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The Impact of Maedi Visna on Breeding Flocks

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CONTENTS

1. Abstract

Small Ruminant lentiviruses (SRLV) including Maedi Visna (MV) and Caprine encephaltitis and arthritis (CAE) are the cause of chronic progressive disease in sheep and goats. The most common clinical signs include lung disease, wasting and mastitis with severe impacts on sheep health and productivity. The disease has a long latent period with clinical signs sometimes not detected until years after the initial infection. There are no vaccines or treatments available, leaving testing and culling of infected animals as the only realistic control option for MV in sheep flocks.

We do not know what the national prevalence rate of the disease is as there is no systematic monitoring and the control programmes (the MV accredited free flock scheme run by Scotlands rural college, and the MV Monitored free scheme run by Axiom laboratories) in place are voluntary. However what data there is suggests the number of infected flocks has risen steeply in recent years. This project examined a number of aspects of MV transmission and impact in UK sheep flocks with a long term goal of improving testing and control strategies.

The first set of experiments examined the risk of sexual transmission of the virus from infected rams to niave ewes using intravaginal insemination as a proxy for natural mating. This 7 week trial demonstrated that several of the 13 naturally infected rams did indeed harbour low levels of virus in their reproductive tracts but that this did not go on to cause a detectable infection in the 12 ewes. This indicates that the risk of transmission from sexual infection is low and that genetic rescue of high value rams via semen collection and AI before culling would be feasible.

Longitudional monitoring records for these rams and 15 others from the same cohort demonstrated that the one animal that was heterozygous for the MV resistant allele of the TMEM154 gene apparently cleared the infection 16 months after initial diagnosis, testing negative via serology and qPCR in all tissues. Nasal swabs collected at post mortem from 13 of the affected rams demonstrated viral RNA detection at quite high loads indicating that this might be a viable route for diagnostic sampling that does not require blood collection. Balancing that, we do not know what the current variability in MV viral sequence is in the UK and whether one qPCR test would detect them all. Multiple published PCR tests for MV failed to detect the virus strain in these rams and deep sequencing was required to identify the virus strain and develop the qPCR tests used in this study.

The final set of work in this project used milk data from a 319 head East Friesian x Lacunae sheep diary herd that had undergone a breakdown in MV control in multiviariable regression models to deterimine the impact of MV infection on milk yield and quality. This demonstrated a 6.60 % drop in milk yield and a paradoxical decrease in SCC, demonstrating that loss in milk yield is a significant factor in production losses due to MV.

2. Introduction

Maedi visna (MV) and caprine arthritis and encephalitis (CAE) are chronic wasting diseases affecting sheep and goats worldwide (Sigurdsson 1954; Sigurdsson et al. 1957). The result of infection by maedi visna virus (MVV) and caprine arthritis encephalitis virus (CAEV), respectively, these viruses are collectively known as small ruminant lentiviruses (SRLVs). To sheep and goats, SRLV infection is a lifelong sentence. An asymptomatic period of around 2 years post infection can result in significant flock wide infection before any indicators are observed.

Following this asymptomatic phase, clinical signs occur gradually and progressively worsen until eventual death (100% fatality rate) in infected animals. Clinical signs in MV infection can vary with strains, presenting mainly in 1 of 2 patterns; respiratory disease or neurological disease (Sigurdsson 1954; Sigurdsson et al. 1957). Within the United Kingdom (UK), the respiratory pattern of disease is most prevalent with clinical signs such as lung disease, wasting and mastitis. Pathologically, lung disease is the result of significant lymphoid infiltration within the lungs, particularly 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). At post mortem, this is observed as firm, dense, enlarged lungs that fail to collapse following opening of the thoracic cavity (Rovid 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.

Interestingly, Herrmann-Hoesing et al. (2009) reported the severity of lesions observed in infected animals to be proportional to the provirus load (quantity of viral genome integrated into host genome), with high blood proviral levels 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 is 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.

Research into prevalence of SRLV infection within the UK suggests the number of infected individuals to be on the rise over recent years (Ritchie et al. 2010). Carried out between 1995

and 2010, the seroprevalence of MVV infections 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). Despite this, the accreditation scheme does 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).

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 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.

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).

Working in such control systems as the MV/CAE accreditation scheme, the use of reliable and efficient diagnostic techniques is critical. Currently the most commonly used diagnostics for detection of SRLV include enzyme-linked immunosorbent assays (ELISAs), agar gel immunodiffusion (AGID) and polymerase chain reaction (PCR) (Herrmann-Hoesing 2010; OIE 2016). Such techniques require components tailor designed to work at peak efficiency with a specific strain of virus (Fevereiro et al. 1999; Carrozza et al. 2010). To date, only a single full genome MVV sequence has been reported within the UK (EV1 strain) (Accession No. S51392) (Sargan et al. 1991). Initial identification occurred in 1991, approximately 30 years ago. Unfortunately, it has been shown that MVV has a tremendous ability for variation between differing strains through multiple mechanisms. For example, it has been estimated that during a single viral lifecycle, between 0.2 – 2 mutations can occur per genome. Given this ability to change, it is possible that the UK EV1 strain as sequenced in 1991 is no longer circulating naturally within the UK population. 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. 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.

Further to factors important for diagnosis of infection is the use of suitable sampling techniques to facilitate reliable detection is of great importance. The diagnostic mentioned previously (ELISA, AGID and PCR), can be used in conjunction with blood and milk samples. Although effective, such sampling techniques hold limitations in their requirement of licenced technicians for collection (blood) and limitation to females postpartum (milk). Previous diagnostic testing of infected animals following death or euthanasia has detected proviral DNA within a wide range of additional 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). Interestingly, 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. This question is further supported by the role of nasal secretions as a medium for virus transmission.

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). In addition, 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 later preferred due to increased pregnancy 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). 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 following insemination 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 therefore necessarily follow that sexual transmission under natural mating or vaginal insemination conditions will also occur.

Further to transmission, identification of genetic factors that are associated with a reduce risk of infection has occurred, with a particular focus on transmembrane protein 154 (TMEM154) (Heaton et al. 2012; Heaton et al. 2013). Within in sheep, an amino acid substitution of glutamate (E) to lysine (K) at position 35 of the TMEM154 gene has been identified. Individuals

homozygous for this substitution have been reported as having resistance to SRLV infection when compared animals heterozygous or lacking said haplotype. In addition to having increased resistance to SRLV infection, sheep homozygous for resistant haplotype have also been suggested as able to control viral replication once infected (Alshanbari et al. 2014). This was following comparison of viral loads in resistant and susceptibles animals, showing significantly reduced viral loads in resistant animals. Together this suggests that resistant animals would not only be more resistant to intial infection, but likely have reduced viral loads when infected and are probably less likely to develop severe lesions. Genetic selection of animals with the MV resistant alleles of TMEM154 could therefore reduce the potential impact of infection on farms and aid in control of infection. Research into the impact of the TMEM154 gene on SRLV in goats showed a lack of indicators supporting a role for TMEM154 in goats for resistance to infection (Heaton et al. 2012).

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

Table 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 showing multiple % 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%

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 somatic cell count (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 as an indicator of infection within mammary tissue. Although once thought possible for use in detection of mastitis in ewes, publications reporting isolation of mastitis pathogen 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 are still used as an estimate of the prevalence of mammary infections within a flock. To date, the majority of threshold values proposed for

differentiating healthy and infected ewes lie within the range of 2.5 x 10^5 and 5.0 x 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).

Over the course of this project, four aims were put forward for investigation. With betterment of the sheep and goat industry a high priority, this projects aims to further upon previous work carried out to expand and increase our knowledge and understanding of SRLV infection and ideally reduce the impact infection currently imposes upon the farming sector.

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 first aim of this study was to quantify the risk of MVV transmission following intravaginal insemination using semen from naturally infected rams to inseminate a group of naïve ewes.

In addition, 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 second aim of this project was to develop a more reliable and cost-effective PCR based diagnostic test for the detection of MVV infection.

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 the disease in these individuals.

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). 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.

3. Materials and methods

3.1. qPCR Design

The virus sequence of the infecting MV strain was determined via Illumina RNAseq of tissue samples from the trial rams. RNA was extracted from the lung and mediastinal lymph node using The RNeasy Mini Kit (Qiagen) according to manufacturers protocol, quantity and quality of RNA extracted was determined using the 2100 Bioanalyzer system (Agilent).

Next generation sequencing (NGS) was carried out by the Imperial BRC Genomics Facility of Imperial College, London. Library preps were completed targeting total RNA with additional ribosomal RNA depletion. 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.

Analysis was carried out using the Cloud Infrastructure for Microbial Bioinformatics (CLIMB) (Pisoni et al. 2007; Ramírez et al. 2013). 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 (Shah et al. 2004). Next, non-sheep sequences were classified against a reference database using Kraken2 software (Leginagoikoa et al. 2010; Illius et al. 2020). Reference database of archaea, bacteria and virus sequences was compiled using the available kraken database. To improve detectability for SRLV sequences, a further 22 full genome and 681 partial sequences were added to the reference database (all that were publically available at the time). 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.

Primers for qPCR were designed using Primer-BLAST free online software (NCBI) targeting the three structural proteins of SRLVs (Pol, Gag and Env). Primers were tested for suitability against RNA extracted from blood samples from seropositive rams and seronegative ewes. RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturers recommended protocol. Reverse transcription was carried out on RNA using moloney murine leukemia virus (M-MLV) (Promega) or avian myeloblastosis virus (AMV) (Promega) and random hexamer primers according to manufacturers instructions. qPCR tests were conducted using the following cycling conditions: 1x qPCRBIO SyGreen Mix Lo-ROX master

mix (PCR Biosystems), 0.04μM forward and reverse primers (Sigma-Aldrich) and 1μl of test DNA or standard in a total volume of 20 μl. Reaction conditions consisted of a starting incubation of 95ºC for 15 minutes followed by 45 cycles of 95ºC for 5 seconds, 60ºC for 30 seconds and 72ºC for 10 seconds. A melt cycle was carried out at reaction end ranging from 65ºC to 95ºC. Reactions were carried out within a CFX Connect Real-Time PCR Detection System (Biorad Laboratories). For positive control, oligonucleotide sequences (50-60 bp) were synthesized for each primer set.

3.2. Artificial Insemination Trial

The trial was conductied under approved by the Home Office under the 'Animals (Scientific Procedures) Act 1986' (Licence no. PPL 30/3367). Thirteen 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 a MVV free flock. 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.

All animals were blood sampled (jugular venepuncture) and screened at the start of the trial with the MVV/CAEV p28 Antibody Screening Test (IDEXX) (serum samples in triplicate) following the manufacturer's recommended protocol and an in-house qPCR test designed for the viral strain present in seropositive rams. DNA and RNA were extracted for PCR testing using the machery nagel Nucleospin® Tissue Kit and the QIAamp Viral RNA Mini Kit (Qiagen) according to manufacturers instructions. Rams were euthanased and post mortems conducted immediately after semen donation, ewes were sacrified at week 7 (with weekly blood collection between insemination and sacrifice). A range of tissue samples were collected at post mortem and stored in either 500μl RNAlater (Sigma-Aldrich) at room temperature for 24 hours and then placed at -20ºC for RNA and DNA extraction and qPCR or in 500ul formalin for later histopathology.

Reverse transcription was carried out on RNA using moloney murine leukemia virus (M-MLV) (Promega) or avian myeloblastosis virus (AMV) (Promega) and random hexamer primers

Table 2. 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	

according to manufacturers instructions. qPCR was conducted using the primers in table 1. Reactions were carried out with the following cycling conditions: 1x qPCRBIO SyGreen Mix Lo-ROX master mix (PCR Biosystems), 0.04µM forward and reverse primers (Sigma-Aldrich) and 1µl of test DNA or standard in a total volume of 20 µl. Reaction conditions consisted of a starting incubation of 95ºC for 15 minutes followed by 45 cycles of 95ºC for 5 seconds, 60ºC for 30 seconds and 72ºC for 10 seconds. A melt cycle was carried out at reaction end ranging from 65ºC to 95ºC. 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 was used as positive control.

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.

MVV positive rams were introduced to 3 'teaser' ewes (hormonally prepared as per the trial ewes) to stimulate mating behaviour. One at a time rams were allowed to mount ewes at which time semen was collected by intercepting the penis and redirecting to within an 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. Remaining semen was pooled for insemination. 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'.

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. Pooled semen was prepared by the addition of ultra-high temperature processed (UHT) milk in equal quantities. 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. Mock inseminated ewes were inseminated with UHT milk containing no semen.

3.3. Longitudinal Study

Thirteen rams from the AI trial and 15 others (27, 6 Aberfield and 21 Abermax rams in total) were previously subjected to longitudional blood sampling and post mortem tissue sampling upon death or euthanasia. Rams were identified as being MVV positive during routine testing as part of the MV/CAE accreditation scheme after which they were acquired by the University of Nottingham in 2015. Animals were held at pasture with available shelter and supplementary

Table 3. 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	

rations. Tissue sampling, blood testing, Serology testing, RNA and DNA extraction and qPCR testing were conducted as for the artificial insemination trial animals.

In addition nasal swabs were collected into 1 ml of RNA later with DNA extracted using the Nucleospin® Tissue Kit (Macherey-Nagel) following the supplementary protocol for purification of genomic DNA from buccal swabs. And RNA extracted from the supernatant using the QIAmp viral RNA mini kit (Qiagen).

Genotyping for the TMEM154 gene was carried out by PCR and sanger sequencing. One 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 2 stating target gene and sequence (Pisoni et al. 2007; Ramírez et al. 2013).

Products were visualed using 0.8% TAE agarose gel electrophoresis and Nancy-520 (Sigma Aldrich) staining. Gels were viewed and photographed by ImageQuant LAS 400 (GE Healthcare Life Science) under ultraviolet (UV) light. PCR products were purified using the Nucleospin® Gel and PCR Clean-up kit (Macherey-Negal) following the recommended protocol for PCR clean-up. Nucleotide sequences were acquired by Sanger sequencing carried out by Source BioScience. Sequence analysis was completed using BioEdit v7.2 and CLC Sequence Viewer software v8.0 (Qiagen).

3.4. Regression Modelling of MVV Impact

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.

Individual 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. Descriptive analysis was carried out using Microsoft Excel 2013 and Graphpad Prism 7.03 (Graphpad Software).

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). 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 to determine optimum model design.

Models took the form:

$$y_i = \beta_{0i} + \beta_1 x_{1i},$$

$$\beta_{0i} = \beta_0 + e_{0i}$$

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.

4. Results

4.1. qPCR Design

To identify the strain on MVV circulating with a group of 27 seropositive rams, RNA extracted from lung and lymph node tissue was sequenced using NGS technologies. Following

compiling, QC and trimming sequences alignment against the sheep genome (v3.1) identified 74106108 'non sheep' sequences. Kraken2 classified 115114 of these sequences successfully to the 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 the SRLV sequence database which provided 2046bp of sequence supported by a read depth >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), doubling that obtained from the *Pol* gene (325 bp). Alignment of sequence to EV1 strain showed 84.5% nucleotide identity.

Phylogenetic trees were generated based on comparison to 862 bp of *Gag* gene (Figure 1), 251 bp of *Gag* gene (Figure 2), 325 bp of *Pol* gene (Figure 3) or 859 bp of *Env* gene (Figure 4).

Obtained sequences were found to be strongly related with genotype A sequences in all trees. 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 (Figures 1+3). 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 the 251 bp region of *Gag* spanned sequences of all genotype subtypes with the exception of A6 (Figure 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* sequence closest match was found to be the UK strain of MVV (EV1).

Primers designed using the obtained sequences are listed in Table 4. Testing of primers against synthesised positive controls gave successful amplification of a single product in Gag and Pol primers (Figure 5a). Negative control showed no amplification with any primers (Figure 5b). All primer sets showed amplification of DNA extracted from seropositive ram blood, but only Pol show amplification of a single product of expected melting temperature (Figure 5c). 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 5d).

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 the qPCR assay

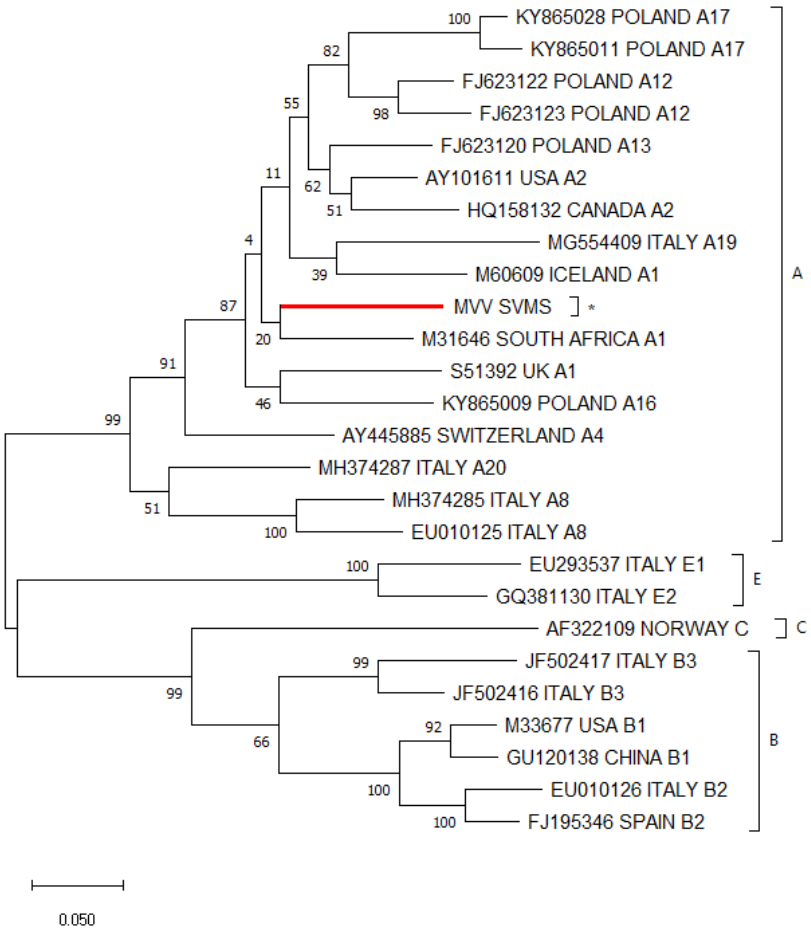

Figure 1. Phylogenetic tree of 862 bp spanning across SRLV Gag gene. Phylogenetic tree was generated using the Maximum Likelihood method and Tamura-Nei model and assessed by bootstrap method. Tree comprises unknown strain and 25 publicly available SRLV genomes listed in Appendix 1. Analysis was carried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

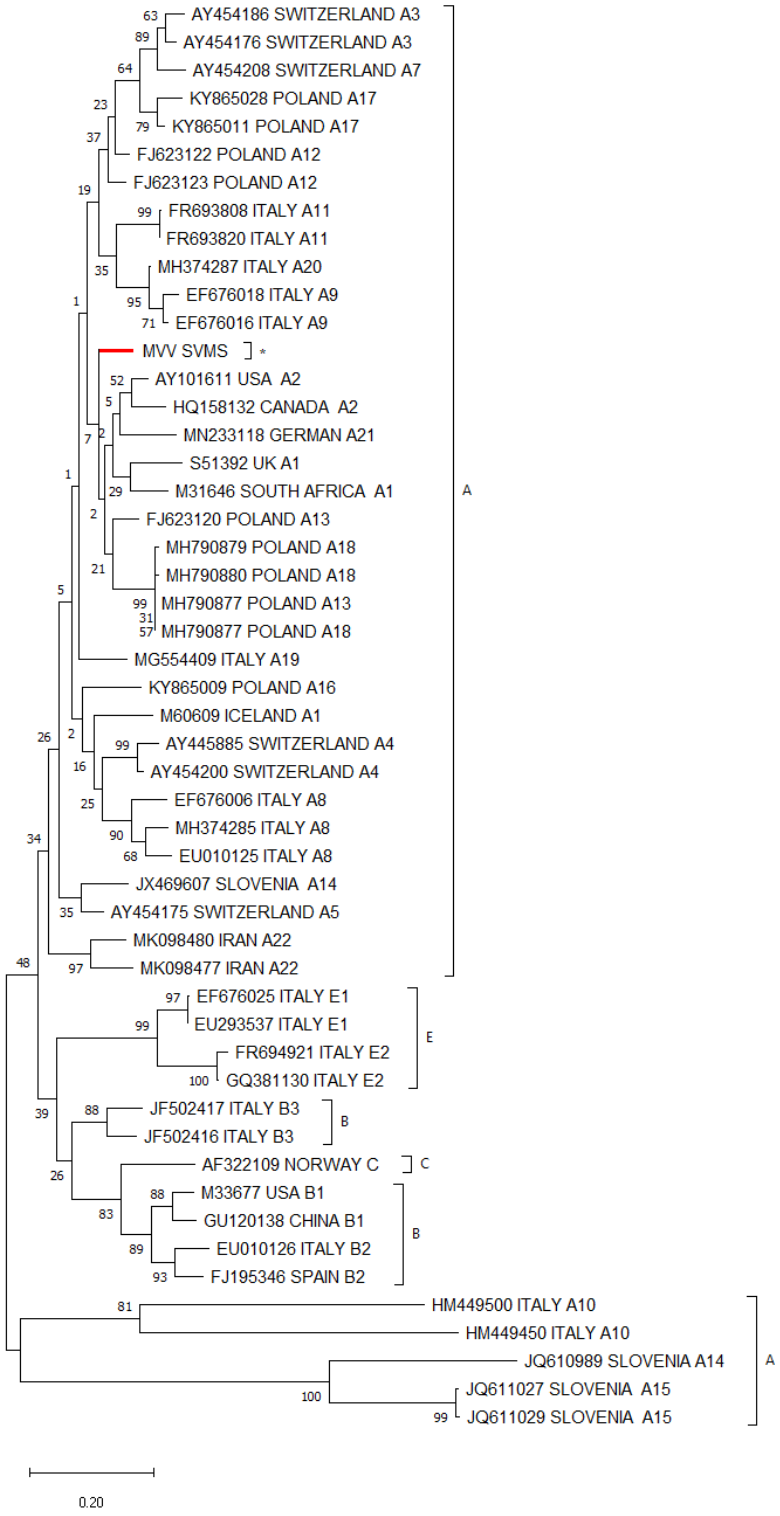

Figure 2. Phylogenetic tree of 251 bp spanning across SRLV* Gag *gene. Phylogenetic tree was generated using the Maximum Likelihood method and Tamura-Nei model and assessed by bootstrap method. Tree comprises unknown strain and 50 publicly available SRLV genomes listed in Appendix 1. Analysis was carried out using MEGA X.

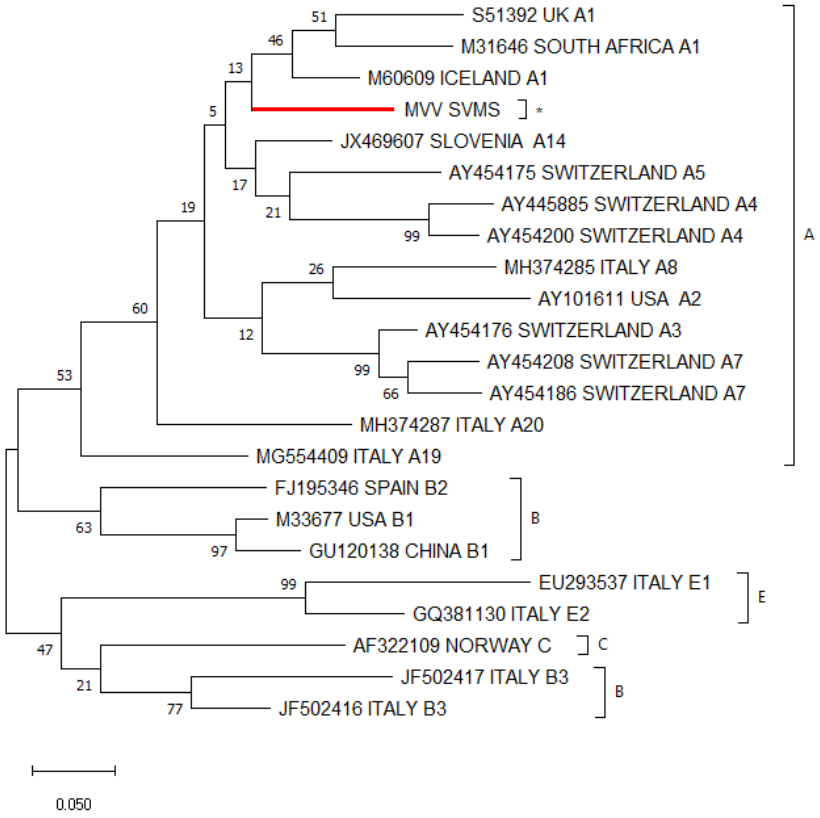

Figure 3. Phylogenetic tree of 325 bp spanning across SRLV* Pol *gene. Phylogenetic tree was generated using the Maximum Likelihood method and Tamura-Nei model and assessed by bootstrap method. Tree comprises unknown strain and 22 publicly available SRLV genomes listed in Appendix 1. Analysis was carried out using MEGA X.

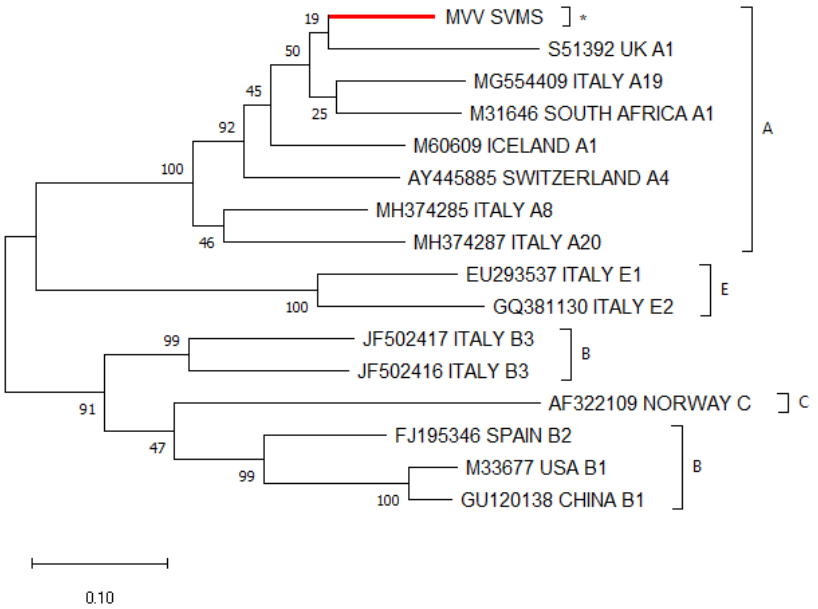

Figure 4. Phylogenetic tree of 859 bp spanning across SRLV* Env *gene. Phylogenetic tree was generated using the Maximum Likelihood method and Tamura-Nei model and assessed by bootstrap method. Tree comprises unknown strain and 16 publicly available SRLV genomes listed in Appendix 1. Analysis was carried out using MEGA X.

Table 4. PCR primers designed from sequences acquired through 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	

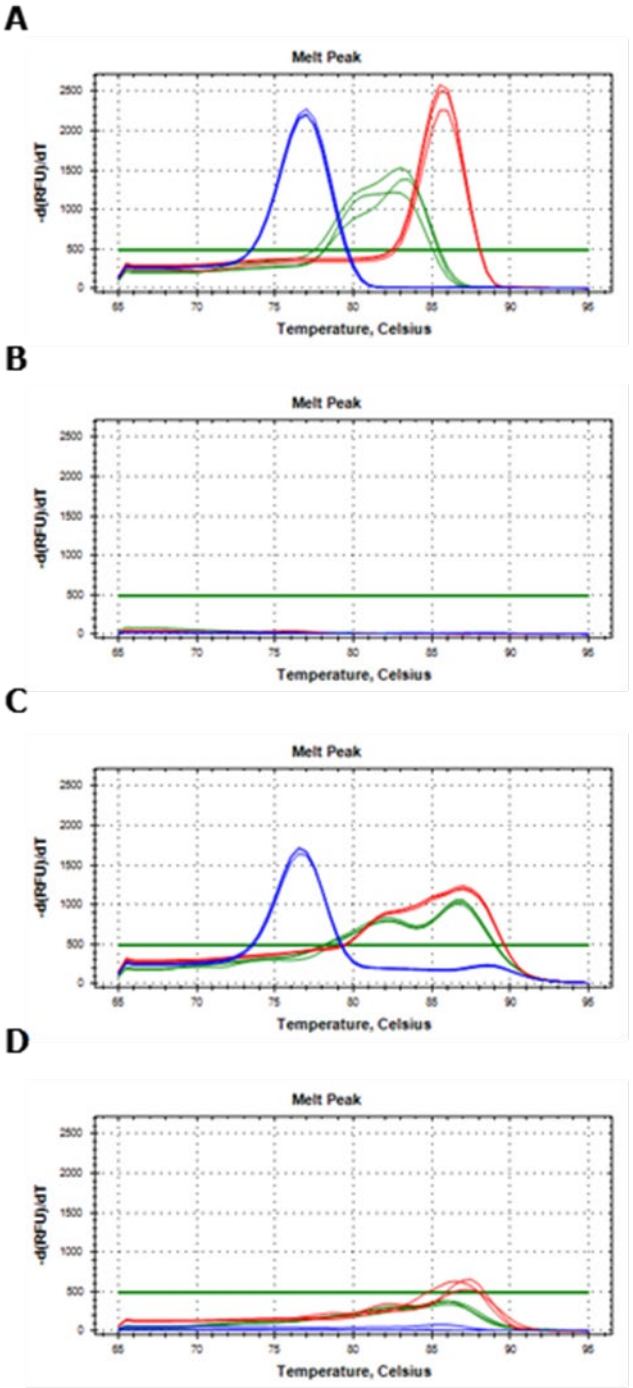

Figure 5. Melt Curves for testing of qPCR primers. 3 primer sets were designed targeting the 3 structural genes of SRLVs: *Gag* (green), *Pol* (blue) and *Env* (red). Primers were tested against synthetic oligo positive control (a), negative water control (b) and DNA extracted from the blood of seropositive rams (c) and seronegative ewes (d).

4.2. Artificial Insemination Trial

To assess the risk of sexual transmission of MVV from seropositive rams to naïve ewes in a natural mating setting, an artificial insemination trial was carried out substituting natural mating for intravaginal insemination. Semen was successfully collected from 11 of 13 rams. DNA and RNA tested by qPCR for the presence of MVV tested negative in all semen samples tested however RNA extracted from epididymal washes obtained at day of slaughter (the day after semen collection) tested positive in 6 of 13 rams (Table 5).

Table 5. 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**	**-**

No ewe blood samples from the inseminated group tested positive for MVV at any stage (serology, RNA or DNA from blood samples or tissue samples at post mortem). A single blood sample from a single control ewe demonstrated a positive serological test at one time point (Week 6). As subsequent samples tested negative for this ewe this was assumed to be a false positive (Figure 6). Histopathology from the majority of affected rams demonstrated typical MV pathology in the lungs whereas that from the ewes did not (Figures 7 + 8).

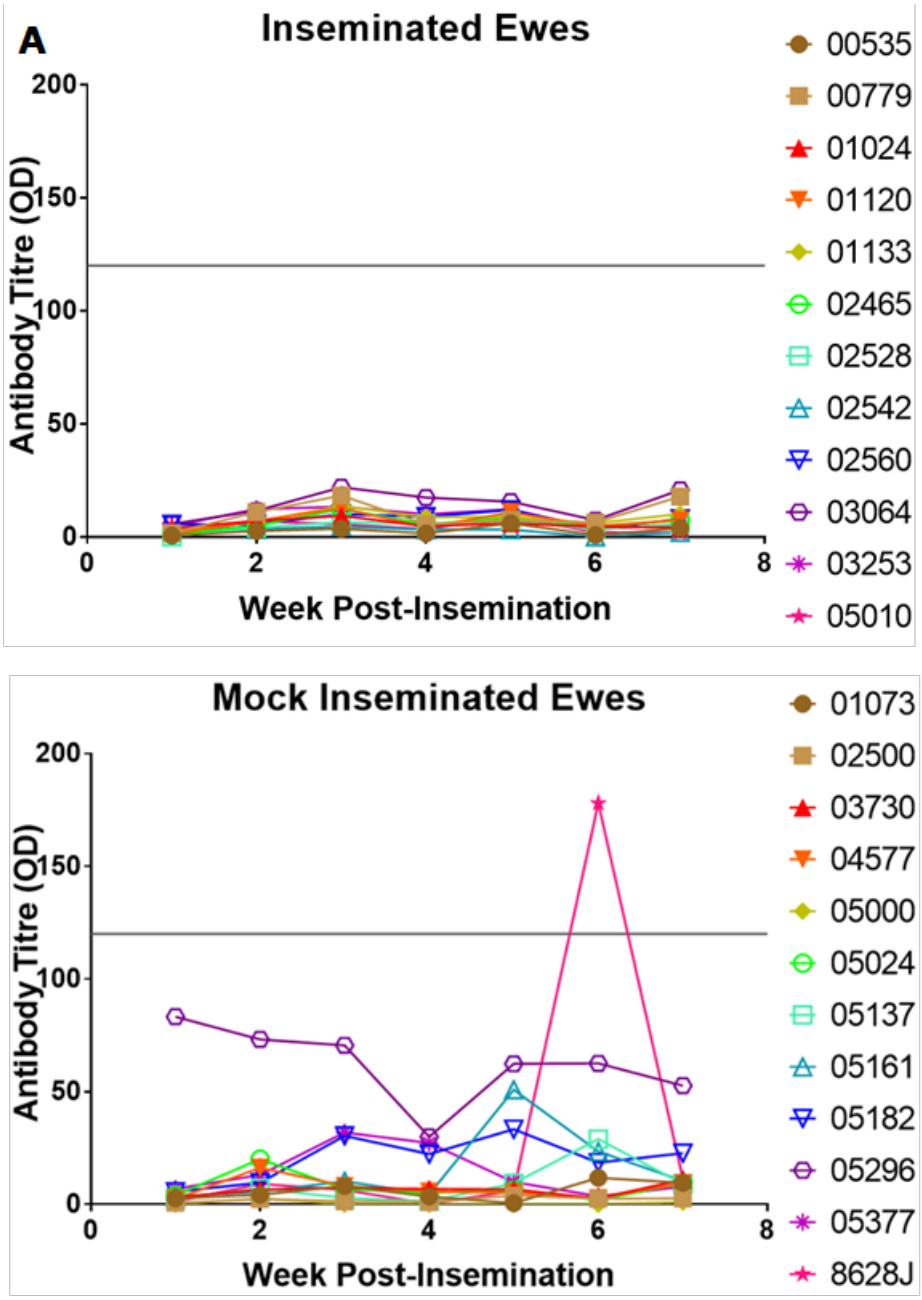

Figure 6. Maedi visna virus serum antibody titres determined by ELISA in ewes. Antibody titres for 12 ewes inseminated (a) with semen from naturally infected rams or (b) 12 mock inseminated ewes over 7 weeks post insemination obtained by ELISA of serum.

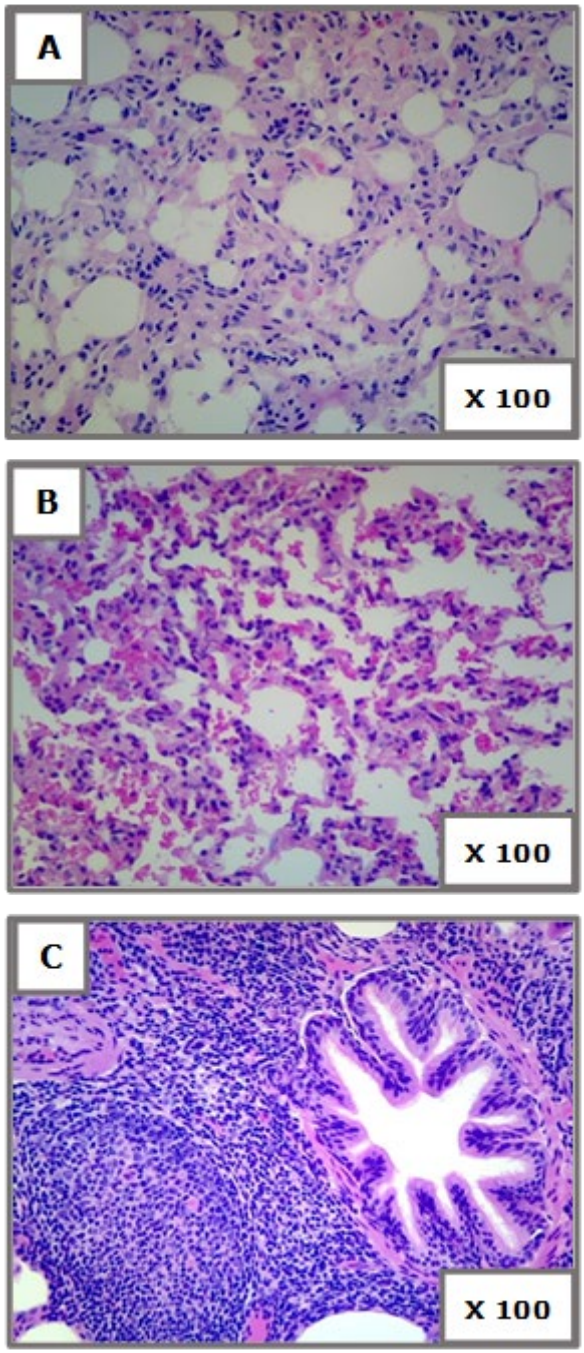

Figure 7. Histology of MVV seropositive rams indicative of infection. Histology observed with lung tissue of seropositive rams known to be associated with infection: (A) thickening of alveolar septa, (B) obliteration of alveolar structures and (C) lymphoid infiltration with occurrence of formation of lymphoid-like follicle.

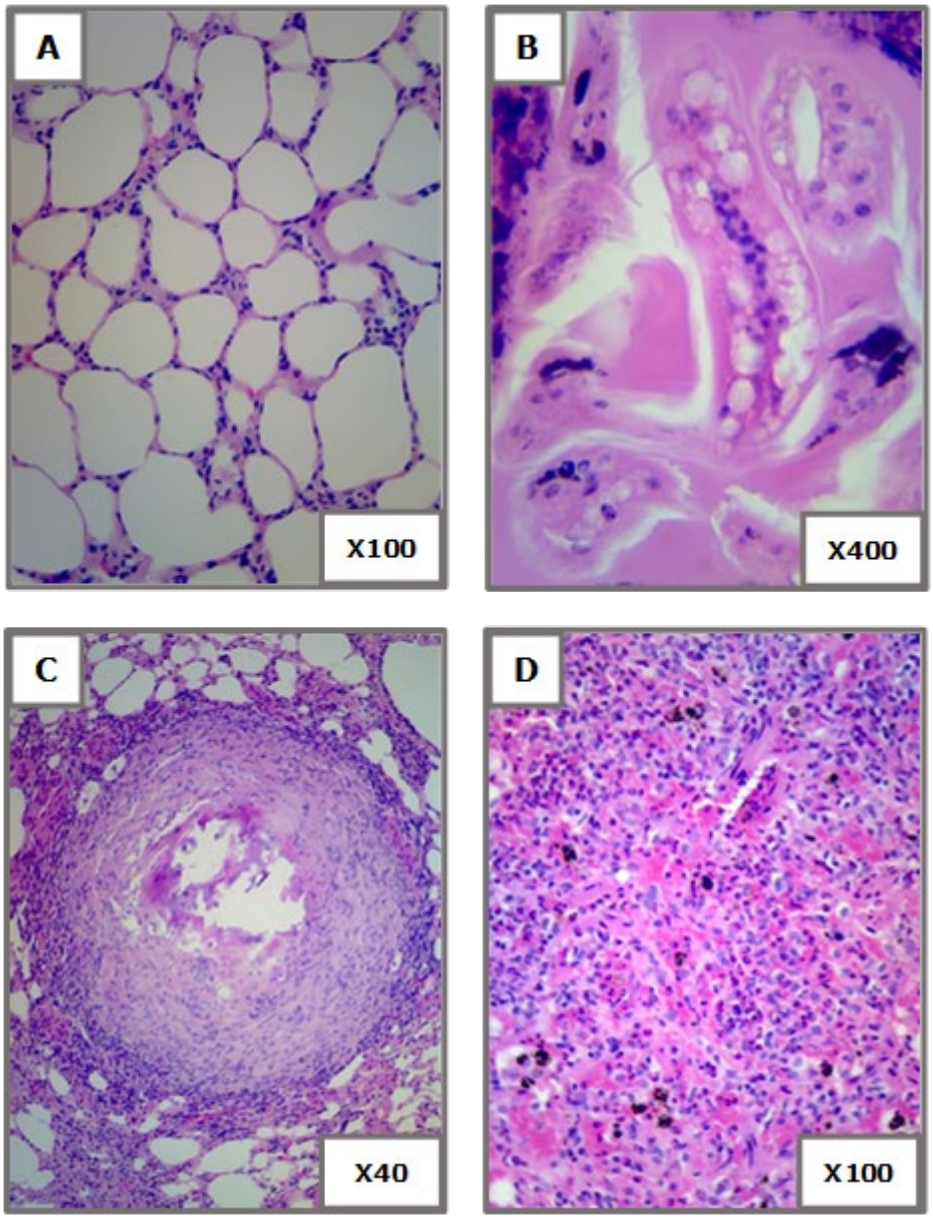

Figure 8. Lung histology of 8 trial ewes. Histology observed in lungs of ewes mock inseminated (n=4) and inseminated (n=4) with semen collected from MVV seropositive rams 7 weeks prior to euthanasia. (A) healthy lung, (B) presence of lung worms, (C) region of scarring and (D) areas of bleeding.

4.3. Longitudinal Study

A longitudinal case study of morbidity and mortality due to MV was carried out in a group of 27 seropositive rams to investigate the impact of MVV on infected hosts. RNA, DNA and ELISA results from testing of blood samples varied over time and were not always concordant with each other (Table 6). These results were found to not display any consistent trend over the three time points. Graphs of these results for individuals alive at all three time points are shown for 10 rams (Figure 9).

Ram 27 (02227) (of all rams tested) was the only ram to demonstrate completely negative results for all three blood tests (ELISA, DNA, RNA) at any time point (Table 6). This occurred on its last testing point in December 2015 and indicates that this ram cleared viral infection (at least to below detectable limits) over the course of this study. Lung and mediastinal lymph samples collected at post mortem (December 2015) also tested negative on RNA and DNA qPCR for this animal.

To identify potential presence of MVV resistant genotypes of TMEM154, genotyping was carried out on DNA extracted from seropositive rams (Table 7). 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.

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 8). 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 an unknown additional product during qPCR testing.

Table 6 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

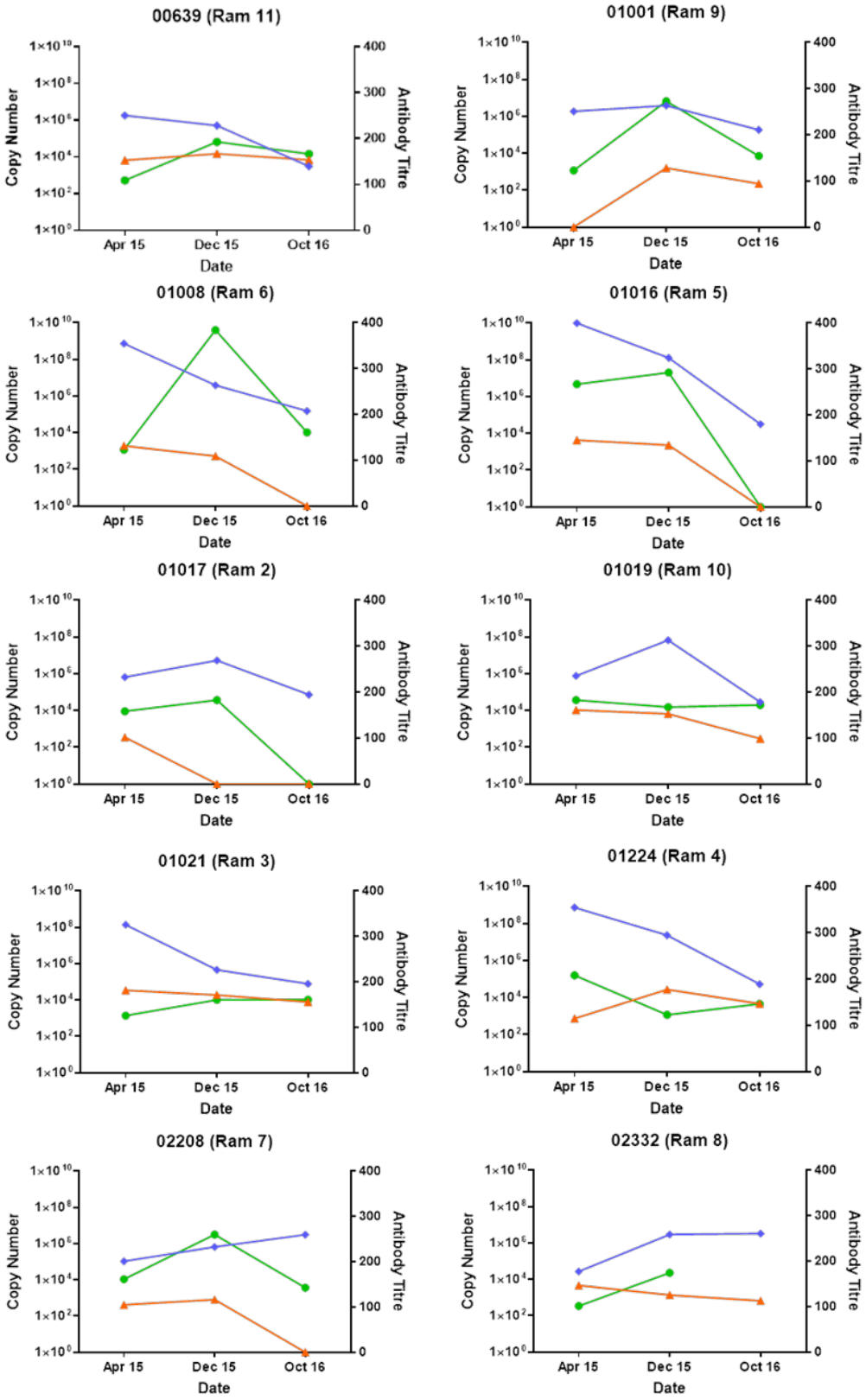

Figure 9. Graphs of MVV antibody titres and copy numbers in RNA of DNA of ten rams. Antibody titres (blue) and copy numbers for DNA (orange: copies per ng of DNA) and RNA (green: copies per µl of sera) determined by ELISA and qPCR, respectively for 3 time points (April 2015, December 2015 and October 2016).

Table 7. 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

Table 8. 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

4.4. Regression Modelling of MVV Impact

To estimate the impact of MVV infection on total milk yield and SCC in dairy ewes in the UK, 2 regression models were constructed using milking data collected from 319 dairy ewes. 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. Parameters estimates and their standard errors are listed in Table 9.

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 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 10). Unpaired t test calculated reduction caused by SRLV infection to be significant ($p<0.005$). Therefore, infection with MVV was predicted to cause a 6.60% reduction in milk yield in dairy ewes.

Table 9. 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

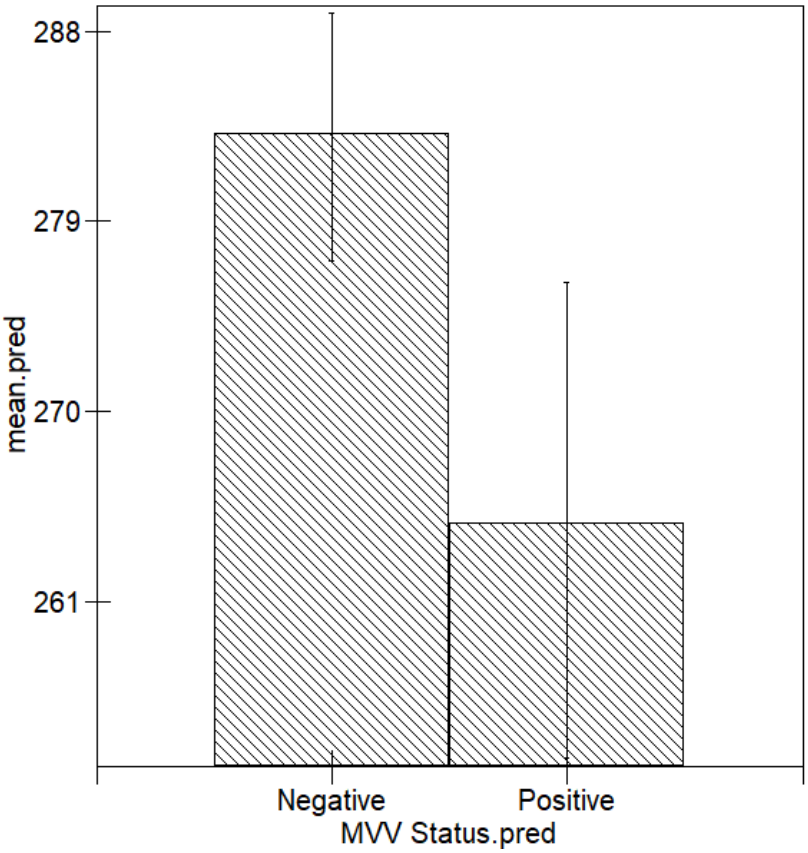

Figure 10. Mean predicted total milk yield in MVV seropositive and seronegative ewes. Predictions of total milk yield (L) in a lactation period based on regression model construction from data collected from 319 dairy ewes. Unpaired t test carried out calculated a p value of 0.0027.

The model constructed for impact of MVV on SCC used data 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 normal distribution indicative of good model fit. Parmeter estimates and their standard errors are listed in Table 10.

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} and 5^{th} lactation periods. 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 11). The model predicted a drop in SCC of 50.93% in animals infected with MVV although unpaired t test calculated this to be non-significant.

Table 10. 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

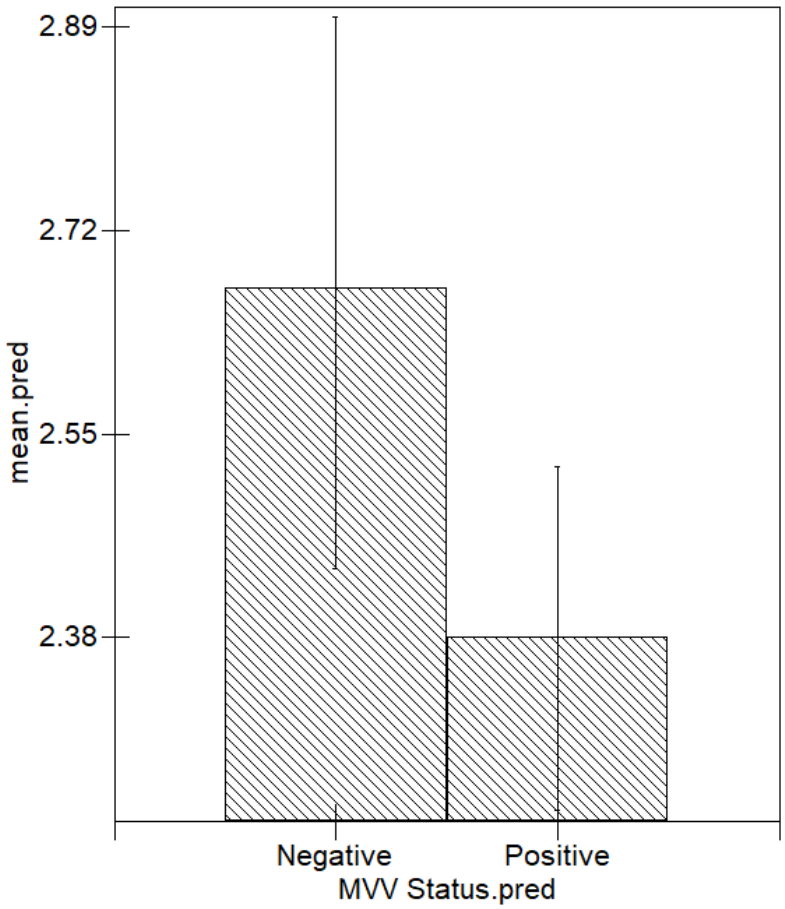

Figure 11. Mean predicted SCC in MVV seropositive and seronegative ewes. Predictions of SCC (Log_{10} 1000 cells/ml) in a lactation period based on regression model construction from data collected from 188 dairy ewes. Unpaired t test carried out calculated a p value of 0.5668.

5. Discussion

MV presents several unique challenges in terms of controlling the viruses spread and impact. One of the primary issues with developing PCR or qPCR based diagnostics is the extreme variability of sequence the virus displays (Pisoni et al. 2007; Ramírez et al. 2013). This was a complicating factor in this project where multiple published PCR protocols for European strains of MV failed to detect the virus strain present in the rams used in this study (despite them being clearly seropositive and displaying typical pathology). Resolving the sequence of the virus strain and developing the qPCR protocols used in this study required the use of deep sequencing technology, not yet a practical or affordable option for production animal diagnostics. The sequences retrieved showed that while the virus present was part of the “A” lineage of SRLV and its env sequence was similar to that of the EV-1 strain detected in the UK 30 years ago the gag and pol sequences were sufficiently different from that strain to no longer be detectable with a PCR developed against it (Shah et al. 2004).

Once a diagnostic qPCR had been established it was used to examine virus loads in a variety of tissues and sample sites. Interestingly viral RNA was detected at a relatively high viral load in nasal swabs collected post mortem into RNA later in taken from the affected rams. This was an unexpected finding as nasal swab detection had not been reported for MV previously and investigations into the beta retrovirus of sheep JSRV had not shown reliably detection in nasal swabs. This sampling site however presents an attractive alternative to the current blood based serology tests for MV that are the mainstay of control programmes, primarily as it is a less invasive sample to collect and would be feasible for farmers to collect themselves rather than requiring a veterinary surgeon to collect blood samples. Not having to pay the veterinary fees for a call out and the time taken blood sampling would considerably lower the cost of MV testing. Nasal swab testing therefore bares further exploration as a diagnostic method for MV. There are however a number of questions that would need to be resolved before routine adoption could be recommended, including whether the test demonstrates positivity before the serology tests (which would be a significant advantage in terms of disease control), how concordant serology and PCR based testing is (which from the results of the blood and tissue sampling would indicate is not always the case) and which strains of virus are currently circulating in the UK (outside of the animals used in this study) and whether a PCR based test can reliably detect them all. All of this would require substantial further work to perform concurrent blood and nasal swab testing from flocks undergoing longitudinal monitoring (likely as part of a control programme) for MV infection.

The results of the artificial insemination trial indicate that transmission of the virus by sexual transmission from rams to ewes via natural mating probably does not present a major

transmission route for the virus. Infected rams may of course still spread the virus to ewes via the respiratory route, particularly if they are housed indoors together (Leginagoikoa et al. 2010; Illius et al. 2020). The results of this trial indicate that sexual transmission is possible, as virus was detected in sperm washed from the epididymis of MV positive rams, albeit at a low level and other studies have shown that rams can excrete virus intermittently in their semen and that intra-uterine insemination with infected semen can transmit infection to niave ewes (Ali Al Ahmad et al. 2008; Peterson et al. 2008). However, the lack of transmission in this study would indicate that the risk of onward transmission from seropositive rams with negative viral qPCR semen tests is low and that genetic rescue via semen collection and artificial insemination from valuable rams could be attempted if necessary.

The milk yield monitoring work from this study confirms other work indicating that MV infection is associated with significantly reduced milk production in affected ewes with a 6.60% lower milk yield evident (Echeverría et al. 2020; Juste et al. 2020). This study was conducted in a diary flock where reduced milk yield has a direct economic impact on the flocks production, however it could be reasonably expected that a lowered milk production in a flock maintained for lamb production would also result in reduced lamb growth rates, survival and production.

The final important observation in this study was a seropositive ram homozygous for the TMEM154 allelle, thought to convey genetic resistance to MV, apparently cleared the infection during the 16 months the animal was monitored. There is a mounting body of evidence that animals that are homozygous for this allelle are less likely to become infected and less likely to show clinical disease if affected (Heaton et al. 2012; Alshanbari et al. 2014). This study however is the first report (that we are aware of) demonstrating a clearly virus positive animal clearing viral infection over time. This piece of data adds to the body of work demonstrating that this allelle conveys resistance to the virus and that selective breeding for this allelle should be considered as part of the control strategies for MV in the future.

6. Industry messages

There are several clear outcomes to this work that are important to the sheep farming industry

1. The risk of sexual transmission from seropositive rams to ewes is low.
2. MV is detectable in nasal swabs from seropositive animals, this requires further investigation into when animals become positive compared with the existing serological tests and whether a qPCR based test for all currently circulating strains of MV can be developed but is promising for the development of a test that does not require blood or tissue sampling
3. Animals genetically resistant to MV infection (with two copies of the resistant allelle of the TMEM154 gene) can clear MV infection to below detectable levels.
4. MV produces a 6.60% loss of milk production in affected ewes.

7. References

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