper reproductive tract, within the uterus.

These studies demonstrated successful transmission of infection to inseminated ewes and does suggesting therefore, that sexual transmission can occur in SRLV infection. However, by inseminating animals directly into the uterus, the lower reproductive tract is bypassed, which in turn bypasses both the physical and immune defences present within the tract. It does not necessarily follow that sexual transmission under natural mating or vaginal insemination conditions will also occur.

In this chapter, a pilot scale study was carried out to assess the risk of SRLV transmission via natural mating (using intravaginal insemination as a proxy for this) within a group of naïve ewes following vaginal insemination with semen harvested from seropositive rams. The project aimed to look at both horizontal transmission to inseminated ewes and vertical transmission to any successfully conceived foetuses.

3.2 Materials and Methods

3.2.1 Ethical Approval

Prior to implementation, all animal and trial procedures were reviewed and approved by the Home Office under the 'Animals (Scientific Procedures) Act 1986' (Licence no. PPL 30/3367).

3.2.2 Animals

13 seropositive rams (6 Aberfield and 7 Abermax) and 30 naïve Exlana ewes participated in this study. Rams were 1 year old when they identified as being MVV positive during routine testing as part of the MVAS after which they were acquired by the University of Nottingham in 2015. Ewes were purchased from an MVV free flock. All animals were retested (as described below) before entering the trial. Ewes and Rams were separated with appropriate husbandry practice to prevent MVV transmission. Animals were held at pasture with available shelter and supplementary rations and separated from other sheep by more than 2 m. In addition, animals were monitored and treated for any signs of clinical disease (including diseases such as foot-rot) and received routine husbandry and preventative treatment. Animals with illness were assessed by a veterinary surgeon.

3.2.3 Blood Collection

Blood was collected in 10ml vacutainer blood tubes and taken from the jugular vein by a qualified veterinarian or home office approved technician. For separation of sera from blood, samples were left at room temperature overnight to allow for clotting after which sera was removed via pipetting. Sera and blood clots were stored at -20^{o}C.

3.2.4 ELISA

Sera was tested for the presence of SRLV specific antibodies using the MVV/CAEV p28 Antibody Screening Test (IDEXX) following the manufacturer's recommended protocol. Repeated in triplicate, seroprevalence was determined by a minimum of two consistent results. Inconclusive results were subjected to further testing.

3.2.5 Hormonal Preparation of Ewes

Synchronisation of the ewe's oestrus cycles was carried out prior to insemination. Progesterone sponges were inserted within the vaginal canal and left in place for 14 days. Following removal of sponges, 3ml of pregnant mare's serum gonadotropin (PMSG) at a concentration of 200 iu/ml was administered intramuscularly in the rump. Insemination was then carried out two days after.

3.2.6 Semen Collection

MVV positive rams were introduced to 3 'teaser' ewes (hormonally prepared as per the trial ewes) to stimulate mating behaviour. Before collection, an artificial vagina was heated to between 40^{o}C and 50^{o}C, which was maintained between rams. Next, one at a time rams were allowed to mount ewes at which time semen was collected by intercepting the penis and redirecting to within the artificial vagina. Upon depositing of semen rams were removed from the ewes. Of the semen collected, up to 400 µl from each ram was stored in RNAlater (Sigma-Aldrich) for nucleic acid extraction at room temperature for 24 hours, after which samples were moved to -20^{o}C. Remaining semen was pooled for insemination and maintained at a temperature of 37^{o}C by use of a water bath. In addition, a sample of pooled semen was stored in RNAlater (Sigma-

Aldrich). Teaser ewes were rehomed in accordance with 'Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012'.

3.2.7 Artificial Insemination

Pooled semen collected from MVV positive rams was used to inseminate 12 hormonally prepped ewes with an additional 12 ewes mock inseminated as a control. Following successful semen collection, semen was immediately transported to the location of naïve ewes whilst being maintained at 37^{o}C (approximately 15-30 minutes). Pooled semen was then prepared by the addition of ultra-high temperature processed (UHT) milk in equal quantities. Mock inseminated ewes were inseminated first with UHT milk containing no semen. To inseminate, the cervix as located using a speculum and 500-750 µl of UHT milk and semen mixture was expelled into the cervix using an insemination pipette heated to a temperature between 40^{o}C and 50^{o}C. Following insemination, groups were separated to remove risk of transmission via other routes and blood collected weekly up to 7 weeks post insemination. Blood collected was tested by ELISA for detection of seroconversion and by qPCR following nucleic acid extraction to detect presence of viral genomic material.

3.2.8 Post-mortem (PM)

Following semen collection and 7 weeks of blood collection rams and ewes, respectively, were euthanised by captive bolt. Visible pathology was documented with blood and tissue samples collected. Table 3.2.8.1 lists tissue samples collected from rams and ewes. Two samples of each tissue were collected, stored in either 500µl RNAlater (Sigma-Aldrich) or 500 µl Formalin at room temperature for 24 hours and then placed at -20^{o}C or

room temperature, respectively. Epididymal washes were obtained by submerging and rinsing a segment of epididymis in RNAlater at least five times after which the segment of epididymis was discarded. Epididymal washes were then stored at room temperature for 24 hours, after which they were stored at -20^{o}C.

Table 3.2.8.1 Tissues sampled from rams and ewes at PM. Listed tissues collected from 13 MVV seropositive rams and 24 naïve ewes 7 weeks post insemination

Animals	**Rams**	**Ewes**
Tissues Sampled*	Trachea Lung Heart Kidney Liver Joint Cartilage Mediastinal Lymph Node Epididymal Wash Nasal Swab Cerebral Swab Oral Swab	Trachea Lung Heart Kidney Liver Joint Cartilage Mediastinal Lymph Node Mammary Tissue Uterus Nasal Swab Cerebral Swab Oral Swab
*** Any additional sites of pathology observed at PM were sampled**		

3.2.9 Histology

Histological analysis was carried out on lung tissue sampled from seropositive rams and trial ewes. Formalin fixed tissue sections were processed and embedded in wax cassettes. Slides were prepared by the University of Nottingham Pathology Service. Slides were wax embedded, sectioned and then hematoxylin and eosin stained.

3.2.10 DNA Extraction – Blood

DNA was extracted from blood using the Nucleospin® Tissue Kit (Macherey-Nagel) for detection of MVV using PCR based methods.

Blood clots were processed by following the supplementary protocol for extraction of genomic and viral DNA from blood samples. Before the protocol was carried out, a small amount (approximately 25 mg) of blood clot was added to 200 µl of PBS. A sterile 5mm steel bead was then added and mixture homogenised by a Retsch MM300 bead mill (Qiagen) at a frequency of 25/s for 2 minutes. The protocol was then followed substituting homogenised blood clot and PBS for 200 µl of fresh blood. DNA was eluted from the column in a final volume of 60 µl. DNA was stored at -20℃ until use.

Successful DNA extraction was confirmed by quantification of DNA content using the NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific).

3.2.11 DNA Extraction – Tissue

DNA was extracted from tissues using the Nucleospin® Tissue Kit (Macherey-Nagel) for detection of MVV using PCR based methods.

Approximately 25mg of tissue was processed following the manufacturers recommended protocol. Following addition of buffer T1 and proteinase k (pre-lyse step) an added step was added. To samples, a sterile 5mm steel bead was added, and mixtures homogenized by Retsch MM300 bead mill (Qiagen) at a frequency of 25/s for 2minutes. Following this, manufacturers protocol was followed as recommended. DNA was eluted from

the column in a final volume of 60 µl. DNA was stored at -20℃ until use.

Successful DNA extraction was confirmed by quantification of DNA content using the NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific).

3.2.12 RNA Extraction

RNA was extracted from sera isolated from blood and epididymal washes using the QIAamp Viral RNA Mini Kit (Qiagen) for detection of MVV using PCR based methods. Extraction was carried out following the manufacturer's recommended protocol. RNA was eluted within a final volume of 60 µl. RNA was stored at -20°C

3.2.13 cDNA Synthesis

Conversion of RNA to cDNA for PCR detection was carried out. One of two RTs were used per reaction; moloney murine leukemia virus (M-MLV) (Promega) and avian myeloblastosis virus (AMV) (Promega) RTs. Reaction mixtures and conditions varied with RT, described in Table 3.2.13.1. Reagents were random hexamer primers (Thermo Scientific), dNTP mix (Thermo Scientific) appropriate buffers supplied by RT manufacturers. Synthesised cDNA was stored at -20°C.

Table 3.2.13.1 cDNA synthesis reaction conditions. Mixtures and reaction conditions for cDNA synthesis from RNA using two RTs; M-MLV and AMV.

RT	**M-MLV**	**AMV**
Primer Binding Step:		
RNA Template	1 µl	1 µl
Primers (1 pmol/µl)	1 µl (per primer)	1 µl (per primer)
Nuclease Free Water	Up to 15 µl	Up to 15 µl
Denaturation	70°C for 5 minutes	
	Store on ice and centrifuge briefly	
Synthesis Step:		
Buffer	5 µl (x5 Conc.)	5 µl (x8 Conc.)
RT	1 µl (100u/µl)	3 µl (10 u/µl)
dNTPs (2mM)	1.25 µl	2.5 µl
Nuclease Free Water	2.75 µl	24.5 µl
Total Reaction Volume	25 µl	40 µl
Incubation	37°C - 60 minutes	42°C - 60 minutes

3.2.14 qPCR

For detection of MVV sequences from extracted nucleic acids, a Sybr green based qPCR procedure was carried out. Reaction mixtures consisted of 1x qPCRBIO SyGreen Mix Lo-ROX master mix (PCR Biosystems), 0.04µM forward and reverse primers (Sigma-Aldrich) (Table 3.2.14.1) and 1µl of test DNA or standard in a total volume of 20 µl. Reaction conditions consisted of a starting incubation of 95°C for 15 minutes followed by 45 cycles of 95°C for 5 seconds, 60°C for 30 seconds and 72°C for 10 seconds. A melt cycle was carried out at reaction end ranging from 65°C to 95°C. Reactions were carried out within a CFX Connect Real-Time PCR Detection System (Biorad Laboratories). A 1:10 standard dilution series of a synthesised PCR product positive control. Analysis was carried out using Biorad CFX Maestro software.

Table 3.2.14.1 qPCR primer sequences. qPCR primers for detection of SRLV, targeting the Pol gene.

Primer	Target Gene	Sequence	Product Size
NGS Pol1 F	SRLV *pol*	AGGGGATGCATACTTTACTATACCA	
NGS Pol1 R		TCTTGTGCATGGCCCTAAAT	

3.3. Results

3.3.1 Pre-Trial Testing of Ewes and Rams

To confirm MVV status of the 30 naïve ewes and 13 'seropositive' rams taking part in this study, blood was tested for MVV infection. Tables 3.3.1.1+2 shows the results from two diagnostic assays carried out, ELISA and qPCR. Of 13 rams, all tested positive by ELISA whilst only 8 and 10 tested positive for DNA and RNA by qPCR, respectively (Table 3.3.1.1). In contrast, all 30 ewes tested negative by both ELISA and qPCR of DNA and RNA extracted from blood samples (Table 3.3.1.2).

In preparation of the trial, the 30 ewes were randomly allocated to three groups, ewes to be mock inseminated, ewes to be inseminated with semen harvested from seropositive rams and teasers ewes to be used in semen collection. Age distribution was dissimilar between inseminated and mock inseminated groups due to random selection at time of insemination (Table 3.3.1.2).

Table 3.3.1.1 Pre-trial diagnostic results of seropositive rams. ELISA and qPCR results for 13 rams to confirm seropositive status prior to start of AI trial.

Animal ID	ELISA	Blood DNA	Blood RNA
Ram 1	Positive	Positive	Positive
Ram 2	Positive	Negative	Positive
Ram 3	Positive	Positive	Positive
Ram 4	Positive	Positive	Positive
Ram 5	Positive	Negative	Positive
Ram 6	Positive	Negative	Positive
Ram 7	Positive	Negative	Positive
Ram 8	Positive	Positive	Positive
Ram 9	Positive	Positive	Negative
Ram 10	Positive	Positive	Negative
Ram 11	Positive	Positive	Positive
Ram 12	Positive	Negative	Negative
Ram 13	Positive	Positive	Positive

Table 3.3.1.2 Pre-trial diagnostic results of naïve ewes.

ELISA and qPCR testing of 30 ewes pre-trial to confirm maedi-visna seronegative status prior to onset of AI trial.

Experimental Group	Animal ID	Year of Birth	ELISA	Blood DNA	Blood RNA
Inseminated	00535	2010	Negative	Negative	Negative
	00779	2010	Negative	Negative	Negative
	01024	2011	Negative	Negative	Negative
	01120	2011	Negative	Negative	Negative
	01133	2011	Negative	Negative	Negative
	02465	2012	Negative	Negative	Negative
	02528	2012	Negative	Negative	Negative
	02542	2012	Negative	Negative	Negative
	02560	2012	Negative	Negative	Negative
	03064	2013	Negative	Negative	Negative
	03253	2013	Negative	Negative	Negative
	05010	2015	Negative	Negative	Negative
Mock Inseminated	01073	2011	Negative	Negative	Negative
	02500	2012	Negative	Negative	Negative
	03730	2013	Negative	Negative	Negative
	04577	2014	Negative	Negative	Negative
	05000	2015	Negative	Negative	Negative
	05024	2015	Negative	Negative	Negative
	05137	2015	Negative	Negative	Negative
	05161	2015	Negative	Negative	Negative
	05182	2015	Negative	Negative	Negative
	05296	2015	Negative	Negative	Negative
	05377	2015	Negative	Negative	Negative
	86281	2008	Negative	Negative	Negative
Teaser Ewes	01174	2011	Negative	Negative	Negative
	01201	2011	Negative	Negative	Negative
	02400	2012	Negative	Negative	Negative
	03141	2013	Negative	Negative	Negative
	04969	2014	Negative	Negative	Negative
	09223	2009	Negative	Negative	Negative

3.3.2 Semen Collection and Testing

To assess the risk of sexual transmission of MVV, semen was collected from 13 seropositive rams for insemination of naïve ewes. Through use of teaser ewes, semen was successfully collected from 11 of 13 rams. At introduction to teaser ewes, rams 4 and 6 showed lack of interest.

DNA and RNA tested by qPCR for the presence of MVV tested negative in all semen samples tested (Table 3.3.2.1). RNA extracted from epididymal washes obtained at day of slaughter (the day after semen collection) tested positive in 6 of 13 rams. Five of these rams had successful semen collection while 1 did not (Ram 6).

Table 3.3.2.1 MVV qPCR testing of DNA and RNA extracted from semen and epdidymal washes. Semen tested intended for insemination of naïve ewes. Epididymal washes were collected the day following semen collection.

Animal ID	Semen DNA	Semen RNA	Epididymal Washes RNA
Ram 1	Negative	Negative	Negative
Ram 2	Negative	Negative	Negative
Ram 3	Negative	Negative	Negative
Ram 4	-	-	Negative
Ram 5	Negative	Negative	Negative
Ram 6	-	-	Positive
Ram 7	Negative	Negative	Negative
Ram 8	Negative	Negative	Positive
Ram 9	Negative	Negative	Positive
Ram 10	Negative	Negative	Positive
Ram 11	Negative	Negative	Negative
Ram 12	Negative	Negative	Positive
Ram 13	Negative	Negative	Positive
Pooled	Negative	Negative	-

3.3.3 A.I. Trial – Blood Sampling

Inseminated ewes were blood sampled weekly up to 7 weeks post-insemination to detect for seroconversion. Animals inseminated with semen from infected rams showed no seroconversion with consistently low antibody titres observed (Figure 3.3.3.1a). Control group animal titres showed greater variation when compared to inseminated ewes. Of these animals, 05296 consistently showed higher antibody titres, within the limits of seronegative results, while 8629J showed a false positive result in week 6 confirmed by a repeat test (Figure 3.3.3.1b). The following week, the sample for this animal was negative.

In addition to ELISA, DNA and RNA extracted from blood was tested for the presence of MVV. All ewes during all three tested weeks (1, 4 and 7) were confirmed negative for both DNA and RNA (Table 3.3.3.2+3). Blood collected from 8628J at week 6 (tested positive by ELISA), when tested by qPCR, tested negative for both DNA and RNA.

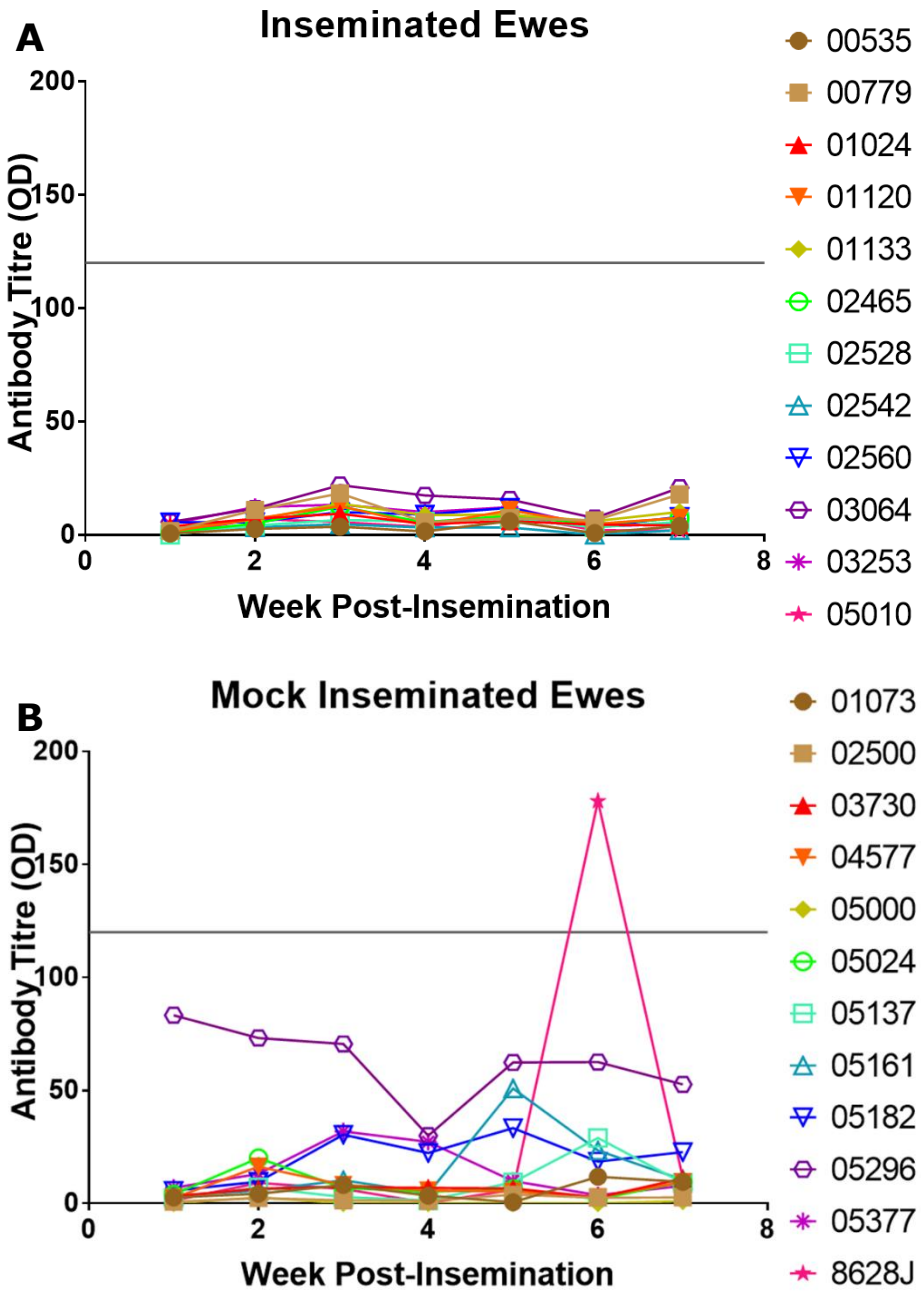


Figure 3.3.3.1 Maedi visna virus serum antibody titres determined by ELISA in ewes. Antibody titres for 12 ewes inseminated (a) with semen from naturally infected rams or (b) 12 mock inseminated ewes over 7 weeks post insemination obtained by ELISA of serum.

Table 3.3.3.2 Blood DNA qPCR results from inseminated ewes. qPCR results for detection of MVV in DNA extracted from blood samples collected at four time points (week 1, 4, 6 and 7) from 12 ewes mock inseminated and 12 ewes inseminated with semen collected from known MVV seropositive rams.

Experimental Group	Animal ID	Week 1	Week 4	Week 6	Week 7
Inseminated	00535	Negative	Negative	-	Negative
	00779	Negative	Negative	-	Negative
	01024	Negative	Negative	-	Negative
	01120	Negative	Negative	-	Negative
	01133	Negative	Negative	-	Negative
	02465	Negative	Negative	-	Negative
	02528	Negative	Negative	-	Negative
	02542	Negative	Negative	-	Negative
	02560	Negative	Negative	-	Negative
	03064	Negative	Negative	-	Negative
	03253	Negative	Negative	-	Negative
	05010	Negative	Negative	-	Negative
Mock Inseminated	01073	Negative	Negative	-	Negative
	02500	Negative	Negative	-	Negative
	03730	Negative	Negative	-	Negative
	04577	Negative	Negative	-	Negative
	05000	Negative	Negative	-	Negative
	05024	Negative	Negative	-	Negative
	05137	Negative	Negative	-	Negative
	05161	Negative	Negative	-	Negative
	05182	Negative	Negative	-	Negative
	05296	Negative	Negative	-	Negative
	05377	Negative	Negative	-	Negative
	8628J	Negative	Negative	Negative	Negative

Table 3.3.3.3 Blood RNA qPCR results from inseminated ewes. qPCR results for detection of maedi visna virus in RNA extracted from blood samples collected at four time points (week 1, 4, 6 and 7) from 12 ewes mock inseminated and 12 ewes inseminated with semen collected from known MVV seropositive rams.

Experimental Group	Animal ID	Week 1	Week 4	Week 6	Week 7
Inseminated	00535	Negative	Negative	-	Negative
	00779	Negative	Negative	-	Negative
	01024	Negative	Negative	-	Negative
	01120	Negative	Negative	-	Negative
	01133	Negative	Negative	-	Negative
	02465	Negative	Negative	-	Negative
	02528	Negative	Negative	-	Negative
	02542	Negative	Negative	-	Negative
	02560	Negative	Negative	-	Negative
	03064	Negative	Negative	-	Negative
	03253	Negative	Negative	-	Negative
	05010	Negative	Negative	-	Negative
Mock Inseminated	01073	Negative	Negative	-	Negative
	02500	Negative	Negative	-	Negative
	03730	Negative	Negative	-	Negative
	04577	Negative	Negative	-	Negative
	05000	Negative	Negative	-	Negative
	05024	Negative	Negative	-	Negative
	05137	Negative	Negative	-	Negative
	05161	Negative	Negative	-	Negative
	05182	Negative	Negative	-	Negative
	05296	Negative	Negative	-	Negative
	05377	Negative	Negative	-	Negative
	8628J	Negative	Negative	Negative	Negative

3.3.4 PM Findings of Ewes

Following euthanasia, PM and sample collection was carried out on trial animals to look for indicators of MVV infection. Of 24 ewes, only two (05377 and 02542) showed any signs of gross pathology (Figure 3.3.4.1). Both ewes showed pathology in the lungs. 05377 (mock inseminated group) showed extensive fibrosis of dorsal medial surface of both lungs, in addition, there were small gritty nodules upon the surface of both lungs. 02542 (inseminated group) had areas of consolidation on approximately a third of the caudal dorsal surface of both lungs (Figure 3.3.4.1).

Histology of lungs was assessed for histopathology indicative of MVV infection. Slides were prepared for four inseminated ewes (00779, 01120, 02542 and 05010) and four mock inseminated ewes (01073, 03730, 05377 and 8628J). No signs indicative of MVV infection were seen in any of the ewe slides, however other pathology (such as evidence of lung worm scarring) was evident (Figure 3.3.4.3).

In addition, during sample collection, the uterus of each animal was inspected for the presence of a foetus. Of 12 inseminated ewes, none were seen to have foetuses.

Animal	Location	Picture	Description
05377			
	Lungs	B	Extensive fibrosis of dorsal medial surface of both lungs, presence of small gritty nodules
02542			
	Lungs	C	Areas of consolidation on caudal dorsal surface of both lungs

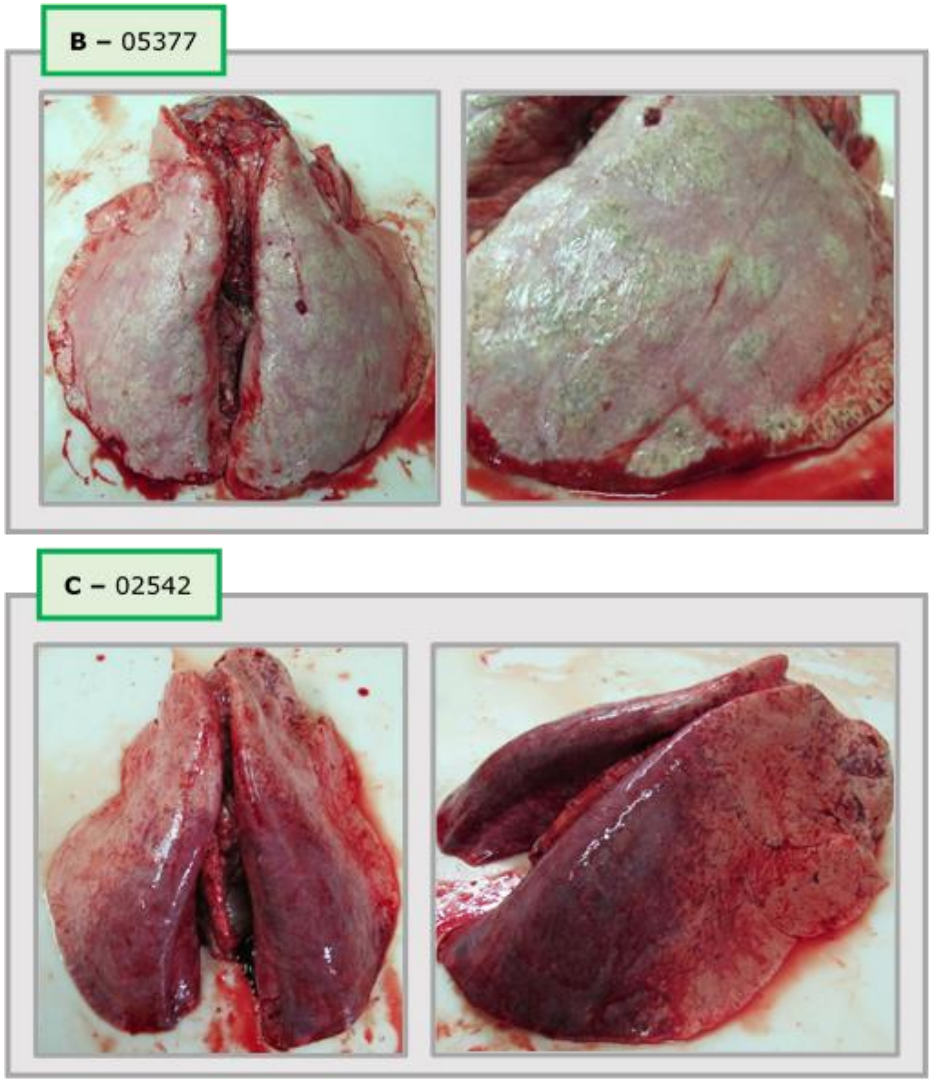


Figure 3.3.4.1 Lung pathology of ewes 05377 and 02542. (A) Pathology observed following euthanisation of 24 ewes 7 weeks post mock insemination (n=12) or insemination (n=12) with semen collected from known MVV seropositive rams. Pathology was seen in two ewes: (B) 05377 (inseminated group) and (C) 02542 (mock inseminated group).

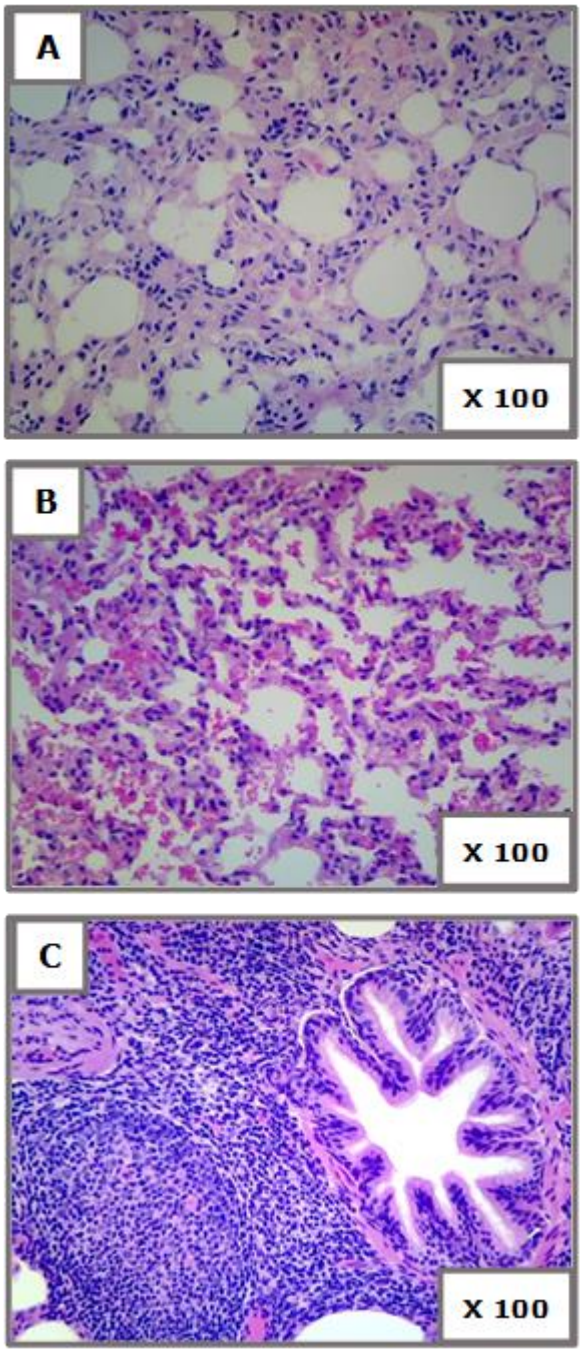


Figure 3.3.4.2 Histology of MVV seropositive rams indicative of infection. Histology observed with lung tissue of seropositive rams known to be associated with infection: (A) thickening of alveolar septa, (B) obliteration of alveolar structures and (C) lymphoid infiltration with occurrence of formation of lymphoid-like follicle.

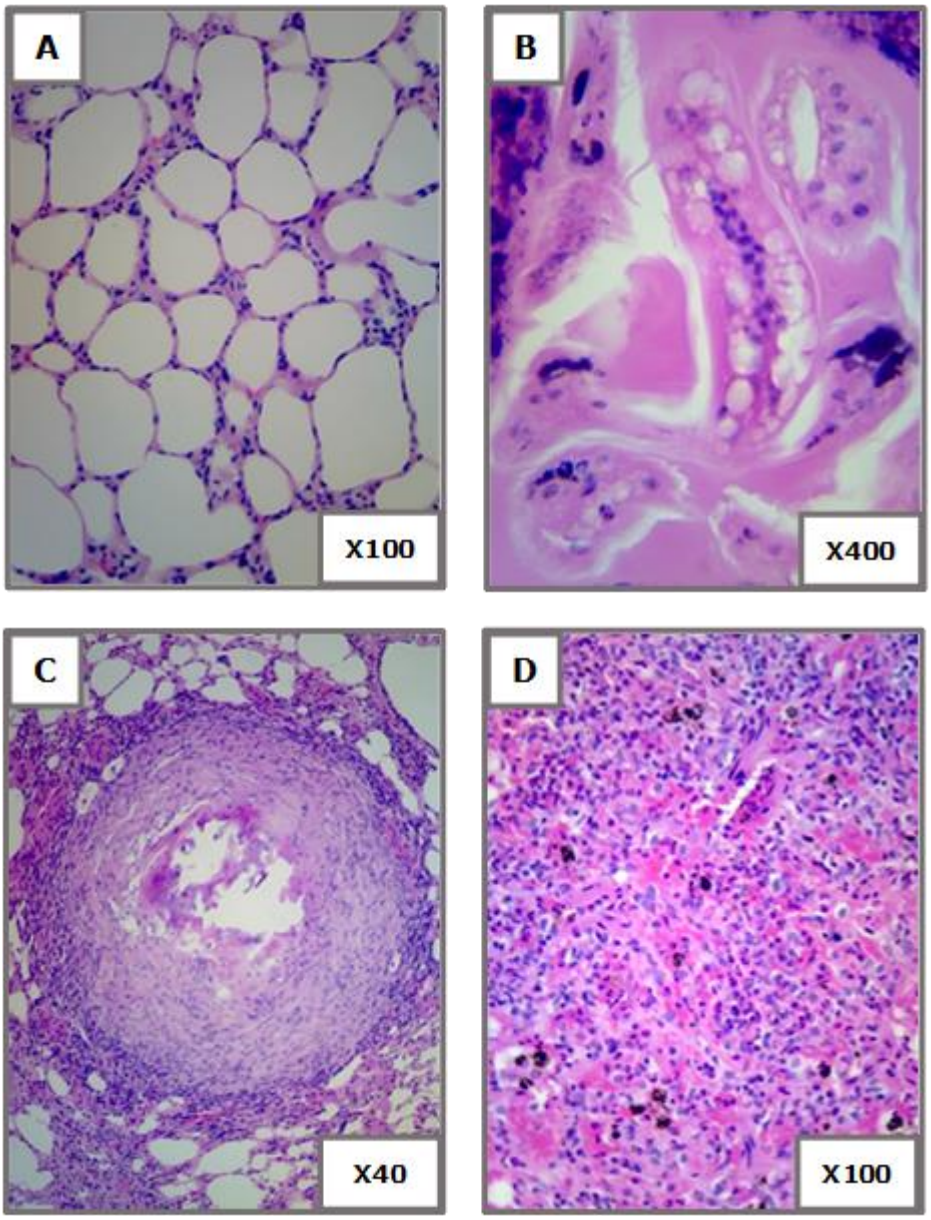


Figure 3.3.4. Lung histology of 8 trial ewes. Histology observed in lungs of ewes mock inseminated (n=4) and inseminated (n=4) with semen collected from MVV seropositive rams 7 weeks prior to euthanasia. (A) healthy lung, (B) presence of lung worms, (C) region of scarring and (D) areas of bleeding.

3.3.5 qPCR Testing of Tissue

To detect for any presence of MVV within trial ewes, DNA was extracted from lung, mediastinal lymph node and uterus tissue. All ewes in both groups tested negative for all tissue types when tested by qPCR (Table 3.3.5.1).

Table 3.3.5.1 qPCR results for DNA extracted from tissue of ewes. Lung, mediastinal lymph node and uterine tissue collected following euthanasia 7 weeks post mock insemination (n=12) or insemination (n=12) with semen collected from known MVV seropositive rams.

Experimental Group	Animal ID	Lung DNA	Lymph DNA	Uterus DNA
Inseminated	00535	Negative	Negative	Negative
	00779	Negative	Negative	Negative
	01024	Negative	Negative	Negative
	01120	Negative	Negative	Negative
	01133	Negative	Negative	Negative
	02465	Negative	Negative	Negative
	02528	Negative	Negative	Negative
	02542	Negative	Negative	Negative
	02560	Negative	Negative	Negative
	03064	Negative	Negative	Negative
	03253	Negative	Negative	Negative
	05010	Negative	Negative	Negative
Mock Inseminated	01073	Negative	Negative	Negative
	02500	Negative	Negative	Negative
	03730	Negative	Negative	Negative
	04577	Negative	Negative	Negative
	05000	Negative	Negative	Negative
	05024	Negative	Negative	Negative
	05137	Negative	Negative	Negative
	05161	Negative	Negative	Negative
	05182	Negative	Negative	Negative
	05296	Negative	Negative	Negative
	05377	Negative	Negative	Negative
	8628J	Negative	Negative	Negative

3.4 Discussion

Ewes naïve to MVV infection in this study were inseminated with semen collected from known seropositive rams to estimate the risk associated with sexual transmission of MVV. Testing prior to the beginning of the trial confirmed the MVV status of all animals. Following this, after a period of 7 weeks post insemination no ewes showed sign of seroconversion by ELISA or presence of viral genome when tested by qPCR. The lack of detectable MVV infection within these ewes, could suggest that the risk of viral transmission following intravaginal insemination to be low. It is important to note that this finding can only be applied in cases where seropositive rams are showing undetectable viral levels within semen. Taking this stipulation into consideration, movement from intrauterine insemination to intravaginal could allow for use of semen collected from seropositive rams (with undetectable semen viral loads) with low risk of transmission. This would potentially allow for genetic rescue of valuable genetics from infected individuals reducing the economic impact of MVV infection on farmers.

It is important to note that both previous studies investigating sexual transmission in SRLVs did not use semen collected from naturally infected animals (Ali Al Ahmad et al. 2012; Souza et al. 2013). Instead, both studies spiked semen samples collected from seronegative animals with virus to a concentration of 10^2-10^6 $TCID_{50}$/ml. In addition, previous studies that have identified the presence of virus within semen have not reported associated viral loads (Ali Al Ahmad et al. 2008; Paula et al. 2009). Therefore, it is unknown if the viral loads utilised within the previously reported insemination trials was indicative of natural levels in infected animals and therefore risk of sexual

transmission by intrauterine insemination using semen from naturally infected bucks/rams could be reduced compared to previous reports.

The seropositive status of the 13 rams was confirmed by ELISA and qPCR. Despite discrepancies in results of qPCR testing of DNA and RNA extracted from ram blood, the results collectively demonstrate infection in all animals. Semen collected from rams and epididymal washes collected at time of euthanasia were tested to discern if MVV was present. While all semen samples tested negative when testing either DNA or RNA, RNA extracted from epididymal washes tested positive in 6 of 13 rams. As testing was carried out on RNA, viral genetic material detected likely represents free virions present within the epididymis. As the epididymis plays a role in sperm transport and maturation, detection of MVV within epididymal washes would therefore suggest the presence of virus within semen, but likely below the detectable threshold for the qPCR assay due to the diluting effect of fluids from the extraseminal vesicles added during natural ejaculation and the pooling of the samples for use in the actual insemination (Cornwall 2009).

Intermittence of detection of MVV within infected hosts has previously been reported (de Andrés et al. 2005; Ali Al Ahmad et al. 2008; Peterson et al. 2008). Discrepancies in detection of MVV in blood by qPCR vs ELISA and the inability to detect virus within DNA and RNA extracted from semen may be attributed to this. In relation to the finding of this study, results suggest low level shedding in semen. Therefore, periods of high shedding may still occur and rams would require further testing over longer time periods to estimate the longer term risk of sexual transmission. In cases of lactogenic transmission, Pisoni et al. (2010) proposed a threshold viral load necessary to

facilitate successful transmission. Further investigation into sexual transmission would be required to ascertain the presence of such a threshold associated with this route of transmission.

Insemination of hormonally prepared ewes with semen collected from seropositive rams did not result in successful conception in any of the 12 ewes. Semen was pooled from 11 rams and the volume increased to by addition of UHT milk before insemination. Success rates of vaginal insemination has reported to vary with breed in the range of approximately 30-65% (Anel et al. 2005; Paulenz et al. 2007). It is not impossible with the small cohort of ewes present within this study for all 12 to have naturally failed to conceive without any external contributary factor. In contrast, random selection of ewes at time of insemination resulted in a large proportion of ewes within the inseminated group being between the ages of 3 and 5 (9 out of 12). This has previously been reported as the age at which ewe fertility is highest and therefore should have aided towards successful conception (Aktas et al. 2015). External factors that could contribute to failure to conceive within this study include, improper insemination, miss handling of semen between collection and insemination or semen viability. The insemination was however conducted by an experienced small ruminant veterinarian. A further external factor potentially contributing to the low conception rates was also the circulation of Schmallenberg virus, an insect borne abortogenic virus of small ruminant that is suspected to also cause failures to conceive during acute infections, during the period of the trial (and detected in these animals) (Veldhuis et al. 2014; Jones et al. 2019).

Unfortunately, the lack of fetuses in this study prevented further investigation of the risk of vertical transmission from the seropositive rams.

During the trial one mock inseminated ewe (8628J) showed a positive result by ELISA at week 6 confirmed by repeat testing. Testing the following week and qPCR testing of DNA and RNA extracted from blood throughout the trial was negative. Together, these results suggest that 8628J showed a false positive result at week 6. Testing further than week 7 was not carried out as ewes were euthanised at week 7 due to insufficient funds and time constraints. In support of this, no gross or histopathological lesions of MVV were seen at PM and no tissue samples from this animal had detectable virus. Souza et al. (2013) reported seroconversion within 30-60 days post-insemination within all trial animals, therefore it is possible that 7 weeks was not sufficient to allow for seroconversion. In addition, as previously stated it is possible that said study utilised significantly enlarged viral loads within semen used which would aid in reduced time for seroconversion. Together this suggests that 7 weeks is in sufficient time to conclusively state transmission has not occurred, but this would require more thorough testing to corroborate.

Overall, the findings of this study suggest the risk of sexual transmission following vaginal insemination in naïve ewes with semen collected from seropositive rams to be low (at least when rams are excreting low levels of virus). As this study represented a pilot scale study, next steps would be to increase the size of the trial cohort whilst also increasing the period of repeated testing following insemination to be longer than 7 weeks. Of most importance, the findings of these results show promise for reducing the impact of MVV infection on farmers of

breeding rams or high value breeds by demonstrating that rescue for genetics from valuable rams without perpetuating virus infection is possible.

Chapter 4: Longitudinal Study of 28 MVV Seropositive Rams over a 28-Month Period

4.1 Introduction

To sheep and goats, SRLV infection is a lifelong sentence. An approximate asymptomatic period of around 2 years post infection can result in significant flock wide infection before any indicators are observed. in addition, with clinical signs overlapping with other respiratory conditions, infection can go unnoticed unless specifically looked for (Sigurdsson 1954).

To date, SRLV infection is typically confirmed by ELISA, AGID or PCR based assays targeting specific antibodies or viral nucleotides present within blood and milk samples (Herrmann-Hoesing 2010; OIE 2016). Although effective, such sampling techniques hold limitations in their requirement of licenced technicians for collection (blood) and limitation to females postpartum (milk). Palsson (1972) once showed successful isolation of virus from nasal swabs taken from seropositive sheep, however, with varying degrees of success between individuals. Such a finding begs the question of whether using present day diagnostics, could virus be detected in such swabs (for which collection can be easily accomplished by farmers themselves) and with what efficacy and reliability.

Diagnostic testing of infected animals following death or euthanasia has detected proviral DNA within a wide range of host tissues such as liver, heart, kidneys, bone marrow, ovaries and even third eyelid tissue (Capucchio et al. 2003; Grossi et al. 2005; Angelopoulou et al. 2006; Brellou et al. 2007; Cortez-Romero et al. 2011). Despite this abundance of virus throughout the body, pathology is typically localised to three

regions of the body: respiratory tract, CNS and mammary glands. In the UK, circulating strains of virus usually present as respiratory and mammary conditions. As such, lungs are typically observed as being noticeably larger (2-3 times) with a slight rigidity (Cutlip et al. 1979). Varying degrees of greyish discoloration can be present, normally localised to coalescing multifocal spots along the surface with areas of consolidation seen on the dorsal surface of both lungs. Adhesion of lung to the thoracic wall and 1-2mm grey fibrous nodules have also been reported, but in less frequency (Christodoulopoulos 2006).

Histologically, significant lymphoid infiltration is often seen in lungs associated with bronchioles and blood vessels (Ellis and DeMartini 1985). In severe cases formation of lymphoid follicle-like structures is seen. This in turn results in thickening of the alveolar septa and obliteration of the alveolar structures present within the lung (DeMartini et al. 1993).

Herrmann-Hoesing et al. (2009) reported that the severity of lesions observed in infected animals is proportional to the provirus load, with high blood proviral levels found in animals showing lesions of greater severity. Interestingly, higher viral loads were shown in blood and tissues of individuals with concurrent inflammatory conditions, such as parasitism and bronchitis in the lungs or orchitis within testicles in a recent study (Grego et al. 2018). In line with this, two studies reported detection of virus within the epididymis or semen only in animals suffering concurrent infections of *Brucella ovis (B. ovis)* (de la Concha-Bermejillo et al. 1996; Preziuso et al. 2002). From this it could be proposed that secondary infection resulting in an inflammatory response, could lead to recruitment of infected macrophages and subsequent 'activation' of latent

virus within these cells, resulting in increased viral loads within cells and surrounding tissues.

In contrast to these findings, it has been suggested that sheep homozygous for TMEM154 haplotype 1 (K35), in addition to having increased resistance to SRLV infection, are also able to control viral replication once infected (Alshanbari et al. 2014). This is following comparison of viral loads in resistant and susceptibles animals, showed significantly reduced viral loads in resistant animals. Together this suggests that resistant animals would not only be more resistant to intial infection, they are likely to have reduced viral loads if infected and are probably less likely to develop severe lesions. Genetic selection of animals with the MV resistant alleles of TMEM154 is likely to reduce the potential impact of infection on farms and aid in control of infection.

In this chapter, a longitudinal study was carried out within a group of 28 MVV seropositive rams following diagnosis during routine testing as part of the MVAS/CAEAS. Rams were maintained for a period 28 months. The study aimed to quantify the long-term impact of MV by a case study of morbidity and mortality due to the disease in these individuals.

4.2 Material and Methods

4.2.1 Ethical Approval

All animal management and procedures were reviewed and approved by the Home Office under the 'Animals (Scientific Procedures) Act 1986' (Licence no. PPL 30/3367).

4.2.2 Animals

28 Aberfield and Abermax MVV seropositive rams were included in this study. Rams were identified as being MVV positive during routine testing as part of the MVAS after which they were acquired by the University of Nottingham in 2015. Animals were held at pasture with available shelter and supplementary rations. In addition, animals were monitored and treated for any signs of clinical disease (including diseases such as foot-rot) and received routine husbandry and preventative treatment. Animals with illness were assessed by a named veterinary surgeon. Animals found to have significant drop in condition likely associated to clinical disease, were humanely euthanised. A number of animals were also culled for flock management purposes. In addition, instances of sudden death were noted. Finally, 13 surviving rams participated in an AI trial study before being euthanised. At time of euthanasia or sudden death, PMs were carried out. Appropriate blood and tissue samples were collected, and any pathology recorded. Tissue samples were collected in 500 µl RNAlater or 500 µl formalin and were then stored at room temperature for 24 hours and then stored at -20^{o}C or room temperature, respectively. Formalin samples were transferred into 70% ethanol after three months. Tissues */collected are listed in Table 4.2.2.2. Rams 1-13 refer to

Table 4.2.2.1 Timetable of longitudinal study. Timetable covering the period of 2014-2016 of (black) initial MVV diagnosis and sample collection and (red) date of sudden death/euthanasia of rams and subsequent post-mortem.

	2014	2015	2016
Jan			
Feb			Rams 20 + 21
Mar			
Apr		Blood Sampling	
May			Rams 22 + 23
Jun	28 Rams tested MVV seropositive		Rams 24-27
Jul		Ram 16	
Aug			
Sep	Ram 14		
Oct			Blood Sampling Ram 1-13
Nov		Ram 17	
Dec	Ram 15	Rams 18 + 19 Blood Sampling	

Rams alive at onset of insemination trial whilst rams 14-27 refer to those euthanised/expired prior to study start (Table 4.2.2.1).

4.2.3 Blood Collection

For detection of MVV, blood was collected from seropositive rams April 2015, December 2015 and at time of death/euthanasia (Table 4.2.2.1). Blood was collected in 10ml vacutainer blood tubes and taken from the jugular vein by a qualified veterinarian or home office licenced technician. For separation of sera from blood, samples were left at room temperature overnight to allow for clotting after which sera was removed via pipetting. Sera and blood clots were stored at -20^{o}C.

4.2.4 ELISA

Sera was tested for the presence of SRLV specific antibodies using the MVV/CAEV p28 Antibody Screening Test (IDEXX, Netherlands) following the manufacturer's recommended

Table 4.2.2.1 Tissues collected from expired/euthanised rams.

Animals	**Rams 1-13**	**Rams 14-27****
Tissues Sampled*	Trachea Lung Heart Kidney Liver Joint Cartilage Mediastinal Lymph Node Sperm (via epididymal wash) Nasal Swab Cerebral Swab Oral Swab	Trachea Lung Heart Kidney Liver Joint Cartilage Mediastinal Lymph Node Sperm (via epididymal wash) Nasal Swab Cerebral Swab Oral Swab Testicle Semen Brain Spleen Joint Fluid

* **Sites of pathology observed at post mortem were sampled**
**** Tissues collected not consistent between animals**

protocol. Repeated in triplicate, seroprevalence was determined by a minimum of two consistent results. Inconclusive results were subjected to further testing.

4.2.5 DNA Extraction – Tissue

DNA was extracted from tissues using the Nucleospin® Tissue Kit (Macherey-Nagel, Hoerdt, France) for detection of MVV using PCR based methods.

Approximately 25mg of tissue was processed following the manufacturers recommended protocol. Following addition of buffer T1 and proteinase k (pre-lyse step) a step was added with a sterile 5mm steel bead added to the samples. The mixtures were then homogenized by Retsch MM300 bead mill (Qiagen) at a frequency of 25/s for 2minutes. Following this, manufacturers protocol was followed as recommended. DNA was eluted from the column in a final volume of 60 µl. DNA was stored at -20 ℃ until use.

Successful DNA extraction was confirmed by quantification of DNA content using the NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific, UK).

4.2.6 DNA Extraction – Blood

DNA was extracted from blood using the Nucleospin® Tissue Kit (Macherey-Nagel, Hoerdt, France) for detection of MVV using PCR based methods.

Blood clots were processed by following the supplementary protocol for extraction of genomic and viral DNA from blood samples. Before the protocol was carried out, a small amount (approximately 25 mg) of blood clot was added to 200 µl of PBS. A sterile 5mm steel bead was then added and the mixture homogenised by a Retsch MM300 bead mill (Qiagen) at a

frequency of 25/s for 2 minutes. The protocol was then followed substituting homogenised blood clot and PBS for 200 µl of fresh blood. DNA was eluted from the column in a final volume of 60 µl. DNA was stored at -20℃ until use.

Successful DNA extraction was confirmed by quantification of DNA content using the NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific, UK).

4.2.7 DNA Extraction – Nasal Swabs

DNA was extracted from nasal swabs using the Nucleospin® Tissue Kit (Macherey-Nagel, Hoerdt, France) for detection of MVV by PCR based methods.

For extraction from nasal swabs, the supplementary protocol for purification of genomic DNA from buccal swabs was followed. Following addition of proteinase k and PBS, a sterile 5mm steel bead was added and the mixture placed in a Retsch MM300 bead mill (Qiagen) at a frequency of 25/s for 2 minutes. For separation of lysate from swab, alternative C of the supplementary protocol was carried out. DNA was eluted in a final volume of 60 µl and stored at -20°C.

Successful DNA extraction was confirmed by quantification of DNA content using the NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific, UK).

4.2.8 RNA Extraction

RNA was extracted from sera isolated from blood, epididymal washes and supernatant of nasal swabs using the QIAamp Viral RNA Mini Kit (Qiagen, Manchester, UK) for detection of MVV using PCR based methods. Extraction was carried out following

the manufacturer's recommended protocol. RNA was eluted within a final volume of 60 µl. RNA was stored at -20°C

4.2.9 cDNA Synthesis

Conversion of RNA to cDNA for PCR detection was carried out. One of two RTs were used per reaction; M-MLV (Promega) or AMV (NEB). Reaction mixtures and conditions varied with RT, described in Table 3.2.13.1. Reagents were random hexamer primers (Thermo Scientific), dNTP mix (Thermo Scientific) appropriate buffers supplied by RT manufacturers. Synthesised cDNA was stored at -20°C.

Table 4.2.8.1 cDNA synthesis reaction conditions. Mixtures and reaction conditions for cDNA synthesis from RNA using two RTs; M-MLV and AMV.

RT	**M-MLV**	**AMV**
Primer Binding Step:		
RNA Template	1 µl	1 µl
Primers (1 pmol/µl)	1 µl (per primer)	1 µl (per primer)
Nuclease Free Water	Up to 15 µl	Up to 15 µl
Denaturation	70°C for 5 minutes	
	Store on ice and centrifuge briefly	
Synthesis Step:		
Buffer	5 µl (x5 Conc.)	5 µl (x8 Conc.)
RT	1 µl (100u/µl)	3 µl (10 u/µl)
dNTPs (2mM)	1.25 µl	2.5 µl
Nuclease Free Water	2.75 µl	24.5 µl
Total Reaction Volume	25 µl	40 µl
Incubation	37°C - 60 minutes	42°C - 60 minutes

4.2.10 qPCR

For detection of MVV sequences from extracted nucleic acids, a Sybr green based qPCR procedure was carried out. Reaction mixtures consisted of 1x qPCRBIO SyGreen Mix Lo-ROX master mix (PCR Biosystems), 0.04µM forward and reverse primers (Sigma-Aldrich) (Table 3.2.14.1) and 1µl of test DNA or standard in a total volume of 20 µl. Reaction conditions

consisted of a starting incubation of 95°C for 15 minutes followed by 45 cycles of 95°C for 5 seconds, 60°C for 30 seconds and 72°C for 10 seconds. A melt cycle was carried out at reaction end ranging from 65°C to 95°C. Reactions were carried out within a CFX Connect Real-Time PCR Detection System (Biorad Laboratories). A 1:10 standard dilution series of a synthesised PCR product positive control was prepared for use as a standard. Analysis was carried out using Biorad CFX Maestro software.

Table 4.2.10.1 qPCR primer sequences. qPCR primers for detection of SRLV, targeting the Pol gene.

Primer	Target Gene	Sequence	Product Size
NGS Pol1 F	SRLV *pol*	AGGGGATGCATACTTTACTATACCA	
NGS Pol1 R		TCTTGTGCATGGCCCTAAAT	

4.2.11 PCR – TMEM154 Genotyping

Amplification of desired TMEM154 sequences for the determination of TMEM154 genotypes present in seropositive rams was carried out by PCR. For template, 1 µl of DNA extracted from the lung tissue of MVV seropositive rams was used in a reaction mixture of 25 µl. Each reaction contained 5 units of *Taq* DNA Polymerase, 1x standard *Taq* (Mg-free) reaction buffer (NEB), 3mM magnesium chloride ($MgCl_2$) (NEB), 0.04 pmol of forward and reverse primers (Table 4.2.11.1) and 0.4mM deoxynucleotide (dNTP) solution mix (Thermo Scientific). Standard PCR cycling conditions consisted of an initial denaturation phase of 95°C for 5 minutes followed by 45 cycles of 95°C, 56/60°C and 68°C, each for 15-60 seconds. Reactions were carried out within a Thermal cycler Life ECO (Bioer Technology). Successful amplification was determined by gel electrophoresis of PCR products. Primers used are listed

within Table 4.2.11.1 stating target gene and sequence (Heaton et al. 2013).

Table 4.2.11.1 Primers for TMEM154 genotyping.

Primer	Target Exon	Sequence	Amplicon Length
84253	Exon 1	GCGAGGCGTGCTAACTG	914 bp
83023		GCTTCATTAGTCACAATCAAC	
86824	Exon 2	TCCATTTCCTTTACCTAAAAGT	1048 bp
86826		ACTGGCCCAAATTACATAAG	

2.2.4 Gel Electrophoresis

To allow identification of products produced by PCR, gel electrophoresis was utilised. Expected PCR products were smaller than 1000 bp, therefore, a 0.8% Agarose gel was prepared to which 1 µl of Nancy-520 (Sigma Aldrich) per 20 ml Tris/acetate/EDTA solution (TAE) had been added. Products were loaded on the gel alongside a 100bp DNA ladder (NEB) and run at 100 V for 45 minutes. Gels were viewed and photographed by ImageQuant LAS 400 (GE Healthcare Life Science, UK) under ultraviolet (UV) light. Confirmed products for which the nucleotide sequence was required were prepared for sequencing using the Nucleospin® Gel and PCR Clean-up kit (Macherey-Negal) following the recommended protocol for PCR clean-up.

4.2.12 Sanger Sequencing and Analysis

Nucleotide sequences were acquired by Sanger sequencing carried out by Source BioScience. 5 µl of product to be sequenced was prepared at 10 ng/µl per 5 µl of primer, at a concentration of 3.2 pmol/µl. Sequence analysis was completed using BioEdit v7.2 and CLC Sequence Viewer software v8.0 (Qiagen).

4.3 Results

4.3.1 Rams

28 MVV seropositive rams were reared together to the age of 1 before they were obtained by the University of Nottingham following diagnosis during routine testing. Thirteen of these rams participated in an AI trial (Chapter 3) which commenced October 2016. Prior to trial commencement 15 rams either died or were euthanised. Of this number, 3 were recorded as sudden death and 10 were euthanised following a drop in body condition. Two rams (14 and 28) were not recorded as either. Of these rams that died or were euthanised, no individual was of the Aberfield breed.

Rams were identified by laboratory number (1-28) or by personal I.D. number (e.g. 00605) (Table 4.3.1.1). Cross referencing of numbers was recorded where possible but loss of ear tags and recording errors resulted in 8 laboratory numbers and I.D. numbers being unable to be paired. Due to labelling at time of collection all results for blood samples will refer to individuals by I.D. number whilst remaining results will refer to laboratory number.

Table 4.3.1.1 Identification of rams. Cross referenced laboratory number and individual ID of 28 rams. Breed and date of death are stated where known.

	Animal ID	Breed	Date of Death
Ram 1	?	Abermax	28/10/2016
Ram 2	01017	Aberfield	28/10/2016
Ram 3	01021	Aberfield	28/10/2016
Ram 4	01224	Abermax	28/10/2016
Ram 5	01016	Aberfield	28/10/2016
Ram 6	01008	Aberfield	28/10/2016
Ram 7	02208	Abermax	28/10/2016
Ram 8	02332	Abermax	28/10/2016
Ram 9	*01001*	Aberfield	28/10/2016
Ram 10	01019	Aberfield	28/10/2016
Ram 11	00639	Abermax	28/10/2016
Ram 12	02371	Abermax	28/10/2016
Ram 13	02316	Abermax	28/10/2016
Ram 14	?	Abermax	??/09/2014
Ram 15	02145	Abermax	15/12/2014
Ram 16	?	Abermax	01/07/2015
Ram 17	?	Abermax	03/11/2015
Ram 18	?	Abermax	15/12/2015
Ram 19	?	Abermax	15/12/2015
Ram 20	?	Abermax	21/02/2016
Ram 21	?	Abermax	21/02/2016
Ram 22	00608	Abermax	23/05/2016
Ram 23	02523	Abermax	23/05/2016
Ram 24	02550	Abermax	02/06/2016
Ram 25	00657	Abermax	02/06/2016
Ram 26	00669	Abermax	20/06/2016
Ram 27	02227	Abermax	20/06/2016
Ram 28	?	Abermax	?
	00605		
	00647		
	00654		
	02107		
	02153		
	02220		
	02296		
	02535		
	02889		

4.3.2 Pathology and Histopathology

At time of death following either sudden death or euthanasia, PMs were carried out to assess for pathology indicative of MVV infection. In addition, sections of lung were prepared from 15 rams (Ram 1-13, 26 and 27) for histology. Table 4.3.2.1 and Figure 4.3.2.2 describe and illustrate observed pathology, respectively. Ten rams showed pathology of the lung, with pathology of liver, kidney, trachea and heart identified in a single ram each.

Of 10 lungs, 8 showed consolidation over the dorsal surface of lungs in varying severities. Gray colouration was noted in multiple lungs (n=7), degree of colouration varied, with colour localised to coalescing multifocal spots along the surface of most affected lungs. One ram showed marked increase in size of the right lung in comparison to a normal sized left lung. Finally, one lung was found to be adhered to the thoracic wall at time of PM.

Histologically, four main abnormalities were clear in the majority of slides prepared (Table 4.3.2.3). Infiltration of immune cells in lung tissue was observed in 14 rams with the formation of lymphoid follicle-like structures being found in 8 rams (Figure 4.3.2.3a). Thickening of the alveolar septa and destruction of alveolar structures was also observed in 11 and 8 rams, respectively (Figure 4.3.2.4b+c). Interestingly, 11 rams showed evidence of lung worm infection (Figure 4.3.2.4c).

Table 4.3.2.1 Gross pathology of rams at PM. Table describes the observed pathology of 11 of 28 rams MVV seropositive following euthanasia or sudden death between June 2014 and October 2016.

Animal	Date	Location	Figure 4.3.2.2	Description
No Tag	Unknown	Lungs		Severe enlargement of right lung
02145 (Ram 15)	Dec 2014	Trachea	A	Diffused darkening and reddening
		Lungs	B	Moderate absence of pulmonary collapse; diffuse, moderately red discolouration of both lungs
		Kidneys		Markedly soft, pulpy
No Tag (Ram 16)	Jul 2015	Lungs	C	Approximately 2/3 of left lung, on cranial surface, covered by abundant amount of fibrin; extensive cranioventral consolidation in apical and middle lobes of pulmonary parenchyma, that secrete pus on compression
		Heart		Fibrin abundant within the pericardial sac
No Tag (Ram 1)	Oct 2016	Lungs	D	Consolidation of craniodorsal surface of right lung
00639 (Ram 11)	Oct 2016	Liver		Slight scarring
01016 (Ram 5)	Oct 2016	Lungs	E	Consolidation of craniodorsal surface of left lung; extensive areas of lumps and scarring throughout lungs
01017 (Ram 2)	Oct 2016	Lungs	F	Slight region of consolidation on craniodorsal surface of left lung
01021 (Ram 3)	Oct 2016	Lungs	G	Slight region of consolidation on craniodorsal surface of right lung
02208 (Ram 7)	Oct 2016	Lungs	H+I	Dark foci of consolidation on craniodorsal surface of left lung; adhesion of right lung to the thoracic wall
02332 (Ram 8)	Oct 2016	Lungs	J	Patches of consolidation over dorsal surface of left lung
02371 (Ram 12)	Oct 2016	Lungs	K+L	Oedema; consolidation of lateral dorsal surface of left lung; coalescing multifocal grey-white nodules/plaques on dorsal medial surface of both lungs

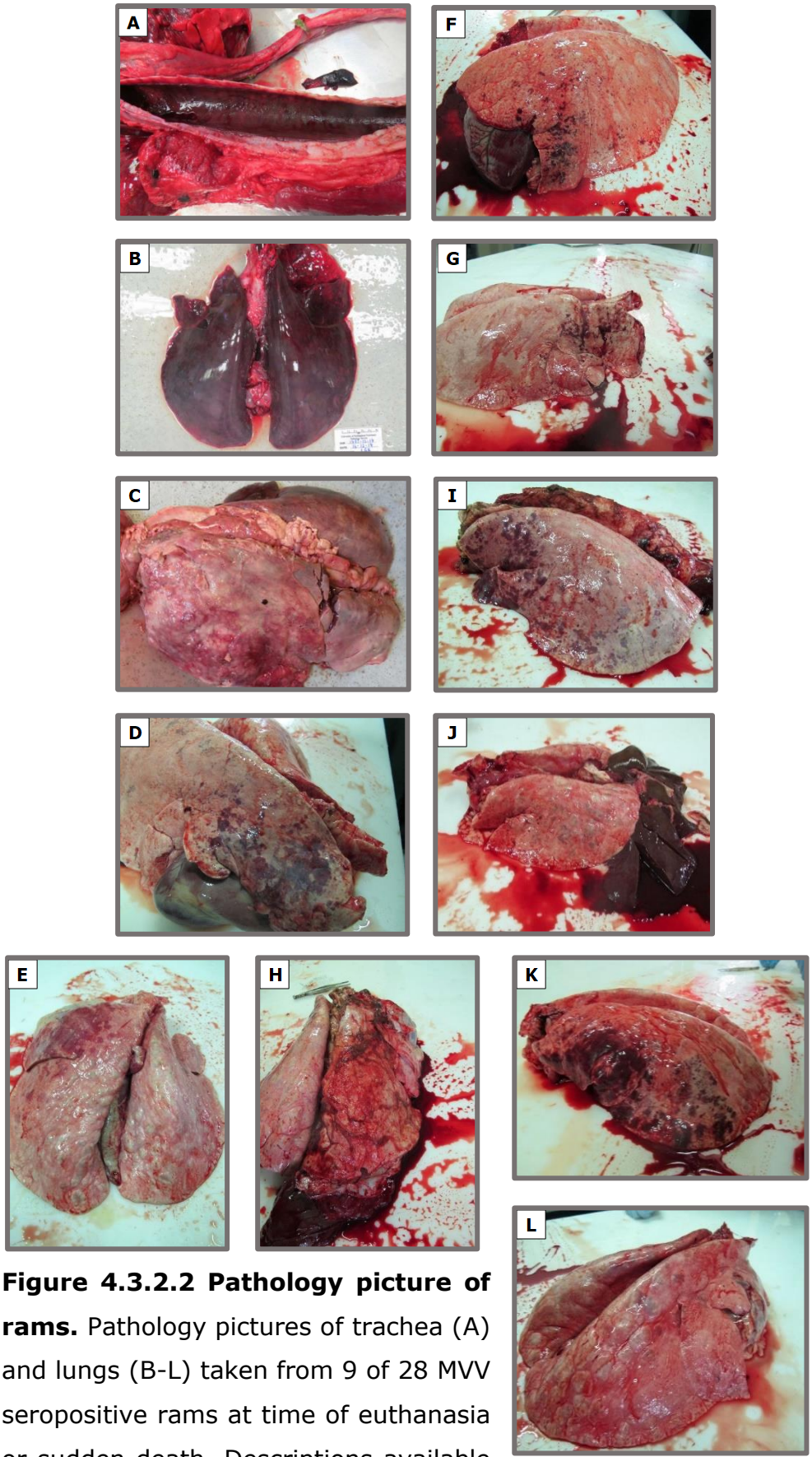


Figure 4.3.2.2 Pathology picture of rams. Pathology pictures of trachea (A) and lungs (B-L) taken from 9 of 28 MVV seropositive rams at time of euthanasia or sudden death. Descriptions available in Table 4.3.2.1.

Table 4.3.2.3 Histopathology of rams in lungs. Table describes the observed histopathology within slides of fixed lung tissue from 15 rams (Ram 1-13, 26, 27) MVV seropositive following euthanasia in June 2016 or October 2016.

Histopathology	Figure 4.3.2.4	Rams														
		1	2	3	4	5	6	7	8	9	10	11	12	13	26	27
Lymphocytic Infiltration	A (8+13)	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Thickening of Alveolar Septa	B (9+12)	+	+	+	-	+	+	-	-	+	-	+	+	+	+	+
Obliteration of Alveolar Structures	D (7+26)	+	+	-	+	+	+	+	-	-	-	-	-	+	+	-
Lung Worm Infestation	C (1)	+	+	+	+	+	-	+	+	+	-	+	+	+	-	-

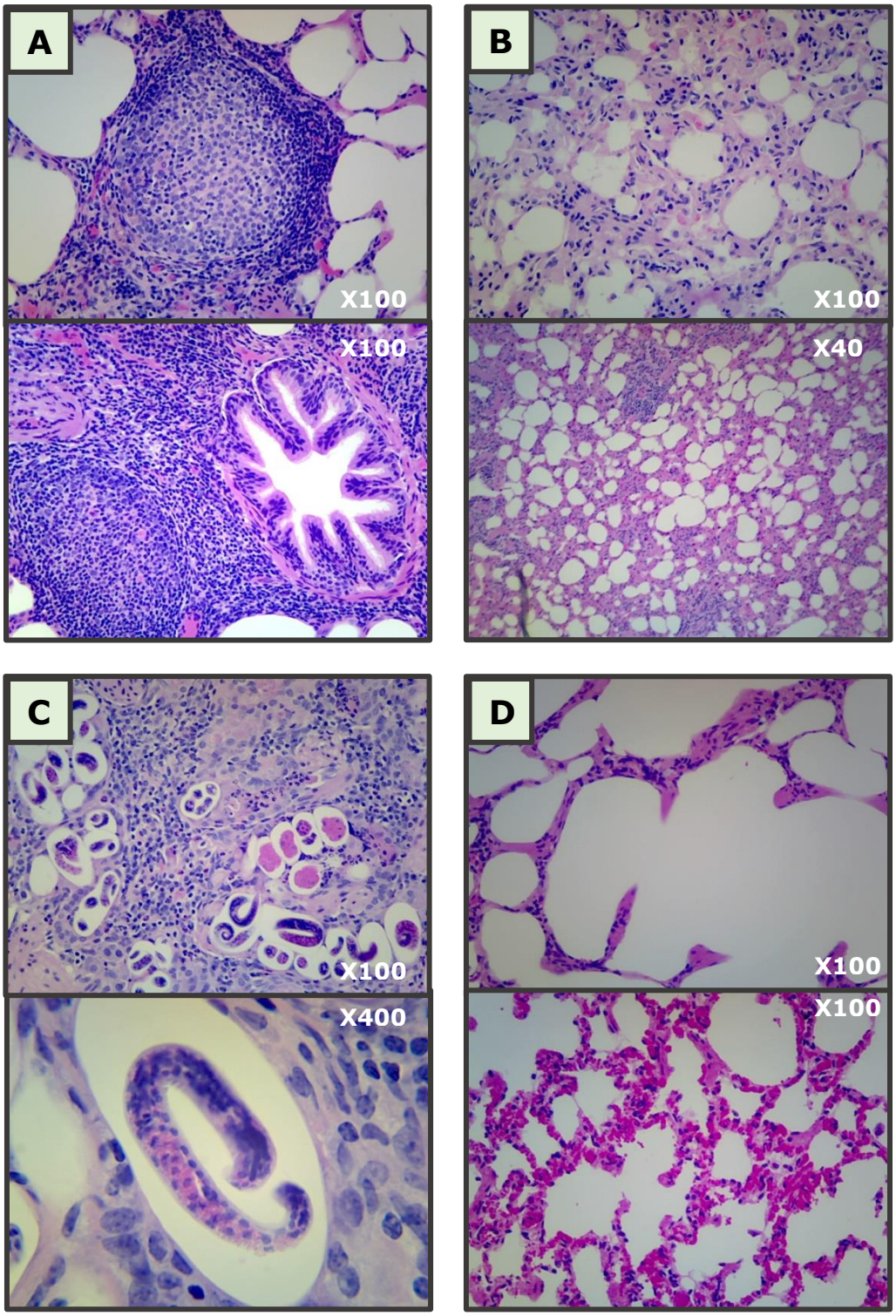


Figure 4.3.2.4 Histopathology of lung tissue from rams seropositive for MVV. (A) Lymphoid follicles with distinct germinal centres present within the lung, (B) thickening of the interalveolar septa, (C) Lung worm larvae within the lung and (D) obliteration of the alveoli structures. Magnification is marked on images Descriptions available in Table 4.3.2.3.

4.3.3 qPCR and ELISA Testing of Blood

Testing for presence of MVV was carried out on blood samples collected from seropositive rams over a period of 18 months at three time points (April 2015, December 2015 and October 2016) (Table 4.3.3.1). Twenty-five blood samples were collected in April 2015 with the majority of animals testing positive by all diagnostics. Negative results were obtained for 4 rams by ELISA, 2 by DNA qPCR and 1 by RNA qPCR, of which no ram tested negative for multiple diagnostics.

December 2015, 19 blood samples were collected, again the majority tested positive. Negative results were seen for 3 rams using ELISA, 2 rams using DNA qPCR and 1 ram using RNA qPCR. Of those testing negative only one showed multiple negative results between diagnostic tests, 02227, which tested negative in December 2015 by all three tests. Comparison of April and December results only showed 2 rams to test negative by ELISA for both dates (02227 and 02550). Remaining negative results were not consistent between dates for any diagnostic test.

Thirteen blood samples were collected in October 2016. All rams tested positive by ELISA, while 5 and 3 rams tested negative by DNA and RNA qPCR, respectively, with the remainder testing positive. Three rams showed consistent negative results by DNA and RNA qPCR (01016, 01017 and 02371). One ram (01017) showed negative results for both December 2015 and October 2016 when tested by DNA qPCR.

Copy numbers were calculated for DNA (copies per ng of DNA) and RNA (copies per µl of sera) qPCR results in rams that tested positive (Table 4.3.3.2). Copy numbers for RNA loads of rams

00605 and 02332 at time points April 2015 and October 2016, respectively, could not be calculated due to presence of additional unknown product (as evidenced by melt curve peak) in qPCR results. Copy counts in DNA showed lesser variation in number ranging from 2.27 x 10^2 to 3.95 x 10^4 copies per ng DNA than RNA copies which ranged from 2.22 x 10^2 to 4.44 x 10^{10} copies per µl of sera.

Figure 4.3.3.3 presents heat maps for antibody titres deduced from ELISA results and calculated copy numbers for DNA and RNA qPCR results for all blood samples collected. Figure 4.3.3.4 shows ten graphs plotting these three values for 10 rams which had values for all three time points.

Overall, no universal trend was observed in the 28 rams of this study. When considering animals present for the full duration of the study, no pattern in antibody or copy numbers was seen between April and December 2015. Between December 2015 and October 2016 copy numbers either dropped or maintained an approximated number uniformly in both DNA and RNA of individual rams. ELISA results also showed this pattern but individual ram ELISA results did not necessarily show concurrence with copy numbers.

→ **Table 4.3.3.1 MVV diagnostic results of blood samples collected from 28 rams over an 18 month period.** ELISA and qPCR diagnostics tested for the presence of MVV within sera, DNA and RNA obtained from blood samples collected at 3 three time points (April 2015, December 2015 and October 2016). 'No Tag' refers to one unidentifiable ram at each time point due to loss of ear tag, '?' denotes possible ram identity at each relative time point.

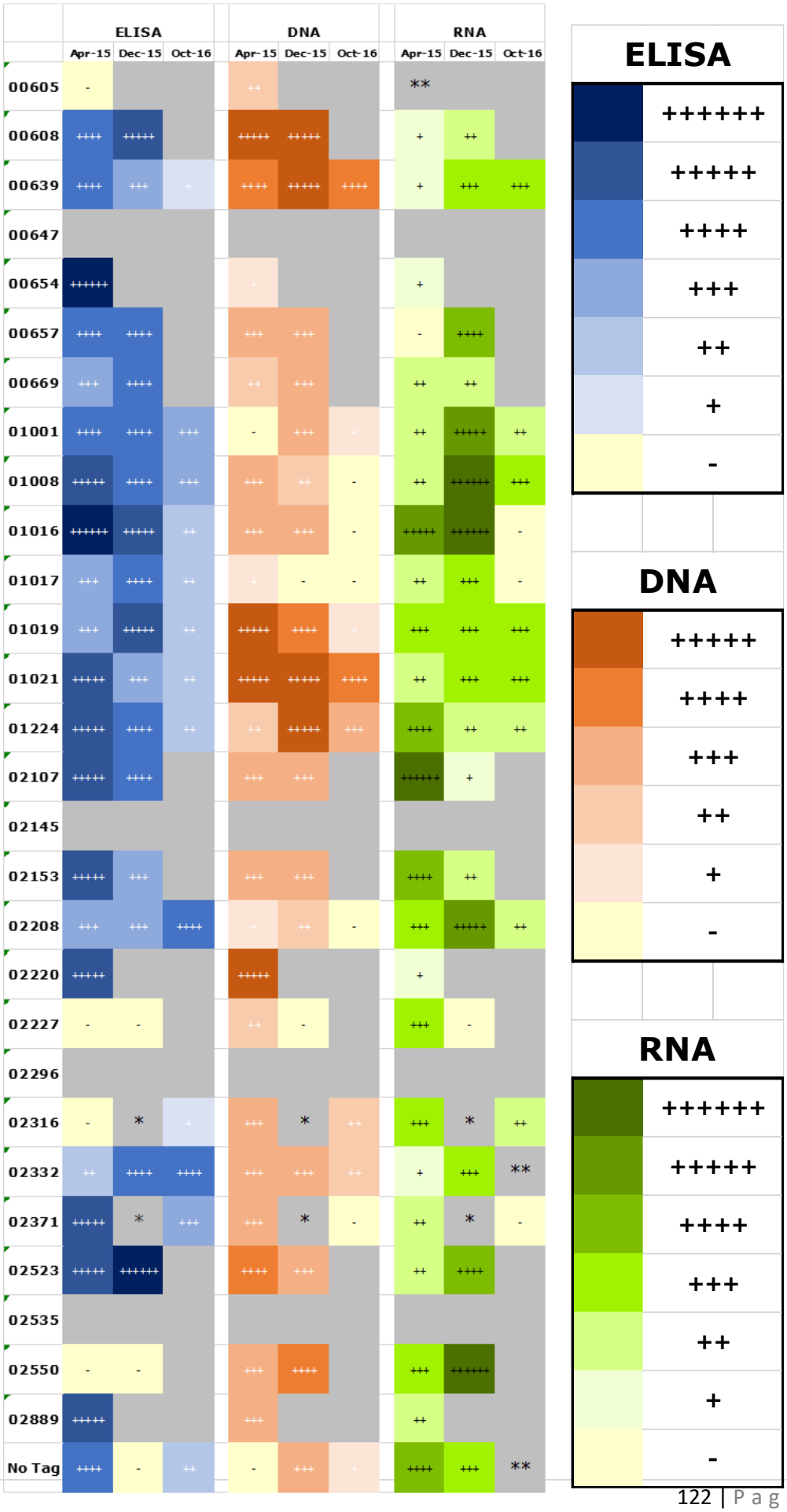
	June 2014	April 2015			December 2015			October 2016		
Animal ID	ELISA	ELISA	DNA	RNA	ELISA	DNA	RNA	ELISA	DNA	RNA
00605	Positive	Negative	Positive	Positive	?	?	?	?	?	?
00608	Positive	Positive	Positive	Positive	Positive	Positive	Positive	-	-	-
00639	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
00647	Positive	?	?	?	?	?	?	?	?	?
00654	Positive	Positive	Positive	Positive	?	?	?	?	?	?
00657	Positive	Positive	Positive	Negative	Positive	Positive	Positive	-	-	-
00669	Positive	Positive	Positive	Positive	Positive	Positive	Positive	-	-	-
01001	Positive	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Positive
01008	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Positive
01016	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative
01017	Positive	Positive	Positive	Positive	Positive	Negative	Positive	Positive	Negative	Negative
01019	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
01021	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
01224	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
02107	Positive	Positive	Positive	Positive	Positive	Positive	Positive	?	?	?
02145	Positive	-	-	-	-	-	-	-	-	-
02153	Positive	Positive	Positive	Positive	Positive	Positive	Positive	?	?	?
02208	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Positive
02220	Positive	Positive	Positive	Positive	?	?	?	?	?	?
02227	Positive	Negative	Positive	Positive	Negative	Negative	Negative	-	-	-
02296	Positive	?	?	?	?	?	?	?	?	?
02316	Positive	Negative	Positive	Positive	*	*	*	Positive	Positive	Positive
02332	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
02371	Positive	Positive	Positive	Positive	*	*	*	Positive	Negative	Negative
02523	Positive	Positive	Positive	Positive	Positive	Positive	Positive	-	-	-
02535	Positive	?	?	?	?	?	?	?	?	?
02550	Positive	Negative	Positive	Positive	Negative	Positive	Positive	-	-	-
02889	Positive	Positive	Positive	Positive	?	?	?	?	?	?
No Tag	-	Positive	Negative	Positive	Negative	Positive	Positive	Positive	Positive	Positive

* No blood was collected at time point

Table 4.3.3.2 MVV copy numbers in DNA and RNA extracted from blood of seropositive rams. Copy numbers calculated from qPCR results for detection of MVV in DNA and RNA extracted from blood collected at 3 time points (April 2015, December 2015 and October 2016). 'No Tag' refers to unidentifiable ram at each time point due to loss of ear tag, '?' denotes possible ram identity.

	DNA (copies per ng of DNA)			RNA (copies per µl of sera)		
Animal ID	Apr '15	Dec '15	Oct '16	Apr '15	Dec '15	Oct '16
00605	9.86×10^2	?	?	**	?	?
00608	2.48×10^4	3.95×10^4	-	6.07×10^2	5.84×10^3	-
00639	6.66×10^3	1.49×10^4	6.94×10^3	5.25×10^2	6.56×10^4	1.45×10^4
00647	?	?	?	?	?	?
00654	4.94×10^2	?	?	7.46×10^2	?	?
00657	2.74×10^3	4.02×10^3	-	Negative	3.50×10^5	-
00669	8.81×10^2	1.73×10^3	-	1.56×10^3	1.80×10^3	-
01001	Negative	1.59×10^3	2.27×10^2	1.18×10^3	6.65×10^6	7.10×10^3
01008	1.94×10^3	5.23×10^2	Negative	1.18×10^3	4.00×10^9	1.05×10^4
01016	4.33×10^3	2.29×10^3	Negative	4.75×10^6	2.05×10^7	Negative
01017	3.51×10^2	Negative	Negative	9.15×10^3	3.69×10^4	Negative
01019	1.06×10^4	6.59×10^3	2.93×10^2	3.70×10^4	1.54×10^4	2.03×10^4
01021	3.42×10^4	1.92×10^4	7.91×10^3	1.40×10^3	1.02×10^4	1.04×10^4
01224	7.43×10^2	2.74×10^4	4.70×10^3	1.59×10^5	1.18×10^3	4.62×10^3
02107	1.01×10^3	1.81×10^3	?	2.50×10^7	2.22×10^2	?
02145	-	-	-	-	-	-
02153	1.81×10^3	1.70×10^3	?	4.65×10^5	2.94×10^3	?
02208	4.17×10^2	8.25×10^2	Negative	1.09×10^4	3.20×10^6	3.71×10^3
02220	1.47×10^4	?	?	5.52×10^2	?	?
02227	6.93×10^2	Negative	-	1.06×10^4	Negative	-
02296	?	?	?	?	?	?
02316	1.08×10^3	*	8.14×10^2	1.21×10^4	*	4.04×10^3
02332	4.65×10^3	1.36×10^3	6.64×10^2	3.44×10^2	2.32×10^4	**
02371	3.64×10^3	*	Negative	2.21×10^3	*	Negative
02523	8.14×10^3	3.25×10^3	-	5.68×10^3	4.51×10^5	-
02535	?	?	?	?	?	?
02550	3.90×10^3	8.86×10^3	-	4.62×10^4	4.44×10^{10}	-
02889	1.53×10^3	?	?	1.89×10^3	?	?
No Tag	Negative	3.23×10^3	4.03×10^2	1.70×10^5	2.26×10^4	**

* No blood was collected at time point
** Presence of unknown products prevented quantification


ELISA
DNA
RNA
Apr-15
Dec-15
Oct-16
00605
00608
00639
00647
00654
00657
00669
01001
01008
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01017
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01021
01224
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02145
02153
02208
02220
02227
02296
02316
02332
02371
02523
02535
02550
02889
No Tag
ELISA
++++++
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+++
++
+
-
DNA
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RNA
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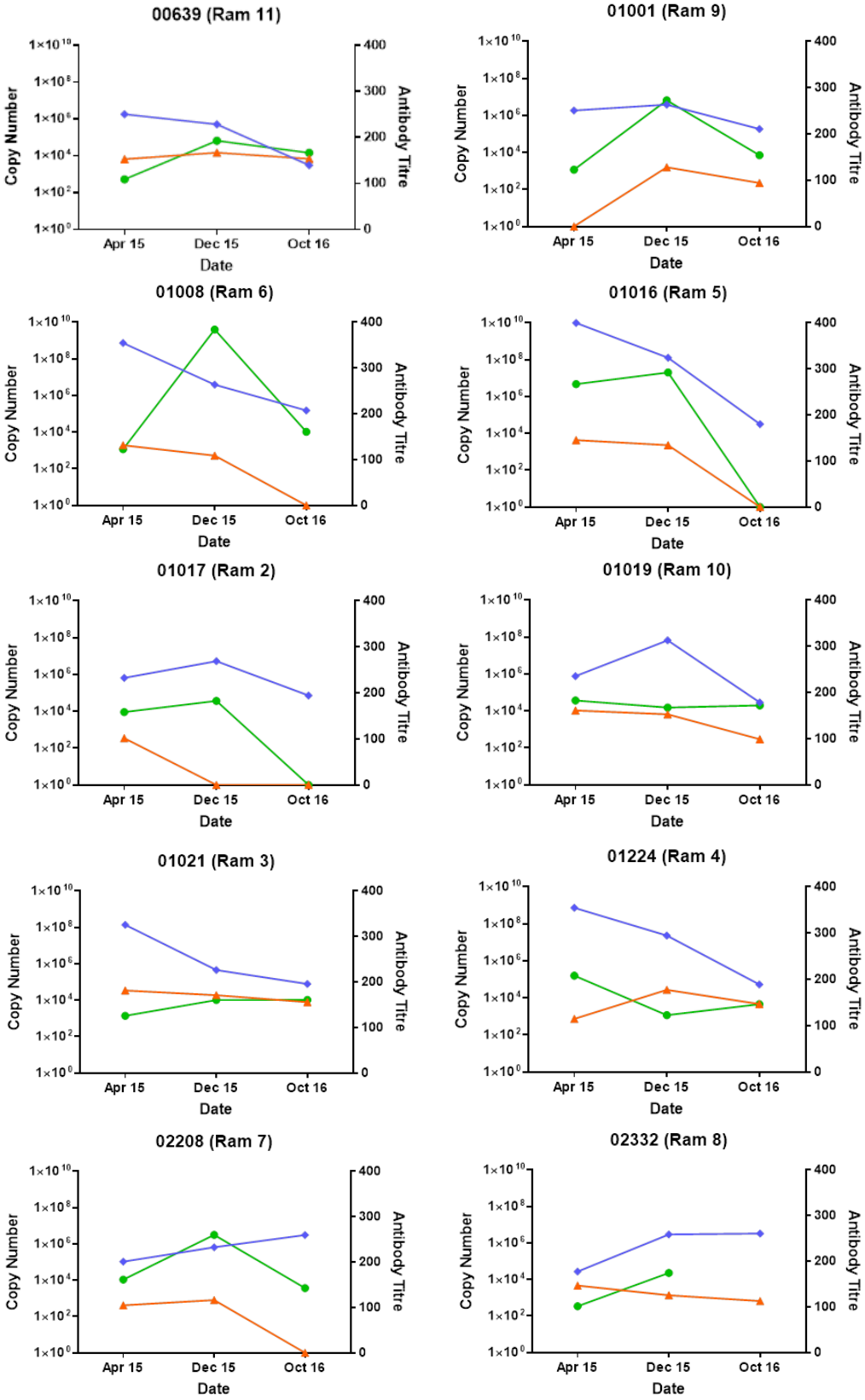
-

← **Figure 4.3.3.3 Heat map of MVV antibody titres and copy numbers in seropositive rams.** Antibody titres and copy numbers for DNA (copies per ng of DNA) and RNA (copies per µl of sera) determined by ELISA and qPCR, respectively for 3 time points (April 2015, December 2015 and October 2016). '*' denotes rams that were present, but no sample of blood was collected. '**' refers copy numbers that could not be calculated due to unknown additional product in sample when tested by qPCR.

→ **Figure 4.3.3.4 Graphs of MVV antibody titres and copy numbers in RNA of DNA of ten rams.** Antibody titres (blue) and copy numbers for DNA (orange: copies per ng of DNA) and RNA (green: copies per µl of sera) determined by ELISA and qPCR, respectively for 3 time points (April 2015, December 2015 and October 2016).


00639 (Ram 11)
01001 (Ram 9)
01008 (Ram 6)
01016 (Ram 5)
01017 (Ram 2)
01019 (Ram 10)
01021 (Ram 3)
01224 (Ram 4)
02208 (Ram 7)
02332 (Ram 8)
Copy Number
Antibody Titre
Apr 15
Dec 15
Oct 16
Date

4.3.4 qPCR Testing of Tissue

Tissue and semen samples were collected from 26 seropositive rams shortly prior or at time of death for diagnostic testing for detection of MVV infection by qPCR. All samples were tested for presence of proviral DNA with the exception of epididymal washes, for which viral RNA was tested for. Semen samples were tested for both proviral DNA and viral RNA.

Positive results were obtained from, samples of lung, mediastinal lymph node and testicle. Twenty-five lung samples were tested of which 19 tested positive and 6 negative (Table 4.3.4.1). In addition, lung pathology samples from 5 rams (2, 3, 7, 8 and 12) were tested of which all bar one (Ram 7) tested positive. Both lung samples tested for Ram 7 tested negative by qPCR. Mediastinal lymph node samples tested by qPCR showed positive results in 22 rams and 3 negative results. Interestingly, all rams that tested negative in lymph node also tested negative in lung. To estimate viral loads within these tissues, copy numbers were calculated. Due to presence of an unknown additional product when testing tissue DNA by qPCR, copy numbers could only be calculated for 10 samples (5 lung samples and 5 lymph node samples) (Table 4.3.4.2). Unknown product was not detected when testing DNA and RNA extracted from blood samples (Chapter 4.3.3). sanger sequencing of this secondary product failed (likely due to the short length of the amplicon).

Testing of DNA extracted from testicles demonstrated proviral DNA in 3 rams out of 11 tested (Rams 17, 22 and 25). Of 11 semen samples tested, no virus was detected in extracted DNA of any ram. In addition to DNA, RNA was also extracted from semen and tested for detection of virus, but again all samples

tested negative (Table 4.3.4.3). Interesting, epididymal washes obtained from these same rams, from which RNA was extracted and tested, were shown to be positive for virus in 6 rams. Copy numbers could not be calculated for any reproductive sample.

Table 4.3.4.1 MVV qPCR results for DNA extracted from tissues of seropositive rams. Results from qPCR testing of samples collected from four sources (lung, mediastinal lymph node, testicles and semen). Additional samples were collected from any areas of observed pathology at time of PM.

Animal ID	Lung	Lung Pathology	Lymph	Testicle	Semen
Ram 1	Positive	-	Positive	-	Negative
Ram 2	Positive	Positive	Positive	-	Negative
Ram 3	Positive	Positive	Positive	-	Negative
Ram 4	Positive	-	Positive	-	-
Ram 5	Negative	-	Positive	-	Negative
Ram 6	Negative	-	Negative	-	-
Ram 7	Negative	Negative	Positive	-	Negative
Ram 8	Positive	Positive	Positive	-	Negative
Ram 9	Positive	-	Positive	-	Negative
Ram 10	Positive	-	Positive	-	Negative
Ram 11	Positive	-	Positive	-	Negative
Ram 12	Positive	Positive	Positive	-	Negative
Ram 13	Positive	-	Positive	-	Negative
Ram 14	Positive	-	-	Negative	-
Ram 15	Negative	-	Positive	Negative	-
Ram 16	-	-	-	-	-
Ram 17	Positive	-	Positive	Positive	-
Ram 18	Positive	-	Positive	Negative	-
Ram 19	-	-	Positive	Negative	-
Ram 20	Positive	-	Positive	Negative	-
Ram 21	Positive	-	Positive	Negative	-
Ram 22	Positive	-	Positive	Positive	-
Ram 23	Positive	-	Positive	Positive	-
Ram 24	Negative	-	Negative	Negative	-
Ram 25	Positive	-	Positive	Positive	-
Ram 26	Positive	-	Positive	-	-
Ram 27	Negative	-	Negative	-	-
Ram 28	-	-	-	-	-

Table 4.3.4.2 MVV proviral copy numbers in DNA extracted from tissues. Calculated copy numbers for samples showing single confirmed product by qPCR. Copy numbers calculated as copies per ng of DNA.

	DNA (copies per ng of DNA)		
Animal ID	Lung	Lung Pathology	Lymph
Ram 8	2.54×10^1	1.22×10^2	1.66×10^3
Ram 14	3.19×10^2	-	-
Ram 18	-	-	3.81×10^2
Ram 19	-	-	4.32×10^1
Ram 20	4.30×10^1	-	5.56×10^1
Ram 22	-	-	5.37×10^4
Ram 26	-	-	7.94×10^2

Table 4.3.4.3 MVV qPCR results for RNA extracted from semen and epididymal washes of seropositive rams.

	RNA	
Animal ID	Semen	Epididymal Wash
Ram 1	Negative	Negative
Ram 2	Negative	Negative
Ram 3	Negative	Negative
Ram 4	-	Negative
Ram 5	Negative	Negative
Ram 6	-	Positive
Ram 7	Negative	Negative
Ram 8	Negative	Positive
Ram 9	Negative	Positive
Ram 10	Negative	Positive
Ram 11	Negative	Negative
Ram 12	Negative	Positive
Ram 13	Negative	Positive

4.3.5 qPCR Testing of Nasal Swabs

To assess the feasibility of nasal swabs as a sampling method for successful virus detection, swabs were taken from 13 known seropositive rams and tested for detectability of MVV. Both DNA and RNA showed detectable virus from swabs but with varying detection rates (Table 4.3.5.1). Virus was detectable in RNA extracted from all rams whilst DNA was detectable in only 6 of 13 rams. Copy numbers were calculated where possible and showed greater RNA copy numbers in all rams. Copy number for DNA extracted from the nasal swab of Ram 11 could not be calculated due to presence of unknown additional product during qPCR testing.

Table 4.3.5.1 MVV qPCR results of DNA and RNA extracted from nasal swabs of 13 seropositive rams. Copy numbers were calculated where possible. '*' denotes a positive result where copy number calculation could not be carried out due to presence of unknown additional product in qPCR.

	Nasal Swabs	
Animal ID	DNA (copies per ng of DNA)	RNA (copies per µl of supernatant)
Ram 1	Negative	2.01×10^4
Ram 2	Negative	2.27×10^4
Ram 3	7.02×10^1	1.46×10^4
Ram 4	1.74×10^2	8.42×10^3
Ram 5	Negative	1.40×10^4
Ram 6	Negative	9.80×10^4
Ram 7	Negative	6.89×10^3
Ram 8	3.38×10^1	3.91×10^4
Ram 9	Negative	4.05×10^3
Ram 10	1.34×10^2	2.36×10^5
Ram 11	*	5.64×10^3
Ram 12	Negative	2.95×10^4
Ram 13	5.08×10^1	5.00×10^3

4.3.6 TMEM154 Genotyping of Rams

To identify potential presence of MVV resistant genotypes of TMEM154, genotyping was carried out on DNA extracted from seropositive rams. Exon 2 of 26 rams was successfully classified, of which 20 were identified as heterozygous for glutamate (E) to lysine (K) substitution at amino acid position 35. Of this number, 11 were also found to be heterozygous for a substitution of asparagine (N) to isoleucine (I) at position 70. The N70I substitution was also present in a ram (Ram 9) which did not possess the E35K substitution. One other heterozygous substitution was identified in Ram 2 at position 44 of methionine (M) to threonine (T).

Only one ram (Ram 27) was homozygous for substitution at position 35 (E to K) indicative of an increased resistance to MVV.

Unfortunately, Exon 1 could not be genotyped to confirm the diplotype of individual rams due to laboratory difficulties. Suspected diplotypes were determined based on the known substitution present and previously described haplotypes in published literature.

Table 4.3.6.1 TMEM154 genotyping of 26 rams. Exon 2 was successfully genotyped for 26 rams to assess for presence of glutamate (E) to lysine (K) substitution at amino acid position 35, which provides resistance to MVV infection. Exon 1 (orange) could not be genotyped due to laboratory difficulties.

		TMEM154 aa Position												Suspected Diplotype
		Exon 1				Exon 2								
Ram	Breed	4	13	14	25	31	33	35	44	70	74	82	102	
Ancestral		R	A	L	T	E	D	E	T	N	I	E	I	
1	Abermax							E/K		N/I				1, 2
2	Aberfield								M/T					3, 4
3	Aberfield							E/K						1, 3
4	Abermax							E/K		N/I				1, 2
5	Aberfield							E/K						1, 3
6	Aberfield							E/K		N/I				1, 2
7	Abermax							E/K						1, 3
8	Abermax							E/K		N/I				1, 2
9	Aberfield									N/I				2, 3
10	Aberfield													3, 3
11	Abermax													3, 3
12	Abermax							E/K		N/I				1, 2
13	Abermax							E/K						1, 3
14	Abermax							E/K		N/I				1, 2
15	Abermax							E/K						1, 3
17	Abermax							E/K		N/I				1, 2
18	Abermax							E/K						1, 3
19	Abermax													3, 3
20	Abermax							E/K						1, 3
21	Abermax							E/K						1, 3
22	Abermax							E/K		N/I				1, 2
23	Abermax							E/K		N/I				1, 2
24	Abermax							E/K		N/I				1, 2
25	Abermax							E/K						1, 3
26	Abermax							E/K		N/I				1, 2
27	Abermax							K						1, 1

4.4 Discussion

In this chapter a longitudinal study was carried out over a period of 28 months including 28 MVV seropositive rams diagnosed in June 2014 following routine testing. Testing of blood and tissue samples collected confirmed MVV status in all rams with exception of one (Ram 28) from which no blood or tissue samples were obtained. Consideration of blood and tissue collection records suggest Ram 28 to have died or been euthanised prior to April 2015.

Difficulties in analysis of results was found when trying to cross reference tissue and blood samples due to a lack of conformity of labelling and loss of ear tags. At each time point of blood collection, one ram was present that could not be successfully identified (Table 4.3.3.1). Should such a study be repeated in the future, emphasis should be made on uniformity of sample labelling and regular checking of animals for lost ear tags.

Pathology observed at PM was mostly localised to the lungs of the rams. Areas of consolidation (n=8), enlargement of lungs (n=1), grey discolouration (n=5), multifocal grey-white nodules (n=1) and adhesion of lung to the thoracic wall (n=1) observed in 9 out 10 rams showing pathology is consistent with that previously reported as being associated with MVV infection (Cutlip et al. 1979; Christodoulopoulos 2006; Gomez-Lucia et al. 2018). Interestingly, 3 of these rams (Ram 5, 7 and 15) presenting with pathology indicative of MVV infection tested negative when extracted DNA was tested by qPCR. In addition, 2 of these rams (Ram 5 and 7) had slides prepared from sections of lung tissue. Again, both rams showed histopathology indicative of MVV infection. This clear presentation of MVV pathology with an inability to detect proviral DNA in blood is in

contrast to previous work which supported severity of lesions in infected rams to be proportional to viral loads within peripheral blood (Herrmann-Hoesing et al. 2009).

Instances of lymphoid infiltration with intermittent formation of lymph follicle-like structures (n=14), thickening of alveolar septa (n=11) and obliteration of alveoli structures (n=8) were found in histological sections of lung tissue collected from 15 rams (Georgsson and Palsson 1971; Lairmore et al. 1986). Eleven rams also showed infestation with lungworm, which has also been previously reported to be histologically characterised by marked lymphoid infiltration and thickening of alveolar septa (Chanie and Ayana 2013). Infestation with lungworm then highlights the question of whether pathology seen in slides can be attributed to lungworm, MVV infection or both. Four rams which showed no sign of infestation, still showed pathology despite no evidence of lungworms supporting that pathology can in part be attributed to MVV infection. In addition, due to the respiratory nature of both conditions, clinical signs are also similar with coughing, increased respiratory rate, dyspnea, nasal discharge, weight loss and fever being reported for both conditions (Chanie and Ayana 2013). The confusion between the two infections clinical signs and pathology has complicated this study's conclusion. Given the prevalence of both infections in the UK this overlap is also likely in many flocks and may well lead to under estimation of MV infection if flocks are being tested on clinical suspicion of lungworm alone.

Testing of blood samples by ELISA and qPCR over three time points showed no trend in changes in antibody titres and copy numbers between time points.

Throughout the study only one ram (02227/Ram 27) was found to test negative by both ELISA and qPCR of DNA and RNA from blood at a single time point (December 2015). Interestingly, TMEM154 genotyping identified Ram 27 to be the only ram homozygous for the substitution of glutamate (E) to lysine (K) at amino acid position 35. This deviation from the ancestral sequence has been reported to convey resistance to MVV infection (Heaton et al. 2012). The presence of only one ram homozygous for a resistance marker within this group is likely to be due the selection criteria of being positive by ELISA during a routine test for the MVAS/CAEAS. Heaton et al. (2012) reported increased resistance in K35 homozygous sheep, therefore the likelihood of seroconversion and detection by ELISA is markedly less than susceptible genotypes.

qPCR testing of DNA and RNA extracted from blood collected in April 2015 tested positive for Ram 27. Copy numbers of 6.93 x 10^2 copies per ng of DNA and 1.06 x 10^4 copies per µl of sera were calculated for viral loads in DNA and RNA, respectively. Proviral DNA copy numbers were lower than the average seen for rams in April 2015 while RNA load was within average values (DNA: mean = 5.67 x 10^3, median = 1.94 x 10^3) (RNA: mean = 1.33 x 10^6, median = 5.68 x 10^3). Transition from positive qPCR results in April 2015 to negative results in December 2015 and negative ELISA results at both times suggests that MVV resistant TMEM154 haplotypes may allow for increased 'control' of infection within such host sheep as has been previously suggested (Alshanbari et al. 2014).

Despite only one ram being homozygous for MVV resistant TMEM154 haplotypes, 20 rams were found to be heterozygous for this haplotype. Although, heterozygous animals have been reported to not benefit from resistance to infection, the

presence of these haplotypes in such frequency indicates that selective breeding could be successful to increase flock resistance to MVV infection, therefore reducing the risk of associated economic impacts (Yaman et al. 2019).

In addition to blood, MVV was also detected in DNA extracted from several tissues (lung, mediastinal lymph node and testicle). MVV was most successfully detected in DNA extracted from lymph node (n=22/25). It was noted that rams that tested negative in DNA, were consistently negative in all tissues tested for that ram. Considering the cellular tropism of MVV to the monocyte/macrophage cell lineage and dendritic cells and their roles during infection, an inability to detect virus within the mediastinal lymph node and subsequent lack of detection in other tissues would not be unexpected (Ramírez et al. 2013).

Interestingly, proviral DNA loads calculated within this study were markedly higher than those previously reported. Within these studies blood proviral DNA loads were reported to range from 1×10^{-2} to 1.6×10^{1} copies per ng of DNA (Herrmann-Hoesing et al. 2007; Crespo et al. 2016; Grego et al. 2018). In comparison, the lowest load within the rams of the current study throughout all three time points was 2.27×10^{2} copies per ng DNA, with the highest load seen 3.95×10^{4} copies per ng DNA. This large difference seen in proviral DNA loads could be attributed to the lung worm infestation diagnosed by histology. Such infestation would typically result in an inflammatory response, resulting in recruitment of latently infected monocyte/macrophages. Such an immune response has previously been suggested to stimulate virus present within these cells resulting in increased replication and proviral loads within surrounding cells and blood (Grego et al. 2018). Alternatively, discrepancies in reported proviral DNA loads may

be the results of differing diagnostics, as qPCR used in this study was designed to target sequences of the Pol gene, whilst the studies mentioned previously, utilised diagnostics targeting the Gag gene.

Testing of DNA extracted from tissues highlighted one limitation of the MVV qPCR designed previously (Chapter 2). Whereby testing of DNA and RNA extracted from blood resulted in amplification of a single correct product, testing of DNA extracted from tissues in addition to producing the correct amplicon, also produced an unknown amplicon consistently in nearly all samples tested (Figure 4.4.1). Attempts at sequencing failed to identify this product, but uniform presence in both positive and negative samples suggests that it is DNA already present within the sheep genome. Therefore, adjustments to this qPCR would be required for future testing of nucleic acids extracted from tissues.

Palsson (1972) reported successful isolation of virus from nasal swabs taken from seropositive animals, but since then and with the rise of PCR diagnostics, the detectability of virus within nasal swabs has not be assessed. In this study, DNA and RNA was extracted from nasal swabs of 13 rams (Rams 1-13) and tested by qPCR. Interestingly, all RNA samples tested positive for MVV whilst only 6 tested positive for DNA samples. Copy numbers were calculated for both and copy numbers in RNA were found to be greater than those in DNA by a minimum factor of 10. The lower copy numbers in DNA may explain the varied ability to detect virus, with viral loads with negative samples being outside of the detection range of the qPCR assay used in this study.

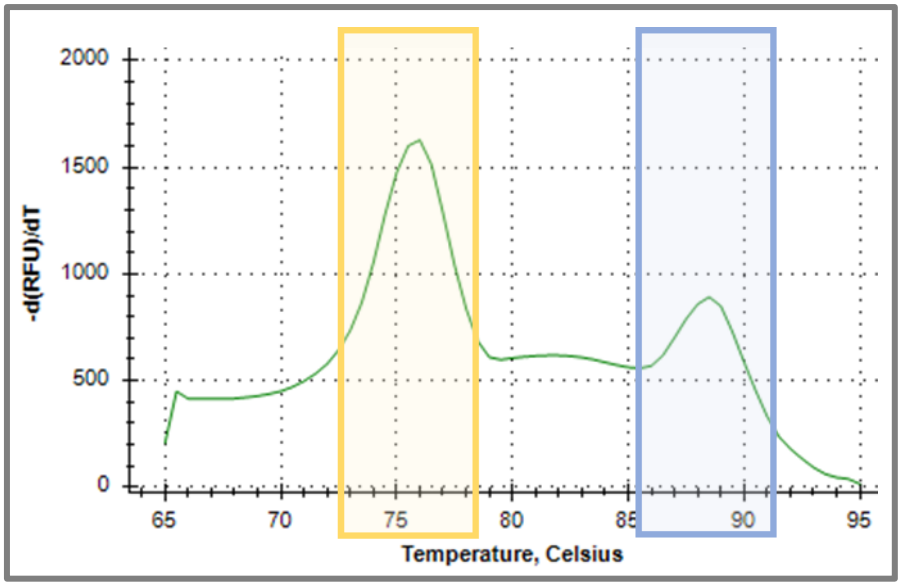


Figure 4.4.1 Melt curve of MVV Pol qPCR products during testing of sheep tissues. qPCR testing of DNA extracted from lung samples using primers designed for amplification of a *Pol* gene nucleotide sequence (Chapter 4.2.10). Two products were produced by assay, the correct MVV *Pol* product at approximately 76°C (yellow) and an unknown product at approximately 89°C (blue).

The 100% detection of virus in RNA could therefore provide a new potential sampling technique for MVV diagnostics in addition to blood and milk. Advantages of this sampling technique being taking swabs is less invasive that blood collection and does not require a trained technician. It has an advantage over milk and colostrum testing in that non-lactating and male animals can also be tested. One consideration that should be made is that the 13 swabbed rams were known to have been seropositive for a period of 28 months. Therefore, sufficient levels of virus within nasal swabs for detection of virus may require a particular stage of infection, which may be longer than desirable for a potential sampling method for diagnostics. Further field testing in live animals that are known to be MV positive is required to properly assess the utility of nasal swabs

as a diagnostic and to assess sensitivity and specificity against the current serological tests. Ideally, a longitudinal study should be carried out with regular nasal swabs taken following experimental infection to determine the length of time post-infection at which virus becomes detectable from nasal swabs. Souza et al. (2015) recently reported detectable virus within saliva of infected animals. Oral swabs therefore may also provide another minimally taxing route of sample collection and could be integrated into the previously suggested study.

The longitudinal study carried out in this chapter has highlighted the variability in detectability of MVV within known seropositive rams between multiple tissues and bodily fluids. Inability to identify any trends within these rams uniformly housed and cared for over a 28 month period suggest that changes in antibody titre and viral loads are strongly influenced by individual factors (e.g. genetic resistance or lung worm burden). The finding of 100% detectability of virus within RNA from swabs is of vital importance. As nasal swabs do not require professional training or qualifications to collect, the use of swabs could significantly reduce costs of monitoring animals. This in turn could incentivise greater uptake in the UK of control programmes for MV aiding in efforts to eradicate MVV within the national flock.

Chapter 5: Regression Modelling of Impact of MVV Infection of Milk Yield and SCC

5.1 Introduction

To date, some of the main identified economic impacts of SRLVs are reductions in birth weight, growth rates and potentially fertility, in sheep and goats (Dohoo et al. 1982; Arsenault et al. 2003; Peterhans et al. 2004). In addition to these, the impact on milk yield has been investigated on several occasions, although there have been inconclusive findings into the differences of milk yield between SRLV seropositive and seronegative animals. Contributing factors for the observed differences in studies have yet to be determined. Similarly, multiple factors have been identified as playing a role in milk yield changes in infected animals such as SRLV induced mastitis and reduced lactation periods (Pekelder et al. 1994; Gregory et al. 2009; Martínez-Navalón et al. 2013).

Of the studies into variation of milk yield during SRLV infection, there have been several studies reporting reduced milk yields in seropositive ewes/does. These studies are summarised in Table 5.1.1, the reduction in milk seen in these studies ranged from 6-30% in seropositive ewes and does.

In contrast, Nord and Dnøy (1997) found there to be no significant difference in milk yield between seropositive and seronegative does under the age of four over two consecutive years. In animals five years of age, they initially reported a significant increase in yield of those seropositive suggesting an age-dependent effect, but this difference was not seen the following year. Similar reports of no significant changes in milk

Table 5.1 Studies of decreased milk yield in SRLV seropositive ewes and does. Listed are 7 studies reporting decreased milk yield in association with SRLV infection. Studies multiple values of change are due to differences viewed in different lactation periods of the same cohort of animals.

		Number			
Species	**Source**	**Flocks**	**Individual**	**Country**	**Seropositive Change in Milk Yield**
Goat	(Greenwood 1995)	1	80	Australia	= 0% - ↓19.8%
	(Bohland and D'Angelino 2005)	1	829	Brazil	↓ 21.5%
	(Leitner et al. 2010)	1	248	Israel	= 0% - ↓22.7%
	(Martínez-Navalón et al. 2013)	22	3913	Spain	↓ 6.3-16.7%
Sheep	(Giadinis et al. 2012)	2	830	Greece	↓ 30%
	(Juste et al. 2020)	3	2146	Spain	↓ 6.7%
	(Echeverría et al. 2020)	4	1497	Spain	↓ 6%

yield in goats are present in the current literature (Smith and Cutlip 1988; Nord and Dnøy 1997; Kaba et al. 2012).

It has been suggested that lower growth rates observed in lambs infected with SRLV can be attributed in part to reduced milk yields and indurative mastitis associated with infection (Keen et al. 1997). Lipecka et al. (2010) investigated the impact on milk yield within two selectively bred meat sheep breeds, from which they found little difference between yields collected from seropositive and seronegative animals. This would therefore suggest that reduction in growth rates of lambs born to seropositive ewes is not a result of reduced milk production. In addition, the study completed by Lipecka et al. also investigated the impact of SRLV infection on SCC in milk (a

marker of udder health commonly used by milk processing companies to set safety limits for human consumption of milk).

Quantifying the number of somatic cells consisting largely of macrophages, leukocytes and lymphocytes, the SCC has been used an indicator of infections within mammary tissue. Although once thought possible for use in detection of mastitis in animals, publications reporting isolation of pathogens causing mastitis from milk samples with low SCC and lack of isolation from milk samples with high SCCs suggest otherwise (Leitner et al. 2001; Albenzio et al. 2002; Nunes et al. 2008), although, SCC of bulk milk samples can be used as an estimate of prevalence of mammary infections within a flock. To date, majority of threshold values proposed for differentiating healthy and infected ewes lie within the range of 2.5×10^5 and 5.0×10^5 cells/ml (Souza et al. 2012).

Lipecka et al. (2010) investigated the impact of natural infection on SCC in two sheep breeds over 2 months of lactation. When comparing seropositive and seronegative animals of both breeds, there was a significant increase in SCC seen during the first month of lactation. A similar increase in SCC has also been reported in CAEV infected goats (Ryan et al. 1993). Despite these studies showing evidence of SRLV infection causing increased SCC, as with milk yield, contrasting results have also been reported in situations where no differences in SCC were seen between seropositive and seronegative does (Turin et al. 2005; Kaba et al. 2012).

In this chapter, the objective was to quantify the impact of SRLV infection within a dairy flock consisting of 319 milking East Friesian X Lacunae ewes, with a focus on milk yield and SCC. To carry this out a multivariable regression model was

constructed using milking data collected over a period of multiple years during and prior to the diagnosis of MVV in some animals within the flock.

5.2 Materials and Methods

5.2.1 Data Collection and Organisation

Individual SCC and milk yield records were analysed from a dairy flock of 319 milking East Friesian X Lacunae ewes recently identified as MV infected via routine serological screening for the presence of MVV antibodies. Data variables collected are listed in Table 5.2.1.1

Table 5.2.1.1 Data variables acquired from dairy flock of 319 milking ewes following MVV outbreak.

Category	Variable
Individual Data	Animal Number Age
Milking History	Lactation Number Milk Last Lactation Total Yield in Lactation Average Daily Yield (Last 14 Days) Average Daily Yield (Last 21 Days) Average Daily Yield (Last 30 Days) Milking Days in Lactation Previous Lambing Date Last Lambing Date
Somatic Cell Counts	SCC 29th June 2015 SCC 15th June 2016 SCC 16th March 2017 SCC 8th May 2017
MVV ELISA Results	Optical Density Value MVV Diagnosis (0.6 Threshold Value)

Individual somatic cell counts (SCC) were calculated from 5ml milk samples collected by the farmer from both mammary glands of each ewe and pooled together in a single collection pot. SCC analysis was conducted by the commercial milk laboratory 'Quality Milk Management Services' (QMMS) according to their standard operating procedures. Individual

milk yield records were collected daily by an automated milk meter system integrated into the parlour management system (DeLaval – DelPro3.0). Individual Maedi Visna ELISA tests were carried out on milk samples collected in the same manner as that described above and analysed by SAC diagnostics service using the *ELITEST-MVV/CAEV (HYPHEN Biomed),* a recombinant ELISA using the capsid p28 core protein and a peptide derived from the immunodominant region of the viral transmembrane protein gp46.

Differentiation of seropositive and negative ewes by ELISA was carried out as recommended by the manufacturer using an optical density threshold of 0.6 for confirmation of positivity.

5.2.2 Descriptive Analysis

Analysis was carried out using Microsoft Excel 2013 and Graphpad Prism 7.03 (Graphpad Software).

5.2.3 Statistical Modelling

Multivariable regression modelling was used to predict the impact of MV status on total milk yield and SCC and estimate variation between ewes seropositive and seronegative for MVV. Model construction and regression analysis were carried out using MLwiN version 3.00 (University of Bristol, Bristol, UK). For these models single level fixed-effect structures were used, with individual ewes as the unit of data. Models were constructed by backwards selection. Variables considered included age (1-9 years), duration of lactation period (milking days), MVV status (positive or negative), somatic cell counts (March and May, 2017) and total yield in lactation. During construction, model fit was assessed by normality of residual histograms, which assesses suitability of model for analysis for associated data set.

Models took the form:

$$y_i = \beta_0 + \beta_1 x_1 + \ldots + e_i$$

Key:

y_i = Outcome variable (i.e. milk production or SCC)

β_0 = intercept

β_1 = effect of explanatory variable on outcome variable (parameter estimates)

x_1 = explanatory variable (e.g. age or lactation number)

e_i = residual term quantifying difference between observed outcome and predicted outcome values

Parameter estimates quantify the change in outcome value associated with a one-unit change in an explanatory variable, whilst all other explanatory variables are held constant. Finally, upon construction of models, predictions were obtained using the 'Customised Predictions' facility in MLwiN estimating the mean predicted values for the total milk yield and May SCC of the two MVV status groups, positive and negative.

5.3 Results

5.3.1 Descriptive Analysis

70 of 319 tested milk samples were confirmed positive for presence of MVV using the ELITEST-MVV/CAEV test (HYPHEN Biomed). Overall, this translated to a seroprevalence of 22%. MVV was seen in all age groups (1-9 years old) with a prevalence range of 18-50% between ages (Figure 5.3.1.1).

In June 2015, SCC measured identified 3 ewes with SCC greater than 250,000 cells/ml (Figure 5.3.1.2a). Number of ewes showing SCC above this level increased in subsequent time points with 7, 12 and 65 ewes showing SCC above 250,000 cells/ml in June 2016, March 2017 and May 2017, respectively. Number of seropositive ewes within these numbers was also seen to increase with time. In June 2015, June 2016, March 2017 and May 2017 seropositive ewes with SCC above this threshold value were 1, 3, 4 and 8, respectively. At the final time point, when comparing MVV seropositive and seronegative ewes, the mean value for seropositive animals (148,800 cells/ml) was lower than that of seronegative animals (383,500 cells/ml) (Figure 5.3.1.2b).

Comparison of the distribution of milk yield (Figure 5.3.1.3) and SCC (Figure 5.3.1.2c) against age of individual ewes showed non-linear relationship between each variable and age. To account for this finding in model construction, age was treated as a categorical variable.

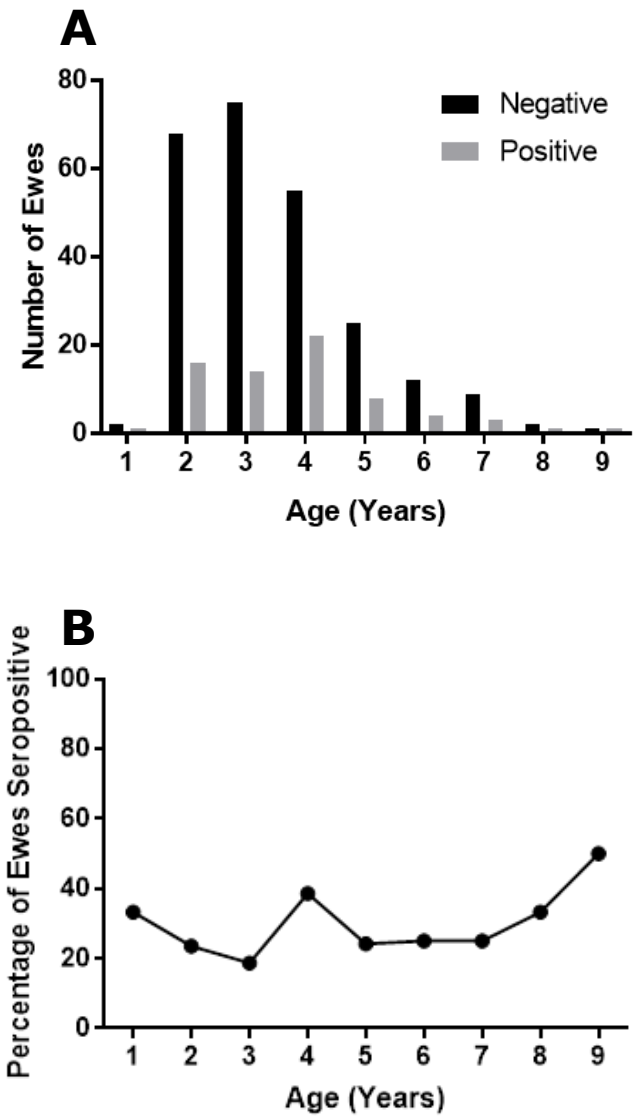


Figure 5.3.1.1 Age distribution and seroprevalence of 319 ewes. Graphs plotting the (A) number of ewes seropositive or seronegative (determined by ELISA) or (B) percentage of seropositive ewes within each age group for a group of 319 dairy ewes.

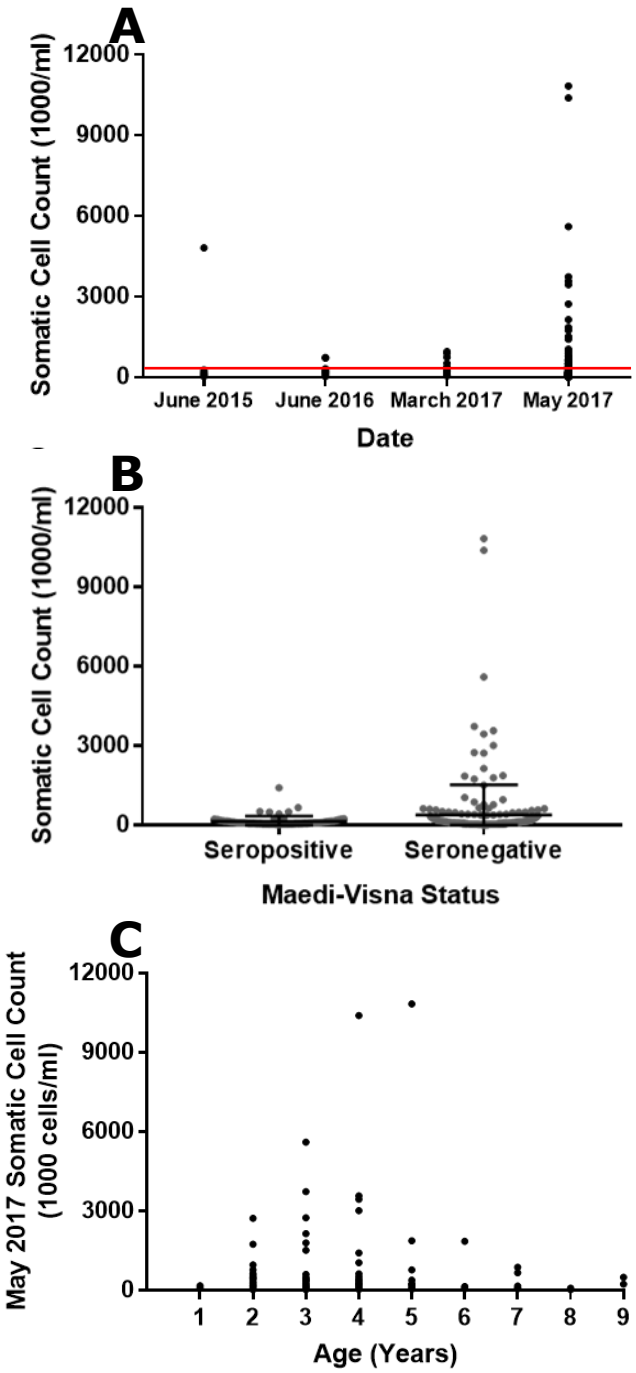


Figure 5.3.1.2 Descriptive graphs of SCC in 319 dairy ewes. Graphs showing (A) distribution of SCC for 319 ewes at four time points (June 2015, June 2016, March and May 2017), red line denotes suggested threshold for indicator of infection at 250,000 cells/ml (B) distribution of SCC at May 2017 in MVV seropositive and seronegative ewes (C) distribution of SCC in May 2017 against age of individual ewes to illustrate non-linear relationship of variables.

Figure 5.3.1.3 Rate of milk production between age groups. Graph plotting the milk yield per milking of 319 dairy ewes against individual age. Milk yield per milking day was plotted instead of total yield as lactations interrupted mid cycle and would therefore not be representative. Graph was plotted to illustrate non-linear relationship between variables of milk yield and age.

5.3.2 Total Milk Yield Model

Construction of the final model for estimation of the impact of MVV on milk yield included the variables lactation number, milking days in current lactation period and MVV status. Data from 319 ewes was included within the model, the explained variance (R^2) was calculated as 0.937 and residual plots showed normal distribution indicative of good model fit (Figure 5.3.2.1). Parameters estimates and their standard errors are listed in Table 5.3.2.2.

In comparison to ewes in their first lactation, the model predicted greater milk yields in ewes during their 2nd to 5th lactation period with a peak in yield seen during the third lactation. For ewes in their 6th to 8th lactation period, the model predicted a reduced milk yield compared to ewes in the first lactation. The reduction observed was greater in later lactation periods, although these predictions were not found to be significant. The number of milking days in the current lactation period showed a positive association with milk yield.

The presence of MVV had showed a negative association with milk yield in dairy ewes within the model. Predictions estimated a total milk yield of 283.282L and 264.589L in seronegative and seropositive ewes, respectively (Figure 5.3.2.3). An unpaired t test using the calculated milk yield showed the reduction caused by SRLV infection to be significant ($p<0.005$) which was further supported by calculated confidence intervals. Therefore, infection with MVV was predicted to cause a 6.60% reduction in milk yield in dairy ewes.

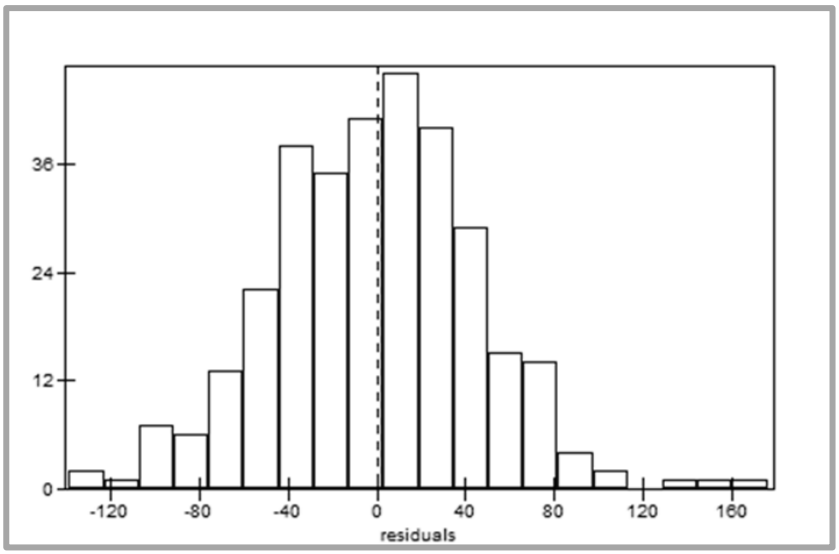


Figure 5.3.2.1Residual histogram for a regression model predicting total milk yield. Normality of distribution used as an indicator of model suitability for data sets.

Table 5.3.2.2 Parameter estimates for a regression model predicting total milk yield. Predictions were based on data from 319 dairy ewes, of which 70 were diagnosed seropositive by ELISA.

Model Term	Coefficient	SE
Total Milk Yield	Outcome	
Intercept	192.931	15.422
Fixed Effects		
Lactation Number 1	Reference	
Lactation Number 2	31.911	8.432
Lactation Number 3	37.322	7.850
Lactation Number 4	32.117	7.106
Lactation Number 5	29.230	11.659
Lactation Number 6	-26.793	13.762
Lactation Number 7	-35.784	33.402
Lactation Number 8	-88.512	47.100
Milking Days	0.557	0.109
MVV Status Negative	Reference	
MVV Status Positive	-18.440	6.530

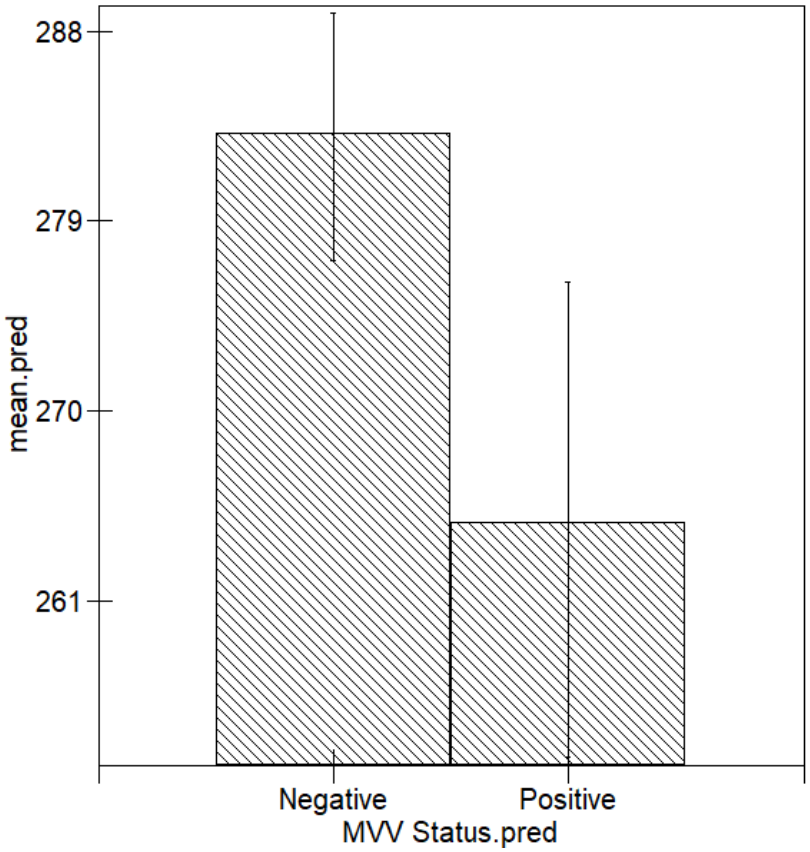


Figure 5.3.2.3 Mean predicted total milk yield in MVV seropositive and seronegative ewes. Predictions of total milk yield (L) in a lactation period based on regression model construction from data collected from 319 dairy ewes. Error bars illustrate 95% confidence intervals. Unpaired t test carried out calculated a p value of 0.0027.

5.3.3 SCC Model

A model was constructed to predict the impact of MVV infection on somatic cell count in May 2017. Data was collected from 188 dairy ewes (seropositive ewes, n=54, 28.7%), the explained variance (R^2) was calculated as 0.936 and residual plots showed weak normal distribution indicative of reduced model fit (Figure 5.3.3.1). Parameter estimates and their standard errors are listed in Table 5.3.3.2.

The model assessed impact of lactation number on the somatic cell count. When compared to ewes in their first lactation period, individuals in lactation period 3-7 showed reduced somatic cell count. Strong association was noted in ewes in the 4^{th}, 5^{th} and 7^{th} lactation periods with SCC. Lactation periods 2 and 8 show an increased SCC when compared to ewes in the first lactation although this was not significant. Days in milk during the current lactation showed a negative association with SCC while March 2017 SCC was predicted as having a significant positive association with SCC in May 2017.

Ewes seropositive for MVV showed a negative association with SCC when compared to seronegative animals. The mean model predictions for MVV status generated SCC of $4.70x10^5$ cells/ml and $2.39x10^5$ cells/ml for seronegative and seropositive ewes, respectively (Figure 5.3.3.3). The model predicted a drop in SCC of 50.93% in animals infected with MVV although confidence intervals suggest this to not be significant.

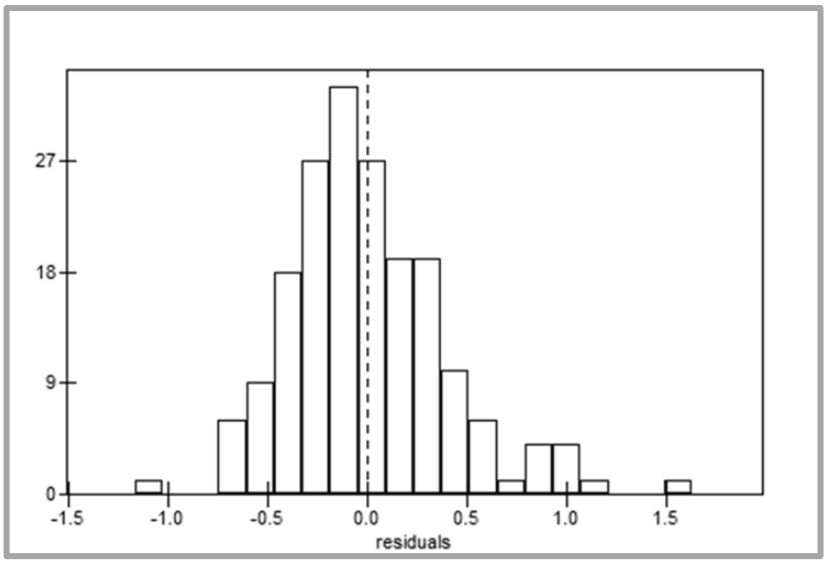


Figure 5.3.3.1 Residual histogram for a regression model predicting total SCC. Normality of distribution used as an indicator of model suitability for data sets.

Table 5.3.3.2 Parameter estimates for a regression model predicting total SCC. Predictions were based on data from 188 dairy ewes, of which 54 were deemed seropositive by ELISA.

Model Term	Coefficient	SE
Log_{10} May 2017 SCC	Outcome	
Intercept	3.300	0.638
Fixed Effects		
Lactation Number 1	Reference	
Lactation Number 2	0.056	0.116
Lactation Number 3	-0.162	0.087
Lactation Number 4	-0.221	0.086
Lactation Number 5	-0.339	0.119
Lactation Number 6	-0.202	0.124
Lactation Number 7	-0.862	0.404
Lactation Number 8	0.710	0.405
Milking Days	-0.008	0.004
March 2017 SCC	0.002	0.000
MVV Status Negative	Reference	
MVV Status Positive	-0.292	0.105

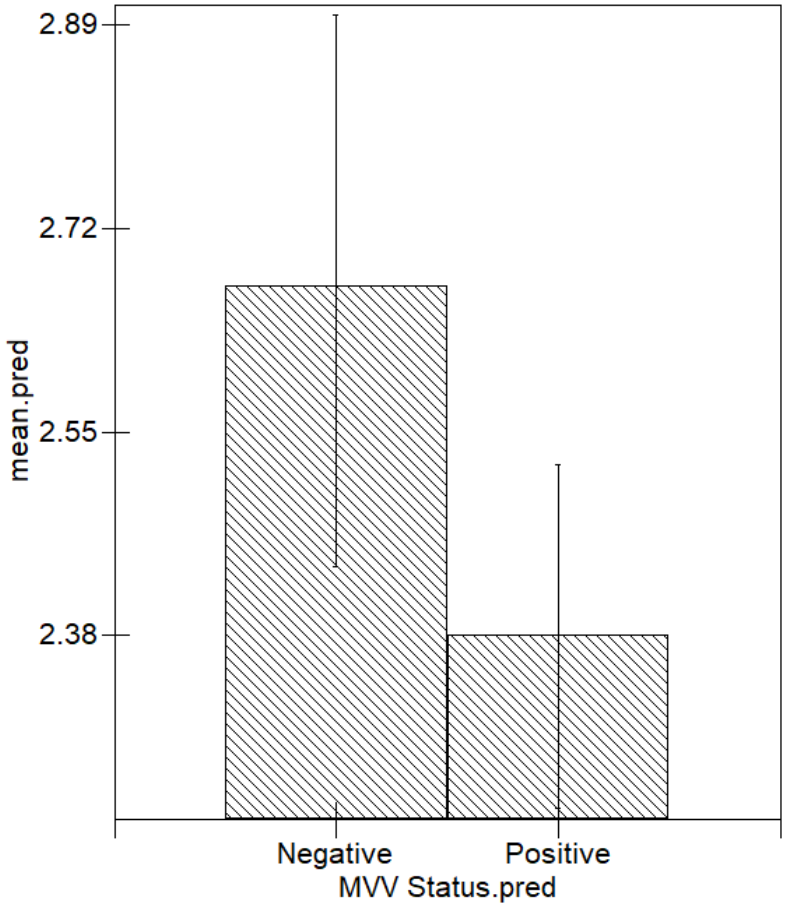


Figure 5.3.2.3 Mean predicted SCC in MVV seropositive and seronegative ewes. Predictions of SCC (Log_{10} 1000 cells/ml) in a lactation period based on regression model construction from data collected from 188 dairy ewes. Error bars illustrate 95% confidence intervals.

5.4 Discussion

Two multivariable regression models constructed to estimate the impact on MVV infection on milk yield and SCC in sheep predicts a significant reduction in both values in seropositive ewes. Models' suitability and fit were assessed upon construction. Residuals were assessed as part of this process and histograms plotted to evaluate normality. Histograms showed normal distribution indicative of model suitability. Models may have been improved with inclusion of further variables not recorded within the present data set such as number of lambs, with ewes bearing more lambs showing increased yields (Pollott and Gootwine 2004), or presence of mastitis, which as previously described has been shown to result in reduced yield and increased SCC.

Reports to date have provided conflicting results to the effect of infection on milk yield with factors such as shorter lactation periods, occurrence of mastitis and breed being identified as explanatory variables for these inconsistencies. Despite this, the majority of studies seem to point towards a reduced milk yield in affected animals. In support, this study reports a model in which MVV infection caused an estimated reduction in milk yield of 6.60% when compared to uninfected individuals using multivariable regression modelling.

With regards to MVV infection in ruminants, another factor of importance to the dairy industry is the reported shortening of lactation period (Martínez-Navalón et al. 2013). Due to the nature of data collection for this study it was not possible to ascertain the complete length of the lactation period for seropositive animals. Therefore, the 6.60% drop in yield

predicted within these models demonstrates an immediate comparable difference between seropositive and seronegative. The true impact of MVV within the ewes of this study may therefore be greater than what has been predicted within this model.

The impact of MVV on SCC was also of interest in this study. The current literature shows little research into the impact of SRLVs on SCC in sheep and goats, although these studies have suggested that infection results in markedly increased SCC in infected animals compared to non-infected individuals (Ryan et al. 1993; Lipecka et al. 2010). The model constructed in this study utilised data collected from 188 dairy ewes undergoing an outbreak of MVV. The model proposed a decrease in SCC in seropositive animals when compared to seronegative. Predictions estimated this decrease to be 50.93%, although this was unlikely to be of significance. The 188 ewes included in this model account for 58.93% of the ewes available. The limiting factor in this model was inclusion of the March 2017 SCC variable. Despite effectively halving the N number for this model, March 2017 SCC variable was necessary as it allowed for accounting of natural individual variation between ewes, improving reliability of model findings. The large predicted drop in SCC in the constructed model, although not significant for this model, is worth further investigation.

The decrease in SCC demonstrated in this model may suggest MVV to possess immunosuppressive effects within infected hosts. Immunosuppressive actions are not uncommon within lentiviruses, HIV being the most widely known example, with other examples include BIV, SIV and FIV (Zagury et al. 1993; Rezikyan et al. 1995; Kalvatchev et al. 1998; Vahlenkamp et al. 2004). Myer et al. (1988), once suggested a small

immunosuppressive role for a South African strain of MVV but no further instances have been reported in recent years.

In addition to the models constructed during this project. The suitability of the threshold value for confirmation of infection for a SRLV diagnostic assay was carried out. Modified models did not show any significant changes in parameter estimates for included variables. Importantly, no marked improvement in model fit was observed that would support adjustment of threshold. Interestingly all modifications resulted in reduced impact of MVV infection on milk yield and somatic cell count.

The results of this study support the findings of several previous studies that state SRLV infection results in a reduced milk yield in infected animals (Smith and Cutlip 1988; Krieg and Peterhans 1990b; Martínez-Navalón et al. 2013). The impact predicted in this model may have been less than previously speculated, but as stated above this may be due to variables that were unable to be accounted for within this model.

Chapter 6: General Discussion

MVV in the UK, is a problem on the rise (Ritchie et al. 2010). An infection with no cure or vaccine. It has an asymptomatic latency period of several months to years in length, in which dissemination throughout a flock can occur unknowingly, culminating in a flock prevalence of up to 85% or higher. On appearance of clinical signs such as fever, laboured breathing or progressive wasting resulting in the animal's condition progressively degrades until eventual death. The economic impacts of infection have been characterised as increased mortality rates, reduced fertility, reduced birth weight and reduced growth rate (Dohoo et al. 1982; Burmeister 2001; Arsenault et al. 2003; Peterhans et al. 2004). Despite these present risks associated with infection, the current control schemes in place to control virus spread (MV/CAE accreditation scheme and MV monitored-free sheep health scheme) have low uptake among farmers with only an approximated 8.5% of holdings taking part in the MV/CAE accreditation scheme (SRUC 2020). To provide farmers a more comprehensive understanding of the current impacts of MVV infection within UK sheep and to identify areas of potential improvement in current monitoring and control systems.

Four aims were put forward for investigation: the design and testing of a qPCR diagnostic assay for detection and quantification of an unknown strain of MVV (Chapter 2), the implementation of an AI trial as a means to assess the risk of sexual transmission of MVV in natural mating behaviour (Chapter 3), the assessment of a longitudinal data set collected from a group of MVV seropositive rams over a period of 28 months as a case study of morbidity and mortality due to the

disease (Chapter 4) and to estimate the impact of MVV infection on milk yield and somatic cell count within a milk production system consisting of 319 dairy ewes suffering a MVV outbreak (Chapter 5). The results of these findings are discussed here.

6.1. Establishment of a Diagnostic for MVV

In Chapter 2, a qPCR assay was designed for detection of an unknown strain of MVV circulating within the UK in 2014. Designed to target proviral DNA within blood samples collected from seropositive rams, testing showed successful amplification of virus within DNA and RNA of blood and DNA of multiple tissues/swabs (Section 4.3.3-5). The use of qPCR-based assay allowed for the quantification of viral loads within tested samples and was successful for DNA and RNA levels within tested blood samples. Unfortunately, it was noted that DNA samples extracted from tissue samples (i.e. lung, mediastinal lymph node and testicle) showed near consistent amplification of an unknown product. The presence of this unknown product was also seen in samples testing negative for MVV infection, therefore suggesting that the product was the result of a random segment of sheep genomic DNA. Unfortunately attempts at identification of product using sanger sequencing techniques failed to retrieve any sequence data.

Proviral loads obtained from testing blood DNA, when compared to those reported in previous studies, were found to be higher by at least a factor of 10^1, and in some cases, by a factor of 10^4 (Reina et al. 2008; Niesalla et al. 2009; Rachid et al. 2013; Crespo et al. 2016). Although this could be attributed to concurrent lungworm infestation or assay targets, it is important to note that should such low proviral loads have been present in this study, the qPCR designed would not have been

able to detect them due to loads being below the threshold range of detection for this assay. This is of even more relevance for the findings of Chapter 3, as studies showing lowest viral loads were from measurements of blood proviral loads in the weeks and months following experimental infection (Reina et al. 2008; Niesalla et al. 2009; Rachid et al. 2013). The highest proviral DNA load seen within blood samples in these studies (approximately 1.6 x 10^1 copies per ng of DNA) were reported in animals from a herd recording 100% seroprevalence and were likely infected for a longer period of time resulting in higher loads (Crespo et al. 2016).

Before future use of this qPCR, it would be recommended that tests be carried out in animals of known infected status during the period following infection. This is important as should the qPCR not be able to identify animals with low level infection, it would likely lead to persistent flock infections, ultimately requiring complete replacement of sheep/goats to ensure complete viral removal. Adaptation of qPCR to a nested PCR (nPCR) set up may combat this problem, for purely diagnostic purposes, although difficulties associated with such a change include increased processing time, occasional difficulty in interpretation and the requirement for a greater comprehension of the target sequence (Demeuse et al. 2016). This later point is of particular relevance in SRLVs due to high degree of variation between strain (Ramírez et al. 2013).

Blood and tissues selected for testing were chosen for the known tropism of SRLV and due to previous reports showing successful detection. One sample, that could not be collected within the group of seropositive rams within this study but has been collected for viral detection is milk (Extramiana et al. 2002b; Mazzei et al. 2005). It would be interesting to determine

if this qPCR could be used for diagnostic testing of milk sample as this is an easily obtained sample from dairy systems. One difficulty that may arise in such a test would be strain specificity of assay. Adjadj et al. (2019) recently compared efficacy of qPCR methods in milk vs serology, although they showed detection in both, milk testing had lower sensitivity and specificity. Despite this, bulk milk testing could be used as an indicator of viral presence within a flock.

Of great interest in this study is the detection of MVV in RNA extracted from 100% of the nasal swabs (n=13) that were collected. Nasal swabs collection has less experience requirements and less individual limitations than either blood or milk sampling and can be collected by farmers. As such, fees associated with MVV testing of animals could be reduced. Souza et al. (2015) has also reported successful detection of SRLV from saliva samples. Oral swabs were collected as part of this study following euthanasia of seropositive rams (nasal and oral swabs collected simultaneously). Due to project restrictions, swabs were not tested for detectable virus. Therefore, future testing of these swabs, which can be cross referenced with results of this study can be carried out at a later date. It is relevant to mention, that techniques used to collect saliva in the previously mentioned study likely provided larger sample size that obtained by swabbing (Souza et al. 2015). In addition, nPCR was carried out on samples, and therefore together, this may suggest that if virus is present within saliva, it could be below the detectable threshold of the designed qPCR although testing would be required for confirmation.

The initial difficulties observed in developing the current qPCR assay highlights one of the key difficulties with using PCR based assays for detection of SRLVs currently in the UK. PCR based

techniques require accurate known sequences for reliable detection of targets, which is difficult for SRLVs due to their high variability (Ramírez et al. 2013). In addition, other than a single full genome subtype A1 virus identified nearly 30 years ago, little is known about current circulating strains within the UK (Sargan et al. 1991). The strain identified in this study, although only partially sequenced could provide some insight. Following phylogenetic analysis, sequences showed similarities with both A1 and A19 subtypes. Similarities with A1 strains is not surprising due to known worldwide prevalence and previous identification in UK (Shah et al. 2004a). What is surprising is clustering observed with subtype A19 (Figure 2.3.4.1), a strain that has only been reported in animals in Italy. Although this could be argued to be due to random bias of sequenced sections, especially as subtype A19 has only been reported in goats (Colitti et al. 2019). It does pose the question if said strain and others are currently present within the national flock.

Overall, this study hoped to put forward a diagnostic for universal detection of SRLV strains, at least within the UK. The results reported together suggest the feasibility of producing such a test is low. For such a test, there a several obstacles tht would have to be addressed. For example, first, all circulating strains would need to be identified and confirmed detectable by test. Second, routine checking for introduction of new or variable strains would be required to maintain efficacy of test. And three, production costs and maintenance costs likely to be greater than economically feasible. Together these factors imply that using current technologies, the production of a universal PCR-based SRLV diagnostic for use in the current market is unlikely.

6.2. Intermittent Infectiousness of Hosts

In Chapter 3, an AI trial to evaluate the risk of sexual transmission of MVV as a model for natural mating was carried out. Semen was collected from 13 seropositive rams and used to inseminate an experimental group of 12 naïve ewes. Bloods were collected weekly for a period of 7 weeks and tissue samples collected at post-mortem were tested for detection of virus by ELISA and qPCR with no detection of virus noted in inseminated ewes (Section 3.3.3+5).

DNA and RNA were extracted from semen for testing by qPCR for presence of virus. None of the 13 rams showed detectable virus within semen samples, although 6 of them showed detectable virus in epididymal washes. Washes were taken at post-mortem of rams, one day post-semen collection therefore sudden changes in viral loads is unlikely to have occurred. Due to the diluting effect of fluids from the extraseminal vesicles added during natural ejaculation, the epididymal wash results suggest that virus is present within semen samples but is below the detectable level of the current qPCR diagnostic (Cornwall 2009). The studies reporting proviral DNA loads within blood sample outside of the detectable range of the diagnostic designed in this study, as previously mentioned above, add support to this statement.

de la Concha-Bermejillo et al. (1996) and Preziuso et al. (2002) have both reported on the detectability of MVV in semen of rams. These studies reported intermittent shedding of virus in semen. Interestingly, they associated presence of virus in semen to detection of *B. ovis* infection of epididymis. These findings suggest that secondary infection within MVV infected individuals can trigger an inflammatory response which may

'activate' infected monocytes resulting in viral production and presence within surrounding tissues. Interestingly, Grego et al. (2018) reported a similar finding in animals suffering lung parasitism, which resulted in high loads when compared to other infected individuals. With this study, evidence of lung worm infestation was present within histological slides prepared from lung sections collected from rams (n=11/15). In addition, reported viral loads within blood samples were higher than reported within the literature. Together, these would align a similar situation to that observed in *B. ovis* infected epididymis, where lung worm infestation produces an inflammatory response within affected lungs. Next, lymphocytic infiltration occurs resulting in recruitment of latently infected monocyte/macrophages resulting in activation of latent virus. Such a model of infection could arguably explain the consistent detectability of viral RNA in nasal swabs seen in this study.

Unfortunately, in this study no epididymal tissue samples were collected from rams at post mortem, only washes. Samples would have allowed for histological assessment of the epididymis to quantify health of tissue within these seropositive rams. Interpretation of these results with regard to previous reported association of epididymitis and increased viral loads would have greatly aided the findings of the AI trial in this study (de la Concha-Bermejillo et al. 1996; Preziuso et al. 2002). The results as they stand suggest that the risk of sexual transmission in a natural mating setting is low when using semen from seropositive rams. But results suggesting low semen viral loads may not represent the whole picture, therefore this study should be repeated using seropositive rams showing high viral load, whether this be the result of natural disease progression or following *B. ovis* inoculation.

6.3 Attempting Control of Transmission

Inability to treat or cure infection of SRLVs in infected animals has shifted focus to preventative measures to control spread of infection. In the UK, two programmes (MV/CAE accreditation scheme and MV monitored-free sheep health scheme) are in place to aid in identification of viral infection and reduce spread. Despite this, prevalence has been reported to be on the rise (Ritchie et al. 2010). Therefore, identification of risk factors for infection can aid in the improvement of control techniques in addition to the current schemes in place. One example of such factors is housing of animals. Illius et al. (2020) highlighted this factor, it was found through mathematical modelling, that housing animals as little as 1 week of the year was sufficient in producing R_o for infection >1. The findings of this study in conjunction with available literature also suggest one such factor to be secondary infection within infected tissues causing increased viral load and potentially leading to increased infectivity of individuals (i.e. increase venereal shedding, or nasal shedding).

TMEM154 genotyping was carried out in 27 seropositives ram as part of this study. One ram (Ram 27) was homozygous K35, which has been reported to provide increased resistance to infection (Heaton et al. 2012; Molaee et al. 2018). In addition, it has been suggested that the K35 TMEM154 genotype is able to control infection as shown by reduced viral loads in homozygous individuals when compared to heterozygous or other individuals (Alshanbari et al. 2014). Results for ram 27 were found to support this finding, with proviral DNA and viral RNA showing a reduction to undetectable levels between April 2015 and December 2015.

Interestingly, out of the remaining 26 rams, 20 (76.9%) were heterozygous for the resistant haplotype (K35). The high frequency of this haplotype in this group suggests that implementation of a selective breeding policy could be successful for selection of TMEM154 resistance. This could be of interest to commercial flock. The high prevalence of the resistance haplotype suggests there would be limited difficulty in finding desired genetics and could add value to animals, especially for breeding ram flocks, although there would be cost associated for genotyping of individuals. Uptake of such a system in breeding flocks alone would provide a lot of aid, as selection of homozygous rams to be consistently used for breeding each year would cause a continual accumulation of homozygous individuals in the national flock, reducing overall risk of infection. In addition, the prevalence of heterozygous animals within the seropositive group when compared to homozygous supports TMEM154 providing resistance to infection with the circulating strain of MVV identified in this study.

6.4 SRLV Impact in Dairy Systems

To date, the impact of SRLV infection on milk yield is a topic of debate (Greenwood 1995; Nord and Dnøy 1997; Juste et al. 2020). In this study, a milk production dataset was received from a flock of ewes currently experiencing a MVV outbreak. Using regression modelling, an estimated decrease of 6.60% in milk yield was calculated.

Interestingly, a regression model estimating the impact of MVV on SCC in milk from infected ewes, calculated infection to cause a 50.93% decrease in SCC, although this was not found to be significant. This suggests an interesting scenario of

immunosuppression, which has once been previously suggested, although not in recent years (Ryan et al. 1993). Immunosuppression is not an uncommon trait of lentiviruses (HIV, SIV, FIV, BIV), but no strong evidence has been provided for such a role in SRLV infection. Although thought not significant for the model produced here the sheer reduction predicted is interesting. In addition, insignificance could be due to insufficient data variables present within the model. It could be interesting to repeat such a model with a greater number of constituent ewes and a greater span of recorded variables.

In dairy systems, the importance of SCC is as an indicator of milk quality. The recommended threshold values for milk have yet to be fully characterised for sheep, although the majority of results suggest a threshold value within the range of 250,000 and 500,000 cells/ml (Souza et al. 2012) In regard to the findings from this study, it could suggest that SCC of milk collected from seropositive ewes may not be representative of the true quality due to an immunosuppressive effect.

6.5 Future Work

Results described here have highlighted areas of interest where further research could provide important insight of SRLV infection within small ruminants. In addition, areas of work that could not be carried out due to various restrictions during this study could show some interesting results.

A 2014 circulating strain of MVV was partially sequenced and characterised as part of this study. Efforts to complete this sequence would provide some critical insight into the current circulating strains within the UK. Sequence data identified during this study came from NGS data of RNA extracted from

lungs and mediastinal lymph node of seropositive rams. Acquisition of complete viral genome could be carried out through further NGS analysis of this data set (e.g. de novo assembly). In addition, gene expression analysis could be carried out using this data set comparing expression with lung and mediastinal lymph of 3 infected rams.

Following the euthanasia or death of seropositive rams, a multitude of tissue samples and swabs were collected for later testing. Of these only lung, lymph, testicle, semen, nasal swabs and epidydimal washes were tested. Testing of remaining samples should be carried out, especially as several of these samples (saliva, heart, kidney, liver, joint cartilage) have been previously reported as showing detectable virus. This would provide a more comprehensive understanding of the complete disease status within the seropositive rams of this study.

The detection of virus with nasal swabs is very important in this study as it provides a non-invasive, potentially cheaper means of testing animals for infection. Animals in this study were infected for a period of at least 28 months and showed signs of lung worm infestation which may have caused a spike in viral load. Therefore, it is unknown how reliable nasal swabs would be for detection of infection during early stages. Future testing of nasal swabs from known infected animals would be prudent, after which if results were promising, a longitudinal study of infection could be carried out with regular nasal swabs to determine the period of time required before detectability.

Genotyping of TMEM154 within UK flocks could prove useful, as it would calculate the prevalence of resistance genes present within the national flock. Should levels be found to be sufficient,

this could pave the way for proposing selective breeding for SRLV resistance.

6.6 Conclusion

In this study we partially characterised the impact of a previously unidentified circulating strain of SRLV within the UK. We developed a qPCR assay that showed efficacy in detecting said strain within both blood and tissue of infected rams. While sequence of studied virus showed similarities with previous UK strains of virus, the similarities seen with virus previously only reported in Italy raises concerns over the current efficacy of preventative measures within the UK. The findings of detectable virus within nasal swabs and the high prevalence of TMEM154 mutations linked to resistance to infection provide important avenues for betterment of control within the UK. Although further work is required to corroborate the results reported in this study, they are still promising. In addition, the evidence that TMEM154 may be able to reduce impact within individuals by reducing viral loads highlights the potential of a selective breeding program for SRLV resistance. Further, our findings of reduced milk yield within ewes infected with a UK strain of virus can be used to provide further clarification of production impacts to farms. With regards to farms, we also showed low risk of sexual transmission via a natural mating route using AI with semen of infected rams. Although, further study is required to look into variable viral loads within semen, the finding that at least a perceived 'low viral load' semen can be used to provide an avenue of genetic rescue for farmers, especially pedigree breeding ram systems where rams suffer large drops in financial worth following diagnosis. Overall it is our hope that the findings of this study can be used to aid in the furthering of

understanding into the current state of SRLV infections in the UK and provide an avenue to address the reported rise in prevalence.

Appendices

Appendix 1: Reference Sequences

Table of SRLV sequence used for qPCR and phylogenetic analysis.

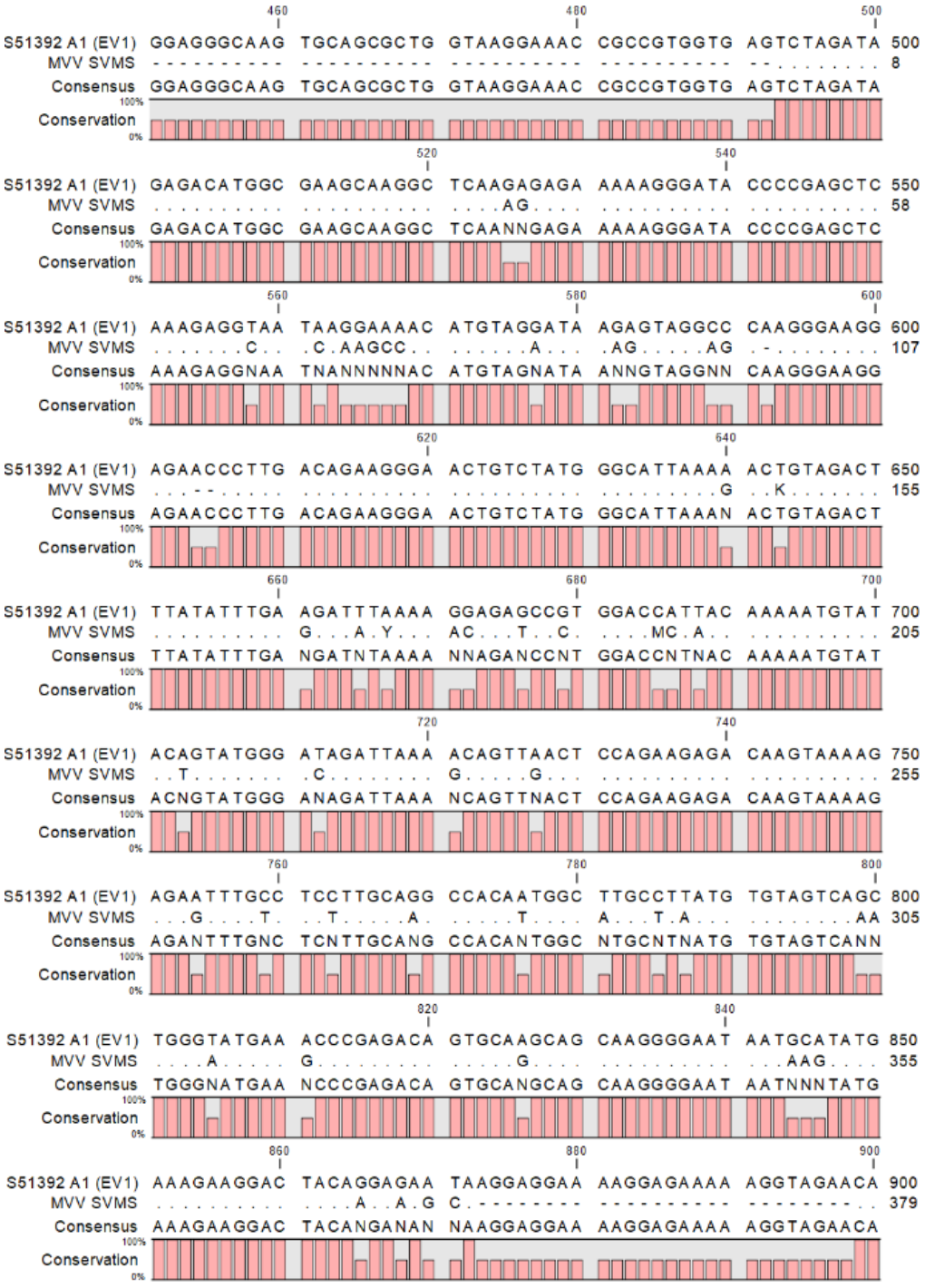
	Accessions	Species	Genomic Region	Country	Year
AFRICA					
	M31646	S	FULL	S AFRICA	1990
	M34193	S	FULL	S AFRICA	2016
	FJ619565-72	G	GAG	SUDAN	2009
ASIA					
	GU120138	G	FULL	CHINA	2009
	AY900630	G	FULL	CHINA	2005
	KT749878-81	G	FULL	CHINA	2016
	AB821356	S	GAG	JAPAN	2014
	AB747557	G	GAG	PHILIPPINES	2013
	LC002526	G	GAG	PHILIPPINES	2010
	GU903321	G	ENV	TAIWAN	2010
	HM237197	G	ENV	TAIWAN	2008
	GQ161209-15	G	GAG	THAILAND	2008
	JF714253-5	G	GAG	THAILAND	2008
	EU983108-9	G	GAG	THAILAND	2008
	EU919141	G	GAG	THAILAND	2008
	FJ167525	G	GAG	THAILAND	2008
	JQ898278-85	S	GAG	TURKEY	2012
	JF502418	S	GAG	TURKEY	2011
	MK098477,80	S	GAG	IRAN	2020
EUROPE					
	JN184351-78	S	GAG	SPAIN	2011
	JN184379-94	S	ENV	SPAIN	2011
	DQ084332-44	S/G	GAG	SPAIN	2005
	DQ632733-35	S/G	POL	SPAIN	2006
	FJ195346	S	FULL	SPAIN	2008
	HQ848062	S	FULL	SPAIN	2011
	AF479638	S	FULL	PORTUGAL	2012
	AM084203-9	S	GAG	FINLAND	2005
	AM084210-15	S/G	ENV	FINLAND	2005
	U35796-801	S/G	ENV	FRANCE	1995
	U35804,811	S/G	ENV	FRANCE	1995
	DQ149845	S	ENV	FRANCE	2005
	AJ400718-21	G	ENV	FRANCE	2000
	AF015181	G	GAG	FRANCE	1997
	AF015182	G	POL	FRANCE	1997
	AF015180	G	ENV	FRANCE	1997
	CAU35813	G	POL	FRANCE	1995
	VVU35803	S	POL	FRANCE	2001
	AJ969032-37	G	POL	IRELAND	2005
	AJ969038-43	G	ENV	IRELAND	2005
	U51910	S	ENV	ICELAND	1996
	NC_001452	S	FULL	ICELAND	1987
	M60609	S	FULL	ICELAND	1991

	L06906	S	FULL	ICELAND	1987
	X54379	S	GAG	NETHERLANDS	1990
	DQ844911-30	S/G	POL	NORWAY	2006
	DQ444931-41	G	ENV	NORWAY	2006
	DQ015910-16	S	ENV	NORWAY	2005
	AF322109	S	FULL	NORWAY	2000
	S51392	G	FULL	UK	1991
	AY45161-232	S/G	GAG	SWITZERLAND	2003
	AY454175-296	S/G	POL	SWITZERLAND	2003
	AY445885	G	FULL	SWITZERLAND	2003
	AY577031-3	S/G	GAG-POL	SWITZERLAND	2004
	AY577034-9	S/G	GAG	SWITZERLAND	2004
	KT453988	G	FULL	SWITZERLAND	2015
	AY53289-93	S/G	GAG	GERMANY	2004
	MN233118	S	GAG	GERMANY	2020
	HM449450,500	G	ENV	ITALY	2010
	EF685749,83	G	ENV	ITALY	2007
	FR687200	S/G	GAG-POL	ITALY	2010
	EF144472,82	G	ENV	ITALY	2006
	EU293537	G	FULL	ITALY	2008
	EU726488-525	G	GAG	ITALY	2008
	EF676000-26	S/G	GAG	ITALY	2007
	EU010120-6	G	GAG	ITALY	2007
	DQ013214-43	S/G	POL	ITALY	2005
	AY044803	G	GAG	ITALY	2001
	GQ381130	G	FULL	ITALY	2009
	GQ428519-36	G	GAG	ITALY	2009
	JF502416	S	FULL	ITALY	2011
	JF502417	S	FULL	ITALY	2011
	JF520393-406	S/G	GAG	ITALY	2011
	EU709743	S	ENV	ITALY	2008
	FR693808,20	S/G	GAG-POL	ITALY	2011
	FR694921	S/G	GAG-POL	ITALY	2011
	MG554409	G	FULL	ITALY	2019
	MH374285,7	S	FULL	ITALY	2019
	KT898826	S	FULL	ITALY	2016
	MH790877-80	S	GAG	POLAND	2018
	FJ623120-25	S/G	GAG	POLAND	2009
	FJ623110-119	S/G	ENV	POLAND	2009
	JN084103	G	GAG	RUSSIA	2011
	JN008914	G	ENV	RUSSIA	2011
	JQ610956-1030	S	POL	SLOVENIA	2012
	JX469600-10	S/G	GAG-POL	SLOVENIA	2012
NORTH AMERICA					
	HQ158122-36	S/G	GAG	CANADA	2010
	HM210570	G	FULL	MEXICO	2010
	HM212792-3	G	ENV	MEXICO	2010
	K03327	G	POL	USA	1985
	NC_001463	G	FULL	USA	1980
	GQ255391-403	S	GAG	USA	2009
	AY362022-35	S	ENV	USA	2003
	M60855	G	ENV	USA	1993
	AY101611	S	GAG-POL	USA	2002
	U64439	S	ENV	USA	1996
	M33677	G	FULL	USA	1990
SOUTH AMERICA					
	AJ30503942	S/G	GAG	BRAZIL	2001
	AF402664-66, 68	G	GAG	BRAZIL	2001
	AY101347-8	G	GAG	BRAZIL	2002
	AY081139	G	GAG	BRAZIL	2002

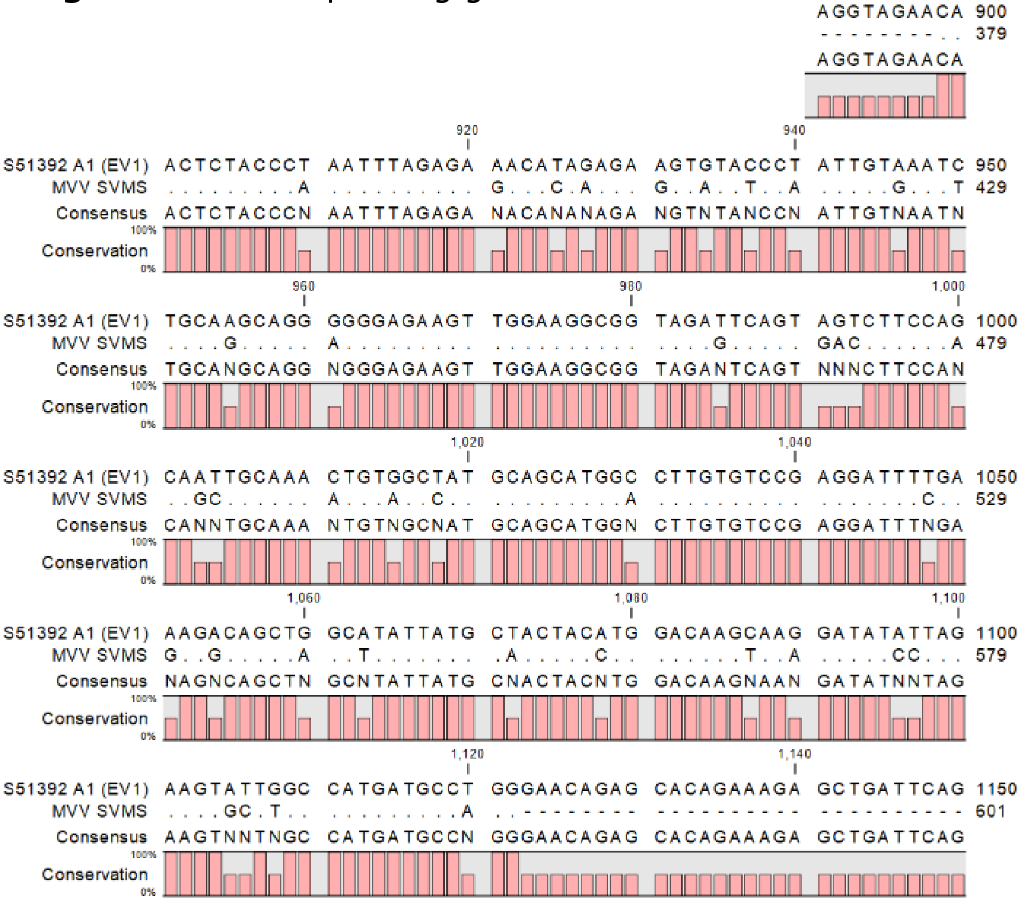
Appendix 2: Unknown Viral Sequence

Alignment of 10 fragment sequences of a circulating SRLV strain within the UK. Sequence is aligned against the previously sequence full genome UK strain of MVV (EV1) (Sargan et al. 1991)

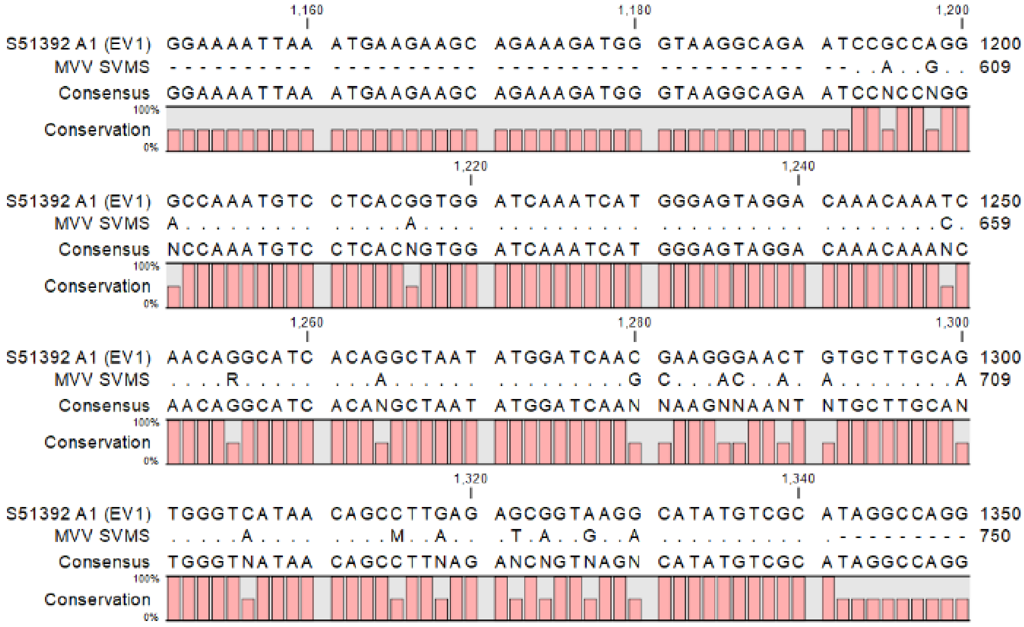
Fragment 1: 377 bp – Gag gene



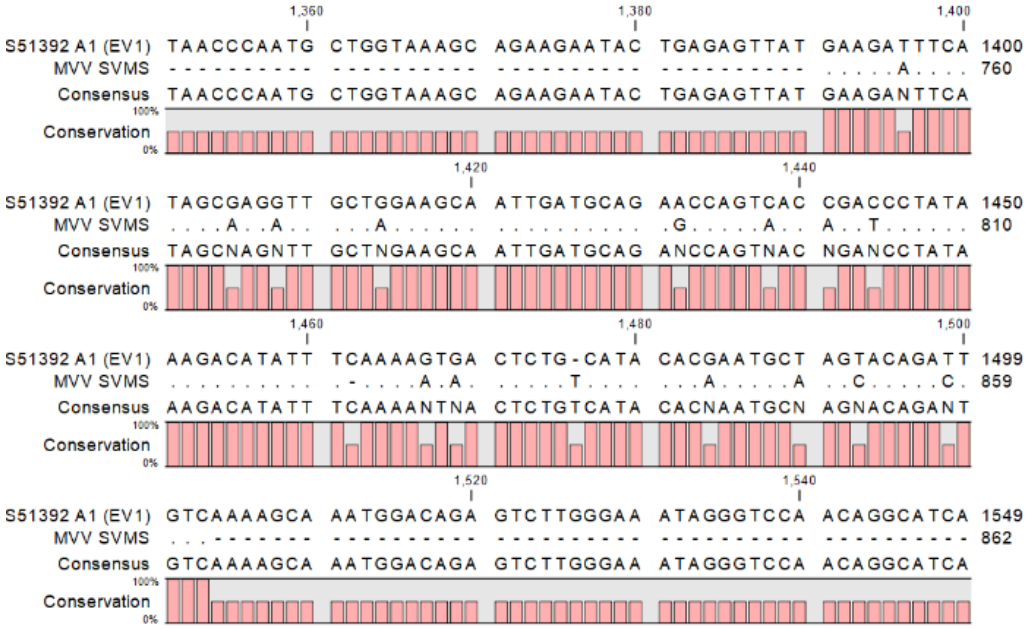
Fragment 2: 224 bp – Gag gene



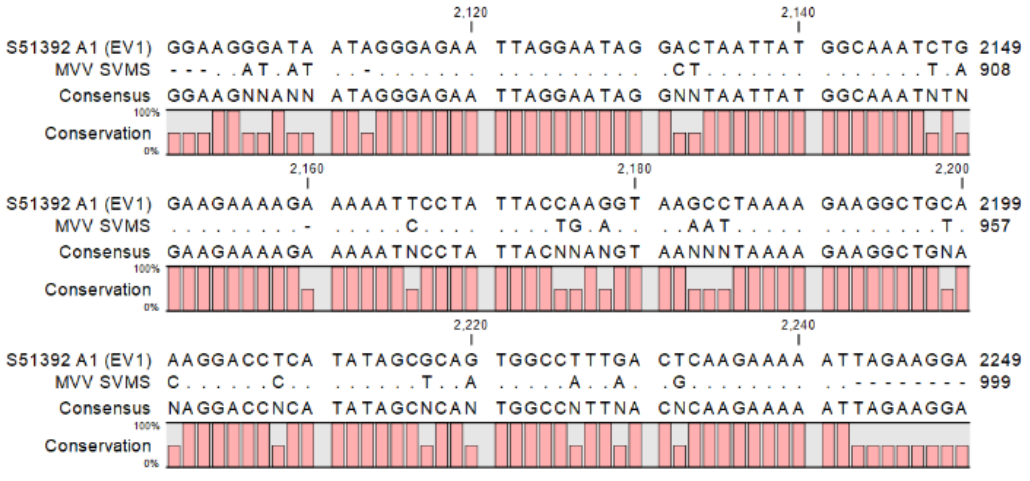
Fragment 3: 149 bp – Gag gene



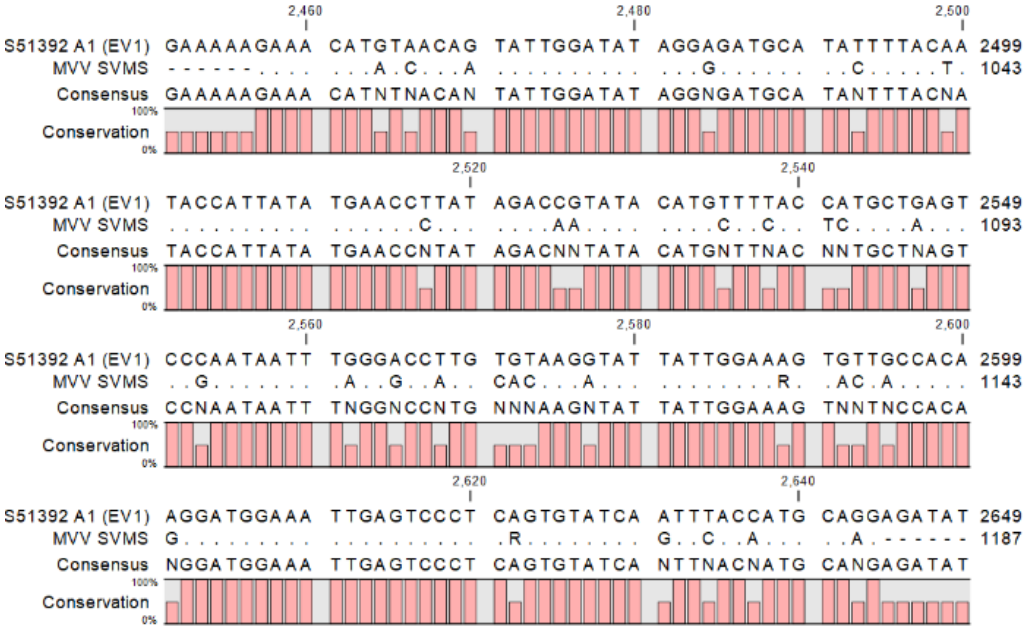
Fragment 4: 112 bp – Gag gene



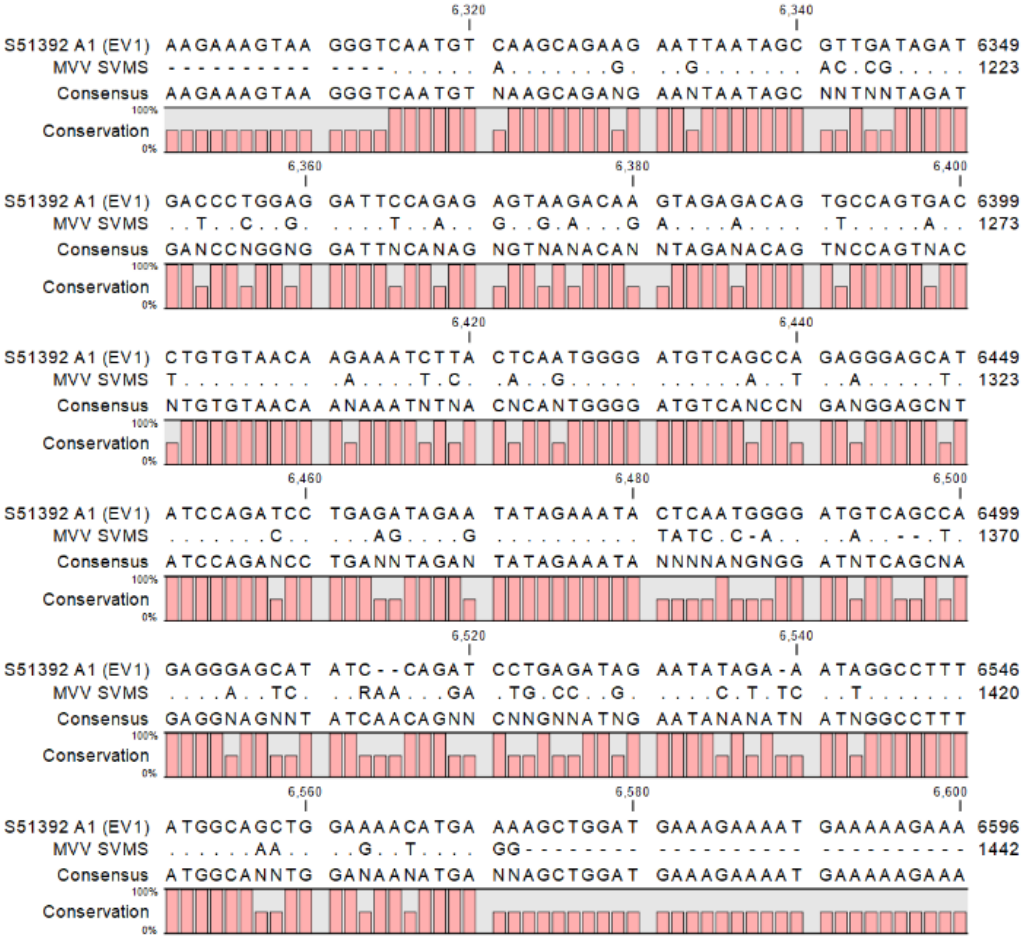
Fragment 5: 137 bp – Pol gene



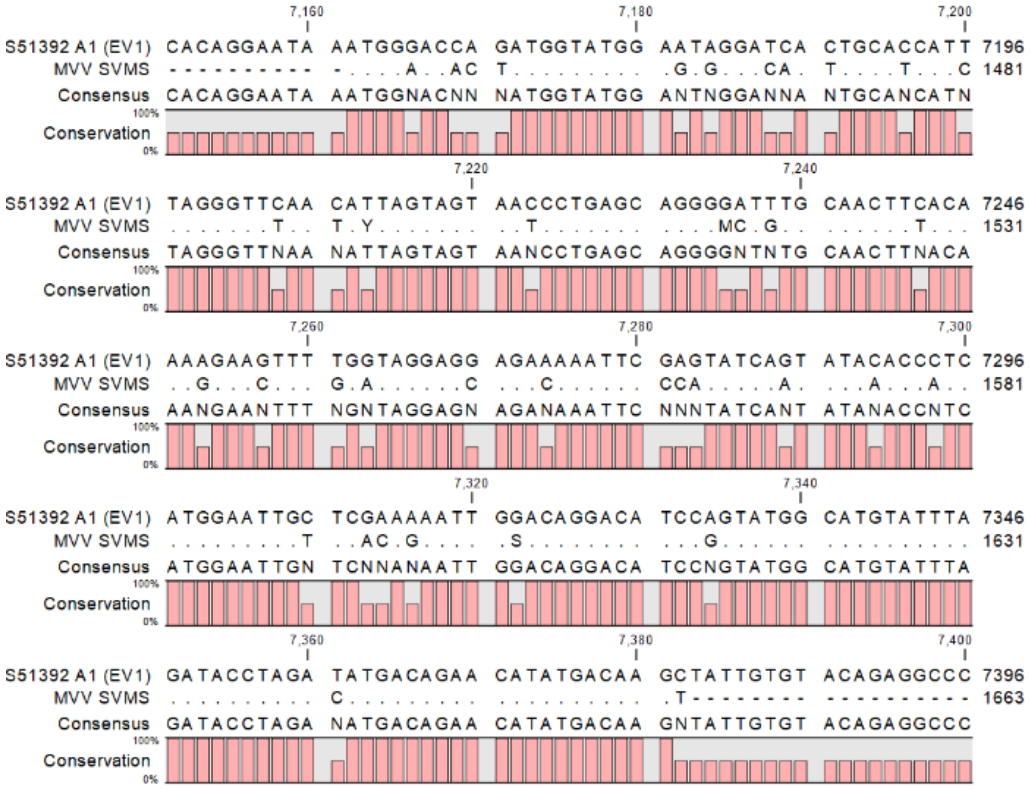
Fragment 6: 188 bp – Pol gene



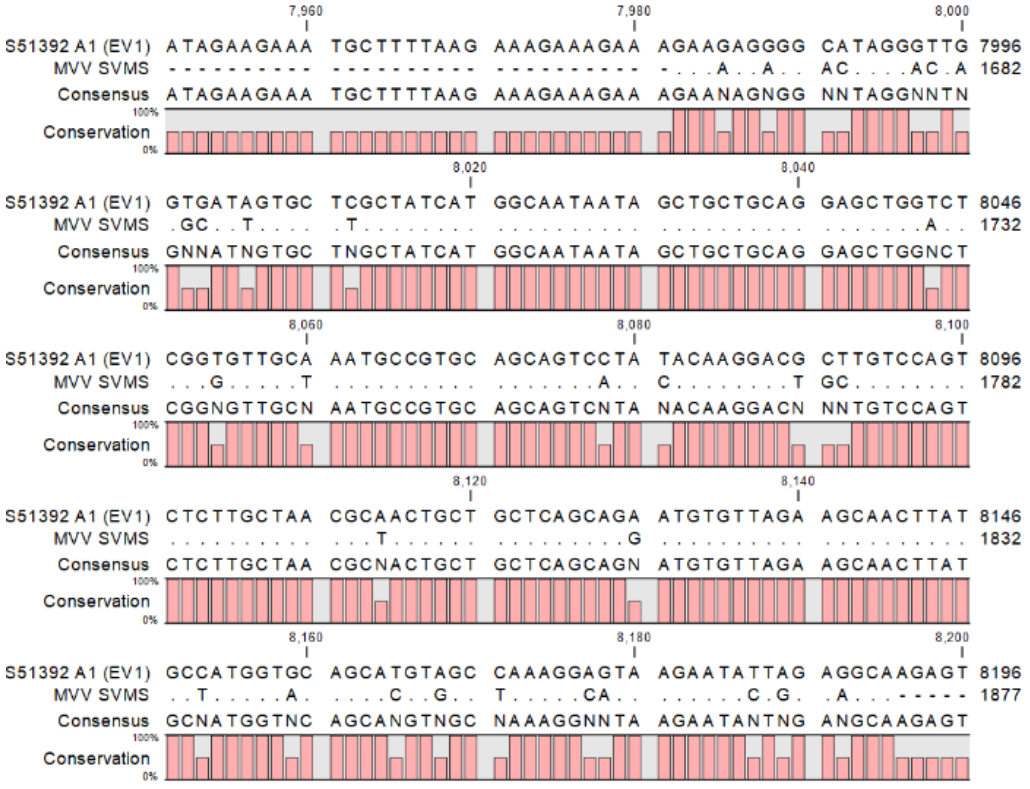
Fragment 7: 254 bp – Env gene



Fragment 8: 221 bp – Env gene



Fragment 9: 214 bp – Env gene



Fragment 10: 169 bp – Env gene

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