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ESTUDIO DE LA PATOGENIA DEL MAEDI-VISNA OVINO Y SU APLICACIÓN EN EL DIAGNÓSTICO Y CONTROL DE LA ENFERMEDAD

PhD THESIS

STUDY OF OVINE MAEDI-VISNA PATHOGENIA AND ITS APPLICATION IN DIAGNOSTIC AND ILLNESS CONTROL

Memoria que presenta para optar al grado de Doctor

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León, julio de 2018

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Memoria presentada por Elena Gayo Roces para optar al grado de Doctor, dentro del Programa de Doctorado ''Ciencias Veterinarias y de los Alimentos'', dirigida por el Dr. Juan Francisco García Marín y la Dra. Silvia Preziuso y tutorizada por el Dr. Juan Francisco García Marín.

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''HIGIA PECORIS, SALUS POPULI''

"LA HIGIENE DEL GANADO, LA SALUD DEL PUEBLO"

Escudo de la Profesión Veterinaria en España

“QUIERO COMPARTIR CON USTEDES EL SECRETO QUE ME HA LLEVADO A ALCANZAR TODAS MIS METAS: MI FUERZA RESIDE ÚNICAMENTE EN MI TENACIDAD”.

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A mi familia

A los que han contribuido a hacer esta tesis doctoral una realidad

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ÍNDICE GENERAL/INDEX

I. Abreviaturas

ADN/DNA: Ácido desoxirribonucleico/ Deoxyribonucleic Acid

APO: Adenomatosis pulmonar ovina

APOBEC3: *Apolipoprotein B editing enzyme catalytic polypeptide-like 3*

ARN/RNA: Ácido ribonucleico/ Ribonucleic Acid

CA: Cápside

CAEV: *Caprine arthritis encephalitis virus,* virus de la artritis y encefalitis caprina

CCR: C-C Motif Chemokine Receptor, Receptor de quimiocinas C-C

DPPA: *Dipeptide-binding Protein*

Gp135: Glicoproteína 135

HIV: *Human immunodeficiency virus*, virus de la inmunodeficiencia humana

HPL: *High Proviral Load*

ICQ/ICC: Inmunocitoquímica/ Immunocitochemistry

IDGA: Inmunodifusión en gel de agar

IFN: interferon

Ig: Immunoglobulin, inmunoglobulina

IHQ/IHC: Inmunohistoquímica/ Immunohistochemistry

IL: interleucina

KIR: *killer Immunoglobulin-like Receptor*

LAMP: *Loop-mediated Isothermal Amplification*

LPL: *Low Proviral Load*

LTR: *Long terminal repit*

LVPR/SRLV: Lentivirus de los pequeños rumiantes/ *Small Ruminant Lentiviruses*.

MA: matriz proteica

MHC: *Major Histocompatibility Complex*, complejo mayor de histocompatibilidad

MV: Maedi-Visna

NK: Células *Natural Killer*

NP: nucleoproteína

OIE: Organización Mundial de Sanidad Animal

PCR: *polymerase chain reaction,* reacción en cadena de la polimerasa

PND: *Principal Neutralization Domain*

RIA: Radioinmunoensayo

RIPA: Radioinmunoprecipitación

RT: Retrotranscriptasa Inversa.

Rt-PCR: *Real Time PCR*, PCR a tiempo real.

SIV: *Simian immunodeficiency virus*, virus de la inmunodeficiencia de los simios

SNC/CNS: Sistema nervioso central/ *Central nervous system*

SU: proteína de superficie

Th: Linfocitos T *Helper*

TLR: *Toll-like receptors*

TM: glicoproteína transmembrana

TMEM: Transmembrane Protein, proteína transmembrana.

TNF: *Tumoral Necrosis Factor*, Factor de necrosis tumoral

TRIM: *Tripartite Motif-containing Protein*

VMV: Visna/maedi virus

WB: *Western Blot*

II. Introducción y objetivos

La enfermedad del Maedi-Visna (MV) ovino está producida por la infección por un retrovirus, lentivirus de los pequeños rumiantes (LVPR), y está ampliamente extendida entre la ganadería ovina a nivel mundial desde hace décadas (Lujan *et al.*, 1993; Luján, 2001). Afecta al ovino adulto y conduce a un síndrome multisistémico caracterizado por una pérdida progresiva de peso y una inflamación intersticial crónica no purulenta del pulmón, glándula mamaria, articulaciones y tejido nervioso (Dawson, 1987). En España tiene una gran importancia, especialmente en la comunidad de Castilla y León, donde es habitual encontrar seroprevalencias superiores al 80%, principalmente en rebaños de producción intensiva de leche (Sotelo 1998; Leginagoikoa *et al.*, 2006). Las formas respiratoria y mamaria son, en general, las de presentación más frecuente, aunque en ovino de producción lechera de Castilla y León se han diagnosticado numerosos casos de la forma nerviosa (Gómez *et al.*, 1999; Benavides *et al.*, 2006c; Benavides *et al.*, 2007b). La intensificación de la producción lechera y la selección genética han dado lugar a un aumento de la prevalencia de algunas enfermedades, especialmente de origen infeccioso, entre las que se encuentra el MV ovino, y que supone pérdidas económicas importantes (Peterhans *et al.*, 2004; Benavides *et al.*, 2013).

Actualmente no hay vacunas eficaces disponibles frente al MV y la mayoría de los métodos de control se basan en la detección precoz de ovinos infectados para limitar la diseminación del virus o incluso su erradicación (Polledo *et al.*, 2013). El test ELISA se ha descrito como el método más eficaz para el diagnóstico precoz de los animales infectados (de Andrés *et al.*, 2005; Minguijón *et al.*, 2015), aunque se conoce que no todos los animales infectados son positivos al mismo, como ocurre en las infecciones recientes, en diferentes cepas víricas o en la utilización de test que utilizan diferente antígenos víricos (de Andrés *et al.*, 2005; Glaria *et al.*, 2012; de Andrés *et al.*, 2013). En estudios llevados a cabo en casos clínicos de la forma nerviosa sea podido comprobar que cada individuo presenta un patrón de lesión diferente, principalmente *histiocítico* o *linfocítico*, que representan distintos estadios inmunopatológicos (Polledo *et al.*, 2012b) y que podrían condicionar la positividad en test serológicos o la

presencia de virus en los tejidos. El *tipo linfocítico* se caracterizada por la presencia de abundantes linfocitos T con predominio del CD8+ y con escasa presencia vírica en el tejido y el *tipo histiocítico* por lesiones más graves y extensas áreas de malacea con predominio de macrófagos y numerosos linfocitos B y con abundante antígeno vírico en la lesión. Estos patrones podrían estar relacionados con la respuesta inmune individual de cada animal (Polledo *et al.*, 2012b) tal y como también se ha descrito en otras infecciones por lentivirus como virus de la inmunodeficiencia humana (HIV) y de los simios (SIV) (Kim *et al.*, 2004; Freel *et al.*, 2011).

Como primer **objetivo, desarrollado en el primer y segundo trabajo** de esta tesis doctoral, se plantea hacer un estudio detallado de los patrones inmunopatológicos del MV en ovinos naturalmente infectados en los tres principales órganos diana del mismo animal, estableciendo un modelo en cada individuo, además de estudiar cómo influyen estos patrones en el desarrollo de la enfermedad y en la respuesta serológica. Para ello, se llevará a cabo una valoración conjunta de los tipos de lesión e intensidad de la misma en SNC, pulmón y mama de cada animal en una selección sistemática de muestras. Asimismo, se comprobará y evaluará la relación entre la respuesta serológica (ELITEST®) y el tipo (linfocítica/histiocítica) e intensidad y extensión de la lesión (mínima, moderada o grave). Los resultados obtenidos se aplicarán en el diagnóstico de la enfermedad y se estudiará su posible aplicación en los programas de control. Este objetivo permitiría, entre otros logros, conocer por qué los animales presentan diferentes niveles de respuestas en test serológicos, aunque la gravedad de la lesión sea similar.

El segundo objetivo, llevado a cabo en el tercer trabajo de esta tesis, se centra en la mama y plantea comprobar qué células participan en la infección inicial de la glándula mamaria y en la progresión de la misma en animales naturalmente infectados, así como en el papel que tiene la mama y leche en la transmisión de la infección. Para ello se estudiarán las células que participan en la infección en las lesiones de mínimas a moderadas o graves,

prestando especial atención a los macrófagos, las células epiteliales acinares y a los espacios perivasculares y valorando el papel de las células del sistema inmune en ambos casos y su relación con la patogenia de la lesión mamaria. La identificación de las células portadoras del VMV en tejido mamario y en leche y la evaluación de la relación entre positividad en leche y el tipo e intensidad de la lesión en la mama permitirá ampliar el conocimiento sobre la patogenia de la enfermedad y sobre la posibilidad de transmisión del virus por vía lactógena.

Un **tercer objetivo**, también llevado a cabo en el tercer trabajo, es determinar la relación entre las diferentes técnicas empleadas en el diagnóstico del MV, tanto en el animal vivo como postmortem, valorando su especificidad y sensibilidad, y establecer si es posible una técnica "gold standard" de referencia.

La técnica de PCR plantea numerosos problemas para la detección de VM, presentando habitualmente numerosos falsos negativos en animales con infección conocida. Por ello se plantea un cuarto objetivo encaminado a resolver este problema mediante el estudio de las cepas de MV circulantes en la región de Castilla y León y su detección mediante técnicas de PCR, así como a la obtención de un árbol filogenético con las cepas de distintos ovinos naturalmente infectados. Ello tiene gran interés por la posibilidad de aplicar estos conocimientos de forma directa en el diagnóstico de la enfermedad mediante la elaboración de nuevos primers específicos para dichas cepas, incrementando la sensibilidad de la PCR, o bien desarrollar test ELISA más específicos frente a determinadas variedades genéticas.

Todo lo expuesto anteriormente hace que los estudios planteados en esta tesis presenten gran interés en el conocimiento de la patología del MV en todas sus formas y su relación con la evolución del curso de la infección, dirigido todo ello al diagnóstico eficaz y específico del mayor número de animales infectados tanto en el animal vivo como post-mortem. Así mismo, podrían constituir una herramienta eficaz para el diagnóstico precoz y para conocer el porqué de los falsos negativos, permitiendo su aplicación en planes de control

más rápidos, económicos y eficaces, que siempre están condicionados por el método de diagnóstico y por la evolución lenta de la infección, que a su vez muestra características diferentes en cada ovino infectado.

III. Revisión bibliográfica

3.1. RESEÑA HISTÓRICA Y SITUACIÓN ACTUAL

La primera descripción del maedi visna (MV) ovino se realizó en el año 1915 en Sudáfrica por Mitchell, que hablaba de una enfermedad pulmonar con lesiones que se correspondían a una neumonía catarral crónica con presencia de folículos linfoides, que asoció a la ya conocida adenomatosis pulmonar ovina (APO). Posteriormente, también en Sudáfrica, Kock observa en 1929 ovinos con una enfermedad similar a la descrita por Mitchell pero que no presenta las características de la APO, lo que le lleva a diferenciarlas como dos entidades diferentes: la ya conocida adenomatosis pulmonar ovina, y una nueva enfermedad caracterizada por neumonía crónica y presencia de folículos linfoides que coincide con la descrita con anterioridad (Dawson, 1980). En la misma época, Marsh (1923) describe en Estados Unidos una enfermedad con lesiones similares a las anteriores que llamó neumonía progresiva ovina. Fue Kock quien sugirió en 1929 que tanto él como Mitchell y Marsh se encontraban ante la misma enfermedad (Dawson, 1980).

El virus de la artritis encefalitis caprina, también perteneciente al grupo de los lentivirus de los pequeños rumiantes (LVPR), se descubre más tarde en Estados Unidos (Cork *et al*., 1974), aislándose por primera vez en 1980 (Crawford *et al.*, 1980). Esta enfermedad pronto se describe en otros países, con unos efectos que parecen ser incluso más graves que en la especie ovina (Minguijón *et al.*, 2015).

La enfermedad cobra entre los años 1939 y 1965 una marcada importancia al introducirse en Islandia a partir de la importación de veinte corderos de raza Karakul procedentes de Alemania (*Animal Breeding Department of the University of Halle*), en cuyo rebaño no se habían observado signos clínicos, y que se introdujeron con la idea de mejorar la producción ovina (Pálsson, 1990). Dichos corderos suponen además la importación de la adenomatosis pulmonar ovina y la paratuberculosis, tres enfermedades que se empezaron a

llamar ``enfermedades lentas´´ (Sigurdsson, 1954a; Sigurdsson, 1954b), y que causaron una importante epizootia con altas mortalidades en la cabaña ovina islandesa, considerándose la importación más nefasta en la historia de la ganadería ovina por muchos autores (Luján, 2001).

Durante esta epizootia se describen las lesiones de las formas pulmonar y nerviosa (Sigurdsson *et al.*, 1952; Sigurdsson, 1954a) y se consigue aislar e identificar por primera vez el agente causal. Así, se aísla en 1957 el virus en cerebro de ovinos con síntomas nerviosos y en pulmón de animales con síntomas respiratorios durante los siguientes años (Sigurdardottir and Thormar, 1964; Thormar, 2013). En 1965 se erradica el MV en Islandia, siendo junto con Nueva Zelanda y Australia los únicos países libres de infección, con la diferencia que en los dos últimos no ha sido erradicada sino que nunca ha sido introducida (Dawson, 1980; Reina *et al.*, 2009a). En honor a los importantes esfuerzos llevados a cabo por los investigadores islandeses en la erradicación, se denominó a la enfermedad con términos propios del país que reflejaban los principales síntomas de la misma en esa época: Maedi (disnea) y Visna (emaciación). Durante los años 80, la infección estaba distribuida por Francia e Italia (Russo *et al.*, 1980; Agrimi *et al.*, 1984; Caporale *et al.*, 1985), mientras que en Noruega y Dinamarca había un bajo grado de infección (Hoff-Jorgensen, 1985 ; Krosgrud, 1985). Por lo tanto, durante la década de los 90 había marcadas diferencias respecto a la epidemiología en los distintos países europeos: en Islandia, Noruega, Suecia e Irlanda la enfermedad había sido prácticamente eliminada o erradicada, mientras que en otros países como Dinamarca, España, Holanda, Reino Unido, Francia o Bélgica los niveles de seropositividad en los rebaños eran elevados (Peterhans *et al.*, 2004).

En España se llevaron a cabo numerosas importaciones de ovinos, principalmente machos mejorantes, durante la década de los años 70, principalmente razas de centroeuropeas como la Fleischschaf, la Landschaf o la Berrichon du Cher, el Merino precoz francés, o la Suffolk inglesa (la Romanoff rusa o la Assaf israelita lo fueron de forma muy

localizada y limitada), siendo a principios de la década de los 80 cuando comienzan a diagnosticarse casos de MV en España. Sin embargo, en los trabajos realizados o dirigidos por el Profesor José María Santiago y Luque en la Facultad de Veterinaria de Zaragoza a finales de los años 50 y principios de los 60 no se observaron casos de esta enfermedad. Estos trabajos incluyeron el estudio pormenorizado de la patología respiratoria de miles de ovinos adultos localizados en Navarra, Aragón, Castellón y Valencia. En los mismos se diagnostican por primera vez en España la APO y varias formas desconocidas de parásitos pulmonares, no describiendo nunca lesiones que pudieran a asociarse a MV, a pesar de que el Profesor Luque conocía bien esta enfermedad, tal y como reflejado en la introducción de las Tesis Doctorales por él dirigidas (Pascual, 1961; Dualde, 1964). Es por ello que pensamos que el MV fue una enfermedad introducida en España a partir de la década de los años 70 del siglo pasado por alguna de las razas mencionadas, extendiéndose rápidamente entre un colectivo ovino autóctono que nunca había tenido contacto previo con la enfermedad, tal y como ocurrió por ejemplo en Islandia.

En la actualidad, la enfermedad del MV está ampliamente distribuida por la geografía mundial, siendo endémica en muchos países. Según datos de la Organización Mundial de Sanidad Animal (OIE), la enfermedad está presente en Estados Unidos y Canadá, así como en América del Sur, aunque los datos disponibles son escasos en este continente al igual que ocurre en Asia y África (Fig.1). A nivel de Europa, no se han detectado casos desde el año 2005 al 2013 únicamente en Croacia, Ucrania, República Checa, Irlanda e Islandia (www.oie.int).

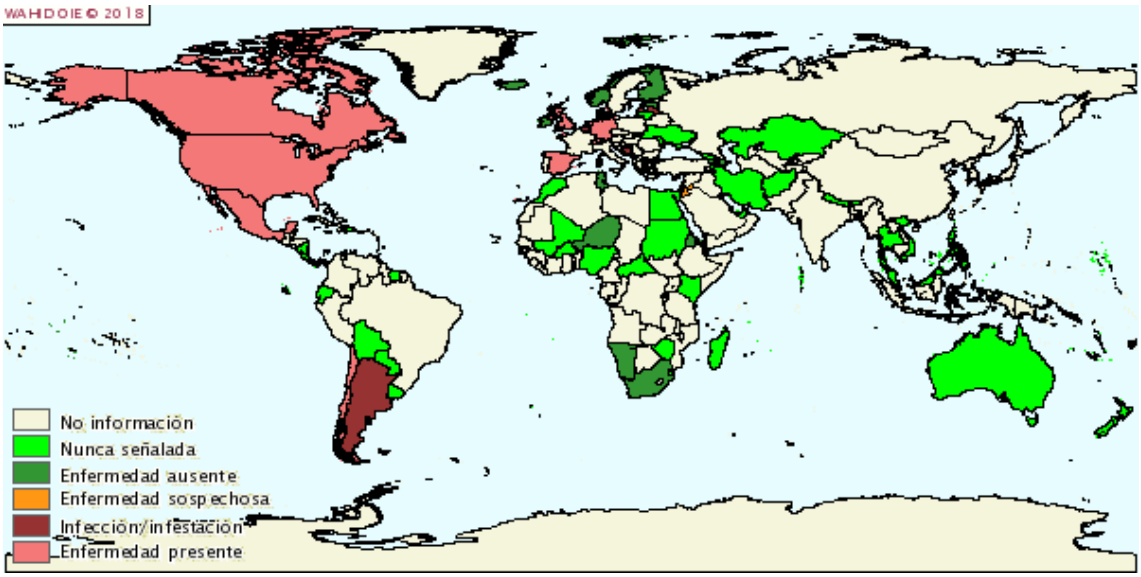

Fig.1: *Mapa de distribución mundial del MV según datos de la OIE recogidos en el primer trimestre de 2017 (www.oie.int).*

En España, la enfermedad se considera endémica aunque los datos de prevalencia varían en función del estudio y del área geográfica estudiada. Fue descrita por primera vez en la década de los 80 (Badiola *et al.*, 1983; González *et al.*, 1984; González *et al.*, 1985), centrándose los primeros estudios en la forma respiratoria de la enfermedad, aunque posteriormente se demostró así mismo la importancia de la forma mamaria con una elevada incidencia (Badiola y González., 1990; Luján *et al.*, 1991; Carey and Dalziel, 1993; Pekelder *et al.*, 1994; Zink and Johnson, 1994). En la década de los 90 se estimó la prevalencia tanto en País Vasco como en el Valle del Ebro entorno al 50% (Badiola y González., 1990), mientras que en otro estudio conjunto con Aragón, sur de Navarra y la Rioja se habla de prevalencias entre el 95-97,5% a nivel de rebaños afectados y entre el 38,6-44,7% a nivel de infección individual en animales adultos de más de dos años de edad (Lujan *et al.*, 1993). Posteriormente, en otro trabajo realizado en Aragón sobre 554 rebaños entre los años 2002 y 2007 se observó una prevalencia del 100% a nivel de rebaño y un 52,8% de prevalencia media (Pérez *et al.*, 2010). En el primer estudio realizado en Aragón se empleó la técnica de inmunodifusión en gel de

agar (IDGA) y en el segundo un test ELISA, que aumentaba la sensibilidad. No obstante, el incremento significativo con respecto al primer estudio ocurrió en la provincia de Huesca, asociado a un notable incremento de explotaciones en estabulación permanente. En Cataluña se estiman como infectados el 85-95% de los rebaños y el 58,3% de los ovinos en un estudio llevado a cabo en 210 rebaños con diferentes sistemas de manejo y número de animales (Alba *et al.*, 2008), mientras que en Galicia se observa un 52,6% de rebaños afectados y 24,8% de prevalencia individual en un estudio realizado en ovino de carne semi-intensivo (Lago *et al.*, 2012). En Asturias se estimó una seroprevalencia individual del 24% (Espí *et al.*, 2001), en Navarra del 89% en rebaños y 26% individual (Ameztoy *et al.*, 1998), en Murcia del 21,7% en rebaños y del 13,9% individual (León and Pats, 1996) o en Sevilla del 31% en rebaños (Artigas *et al.*, 1999).

En Castilla y León, se ha observado una seroprevalencia del 77% (Leginagoikoa *et al.*, 2006), estimándose que la prevalencia en el norte de España es del 80% si se consideran únicamente rebaños lecheros de raza Assaf Española, y llegando en particular en la provincia de León a prevalencias de 96,8% en rebaños de producción intensiva de la misma raza (Sotelo, 1998). En el mismo año Reviriego realizó un estudio epidemiológico en la provincia de Ávila, en el que se observó una prevalencia de rebaños del 30% e individual del 14,95% (Reviriego, 1998). Las presentaciones más frecuentes de la enfermedad en esta zona geográfica son la respiratoria, la mamaria, y especialmente la nerviosa (Gómez *et al.,* 1999; Benavides *et al.*, 2006c; Benavides *et al.*, 2007a; Benavides *et al.*, 2009; Benavides *et al.*, 2013; Polledo *et al.*, 2013). La enfermedad del MV ovino tiene por tanto una elevada importancia en nuestra región, suponiendo pérdidas económicas destacables, pudiendo considerarse como la enfermedad de origen infeccioso de mayor prevalencia en ganado ovino de producción lechera intensiva (Benavides *et al.*, 2013; Polledo *et al.*, 2013).

Todos estos estudios de prevalencia realizados desde principios de los años ochenta demuestran que la infección está ampliamente extendida y con una elevada prevalencia en los rebaños afectados, siendo, probablemente una de las enfermedades infecciosas más comunes en ganado ovino adulto en España.

3.2. ETIOLOGÍA

3.2.1 Clasificación

El virus Visna/maedi (VMV) forma parte de la familia *Retroviridae,* subfamilia *Orthoretrovirinae,* género *Lentivirus*, y subgénero *Lentivirus de los pequeños rumiantes* (LVPR) (Buitrago *et al.*, 2012) que engloba las distintas cepas de MV y de la Artritis Encefalitis Caprina (CAEV). Dentro del género *Lentivirus*, se encuentran otros virus como el HIV o el SIV con los que comparte características patológicas similares (Pétursson *et al.*, 1991; Thormar, 2005; Leroux *et al.*, 2010), resultando su estudio de gran interés.

Los LVPR son un grupo heterogéneo de virus que actualmente se clasifican filogenéticamente en cinco genotipos designados con las letras de la A a la E (Fig.2), que difieren en un 25-37% en las secuencias de nucleótidos (Reina *et al.*, 2006; Glaria *et al.*, 2012; Ramírez *et al.*, 2013; Minguijón *et al.*, 2015; Crespo *et al.*, 2016). Los estudios filogenéticos y aislados ovinos y caprinos realizados en las últimas décadas en distintas áreas geográficas demuestran la existencia de una transmisión vírica entre ambas especies e incluso a rumiantes salvajes (Shah *et al.*, 2004a; Shah *et al.*, 2004b; Pisoni *et al.*, 2007; Erhouma *et al.*, 2008; Glaria *et al.*, 2009; Olech *et al.*, 2012; Fras *et al.*, 2013; Kuhar *et al.*, 2013; Santry *et al.*, 2013). El genotipo A es el que ha sido más habitualmente identificado en los casos de MV, y se

subdivide a su vez en los subgrupos A1-A4, donde se englobarían las cepas respiratorias y nerviosas aisladas en el norte de España (Glaria *et al.*, 2012). El B incluye las formas del CAEV y se subdivide en B1, B2 y B3, aislándose el B2 en ovinos con lesiones artríticas estudiados en Aragón, aunque con una variación en el genoma a nivel del gen *pol*, de tipo maedi (Glaria *et al.*, 2009). En el grupo C se encuentran las cepas noruegas aisladas en caprino y rumiantes salvajes (Gjerset *et al.*, 2009) y el grupo D contiene secuencias de ovinos Suizos y Españoles caracterizados por particularidades en el gen *pol*, siendo este último el grupo el filogenéticamente más distante (Shah *et al.*, 2004a; Shah *et al.*, 2004b; Reina *et al.*, 2006). El genotipo E ha sido descrito en Italia y está confinado por el momento a esta área geográfica (Gjerset *et al.*, 2009).

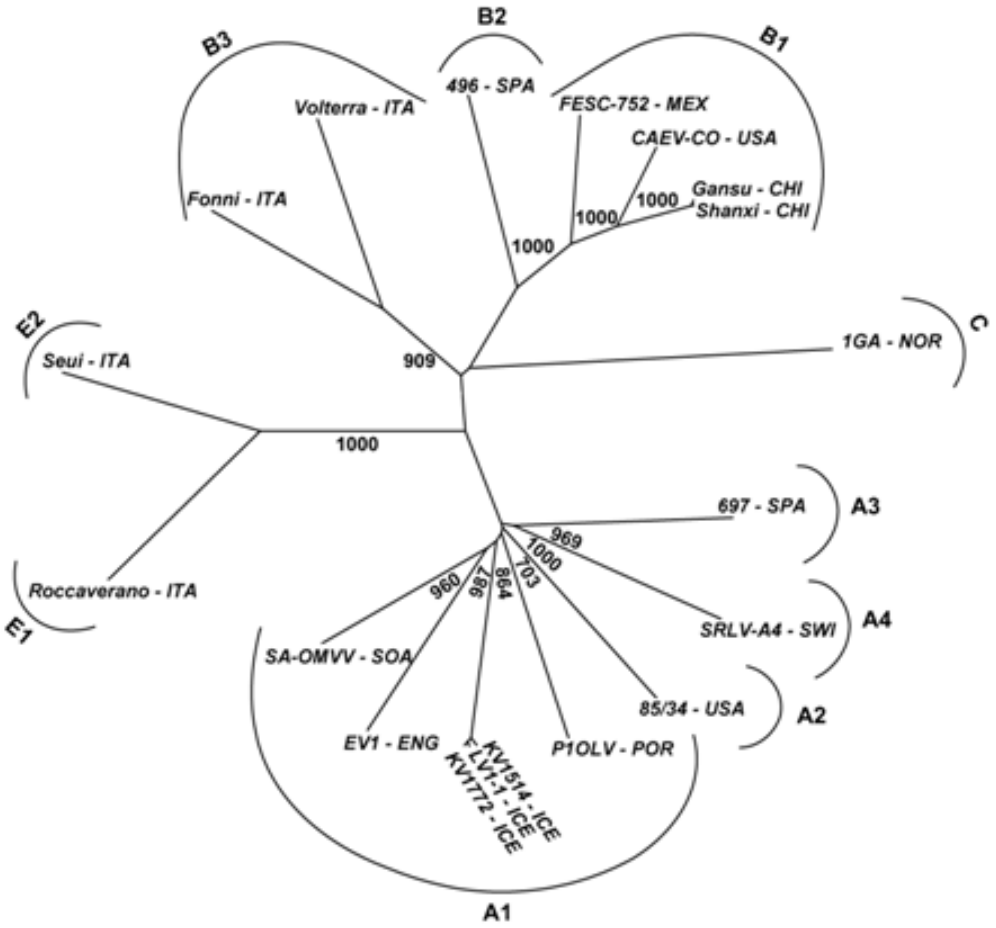

Fig. 2. *Árbol filogenético que comprende las secuencias de LVPR del GenBank (Ramírez et al., 2013).*

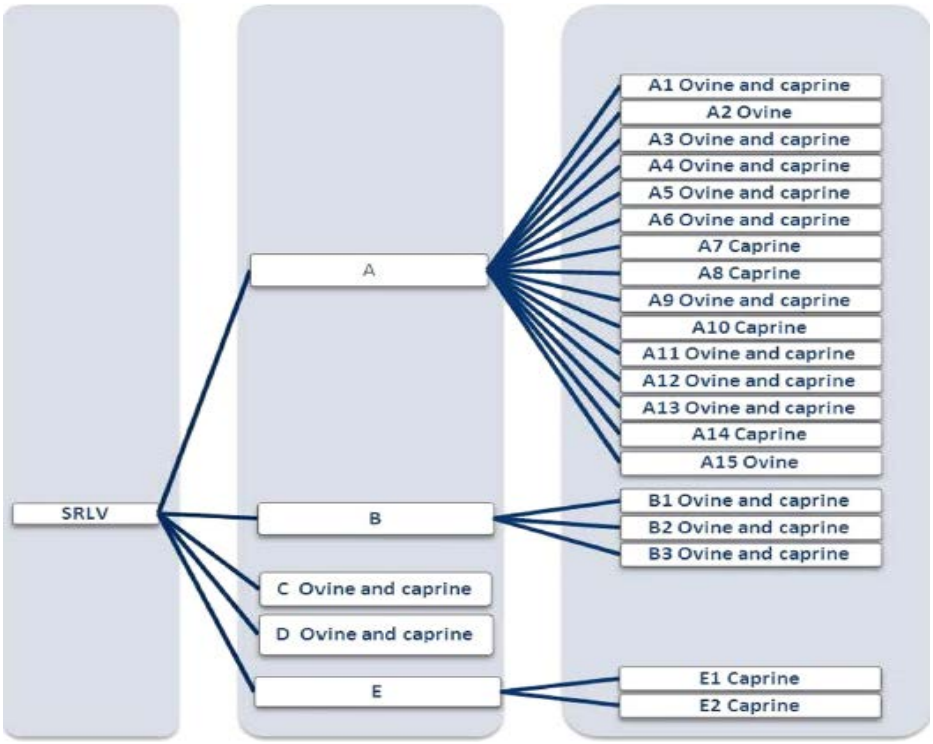

Fig. 3: *Tipos y subtipos de LVPR detectados en ovinos y caprinos (Minguijón et al., 2015).*

3.2.2 Estructura vírica

El VMV se trata de un virus ARN cuyos viriones son partículas esféricas de 80-100 nanómetros de diámetro y que presentan una estructura típica en la que se diferencian tres capas: un complejo nucleoprotéico central de geometría helicoidal donde se localiza el genoma vírico (8.4–9.2 kb) constituido por dos cadenas monocatenarias de ARN de sentido positivo, y numerosas copias de la retrotranscriptasa inversa junto con otras enzimas como la proteasa o la integrasa; una cápside proteica de forma icosaédrica de 60 nm de diámetro que posee la proteína immunogénica p25; y una envoltura externa que se trata de una bicapa lipídica derivada de la célula huésped y de la inserción de glicoproteínas del virus, compuesta principalmente por dos subunidades, gp135 de superficie (SU) o gp46 (TM). Entre las dos últimas capas descritas se halla una matriz proteica que favorece la unión de las mismas (Fig.4)

(Pétursson and Georgsson, 1992; Coffin *et al.*, 1995; Murphy *et al.*, 1999). Los lentivirus están compuestos aproximadamente por un 60% de proteína, 35% de lípidos, 3% de carbohidratos y 1% de ácidos nucleicos (ARN) (Pétursson and Georgsson, 1992). Los viriones son relativamente poco resistentes a agentes químicos y bastante a las radiaciones ultravioleta, la congelación, la sonicación y a la desoxirribonucleasa (Thormar, 1961; Thormar, 1965). El VMV tiene la capacidad como el resto de retrovirus de utilizar un intermediario de ADN para dirigir la síntesis de sus proteínas desde su ARN genómico a través de la enzima transcriptasa inversa (Coffin, 1996). Los lentivirus en particular, contienen de 3 a 6 genes accesorios y exclusivos de este grupo que los hace más complejos que otros retrovirus (Clements and Gabuzda, 1989).

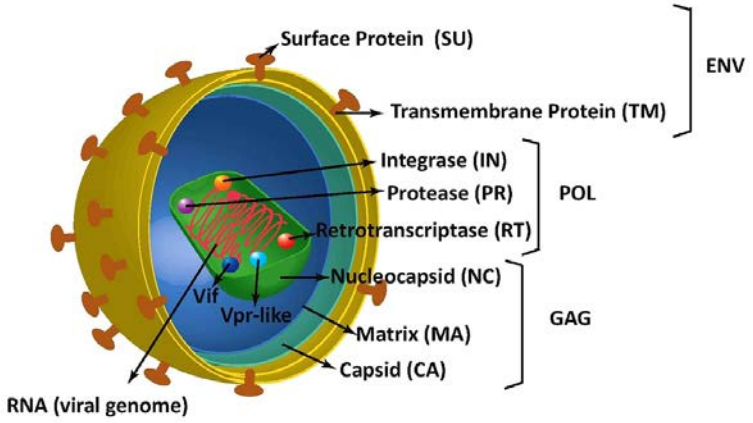

Fig.4: *Esquema de la estructura vírica de un lentivirus (Minguijón et al., 2015).*

El genoma del VMV está compuesto por 6 genes, que del extremo 5' a 3' son: *gag, pol, vif, vpr,* rev y *env*, codificados por dos moléculas iguales de ARN monocatenario de unos 9000-10000 pares de bases, con un extremo 5' metilado y uno 3' poliadenilado (Coffin, 1996). Los genes principales son: 5'- *gag- pol- env*- 3', que codifican los componentes de la partícula vírica (genes estructurales), y los genes reguladores *vpr, vif* y *rev*, encargados de regular la expresión de los mismos (genes reguladores) (Clements and Wong-Staal, 1992; Pépin *et al.*, 1998; Leroux

et al., 2010; Minguijón *et al.*, 2015) (Fig. 5). El gen *gag* codifica tres proteínas estructurales internas de las que derivarán proteínas de la nucleocápside, de la matriz proteica y de la cápside, como la proteína p25 y p28 de la cápside que participan en la estimulación de la respuesta inmune humoral durante la infección. El gen *pol* contiene fundamentalmente enzimas como la transcriptasa inversa y la integrasa responsables de la integración del genoma vírico en la célula hospedadora, mientras que el gen *env* codifica las glicoproteínas de superficie, gp135 (SU) y transmembrana gp46 (TM) de la estructura proteica de superficie, participando de este modo en la asociación y entrada del virus en la célula huésped. En los extremos del ARN se encuentran dos secuencias largas de bases repetidas (LTR) que participan en la integración del genoma vírico en el de la célula hospedadora (Leroux *et al.*, 2010).

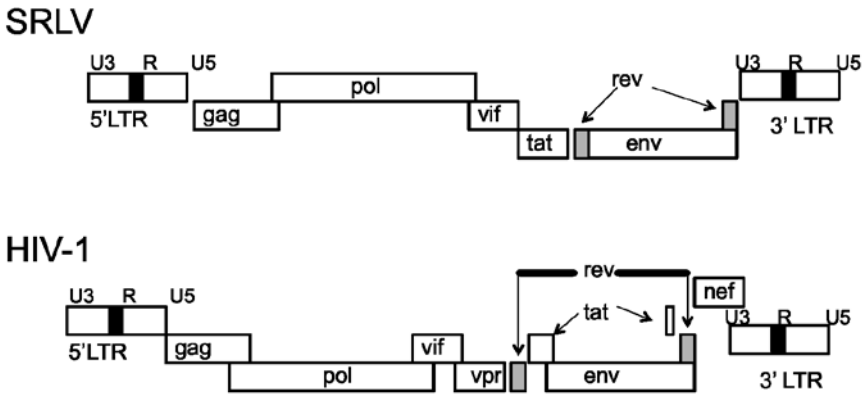

Fig. 5: *estructura del genoma de LVPR y HIV-1, incluido para su comparación (Stonos et al., 2014).*

3.2.3 Variabilidad genética vírica

Mientras que los genes *gag* y *pol* y algunas regiones del *env* están bastante conservados, otras regiones como las del gen *env* que codifican proteínas de superficie donde se unen los anticuerpos, presentan una gran variabilidad, contribuyendo al fallo de la

respuesta inmune humoral frente al VMV (Ramírez *et al.*, 2013), y a la variabilidad genética asociada a las diferente cepas (Carey and Dalziel, 1994). En los LVPR, se han identificado 5 regiones variables (V1-V5) y 4 regiones conservadas (C1-C4) en la proteína *env*. La proteína V4 de los LVPR, estructural y funcionalmente análoga a la V3 del HIV se ha asociado a la colonización de varios órganos diana (pulmón, glándula mamaria, encéfalo y articulaciones) (Minguijón *et al.*, 2015). Se ha descrito que las mutaciones en V4 dan lugar a diferentes subpoblaciones, como ocurre en otros lentivirus (Ramírez *et al.*, 2012). Una de las regiones más conservadas del genoma de los lentivirus es el primer punto de unión al ARN^{lys}, (PBS-GAACAGGGACUUGAA), donde la lisina del hospedador transfiere híbridos de ARN al ARN genómico del virus, sirviendo como *primer* para la transcriptasa inversa (Foley, 2000). El tracto de polipurina, *Rev responsive element* (RRE) y otros elementos involucrados en la replicación y empaquetamiento del genoma vírico presentan también un alto grado de conservación entre los lentivirus (Foley, 2000).

La variabilidad genética típica de los retrovirus da lugar a la formación de diferentes *quasispecies*, descritas por primera vez por Manfred Eigen (Eigen, 1971), y que se define como al conjunto de virus presentes en un individuo infectado (Ojosnegros *et al.*, 2011). Mediante mutación, recombinación y presión selectiva por parte del hospedador aparecen constantemente nuevas *quasispecies* en los individuos infectados (Ramírez *et al.*, 2013). Se puede producir una variación en la frecuencia de las formas genéticas en una población vírica por diferentes circunstancias como la presión selectiva ejercida por el sistema inmune, produciéndose un ‘’archivo’’ de secuencias víricas en forma de provirus que pueden re-emerger. La variabilidad genética entre *quasispecies* depende de un conjunto complejo de factores como una elevada re-infección vírica, mutaciones, recombinación retroviral o selección por el sistema inmune del hospedador (Pasick, 1998; Korber *et al.*, 2001; Ojosnegros *et al.*, 2011; Smyth *et al.*, 2012).

La recombinación es un evento temprano que se produce antes de la integración, durante la retrotranscripción. De forma conjunta la recombinación y mutación de los retrovirus supera con creces las de otros virus animales (Murphy *et al.*, 1999). Se trata de una herramienta evolutiva importante, al producir un beneficio genético que facilita la adaptación, integración y persistencia del virus. En ausencia de recombinación los lentivirus tienden a acumular mutaciones deletéreas (Minguijón *et al.*, 2015). La hipermutación se debe a la falta de capacidad de ''lectura de prueba'' de la retrotranscriptasa y de la deaminación citosina a uracilo en la cadena simple de ADN que se transcribe (Minguijón *et al.*, 2015). Esta variabilidad permite a los LVPR evadir la respuesta inmune del hospedador (Narayan *et al.*, 1977b) y pasar la barrera interespecie (Murphy *et al.*, 1999).

3.3. PATOGENIA

3.3.1. Infección, replicación y diseminación del virus

Cuando el VMV alcanza el organismo, los mecanismos de replicación son similares a los de otros retrovirus (Pétursson *et al.*, 1991). El tropismo de los LVPR depende de las interacciones virus-hospedador a diferentes niveles: celular (receptor, pre-integración e integración, post-integración del genoma vírico en el ADN del hospedador); del órgano o tejido y del hospedador (individuo, raza o especie) (Minguijón *et al.*, 2015).

La célula diana principal es el monocito (Gendelman *et al.*, 1985; Gendelman *et al.*, 1986), que transporta el virus sin que pueda ser detectado por el sistema inmune, produciéndose la replicación vírica fundamentalmente en la maduración de esta célula a macrófago (Narayan, 1983; Narayan *et al.*, 1983; Peluso *et al.*, 1985; Clements *et al.*, 1994;

Zhang *et al.*, 2000), aunque también se ha demostrado la replicación vírica *in-vivo* en otras células como las células dendríticas aferentes a ganglios linfáticos (Gorrell *et al.*, 1992; Ryan *et al.*, 2000).

El mecanismo de infección comienza con la interacción y fusión de la glicoproteína gp135 de la envoltura viral externa con la membrana celular de la célula huésped (Crane *et al.*, 1991), liberándose el ARN en el citoplasma de la misma una vez que la partícula viral se encuentra en el interior. La transcriptasa inversa comienza la transcripción a una doble cadena de ADN, creándose en un primer momento una cadena de ARN y otra de ADN, y posteriormente, actuando como polimerasa, dos cadenas de ADN, como ocurre en el resto de los retrovirus. Este ADN viral se fusiona con el ADN genómico con la ayuda de la integrasa vírica, quedando la célula diana infectada por el virus (provirus) (Fig.6).

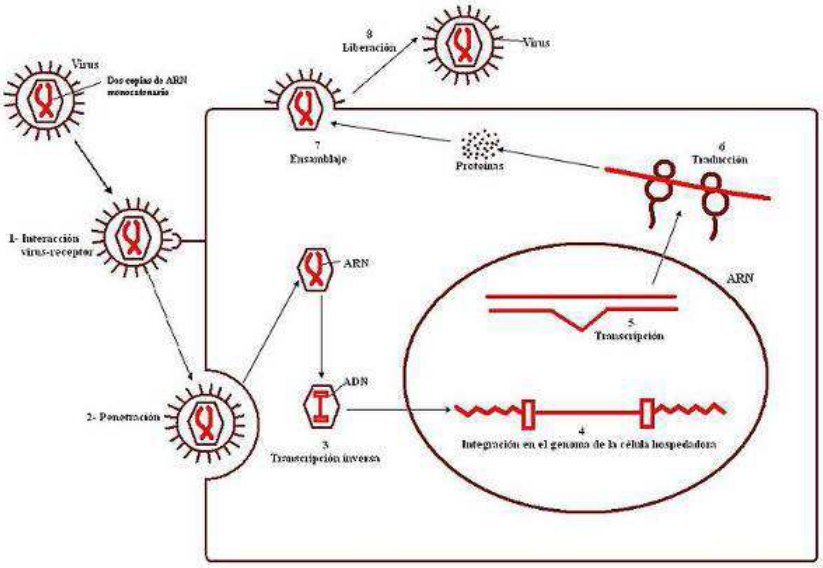

Fig.6: *Ciclo biológico del virus del maedi visna. Imagen procedente de:http://upload.wikimedia.org/wikipedia/commons/1/15/Ciclo_de_replicaci%C3%B3n_de_un_retrovirus.JPG*

En este momento, el virus puede entrar en fase de latencia, permaneciendo en las células y eludiendo la respuesta inmune, o replicarse activamente liberando viriones capaces de infectar otras células utilizando los sistemas enzimáticos de la célula huésped (Zink *et al.*, 1987). Normalmente, los lentivirus ovinos se replican rápido, dando lugar a sincitios y lisis celular, por lo que se clasifican como de replicación *rapid/high*, mientras que los de caprino suelen ser *slow/low*. Los LVPR de fenotipos intermedios se han aislado tanto en ovejas como en cabras.

En cuanto a la diseminación del virus a partir de las células diana (monocitos/macrófagos y células dendríticas), se han planteado diferentes hipótesis. Por un lado, se plantea que esas células podrían migrar a los ganglios linfáticos regionales a partir de los cuales se produciría una diseminación sistémica (Bird *et al.*, 1993; Blacklaws *et al.*, 1995b), o bien los macrófagos/monocitos infectados podrían alcanzar la médula ósea e infectar otras células precursoras de la estirpe mieloide, de forma que con la maduración y multiplicación de estas células inmaduras, el propio virus favorecería su replicación y diseminación, permitiendo el paso de células infectadas a sangre periférica de forma crónica (Gendelman *et al.*, 1985; Blacklaws, 2012).

Durante la viremia el virus se distribuye por todo el organismo, principalmente SNC, pulmón, articulaciones o glándula mamaria (Narayan *et al.*, 1983), de donde puede ser aislado (Cadoré *et al.*, 1993). Al no expresar las células infectadas proteínas víricas, no es reconocido por la respuesta inmune, conociéndose este mecanismo de evasión como “caballo de Troya” (Peluso *et al.*, 1985). El paso de estos monocitos infectados desde sangre periférica a otros tejidos propicia la replicación del virus en su diferenciación a macrófagos tisulares (Narayan, 1983; Narayan *et al.*, 1983; Peluso *et al.*, 1985; Clements *et al.*, 1994; Zhang *et al.*, 2000).

Se ha demostrado que la activación de macrófagos (principalmente por citoquinas) supone un incremento de la replicación vírica, al existir una asociación entre la inducción

enzimática de la célula huésped y la expresión vírica (Blacklaws, 2012). Sin embargo, a nivel tisular existen también mecanismos de restricción de la replicación vírica consistentes en la limitación de la maduración de monocito a macrófago y en la alteración del ensamblaje de las partículas virales (Kennedy-Stoskopf and Narayan, 1986).

3.3.2. Respuesta inmunitaria

El MV es una enfermedad de progresión lenta con una etapa subclínica que puede durar meses o años, en los que la carga viral es baja (Sigurdsson and Palsson, 1958; Haase *et al.*, 1977). Cuando el virus alcanza el organismo produce un pico de viremia inicial que induce una respuesta inmune de forma rápida a las 2-4 semanas post-infección, que permanece durante unas 6-8 semanas (Begara *et al.*, 1996). Sin embargo, aunque la infección induce una respuesta inmunitaria tanto innata como adquirida, el virus interfiere en la correcta regulación y eficacia de la misma (Reina *et al.*, 2007; Blacklaws, 2012) y es capaz de evadirla permaneciendo de forma crónica en el organismo, pudiendo progresar con el tiempo y dar lugar a manifestarse la enfermedad (Pépin *et al.*, 1998).

3.3.2.1 Respuesta inmune innata

En la respuesta inmune innata frente a los LVPR participan los receptores *toll-like* (TLR), péptidos antivirales, células *natural killers* (NK) y células T *γ δ* (Stonos *et al.*, 2014).

El papel de los **TLR** no está claro en ovinos y caprinos, pero se ha descrito una activación de los TLR 7 y 8 durante la infección por LVPR, induciendo la producción de IFN-α, IL-6, TNF-α y produciendo un aumento de la expresión de proteínas antivirales (Blacklaws,

2012). También se han descrito en LVPR diferentes **factores de restricción vírica**. Los factores de restricción descritos en HIV-1 como axis de este camino antiviral son la proteína *tripartite motif-containing* (TRIM) 5α, las proteínas de la familia *apolipoprotein B editing enzyme catalytic polypeptide-like 3* (APOBEC3) y las BST-2/ teterinas (Malim and Bieniasz, 2012). Se trata de proteínas presentes en las células de mamíferos frente a las cuales los lentivirus cuentan con diferentes mecanismos de evasión de la restricción vírica, relacionados con las proteínas víricas Vif, Nef, Vpv y Vpr en el caso del HIV-1. De esta manera, el HIV-1 es capaz de evadir los factores de restricción en células humanas permitiendo la replicación viral, mientras que la replicación se ve comprometida en otras especies por la acción de los factores de restricción frente a los que el virus no dispone de mecanismos de evasión específicos (Malim and Bieniasz, 2012; Crespo *et al.*, 2016;).

Dichos factores de restricción también se han descrito en los LVPR (LaRue *et al.*, 2008; Arnaud *et al.*, 2010; Jáuregui *et al.*, 2012;). Así mismo, se ha realizado un estudio genético detallado de los factores de restricción en cabras procedentes de un rebaño altamente infectado (Crespo *et al.*, 2016). En dicho estudio se distinguieron dos grupos, los animales LPL (*low proviral load*), con baja carga viral y ausencia de eliminación vírica; y los HPL (*high proviral load*), con alta carga viral y eliminación vírica. Tras el cultivo de fibroblastos de la piel de un animal de cada grupo, se observó una mayor expresión de los factores de restricción TRIM5α, APOBEC3 (A3Z1) en células con infección LPL, así como una mayor actividad proteosómica y una menor presencia de proteínas víricas mediante inmunocitoquímica en este grupo. Estos resultados parecen indicar que animales con una mayor expresión de los genes que codifican estos factores de restricción presentan una menor carga viral y suponen un menor riesgo de diseminación vírica. Los animales LPL, descritos previamente como *long term non-progressors* (Stonos *et al.*, 2014), muestran una respuesta serológica competente aunque son poco eficientes en la replicación vírica, aunque no se consideran actualmente en el control de la enfermedad.

Así mismo, se ha descrito un posible rol de las **células NK** en la infección por LVPR (Stonos *et al.*, 2014) debido a su importancia en el control del HIV-1, donde se cree que controlan la replicación viral previamente a la inducción de linfocitos CD8+ específicos (Alter *et al.*, 2007).

Por otro lado, se ha descrito el rol de las **células *γδ*** en el control de las infecciones por LVPR, ya que constituyen aproximadamente el 70% de todos los linfocitos de rumiantes jóvenes y son una parte importante del sistema inmune innato (Jolly *et al.*, 1997; Ponti *et al.*, 2008; Kaba *et al.*, 2011). Se ha observado que animales infectados por CAEV muestran una cantidad de linfocitos *γδ* significativamente más alta que cabras sanas, sugiriendo su posible papel en el control de la enfermedad (Jolly *et al.*, 1997; Ponti *et al.*, 2008; Kaba *et al.*, 2011). Dichas células se localizan en las superficies mucosas, por lo que podrían ser cruciales en la limitación de la entrada del virus y la respuesta inmune temprana (Stonos *et al.*, 2014).

3.3.2.2 Respuesta inmune adquirida

La respuesta inmune adquirida comprende la respuesta inmune mediada por células y mediada por anticuerpos. Durante la infección por LVPR ambas vías se activan aunque no está claro cuánto influyen en la protección del hospedador y cuánto en la progresión de la enfermedad (Reina *et al.*, 2008; Stonos *et al.*, 2014). Al igual que ocurre en el HIV-1, el grado de la respuesta inmune influye en la carga viral, la cual está relacionada con la gravedad de la enfermedad y la aparición de signos clínicos (Cheevers *et al.*, 2000; Cheevers *et al.*, 2001). Los animales que responden con una inmunidad celular se suelen llamar *long term progressors* ya que presentan una infección persistente pero poca sintomatología clínica y baja carga viral (Stonos *et al.*, 2014). Estos animales producen altas dosis de anticuerpos IgG2 específicos frente a la gp135 y una respuesta Th con alta producción de IFN-γ (Cheevers *et al.*, 2000;

Cheevers *et al.*, 2001). Los animales con una respuesta humoral se caracterizan por la presencia de altos títulos de anticuerpos IgG1 y un predominio de células Th2 con poca producción de Il-4 (Cheevers *et al.*, 2001; Stonos *et al.*, 2014). Asimismo, las diferencias entre ambos tipos de respuesta inmune se han descrito en relación con la presencia de lesiones y de antígeno vírico mediante inmunohistoquímica en tejido nervioso, observándose de forma subjetiva una mayor cantidad de virus en animales con patrón histiocítico o respuesta inmune de tipo celular que en los que presentaban un patrón de tipo linfocítico, con predominio de la respuesta humoral (Polledo *et al.*, 2012b).

3.3.2.2.1 Respuesta inmune humoral

La infección por VMV induce una intensa respuesta inmune humoral en el hospedador con producción de anticuerpos desde los 15 días a varios meses post-infección, tendiendo a fluctuar durante los primeros 6 meses de infección (Lacerenza *et al.*, 2006; Rachid *et al.*, 2013). Al igual que ocurre en el HIV-1 estos anticuerpos tempranos no son neutralizantes, pero podrían jugar un papel importante en la inmunidad mediada por células. (Perry *et al.*, 1995; Cheevers *et al.*, 2000; Singh *et al.*, 2006). Las células presentadoras de antígeno interaccionan con los linfocitos T CD4+, que inducen la proliferación de linfocitos Th, los cuales a su vez dan lugar a la producción de anticuerpos al interactuar con las células B (Woodall *et al.*, 1997; Mariotti *et al.*, 2007).

La respuesta mediada por anticuerpos de los LVPR normalmente se relaciona con los epítopos gp135, gp38 y proteínas de la cápside (Bertoni *et al.*, 2000). Aunque se secretan anticuerpos frente a todas las proteínas virales, son generalmente los que se producen frente a proteínas de la envoltura los que son capaces de neutralizar el virus (Blacklaws, 2012). Los anticuerpos neutralizantes pueden tardar hasta dos años en aparecer y pueden controlar la

infección, aunque en la mayoría de los casos se produce una limitación de la diseminación vírica sin producirse una neutralización completa (Narayan *et al.*, 1981; Pépin *et al.*, 1998; Lacerenza *et al.*, 2006).

El fracaso de la respuesta inmune humoral en la neutralización del virus puede explicarse debido a las diferentes estrategias de **evasión** de la misma que el virus ha desarrollado, provocando una persistencia de la infección, y haciendo que la presencia de anticuerpos frente a VMV sean indicativos de infección, pero no de niveles altos de protección:

a. **Estrategia del ``caballo de Troya´´,** ya mencionado, en el que el genoma vírico persiste de forma silente en forma de provirus integrado en el genoma del monocito, evadiendo la respuesta inmune y consiguiendo la dispersión por el organismo (Narayan *et al.*, 1983).
b. **Variación antigénica del virus**, debido a mutaciones y recombinaciones genéticas. Las mutaciones se producen sobre todo a nivel del gen *env*, dando lugar a nuevos epítopos que los anticuerpos no son capaces de neutralizar (Narayan *et al.*, 1977a; Narayan *et al.*, 1977b; Haflidadóttir *et al.*, 2008; Arnarson *et al.*, 2017). De esta forma, se produce una selección positiva del gen, especialmente en aminoácidos del *principal neutralization domain* (PND) en *env,* que se asocia a persistencia vírica adquirida a partir de una selección de variantes antigénicas de los reservorios de virus latentes que se transmiten de forma natural (Arnarson *et al.*, 2017). Las recombinaciones genéticas son también frecuentes en el gen *env* de los lentivirus, como se ha estudiado en la cepa 1514 del VMV tanto *in vivo* como *in vitro* (Andrésdóttir *et al.*, 2002; Andrésdóttir, 2003). Una de las principales fuentes de variación se atribuye a la retrotranscriptasa inversa (RT) en sí, que tiene una tasa de error de 0,2-2 mutaciones por genoma y ciclo (Roberts *et al.*, 1988; Ojosnegros *et al.*, 2011).

c. **Alta afinidad del virus por las células diana**, pudiendo existir una alta absorción del virus por parte de monocitos/macrófagos que no se ve compensada con la capacidad de las defensas humorales para neutralizar el virus (Kennedy-Stoskopf and Narayan, 1986).

d. **Infección de los precursores celulares de monocitos** en la médula ósea, sin producirse respuesta inflamatoria, de tal forma que el virus no es reconocido por el sistema inmune (Gendelman *et al.*, 1985), además de producirse un reservorio para la formación de monocitos infectados (de la Concha-Bermejillo, 1997). También se ha descrito que las células del epitelio bronquioalveolar, con baja tasa de replicación vírica, podrían desempeñar un papel similar actuando como un reservorio poco accesible al sistema inmune (Staskus *et al.*, 1991).

La respuesta mediada por anticuerpos podría contribuir a la progresión de la infección por LVPR, ya que en un estudio de CAEV se observó que los animales asintomáticos tienden a desarrollar una respuesta de tipo celular con título bajo de IgG2 específicas frente a gp135, mientras que animales con sintomatología clínica (artritis) muestran niveles elevados de IgG1 y un mayor ratio IgG1/IgG2 que los anteriores (Trujillo *et al.*, 2004; Stonos *et al.*, 2014).

3.3.2.2.2 Respuesta inmune celular

La respuesta inmune celular es probablemente la más eficiente en el control de la carga viral de los lentivirus (Stonos *et al.*, 2014). Además de intervenir en la respuesta inmune humoral, los linfocitos T CD4+ participan en mantener activos los linfocitos T citotóxicos, siendo imprescindibles también para el desarrollo de la respuesta inmune celular.

Se ha observado que una semana después de la infección, se produce a nivel periférico una intensa activación linfocítica en respuesta al antígeno *gag* que, aunque no consigue

eliminarla, controla de forma efectiva la viremia inicial (Narayan and Clements, 1989; Blacklaws *et al.*, 1995b), produciéndose una disminución del número de células infectadas cuando aparece una respuesta inmune específica de tipo T (Bird *et al.*, 1993). En concreto, se ha relacionado la presencia a nivel periférico de un número elevado de linfocitos T citotóxico, especialmente CD8+, con un descenso en los niveles víricos plasmáticos (Lichtensteiger *et al.*, 1993; Blacklaws *et al.*, 1994; Blacklaws, 2012), tal y como ha sido demostrado en otros lentivirus como VIS (Schmitz *et al.*, 1999). Se ha descrito una disminución de la cantidad de linfocitos CD4+ en animales con infección por CAEV y sintomatología clínica artrítica con respecto a caprinos asintomáticos (Perry *et al.*, 1995), al igual que en VMV ovino, donde se observó una disminución de la proliferación linfocítica en ovejas con sintomatología clínica en comparación con ovinos asintomáticos (Reina *et al.*, 2007). Este hecho parece indicar que la infección por LVPR interfiere en el procesamiento y presentación de antígenos, limitando la acción de activación de células T CD4+ por parte de las células presentadoras de antígeno y por lo tanto la inducción de una respuesta citotóxica (Stonos *et al.*, 2014).

Con la replicación vírica a nivel tisular, se induce una respuesta inflamatoria basada en la secreción intensa de linfoquinas que estimularán la diferenciación de monocitos a macrófagos. Debido a la continua presentación de antígeno, se produce una sobreexpresión del complejo mayor de compatibilidad (MHC) tipo 2 que da lugar a un aumento en la activación tanto de linfocitos-T como B (Torsteinsdóttir *et al.*, 2007b). Sin embargo, a pesar de que la proliferación de linfocitos-T CD8+ citotóxicos controlan la replicación vírica inicial, no se alcanza una eliminación completa de la infección de forma similar a como ocurre a nivel periférico (Torsteinsdóttir *et al.*, 1992; Bird *et al.*, 1993; Blacklaws *et al.*, 1994; Blacklaws *et al.*, 1995a; Blacklaws *et al.*, 1995b; Luján *et al.*, 1995).

Esta regulación defectuosa de la respuesta inmunitaria se ha atribuido tanto a una respuesta improductiva de los linfocitos T como a una alteración en la regulación de la

secreción de citoquinas (Tompkins and Tompkins, 2008), pudiendo permanecer los linfocitos T en un estado de anergia clonal en animales en fases clínicas, conduciendo a una activación crónica y ausencia de respuesta de recuerdo antígeno-específica y dando lugar a un control ineficaz de la infección viral y un desequilibrio en la regulación de la respuesta inmunitaria (Ellis and DeMartini, 1985; Begara *et al.*, 1995; Mariotti *et al.*, 2007; Tompkins and Tompkins, 2008).

Una de las citoquinas mediadoras más importantes que participan en la inmunidad celular frente a LVPR es el interferón (IFN), tanto de tipo I como de tipo II o INF-γ. Éste es sintetizado únicamente tras la interacción con determinados linfocitos T, ya que las células fagocíticas infectadas no son capaces de sintetizarlo por sí mismas (Woodall *et al.*, 1997; Blacklaws, 2012). Mientras que el IFN tipo I tiene un papel fundamental en la respuesta antiviral, el IFN tipo II participa en la regulación de la respuesta inmune (Wood and Seow, 1996). Sin embargo, al igual que ocurre en los casos anteriores parece que los LVPR han desarrollado vías de escape a la acción del mismo, incluso que en la infección por CAEV, el IFN-γ podría llegar a jugar un papel antagónico al habitual, produciendo una activación de la replicación vírica en monocitos tras interaccionar con la región genómica LTR del virus (Tong-Starksen *et al.*, 1996).

3.3.3 Génesis lesional

La génesis lesional en las infecciones por LVPR se relaciona con una desregulación de la respuesta inmune. Las lesiones que se desarrollan en el VMV se caracterizan por una infiltración de macrófagos, linfocitos, células plasmáticas, en los órganos diana que gradualmente serán los responsables de la manifestación clínica de la enfermedad. El

fenómeno iniciador es la infección de macrófagos, que una vez infectados desencadenan una respuesta inmune mediante la liberación de citoquinas como la interleuquina 8 (Cadoré *et al.*, 1993; Cadoré *et al.*, 1996), que atraen más monocitos y células inflamatorias a la zona de la lesión, que a su vez secretan diferentes productos como el IFN-OvLV y otras linfoquinas, activando y atrayendo más macrófagos y linfocitos, de tal forma que se mantiene una estimulación continua del sistema inmune, amplificándose la inflamación y dando lugar al daño tisular (Narayan, 1983; Nathason *et al.*, 1985; Georgsson, 1994). Junto a la liberación de estos activadores tisulares por parte de linfocitos y macrófagos, se liberan otros mediadores como los factores de necrosis tumoral α y β, que podrían contribuir al daño tisular (Georgsson, 1994).

La caquexia marcada que presentan los animales que padecen esta enfermedad podría relacionarse con la infección de monocitos y macrófagos, que liberan caquexina, enzima que induce la producción y liberación de otras enzimas lipolíticas (Petursson *et al.*, 1976).

Otro factor que influye en la génesis lesional son las propias cepas víricas, ya que no todas presentan la misma patogenicidad tanto *in vivo* como *in vitro.* Se ha observado que parece haber una relación entre los efectos que estas cepas *in vitro* y la gravedad de las lesiones que causan *in vivo* (de la Concha-Bermejillo, 1997).

3.3.4 Genética del hospedador

Existe poca información acerca de los factores genéticos que pueden afectar a la susceptibilidad o resistencia frente a los LVPR. En el HIV-1 se han descrito diferentes polimorfismos genéticos que parecen estar asociados a resistencia frente a la enfermedad o a una progresión más lenta de la misma (Stonos *et al.*, 2014) . La mayoría de los genes asociados a resistencia genética frente a HIV-1 codifican una variedad de moléculas inmunes descritas

anteriormente como factores de restricción, entre las que se encuentran el MHC tipo 1, receptor de quimiocinas C-C (CCR) 5, *killer immunoglobulin-like receptor* (KIR) o *toll-like receptors* TLRs (Kuniholm *et al.*, 2011), aunque se han observado diferencias entre poblaciones (Brinkhof and Van Maanen, 2007; An *et al.*, 2009; Naruto *et al.*, 2012).

Se han descrito diferencias similares en el caso de los LVPR, donde diferentes razas muestran distinta resistencia a dichos virus, identificándose polimorfismos en TLR 7 y 8 y en genes de CCR5 y MHC asociados a resistencia vírica (Fluri *et al.*, 2006; White *et al.*, 2009; Mikula *et al.*, 2010; Larruskain *et al.*, 2013; Bowles *et al.*, 2014). Así, se ha observado una mayor proporción de polimorfismos en TLR 7 y 8 en ovinos infectados por MV (Mikula *et al.*, 2010), mientras que una delección en el gen CCR5 ovino se ha relacionado con una menor carga viral en las razas Ramboullet, Polypay y Columbia (White *et al.*, 2009). También se ha relacionado una mutación (glicina a arginina) en el codón 250 del TLR 9 en ovinos con seropositividad frente a LVPR (Sarafidou *et al.*, 2013).

Se ha descrito el gen de la proteína transmembrana TMEM154 como posible factor de resistencia frente a los LVPR (Heaton *et al.*, 2012; Alshanbari *et al.*, 2014; Clawson *et al.*, 2015). Aunque no se conoce actualmente el papel de dicha proteína, se ha observado una elevada expresión de la misma en monocitos y linfocitos B, lo que sugiere que podría tener importancia inmunológica (Heaton *et al.*, 2012; Stonos *et al.*, 2014). Un estudio reciente ha demostrado una mayor frecuencia de la mutación de dicho gen en las razas Dalsebred, Herdwick y Rough Fells asociada a resistencia genética frente a LVPR (Bowles *et al.*, 2014). Así mismo se ha observado en un rebaño ovino que la presencia de dos copias del halotipo 1 de TMEM154 se asocia a una menor carga proviral (Alshanbari *et al.*, 2014). Por otra parte, el gen TMEM38A se ha asociado a resistencia genética y la *dipeptide-binding protein* (DPPA) 2 a susceptibilidad frente a LVPR (White *et al.*, 2012).

La proteína TRIM5 es otro factor de restricción vírica que se ha asociado con la restricción de la síntesis de ADN viral de LVPR en ovino (Jáuregui *et al.*, 2012). En dicho estudio, se caracterizaron así mismo las proteínas TRIM5 presentes en ovino y caprino, siendo todas ellas TRIM5α.

Lo mismo ocurre con la familia de proteínas del sistema inmune innato APOBEC3, relacionada con la inhibición de la replicación de diferentes familias víricas (Harris and Dudley, 2015). La proteína A3Z1 codificada por un gen APOBEC3 se ha asociado con menor replicación vírica de LVPR en monocitos de oveja mientras que la variante A3Z1Tr obtenida tras la estimulación con interferón gamma permite la infección (De Pablo-Maiso *et al.*, 2017).

Estos estudios parecen abrir la posibilidad de una selección genética de razas ovinas con mayor resistencia frente a SRLV (Stonos *et al.*, 2014). Sin embargo, se ha propuesto que la selección genética de pequeños rumiantes para evitar enfermedades podría no ser del todo práctica (Stonos *et al.*, 2014) ya que, al igual que ocurre en el HIV-1, la resistencia o progresión más lenta del virus es poligénica, es decir, envuelve una interacción compleja entre una variedad de genes de la respuesta inmune innata y adquirida (Petrovski *et al.*, 2011; Luo *et al.*, 2012). Una selección genética de este tipo podría aumentar la susceptibilidad a otras enfermedades e incluso podría haber una rápida adaptación de los LVPR, al tratarse de virus con una alta tasa de mutación. Por lo tanto, se ha planteado que podría ser más útil y sencillo realizar una selección fenotípica en vez de genotípica (Stonos *et al.*, 2014). Otra opción que se ha descrito es la selección por respuesta inmune mejorada tras medir las respuestas inmunes celular y humoral de los individuos frente a diferentes antígenos. Por ejemplo, en la especie bovina se han encontrado diferentes polimorfismos de un solo nucleótido (SPS) en el locus MHC asociados a ambos tipos de respuesta inmune (Stonos *et al.*, 2014). En caprino, se ha asociado el halotipo Be10-D2 con una seroconversión más rápida y títulos de anticuerpos más altos que en el halotipo Be1-D5 (Fluri *et al.*, 2006), lo que podría abrir la posibilidad de

seleccionar animales con ciertos SPS que permitan una respuesta inmune más efectiva en el control de la enfermedad (Stonos *et al.*, 2014).

3.4. TRANSMISIÓN Y VÍAS DE CONTAGIO

Se considera que las formas principales de transmisión del MV de forma natural son dos: la transmisión horizontal y la lactógena, habiendo sido debatida su contribución a la diseminación de la infección en diversos estudios (Berriatua *et al.*, 2003; Blacklaws *et al.*, 2004; Álvarez *et al.*, 2005; Leginagoikoa *et al.*, 2010). Además de estas dos vías, se ha conseguido experimentalmente la transmisión de la infección mediante diferentes inóculos como secreciones de animales infectados, agua contaminada, homogeneizados de órganos o aislamientos víricos, por vía respiratoria (intratraqueal, intranasal, intrapulmonar e intratorácica), intracerbral, endovenosa, digestiva, intramuscular, subcutánea, intrarticular e incluso mediante inoculación en el saco amniótico (Badiola y González, 1990; Torsteinsdóttir *et al.*, 2003).

La transmisión horizontal se produce por el contacto directo entre animales sanos e infectados, mediante la aspiración de aerosoles u otras secreciones (Luján *et al.*, 1994; Blacklaws *et al.*, 2004; Peterhans *et al.*, 2004), considerándose la vía oronasal, sobre todo entre cordero sano y madre infectada, como la forma más efectiva de transmisión de la enfermedad (Houwers *et al.*, 1989; Blacklaws *et al.*, 2004; Leginagoikoa *et al.*, 2006; Leginagoikoa *et al.*, 2010). Se ha demostrado la presencia de virus libre en el fluido respiratorio (McNeilly *et al.*, 2007), y se ha reproducido experimentalmente la enfermedad a través de la infección por vía intranasal, intratraqueal e intrapulmonar (Torsteinsdóttir *et al.*, 2003; Niesalla

et al., 2008). Así mismo, se ha hallado la presencia de ácidos nucleicos del virus en agua de bebederos y en el exhalado de animales infectados (Villoria *et al.*, 2013).

En cuanto a la transmisión lactógena, parece estar relacionada con la entrada del virus en el organismo del cordero a través del tracto digestivo (Cutlip *et al.*, 1985a; Blacklaws *et al.*, 2004; Preziuso *et al.*, 2004). Así, se han detectado LVPR en tejido mamario de hembras lactantes mediante PCR *in situ* (Carrozza *et al.*, 2003; Preziuso *et al.*, 2003b) y en macrófagos en leche y calostro mediante PCR (Sihvonen, 1980; Leroux *et al.*, 1997; Extramiana *et al.*, 2002; Blacklaws *et al.*, 2004; Álvarez *et al.*, 2006; Brinkhof *et al.*, 2010), y se ha demostrado la presencia de VMV en células mononucleares del tejido linfoide de la submucosa intestinal en corderos alimentados con calostro infectado (Preziuso *et al.*, 2004). También se ha comprobado que las corderas nacidas de madres seronegativas tienen menor tendencia a seroconvertir en el futuro que las de madres seropositivas por la no ingestión de calostro y leche infectada, aunque también se ha relacionado con una posible mayor resistencia genética a la infección transmitida por la madre (Blacklaws *et al.*, 2004; Leginagoikoa *et al.*, 2006). Igualmente, se han asociado positivamente los niveles de seroconversión con el tiempo de vida que pasa el cordero con la madre infectada seropositiva. En esta situación, el contagio horizontal tiene mayor importancia que el que se establece a través de calostro (Berriatua *et al.*, 2003). Sin embargo, en condiciones naturales esta vía de contagio parece tener una importancia relativa menor en la diseminación de la infección en comparación con la transmisión por vía aerógena entre la madre infectada y el cordero, donde la duración del estrecho contacto entre ambos parece jugar un papel decisivo (Álvarez *et al.*, 2005; Leginagoikoa *et al.*, 2006; Broughton-Neiswanger *et al.*, 2010).

También se ha propuesto la vía vertical como posible vía de contagio de los LVPR, aunque actualmente se considera una vía de escasa importancia epidemiológica (Blacklaws *et al.*, 2004; Peterson *et al.*, 2008; Broughton-Neiswanger *et al.*, 2010; Romero *et al.*, 2010;

Cortez-Romero *et al.*, 2011; Cortez-Romero *et al.*, 2013). Se ha demostrado la presencia de ADN proviral de LVPR en el aparato genial de hembras infectadas, lo que explica la posible transmisión de la madre al embrión o feto *in utero* (transmisión vertical). Así mismo, se han descrito un cierto número de casos de infección *in utero* y postnatal durante la transferencia de embriones. Se ha identificado el VMV mediante técnicas de PCR en ovario, oviducto y útero, y se ha observado que las células de los folículos ováricos y ovocitos permanecen libres de infección (Cortez Romero *et al.*, 2006; Cortez-Romero *et al.*, 2011). Lamara y colaboradores (Lamara *et al.*, 2001; Lamara *et al.*, 2002b) observaron que células epiteliales del oviducto de cabras infectadas podrían ser infectadas *in vitro* con virus CAEV, aunque no hay evidencias de la infección de estas células *in vivo*. Mediante PCR se ha demostrado la presencia de LVPR en lavados de oviducto de 11/25 cabras infectadas con CAEV así como en muestras de tejido de útero y oviducto (Fieni *et al.*, 2002; Fieni *et al.*, 2003), sugiriendo que los fetos puedes estar expuestos al virus *in vivo*. En cuanto a la infección directa de embriones con LVPR, se ha demostrado la posibilidad de infección *in vitro* con CAEV en embriones de 8-16 células si se eliminaba la zona pelúcida, pero no si se mantenía intacta, pareciendo que supone una barrera efectiva contra la infección de los embriones (Lamara *et al.*, 2002a). Además, el momento de la gestación en el que se produce la infección parece influir en la aparición de reabsorciones o abortos, desarrollo de fetos resistentes a LVPR o nacimiento de animales infectados (Blacklaws *et al.*, 2004).

Se ha propuesto el contagio vía seminal a través de machos infectados, habiéndose detectado con PCR ADN proviral de LVPR en testículos, epidídimo y en todas las glándulas sexuales accesorias, además de en semen eyaculado y recogido del epidídimo post-mortem (Peterson *et al.*, 2008). En dicho estudio, se separó el semen diluido 1:1 en PBS en 4 fases mediante un gradiente doble de Percoll (35/85 %). En la fase superior, en la que se obtuvo el plasma seminal, no hubo positividad mediante PCR a tiempo real (rt-PCR) a LVPR; en la segunda fase, en la que se encontraron principalmente macrófagos y algunos restos de

espermatozoides, se obtuvo positividad en un 55% de las muestras; en la tercera fase, compuesta por espermatozoides no viables y algunas células procedentes de los órganos sexuales, se obtuvo positividad únicamente en una muestra, mientras que la última fase en la que se encontraban los espermatozoides viables no pudo ser estudiada debido a problemas en la extracción de ADN por la elevada densidad celular. Estos datos confirman que los macrófagos actúan como células portadoras de los LVPR en forma de provirus de ADN. En el estudio anterior el semen eyaculado presentó una eliminación discontinua de ADN proviral, que se produjo de forma estacional y no en todos los animales, coincidiendo la mayor positividad en la parte media del periodo de extracción. Este hecho se observó también en casos de coinfección con *B.ovis*, lo que podría considerarse un factor de riesgo de infección tanto para la madre como para los corderos (Preziuso *et al.*, 2003a). No se ha podido relacionar la presencia de un mayor número de monocitos/macrófagos en sangre o semen con una mayor positividad en rt-PCR (Peterson *et al.*, 2008), a pesar de que en estudios previos se propone un aumento de la infección por MV en casos de coinfección con *B.ovis* debido al aumento de proliferación de monocito a macrófago causado por la respuesta inflamatoria producida en el tejido frente a la brucelosis (Preziuso *et al.*, 2003a). En cabras, se ha demostrado recientemente la posibilidad de transmisión del CAEV a través de inseminación artificial con semen infectado en cabras seronegativas (Souza *et al.*, 2013). En dicho estudio, en el que se utilizaron 20 cabras seronegativas, se observó una seroconversión del 60% de los animales a los 30 días de la inseminación, y del 100% a los 60 días, independientemente de la dosis viral introducida en el semen (alta carga viral o baja carga viral). No se observaron diferencias en los parámetros reproductivos en las hembras utilizadas como control negativo, las inseminadas con semen con alta carga viral o con baja carga viral (Souza *et al.*, 2013).

3.5. FORMAS CLÍNICAS

La enfermedad del MV se caracteriza por un síndrome multisistémico que cursa con pérdida progresiva de la condición corporal e inflamación crónica no purulenta de los pulmones, glándula mamaria, SNC y articulaciones. Aparece en ovinos de edad adulta debido a su progresión lenta, aunque se han dado casos en corderos de tan sólo 4-6 meses de edad (Benavides *et al.*, 2007a).

3.5.1 Forma respiratoria

En la forma respiratoria, los animales presentan inicialmente una ligera apatía y menor ganancia de peso, que puede progresar a disnea y pérdida de condición corporal (Dawson, 1987), incluso intolerancia al esfuerzo físico (De Boer *et al.*, 1979). Estas manifestaciones clínicas se deben a una neumonía intersticial crónica no purulenta que dificulta el intercambio normal de gases (Georgsson and Pálsson, 1971).

Macroscópicamente, se observan unos pulmones más voluminosos y pesados de lo normal, que no colapsan o lo hacen sólo ligeramente, y con una consistencia gomosa y color grisáceo (Sigurdsson *et al.*, 1952; Cutlip *et al.*, 1988; Luján *et al.*, 1991), con los nódulos linfáticos regionales muy aumentados de tamaño y de color blanquecino (Badiola y González, 1990).

Histológicamente, la forma pulmonar del MV se caracteriza por un infiltrado inflamatorio intersticial en las paredes alveolares de células mononucleares, principalmente linfocitos, macrófagos y células plasmáticas, que dan lugar a una neumonía intersticial crónica no purulenta (Sigurdsson *et al.*, 1952; Georgsson and Pálsson, 1971; Cutlip *et al.*, 1988; Badiola

et al., 1990). Suele acompañar a estos cambios una hiperplasia linfoide difusa, formándose agregados e incluso folículos rodeando bronquiolos y vasos sanguíneos (Palsson, 1976; Badiola *et al.*, 1990; Watt *et al.*, 1992b), que pueden verse macroscópicamente como un punteado grisáceo subpleural de 0,5-1 mm (Cutlip *et al.*, 1988; Luján *et al.*, 1991). Generalmente estos cambios se acompañan de hiperplasia de las fibras musculares lisas, así como de una proliferación de fibroblastos y depósito de colágeno que producen un aumento de la consistencia (Badiola y González, 1990).

3.5.2 Forma mamaria

La forma mamaria se caracteriza por una atrofia e induración progresiva bilateral de las mamas (Cutlip *et al.*, 1985a; Houwers *et al.*, 1988; Luján *et al.*, 1991; Pekelder *et al.*, 1994; van der Molen and Houwers, 1987; Watt *et al.*, 1992a), con incremento de tamaño de los ganglios linfáticos retromamarios (Badiola y González, 1990). La secreción de leche puede verse disminuida notablemente e incluso producirse agalactia, aunque el aspecto de la misma no sufre alteraciones (Dawson, 1987).

Histológicamente, se observa en el parénquima mamario un infiltrado inflamatorio con predominio linfocítico que se dispone entre túbulos y alvéolos mamarios y alrededor de conductos galactóforos, pudiendo dar lugar a agregados o folículos linfoides (van der Molen *et al.*, 1985; van der Molen and Houwers, 1987; Luján *et al.*, 1991), además de degeneración de células secretoras y alteración de la estructura túbulo-alveolar normal de la glándula (Luján *et al.*, 1991).

3.5.3 Forma nerviosa

En general es poco frecuente, aunque en ovino intensivo de leche en Castilla y León, especialmente de raza Assaf Española, se han detectado numerosos casos (García Marín *et al.*, 1998; Gómez *et al.,* 1999; Benavides *et al.*, 2006a; Benavides *et al.*, 2006c; Benavides *et al.*, 2009; Glaria *et al.*, 2012; Polledo *et al.*, 2012b).

Los principales signos clínicos que se observan son debilidad de las extremidades, ataxia, decúbito y postración, permaneciendo el animal alerta y atento a estímulos externos (Sigurdsson *et al.*, 1957; González *et al.*, 1985; Gómez, 1999; Benavides *et al.*, 2006a; Christodoulopoulos, 2006; Benavides *et al.*, 2009). Como síntomas precoces puede apreciarse que los animales se separan del resto del rebaño, inestables en los miembros posteriores (de la Concha-Bermejillo, 1997), cayéndose o tropezando sin razón aparente, o caminando en círculos (Watt *et al.*, 1990).

Macroscópicamente, las lesiones en el encéfalo y en la médula espinal suelen ser inaparentes, pero en ocasiones se observan áreas de malacia en la sustancia blanca del encéfalo, principalmente en áreas periventriculares y en puente y pedúnculos cerebelosos, y zonas en forma de cuña de necrosis por licuefacción en la sustancia blanca de secciones transversales de la médula (Sigurdsson *et al.*, 1957; Sigurdsson and Palsson, 1958; Gómez *et al.*, 1999; Benavides *et al.*, 2006a; Benavides *et al.*, 2009; Polledo *et al.*, 2012b) .

Histológicamente, se caracteriza por desmielinización y meningoencefalitis no purulenta. Se observa un infiltrado inflamatorio formado principalmente por células mononucleares, siendo característica la presencia de células “gitter” o macrófagos de citoplasma abundante, claro y espumoso encargados de fagocitar la mielina (Gómez *et al.*, 1999; Benavides *et al.*, 2006a; Benavides *et al.*, 2009; Polledo *et al.*, 2012b). Los plexos coroideos también suelen

presentar un infiltrado inflamatorio no purulento de células mononucleares que se disponen formando agregados o folículos linfoides (Cutlip *et al.*, 1979). Estas lesiones histológicas se han sido clasificado en función de la localización la población celular predominante en la lesión en (Benavides *et al.*, 2009):

- Tipo vascular, donde las células inflamatorias se disponen formando manguitos perivasculares.
- Tipo infiltrativo, donde además se observa en el neuroparénquima abundante infiltrado inflamatorio.
- Tipo malácico, donde la característica principal es la presencia de abundante desmielinización.

Así mismo, Polledo et al. (Polledo *et al.*, 2012b) realizan una clasificación de las lesiones nerviosas en formas histiocíticas, con predominio de macrófagos; y formas linfocíticas, en las que el tipo celular predominante son los linfocitos. Establece una relación de dichos patrones lesionales con la respuesta inmune celular individual mediante la realización de estudios histológicos e inmunohistiquímicos. Observa que en el tipo linfocítico las lesiones parecen representar una forma inicial o de latencia en la que los animales tienen cierta resistencia natural a la infección mientras que en el patrón histiocítico podría reflejar una respuesta inmune ineficaz frente al virus o una mayor virulencia de la cepa vírica que está afectando al animal.

Dentro de las lesiones nerviosas (linfocíticas o histiocíticas), se hace una subclasificación en tipo A o tipo B. Así se consideran de tipo A los casos en los que la lesión principal se localiza en las meninges y plexos coroideos, mientras que las de tipo B son aquellas en las que también hay una afectación grave del neuroparénquima (Polledo *et al.*, 2012b). En las lesiones linfocíticas de tipo A, se observa mediante inmunohistoquímica (IHQ) un predominio de linfocitos T CD4+, mientras que en las de tipo B predominan los linfocitos T CD8+, cuya acción

citotóxica mediante la liberación de citoquinas con el objetivo de impedir la replicación viral y controlar la infección produce un efecto agresivo sobre el tejido (Torsteinsdóttir *et al.*, 2007; Polledo *et al.*, 2012b). Las lesiones histiocíticas, en las que siempre hay afectación más o menos grave del neuroparénquima, podrían representar una fase avanzada de la enfermedad en la que se reclutan monocitos y macrófagos, debido a una respuesta inmune exagerada pero inefectiva (Polledo *et al.*, 2012b). En otro estudio del mismo grupo se examinó mediante IHQ la distribución de antígeno de VMV, células T (CD3+, CD4+ y CD8+), células B y macrófagos en 22 casos de encefalitis con infección natural de MV. En las lesiones leves de tipo linfocítico el antígeno vírico se observó en los manguitos perivasculares donde hubo predominio de células CD8+, mientras que en lesiones graves el antígeno de VMV se encontró de forma dispersa y las células CD4+/CD8+ aparecieron en proporciones similares en los manguitos perivasculares. En las lesiones histiocíticas, se observó un elevado número de macrófagos rodeando los vasos sanguíneos con abundante presencia vírica, situándose los linfocitos CD8+ y CD4+ en la periferia junto con las células B (Polledo *et al.*, 2012a).

3.5.4 Forma articular

Las lesiones articulares producidas por el VMV son muy similares a las producidas por el CAEV. Clínicamente se caracteriza por una tumefacción de las articulaciones, siendo el carpo la localización más frecuente de lesiones. También pueden observarse en las articulaciones del tarso, metacarpo, metatarso, ligamento nucal o uniones vertebrales, siendo en ovino poco frecuentes. Se produce una sinovitis proliferativa junto con fibrosis y calcificación de los tejidos blandos, y necrosis y degeneración del cartílago articular que dan lugar a la aparición de cojera de forma progresiva (Cutlip *et al.*, 1985b; Pérez *et al.*, 2012; Minguijón *et al.*, 2015). Histológicamente, esta lesión crónica se caracteriza por la infiltración de la sinovia por células

mononucleares con la formación de agregados y folículos linfoides (Cutlip *et al.*, 1985b; Minguijón *et al.*, 2015).

En España se ha diagnosticado un brote de forma artrítica en ovejas de raza Rasa Aragonesa, correspondiente a la cepa B2 de los LVPR con genoma completo de CAEV pero con la región pol de la integrasa de tipo VMV. Aunque en dichos animales faltaba una repetición de la secuencia U3 en LTR y había una delección en la región R que se asocia a baja replicación vírica, el fenotipo en fibroblastos de piel de ovino era de tipo *rapid/high* por lo que parece encontrarse adaptado a células ovinas (Glaria *et al.*, 2009).

3.6. DIAGNÓSTICO

Llevar a cabo un buen diagnóstico de la enfermedad es fundamental al no existir vacunas comerciales efectivas frente al MV (Peterhans *et al.*, 2004). Actualmente, la mayoría de los métodos de diagnóstico se basan en la diferenciación de animales infectados y libres de infección.

3.6.1 Clínico y anatomopatológico

Se basa en la observación de los signos clínicos descritos en el apartado anterior junto con la realización de necropsias para la confirmación de los mismos.

Hasta que se produjo el aislamiento vírico, el diagnóstico de los LVPR se basaba en observaciones clínicas y patológicas, que en numerosos casos dieron lugar al nombre de la enfermedad: neumonía ovina progresiva, *bouhite* o maedi referentes al síndrome respiratorio,

o CAEV, referente a la artritis y encefalitis producida por dicha infección en cabras (Minguijón *et al.*, 2015).

Los síntomas clínicos junto con la presencia de lesiones macroscópicas permiten llevar a cabo un diagnóstico fiable en los casos moderados o graves (Luján *et al.*, 1991). Sin embargo, los LVPR pueden extenderse ampliamente en un rebaño o región antes de que se puedan observar signos clínicos, al tratarse de enfermedades de naturaleza lenta y progresiva y solo se detecta la infección en estados avanzados de enfermedad, por lo que es necesaria la realización de otros métodos de diagnóstico más precoces (Minguijón *et al.*, 2015).

3.6.2 Histopatológico e inmunohistoquímico

El diagnóstico histopatológico del MV se basa en la observación de las lesiones descritas anteriormente en el apartado de formas clínicas en pulmón, SNC, glándula mamaria y articulaciones, consistentes en neumonía intersticial, meningoencefalitis y/o mielitis no purulenta, mamitis intersticial o artritis no purulenta, normalmente con la presencia de folículos linfoides. Se han descrito distintas clasificaciones de dichas lesiones en los diferentes órganos diana en función de la gravedad de la inflamación intersticial y de la proliferación linfoide, clasificándose normalmente como lesiones leves (+), moderadas (++) y graves (+++) (Luján *et al.*, 1991; Benavides *et al.*, 2013). En dichos estudios, los porcentajes de lesiones producidas por MV en pulmón y mama de animales seleccionados al azar fueron 32,3% y 35.6% respectivamente considerando únicamente lesiones moderadas y graves (Luján *et al.*, 1991) y 79,3% y 46,7% respectivamente considerando también lesiones de menor gravedad (Benavides *et al.*, 2013).

La IHQ se ha descrito como una técnica eficiente para la detección de proteínas de LVPR en muestras de tejido. Se ha observado positividad a VMV en macrófagos, células epiteliales, células dendríticas y células endoteliales utilizando distintos anticuerpos monoclonales y policlonales frente a proteínas *env* del VMV con diferentes protocolos (Gómez *et al*., 1999; Gelmetti *et al.*, 2000; Preziuso *et al.*, 2003b; Benavides *et al.*, 2006b). La técnica de IHQ resulta muy útil no solo para la identificación vírica y diagnóstico de la enfermedad, sino también para el estudio de la patogenia de la misma. Se ha descrito positividad a VMV mediante IHQ no solo en los órganos diana, sino también en otras localizaciones como linfonodos, (en zona subcapsular y corteza, en macrófagos y células dendríticas), bazo (en la periferia de los folículos) o en médula ósea (en células grandes), órganos que podrían ser reservorios de lentivirus como ocurre en el HIV (Preziuso *et al.*, 2003b). Así mismo, se ha descrito un aumento de la positividad en IHQ en coinfecciones como APO, neumonías crónicas o parasitarias en pulmón (Gelmetti *et al.*, 2000; Preziuso *et al.*, 2003b) o en el testículo en casos de coinfección con *Brucella ovis* en machos (Preziuso *et al.*, 2003a).

Se ha estudiado el efecto de la fijación en el desarrollo de la técnica de IHQ, haciéndose una comparación de 3 anticuerpos monoclonales en muestras fijadas en formol, zinc y Bouin en muestras de pulmón. No se observó diferencia en la intensidad de la señal en los dos primeros fijadores, independientemente del tiempo de fijación, mientras que en el caso del Bouin la IHQ solo fue evaluable con una fijación inferior a 4 días. Así mismo, se observó un mayor número de células descamadas en el tejido fijado con zinc (Benavides *et al.*, 2006b).

3.6.3 Inmunológico

Este método de diagnóstico se basa en la detección de anticuerpos neutralizantes producidos frente al VMV, que son detectables a las 2-3 semanas post-infección (Narayan *et al.*, 1977b; Houwers and van der Molen, 1987), aunque en algunos casos esta respuesta puede tardar varios años en ser detectada (Narayan *et al.*, 1981) o incluso existir animales permanentemente infectados sin presencia de anticuerpos neutralizantes (Narayan *et al.*, 1984). El diagnóstico serológico se considera el más conveniente para la detección de LVPR (de Andrés *et al.*, 2005).

La producción de anticuerpos de forma cruzada frente a antígenos VMV y CAEV ha permitido el diagnóstico de los LVPR durante años, a pesar de la variabilidad antigénica de ambos virus (Gogolewski *et al.*, 1985). Tanto ovejas como cabras permanecen infectados de por vida aunque en ocasiones nunca desarrollen signos clínicos. La presencia de anticuerpos frente a LVPR indica infección pero no es sinónimo de protección, al escapar dichos virus de la respuesta inmune integrándose en el genoma del hospedador en forma de provirus de ADN y producirse mutaciones durante la replicación viral, dando lugar a falsos negativos (Cheevers *et al.*, 1999). Otra causa se la obtención de falsos negativos en un test serológico es el espectro de especificidad del antígeno/anticuerpo involucrados en el test. Así mismo, se pueden obtener falsos positivos a lo largo de la vida de un animal, para lo que no se ha encontrado una explicación (Brinkhof and Van Maanen, 2007), o se pueden producir interferencias debido a la adquisición pasiva de anticuerpos maternos durante la lactación (Cutlip *et al.*, 1988).

Las pruebas más comúnmente utilizadas y propuestas por la OIE (Epizootias., 2008) para el comercio internacional de animales son la IDGA y las pruebas serológicas de inmunoensayo (ELISA). También pueden detectarse anticuerpos frente al VMV mediante otras técnicas tales como la radioinmunoprecipitación (RIPA), radioinmunoensayo (RIA) o el Western

Blot (WB), aunque éstas suelen emplearse como técnicas de confirmación (de Andrés *et al.*, 2005; Herrmann-Hoesing, 2010; Ramírez *et al.*, 2013). En cualquier caso, el diagnóstico viene determinado por la sensibilidad y especificidad de la técnica empleada, la duración de la infección, los títulos de viremia, el estado inmune del hospedador y el fenotipo del virus (Brodie *et al.*, 1998).

3.6.3.1 Pruebas serológicas de tipo ELISA

Es la más utilizada hoy en día al tratarse de una técnica económica de la que existen test comerciales con buena especificidad y sensibilidad, que a su vez dependen en gran medida de la homogeneidad entre el antígeno comercial del kit y el genotipo del VMV (Reina *et al.*, 2009b). Se ha estimado que para conseguir una sensibilidad óptima la mejor combinación de antígeno sería una mezcla de proteínas del núcleo y de la envoltura vírica (de Andrés *et al.*, 2005). Se han descrito diferencias en la sensibilidad dependiendo del status de la enfermedad cuando se evalúan anticuerpos frente a *env* TM (Bertoni *et al.*, 1994; de Andrés *et al.*, 2013) y *env* SU (Knowles Jr *et al.*, 1990; Minguijón *et al.*, 2015) en ovejas y cabras. Los ELISA más utilizados para el diagnóstico de los LVPR son los indirectos, aunque también se han diseñado diferentes ELISA competitivos (de Andrés *et al.*, 2005). De todas formas, en un estudio en el que se valoran dos ELISA de cada tipo, se observan valores de especificidad y sensibilidad superiores al 95% (Herrmann-Hoesing, 2010).

Existe actualmente variedad de test de tipo ELISA disponibles en el mercado frente a VMV/CAEV. Algunos estudios describen los ELISA de virus entero o primera generación como más sensibles y menos específicos que los ELISA de proteínas recombinantes o segunda generación (Zanoni *et al.*, 1994). Los antígenos *gag* CA y *env* TM están muy conservados entre

los LVPR (Grego *et al.*, 2002). Normalmente dichos antígenos se emplean juntos para el desarrollo de ELISA de segunda generación (Saman *et al.*, 1999), aunque también se ha descrito el uso de antígenos *env* TM por separado (Rosati *et al.*, 1994), incluyendo algunos ELISA también *gag* MA (Minguijón *et al.*, 2015). Se han descrito así mismo test ELISA específicos para una determinada cepa que emplean diferentes proteínas recombinantes, péptidos sintéticos o proteínas de regiones variables de *gag* CA, MA y NP (Grego *et al.*, 2002; Lacerenza *et al.*, 2008), *env* TM (de Andrés *et al.*, 2013) o *env* SU (Glaria *et al.*, 2012). Se considera de gran importancia el contar con una combinación de test ELISA que sea capaz de identificar la cepa vírica circulante en una región para evitar falsos negativos para lo que sería necesario profundizar en este tipo de estudios (Cardinaux *et al.*, 2013; Minguijón *et al.*, 2015). Algunos test comerciales disponibles en el mercado actualmente son el test ELITEST® de HYPHEN Biomed que utiliza una combinación de proteínas recombinantes del MVV (Ramírez *et al.*, 2013), los test de IDEXX (screening y verificación) que utilizan proteína recombinante p28 y un péptido TM o los test de Noack que emplean diferentes proteínas recombinantes (p25, gp135) o péptidos sintéticos (TM1). En esta tesis doctoral se utiliza el test ELITEST®, con sensibilidad de 99,4% y especificidad 99,3% (Saman *et al.*, 1999; de Andrés *et al.*, 2005), que emplea el antígeno recombinante formado por un péptido inmunogénico de la glicoproteína transmembrana gp46 y de la proteína recombinante p25.

Además de en sueros individuales, se puede detectar antígeno frente al VMV en leche, calostro o tanque de leche (Julian Motha and Ralston, 1994; Keen *et al.*, 1996; Plaza *et al.*, 2009; Brinkhof *et al.*, 2010; Reina *et al.*, 2010), en un pool de sueros (Brinkhof *et al.*, 2007), o en muestras de semen (Ramírez *et al.*, 2009), lo que permite un diagnóstico rápido y de bajo coste de la infección en el rebaño, especialmente útil para el control de la enfermedad en regiones de escasos recursos.

En general se han descrito los test ELISA como más sensibles que la técnica de la PCR, excepto en animales jóvenes o en infecciones recientes (Álvarez *et al.*, 2006). En estos casos es de gran utilidad la combinación de ambas técnicas, para evitar dejar animales infectados en el rebaño que puedan transmitir la infección (Minguijón *et al.*, 2015).

Sin embargo, como se ha explicado anteriormente en el apartado de inmunología, la presencia de anticuerpos indica infección pero no protección frente a la enfermedad (Cheevers *et al.*, 1999). Por otro lado, animales seronegativos no deben considerarse estrictamente libres de infección ya que el título de anticuerpos puede ser negativo previamente a la seroconversión o a la fluctuación de los mismos que puede suceder durante la vida del animal. Los animales recién nacidos presentan anticuerpos maternos que consumen con el calostro o la leche y que están presentes durante al menos dos o tres meses, siendo normalmente seronegativos hasta que seroconvierten entre los seis y doce meses de edad. Así mismo, llevar a cabo test diagnósticos inmediatamente antes de la gestación no garantiza que las madres no puedan seroconvertir después del parto, siendo siempre los animales seronegativos una fuente potencial de infección tanto para la transmisión horizontal como vertical (de Andrés *et al.*, 2005; Herrmann-Hoesing, 2010; Ramírez *et al.*, 2013). Se han descrito reacciones cruzadas en test ELISA frente a LVPR con diferentes genotipos. Así, el genotipo A codifica antígenos que tienden a detectar mediante reacción cruzada anticuerpos frente al genotipo B con mayor facilidad que en sentido inverso (de Andrés *et al.*, 2013). También se han descrito reacciones cruzadas en animales vacunados contra lengua azul en distintos países europeos utilizando vacunas inactivadas escasamente purificadas, que han dado falsos positivos a test ELISA frente a LVPR (Valas *et al.*, 2011).

3.6.3.1 Pruebas serológicas de tipo IDGA

La inmunodifusión en gel de agar es una prueba que consiste en la difusión a través de un gel de agarosa del suero problema y el antígeno, con sueros control que determinarán la presencia o ausencia de anticuerpos anti-VMV en el suero problema. En el caso del VMV se suele estudiar a simple vista la precipitación de anticuerpos frente a proteínas CA y SU (Winward *et al.*, 1979). Aunque es fácil de realizar y presenta una alta especificidad, su sensibilidad es más baja que la del ELISA (Celer Jr *et al.*, 1998; Synge and Ritchie, 2010) y el hecho de que sea difícil de automatizar y de interpretación subjetiva hace que no sea la técnica más utilizada actualmente para la detección del MV (de Andrés *et al.*, 2005). También se ha evaluado para el diagnóstico de CAEV, observándose resultados similares a los obtenidos en ovino (Knowles Jr *et al.*, 1994). Es menos precoz a la hora de detectar la seroconversión que el test ELISA, por lo que animales recientemente infectados se detectan con mayor dificultad (Luján *et al.*, 1991; Saman *et al.*, 1999; Varea *et al.*, 2001).

La inmunodifusión en gel de agar se ha descrito en muestras de semen mediante la utilización de un test comercial que utiliza anticuerpos frente a la glicoproteína gp135 de la envoltura vírica y la proteína estructural p28 de la cápside (Paula *et al.*, 2009). En dicho estudio experimental se observó la seroconversión en la semana 16 en 4 animales y en la semana 32 en 1 animal, y se obtuvo positividad por PCR anidada en muestras de semen en 2 ovinos previamente a la seroconversión, indicando que la ausencia de positividad en inmunodifusión no descarta la infección y por tanto el riesgo de excreción vírica.

3.6.4 Cultivo

La forma más frecuente de cultivo de VMV es en fibroblastos procedentes de membrana sinovial de cabra y de plexos coroideos de oveja (Minguijón *et al.*, 2015) También algunas líneas celulares continuas de varios tejidos permiten la infección por LVPR (Matsuura *et al.*, 2011).

Otras células que se han descrito como células permisivas al cultivo de VMV son las células dendríticas (Ryan *et al.*, 2000), microglia (Adebayo *et al.*, 2008), células epiteliales de glándula mamaria (Bolea *et al.*, 2006), pulmón (Carrozza *et al.*, 2003), tercer párpado (Capucchio *et al.*, 2003), riñón (Angelopoulou *et al.*, 2006b), útero y epidídimo (Ali Al Ahmad *et al.*, 2012; Lamara *et al.*, 2013), endotelios, miocitos (Leroux *et al.*, 1995; Carrozza *et al.*, 2003), células de la granulosa (Lamara *et al.*, 2001) y células del parénquima del hígado o corazón.

La infección produce un efecto citopático característico y diferentes grados de lisis celular, aunque la infección por VMV se debe confirmar por otras técnicas como microscopía electrónica, IHQ o PCR (Molitor *et al.*, 1979).

3.6.5 Amplificación de ácidos nucleicos

Las técnicas de diagnóstico mediante la amplificación de ácidos nucleicos se basan en la detección de secuencias específicas de ADN o ARN viral. La más comúnmente utilizada es la técnica de la reacción en cadena de la polimerasa o PCR, que permite la detección del virus integrado en el genoma de las células hospedadoras mediante la amplificación de ADN (Brodie *et al.*, 1995; Extramiana et al., 1998). También puede emplearse la variedad rt-PCR, que aunque ha sido menos estudiada que la forma clásica supone un importante progreso en el diagnóstico molecular (Zhang *et al.*, 2000; Gudmundsson *et al.*, 2003).

Se han descrito diferentes *primers* para la detección de LVPR mediante PCR, principalmente de las regiones LTR, tanto en PCR clásica (Extramiana *et al.*, 2002; Benavides *et al.*, 2006b) como anidada (Ryan *et al.*, 2000; Reina *et al.*, 2006), *gag* (Reina *et al.*, 2006) y *pol* (Pisoni *et al.*, 2005; Reina *et al.*, 2006).

La detección mediante PCR de VMV suele hacerse a partir de células mononucleares sanguíneas (de Andrés *et al.*, 2005; Reina *et al.*, 2009a), aunque en ocasiones el bajo número de células infectadas circulantes reduce la sensibilidad (Grego *et al.*, 2007). La técnica de la PCR ha descrito en diferentes tejidos, en sangre, leche o semen (Extramiana *et al.*, 2002; Peterson *et al.*, 2008; Cortez-Romero *et al.*, 2011). También es frecuente la realización de la PCR en bloques de parafina de archivo (Gómez *et al*., 1999; González *et al.*, 2001). Se ha estudiado la influencia de diferentes fijadores en los resultados de las PCR realizadas en este tipo de muestras. Así, los bloques de tejido fijado en formol dieron resultados positivos con una fijación inferior a 14 días. Esto podría deberse a que el formaldehido produce la desnaturalización del ADN en zonas ricas en AT creando mutaciones artificiales y aumentando estos efectos secundarios con el tiempo. Los bloques de tejido fijados en Zn dieron resultados positivos hasta con 30 días de fijación, mientras que en los bloques de tejido fijado en Bouin no se pudo amplificar ADN proviral por PCR (Benavides *et al.*, 2006b).

Actualmente se están empleando otras técnicas de amplificación de ácidos nucleicos como la `` heteroduplex mobility assay´´ o HMA (Germain and Valas, 2006), que se utiliza normalmente en combinación con la PCR para ayudar a la caracterización genotípica de las cepas víricas que circulan en un rebaño (Ramírez *et al.*, 2013) o la ``loop-mediated isothermal amplification´´ (LAMP) que se ha desarrollado para la detección de ADN de provirus de CAEV (Huang *et al.*, 2012).

3.7. PREVENCIÓN Y CONTROL

El gran impacto económico que causa el MV, especialmente en la producción ovina de leche, hace que sea fundamental llevar a cabo estrategias de prevención y control para evitar la diseminación de la infección (Polledo *et al.*, 2013).

Actualmente, no existen vacunas disponibles en el mercado frente a VMV y, aunque se han probado vacunas con el virus completo atenuado o vacunas recombinantes, con plásmidos o proteínas víricas, aún no ha habido ninguna que haya resultado eficaz en la protección frente al virus (Pépin *et al.*, 1998; Torsteinsdottir *et al.*, 2007b; Reina *et al.*, 2008). Se ha observado tras la infección por vía intratraqueal con VMV atenuado que, aunque la vacuna no produce protección total frente a la infección, sí induce una protección parcial, aislándose hasta 10 veces más virus en animales no vacunados que en animales protegidos por la vacuna (Pétursson *et al.*, 2005). También se ha probado en ratón una vacuna ADN cuyo plásmido codifica la proteína inmunogénica p25 del VMV que parece obtener una buena respuesta humoral con un alto título de anticuerpos (Henriques *et al.*, 2009), además de haberse realizado pruebas con diferentes adyuvantes para la estimulación de la respuesta inmune y una mejor respuesta a la vacuna (de Andrés *et al.*, 2009). Sin embargo, aún no se han aplicado en condiciones de campo y su elaboración supone unos costes elevados, por lo que económicamente no suponen una alternativa viable en la lucha frente a la enfermedad.

Debido a la ausencia de tratamientos y vacunas efectivas frente al MV, los programas de control, como los que se llevaron a cabo en el brote de Islandia y que consiguieron la erradicación de la enfermedad, son la herramienta básica para el control del VMV en la actualidad. En esta época, las medidas que se tomaron para eliminar la infección fueron fundamentalmente dos: el sacrificio de los rebaños infectados y el reemplazo de los mismos con animales procedentes de rebaños libres de infección tras un tiempo prudente de vacío

sanitario (Houwers *et al.*, 1984; Pétursson, 1994), y la división de los rebaños en dos grupos, uno compuesto por el rebaño adulto, y el otro compuesto por el grupo de reposición cuyos corderos están retirados inmediatamente después del parto (Pétursson, 1994). Sin embargo, es importante conocer la prevalencia inicial de una explotación a la hora de llevar a cabo estrategias de sacrificio de animales como las que se siguieron en el brote de Islandia. Así, el sacrificio es útil en rebaños de baja o moderada prevalencia (<50%), donde el número de ovejas que pueden seroconvertir es mayor al de ovinos que se sacrifican y reemplazan anualmente (Berriatua *et al.*, 2003), mientras que en zonas como Castilla y León donde la prevalencia supera el 80% esta estrategia presenta serias dificultades al ser económicamente inviable.

En países como Holanda, Alemania, Francia o Italia, se realizaron programas de control menos drásticos, basados en la aplicación de medidas combinadas (Luján et al., 2001), entra las que se encontraron la eliminación de animales seropositivos, junto con la separación de los corderos nada más nacer para su alimentación con calostro/leche de hembras seronegativas o calostro bovino (Houwers *et al.*, 1983; Houwers *et al.*, 1989) ; el sacrificio de animales seropositivos tras la realización periódica de estudios serológicos (Cutlip and Lehmkuhl, 1986); el sacrificio de la progenie de madres seropositivas (inmediatamente después nacimiento) si el porcentaje de prevalencia y la tasa de reposición lo permitían, por las altas probabilidades de infección madre-cordero (Houwers *et al.*, 1989); y formación de dos rebaños uno seropositivo y otro seronegativo si la medida anterior no era posible, y las instalaciones y manejo lo permitían (Luján *et al*., 2001; Pérez *et al*., 2013).

En el valle del Ebro se ha ideado un método de control aplicado con éxito en rebaños de prevalencias moderadas (alrededor del 50%). Consiste en un único muestreo serológico de todo el rebaño y el marcado indeleble de las ovejas seropositivas para posteriormente utilizar como reposición los animales seronegativos e hijas de seronegativas, junto a la vigilancia de

cualquier signo clínico característico de la enfermedad y que conducirá al sacrificio precoz del ovino afectado (Luján et al., 2001).

Como se ha mencionado anteriormente, las dos fuentes más importantes de contagio del VMV entre animales son la vía horizontal, por contacto con ovejas infectadas, y la lactación natural, a través de calostro o leche (Berriatua *et al.*, 2003; Blacklaws *et al.*, 2004; Peterhans *et al.*, 2004; Leginagoikoa *et al.*, 2010). Por lo tanto, las prácticas de manejo, especialmente las relacionadas con el alojamiento de los animales y la densidad de los mismos, están altamente asociados con la prevalencia del MVV en el rebaño (Leginagoikoa *et al.*, 2006; Pérez *et al.*, 2010; Polledo *et al.*, 2013) Así, Polledo *et al*., han demostrado mediante un estudio realizado con 197 ovejas de raza Assaf Española pertenecientes a un rebaño con una seroprevalencia de VMV superior al 90%, que la simple colocación de una valla de metal de 1,20 metros que impida el contacto físico entre los corderos y las madres a pesar de compartir la misma nave, es suficiente para bajar la prevalencia de forma notable (de 93,1% a 54,2% en 4 años).

IV. Estandarización y puesta a punto de técnicas

4.1 INMUNOHISTOQUÍMICA

Para la realización de los estudios incluidos en esta tesis doctoral fue necesaria la detección de antígeno vírico de VMV en muestras de tejido incluido en parafina. Hasta la realización de esta tesis, la detección del VMV en nuestro laboratorio se realizaba utilizando los anticuerpos elaborados por nuestro grupo anti-gp135 y anti-p28, ambos policlonales. Ambos anticuerpos caseros se obtuvieron inmunizando conejos blancos de Nueva Zelanda con las proteínas gp135 y p28 de Pourquier (1,6 g) emulsionadas con 1 ml de adyuvante completo de Freund en los días 1, 14, 21 y 28. En el día 35 se administró por vía intravenosa la proteína en ausencia de adyuvante. Se utilizaron los sueros del día 38 y el pre-inmune del mismo conejo para los estudios inmunohistoquímicos. Se evaluó la sensibilidad y la especificidad de ambas técnicas en 10 ovinos con resultados positivos y negativos conocidos a MV.

Sin embargo, dichos anticuerpos funcionaban únicamente en nuestro laboratorio únicamente en muestras de tejido fijadas en semifijadores como Zinc y Bouin o en muestras congeladas y no en muestras fijadas rutinariamente en formol.

Durante esta tesis doctoral, en la primera colaboración con la Universidad de Camerino realizada en 2016, se pusieron a punto las técnicas de inmunohistoquímica para la detección de VMV en muestras fijadas en formol con el equipo de la profesora Preziuso y del profesor Rossi. En concreto, se utilizó el anticuerpo monoclonal comercial aniti-p28 (CAEP5A1, VMRD), así como los anticuerpos policlonales anti-gp135 y anti-p28 disponibles en nuestro laboratorio.

Después de diferentes pruebas, se estableció el protocolo de IHQ descrito a continuación, válido para los tres anticuerpos descritos anteriormente:

1. **Fijación, tallado e inclusión de las muestras:** recogida de las muestras de forma rutinaria y fijación durante 48 horas en formol tamponado al 10%. Posteriormente se tallan secciones de unos 0,5 cm de grosor y se introducen en un inclusor donde se

deshidratan con un ciclo rutinario de inclusión histológica mediante pases por alcoholes de graduación creciente, xilol y parafina a 60°C.

2. **Corte de las muestras:** se realizan secciones de 3 micras con microtomo, que se colocan sobre portaobjetos previamente tratados con un homopolímero (Poli-L-lysine, Sigma-Aldrich) para evitar el desprendimiento del tejido durante la realización de la IHQ. Se dejan secar las preparaciones en una estufa a 37°C durante 24 horas.
3. **Desparafinado:** se desparafinan las preparaciones dejándolas 15 minutos en xilol (x2), 15 minutos en alcohol de 100° (x2), 5 minutos en alcohol de 96°, 5 minutos en alcohol de 60° y 5 minutos en agua.
4. **Inactivación de la peroxidasa endógena:** se realiza mediante un tratamiento con peróxido de hidrógeno al 5% en agua destilada durante una hora.
5. **Desenmascaramiento antigénico:** tras el lavado de las preparaciones se realiza el desenmascaramiento antigénico en una solución de citrato a pH6, durante 11 minutos en microondas a 750W al baño María, y con un enfriamiento posterior de 10 minutos en agua fría.
6. **Bloqueo de uniones inespecíficas:** después de lavar las preparaciones en TBS se incuban con suero normal de cabra diluido a 1:10 en TBS+BSA1%+PVP1% durante una hora en cámara húmeda a temperatura ambiente.
7. **Incubación con el anticuerpo primario:** se incuba durante toda la noche en cámara húmeda a 4°C con el anticuerpo primario. El anticuerpo monoclonal anti-p28 (CAEP5A1, VMRD) se usa a una dilución de 1:100 en TBS+BSA1%+PVP1%; el anticuerpo policlonal anti-p28 (elaboración propia) se usa a una dilución de 1:10.000 en TBS+BSA1%+PVP1%, y el anticuerpo policlonal anti-gp135 (elaboración propia) se usa a una dilución de 1:20.000 en TBS+BSA1%+PVP1%.
8. **Incubación con el anticuerpo secundario:** después de hacer dos lavados de las preparaciones en TBS, se incuban durante una hora en cámara húmeda a temperatura

ambiente con el anticuerpo secundario. En el caso del anticuerpo monoclonal comercial se emplea un anticuerpo secundario biotinilado anti-mouse hecho en cabra, mientras que en los dos anticuerpos policlonales de fabricación propia se utiliza un anti-rabbit hecho en cabra, todos ellos a una dilución de 1:200 en TBS+BSA1%+PVP1%.

9. **Incubación con solución ABC:** se incuba durante 45 minutos con la solución ABC (Elite, VECTOR), preparada con 45 minutos de antelación siguiendo las instrucciones del fabricante.
10. **Revelado:** tras lavar las preparaciones en TBS se hace el revelado utilizando la solución DAB (VECTOR) durante 10 minutos.
11. **Tinción y montaje:** se tiñen las preparaciones con hematoxilina durante 30 segundos, se lavan, se deshidratan en pases sucesivos de alcoholes de graduación creciente y en xilol y se montan siguiendo el procedimiento rutinario.

4.2 PCR

Para la realización de esta tesis doctoral fue necesaria la puesta a punto de una técnica de PCR que permitiera la detección del VMV en muestras de tejido congelado, en bloques de parafina, y en muestras de leche, para lo que se trabajó con el equipo de la doctora Preziuso en la Universidad de Camerino. Se estudiaron las secuencias víricas circulantes en la región de Castilla y León y se hicieron diferentes pruebas, en tejido congelado y en cortes de bloques de parafina, utilizando diversos *primers* descritos hasta el momento y utilizados por el equipo de la doctora Preziuso con anterioridad.

4.2.1 Extracción de ADN proviral.

En muestras de **tejido congelado** se usó el kit Norgen (Biotek Corporation) mediante el siguiente protocolo:

1. Preparación de la muestra: corte con material estéril de un fragmento de tejido de aproximadamente 20 mg, que se coloca en un eppendorf estéril libre de ADNsas.
2. Lisis: incubación de cada muestra con 300 µl de *digestion buffer* y 12 µl de proteinasa K a 55° hasta la disolución completa del tejido. Posteriormente, se añaden 300 µl de *binding solution* y 300 µl de etanol al lisado y se mezcla bien mediante vórtex.
3. Unión a la columna: se añaden 500 µl del preparado anterior a la columna, situada sobre un tubo estéril y se centrifuga durante 3 minutos a 8.000 rpm. Se elimina el sobrenadante y se repite el proceso, añadiendo el resto del preparado obtenido en el paso 2. Centrifugación final a 14.000 rpm durante dos minutos.
4. Lavado del ADN: se añaden 500 µl de *wash solution* a la columna y se centrifuga 2 minutos a 14.000 rpm. Se repite el proceso y se desecha el sobrenadante.
5. Elución del ADN puro: tras el paso de la columna a un nuevo tubo estéril, se añaden 200 µl de *elution buffer,* previamente calentado a 55°, al centro de la columna. Después de una incubación de 5 minutos a temperatura ambiente, se centrifuga durante un minuto a 6.000 rpm y posteriormente durante dos minutos a 14.000 rpm. Una vez eliminada la columna, se puede conservar el ADN extraído a 2-8 °C para su uso inmediato o a -20°C para su conservación a largo plazo.

En muestras de **tejido embebidas en parafina** se empleó el kit Ambion RNA (Life Technologies), utilizando el protocolo que se explica a continuación:

1. Preparación de la muestra: corte con microtomo de 6 secciones de 10 micras, que se colocan en un eppendorf estéril libre de ADNsas.
2. Desparafinado: se realiza mediante la incubación en 1 ml de xilol durante 5 minutos y centrifugado a 14.000 rpm durante 2 minutos, tirando el sobrenadante y conservando el pellet de tejido (x2).
3. Lavado de la muestra: se añade 1 ml de etanol, se mezcla mediante vórtex y se centrifuga durante 2 minutos a 14.000 rpm, eliminando el sobrenadante (x2). Se deja secar durante 4 horas para que se evapore todo el alcohol restante.
4. Lisis: incubación de cada muestra con 200 µl de *digestion buffer* y 4 µl de proteinasa K a 50° durante toda la noche (mínimo 16 horas), moviendo de vez en cuando para facilitar la digestión.
5. Aislamiento del ADN: se añaden 240 µl de *isolation additive* y 550 µl de etanol a cada eppendorf.
6. Unión a la columna: se pasan 700 µl del preparado anterior por la columna, situada sobre un tubo estéril, y se centrifuga durante 1 minuto a 10.000 rpm, descartando el sobrenadante.
7. Lavado del ADN: se añaden 700 µl de *wash solution* 1 a la columna y se centrifuga 1 minuto a 10.000 rpm, descartando el sobrenadante. Se realiza un segundo lavado añadiendo 500 µl de *wash solution* 2/3 y se centrifuga 1 minuto a 10.000 rpm. Después de eliminar el sobrenadante, se hace una última centrifugación igual a las anteriores para eliminar cualquier resto de solución de lavado.
8. Elución del ADN puro: tras el paso de la columna a un nuevo tubo estéril, se añaden 60 µl de *elution buffer,* previamente calentado a 95°, al centro de la columna. Después de una incubación de 1 minuto a temperatura ambiente, se centrifuga durante 2 minutos a 14.000 rpm. Se elimina la columna y se conserva el ADN extraído a 2-8 °C para su uso inmediato o a -20°C para su conservación a largo plazo.

4.2.2 Obtención de células blancas de leche para extracción de ADN.

Para la separación de las células blancas de muestras de **leche** se utilizó el protocolo que se describe a continuación:

1. Se añaden 50 ml de leche de tanque o 15 ml de leche de animales individuales en un Falcon.
2. Se centrifuga a 2.000 rpm durante 20 minutos a 10°C.
3. Se elimina la nata y parte del líquido con pipeta Pasteur, dejando el pellet y unos 10 ml de leche.
4. Se centrifuga a 2.000 rpm durante 10 minutos a 10°C.
5. Se desecha el sobrenadante dejando el pellet y unos 2 ml de leche. Se resuspende el pellet con pipeta Pasteur.
6. Se transfieren 1,5 ml del líquido anterior a un eppendorf de 2 ml estéril y libre de ADNsas.
7. Se centrifuga a 14.000 rpm durante 10 minutos a 10°C.
8. Se elimina todo el líquido posible con micropipeta, dejando el pellet.
9. Se resuspende el pellet en 1,5 ml de PBS 1X estéril mediante pipeta Pasteur.
10. Se centrifuga a 14.000 rpm durante 5 minutos a temperatura ambiente. Se elimina el sobrenadante.
11. Se repiten los pasos 9 y 10 hasta un total de 3 lavados.
12. Se congela el pellet a -20°C o -80°C.

4.2.3 PCR clásica y anidada.

Se probaron diferentes protocolos de PCR clásica y anidada utilizando distintos primers de las regiones LTR, *gag* y *pol*. Se utilizaron los siguientes protocolos ya descritos con anterioridad en muestras de tejido congelado de 6 animales y un control positivo y en muestras de tejido embebido en parafina de 9 animales y un control positivo.

LTR (Extramiana et al., 2002, Benavides et al., 2006b)

Mastermix	25
LTR_{fw}	2,5
LTR_{rv}	2,5
H_20	18
ADN	2

Termociclador (temperatura en °C/tiempo):

95x5min

95x30s 58x30s72x40s (35 ciclos)

72x7min

LTR externa (Ryan et al., 2000, Reina et al., 2006)

Mastermix	25
LTR_{e-f}	2,5
LTR_{e-r}	2,5
H_20	18
ADN	2

Termociclador (temperatura en °C/tiempo):

94x5min

94x30s 55x1min 72x1min (40 ciclos)

72x7min

LTR interna (Ryan et al., 2000, Reina et al., 2006)

Mastermix	25
LTR_{i-f}	2,5
LTR_{i-r}	2,5
H_20	18
ADN	2

Termociclador (temperatura en °C/tiempo):

94x5min

94x30s 50x30s 72x1min (40 ciclos)

72x7min

LTR anidada (Ryan et al., 2000, Reina et al., 2006)

Mastermix	25
LTR_{e-f}	2,5
LTR_{e-r}	2,5
H_20	18
ADN	2

Termociclador (temperatura en °C/tiempo):

94x5min

94x30s 55x1min 72x1min (40 ciclos)

72x7min

Mastermix	25
LTR_{i-f}	2,5
LTR_{i-r}	2,5
H_20	19
ADN	1

Termociclador (temperatura en °C/tiempo):

94x5min

94x30s 50x30s 72x1min (35-40 ciclos)

72x7min

LeNestup (Elthair et al., 2006)

Mastermix	25
$LeNest_{f}$	2,5
$LeNest_{r}$	2,5
H_20	18
ADN	2

Termociclador (temperatura en °C/tiempo):

94x5min

94x30s 60x30s 72x40s (45 ciclos)

72x7min

Pol (Reina et al., 2006)

Mastermix	25
Pol_{-4231}	2,5
Pol_{-4448}	2,5
H_20	18
ADN	2

Termociclador (temperatura en °C/tiempo):

94x5min

94x30s 55x30s 72x40s (45 ciclos)

72x7min

Pol EFW

Mastermix	25
Pol_{-fw}	2,5
Pol_{-rv}	2,5
H_20	18
ADN	2

Termociclador (temperatura en °C/tiempo):

94x5min

94x30s 55x30s 72x40s (45 ciclos)

72x7min

Gag-Pol externo (Grego et al., 2007)

Mastermix	25
M1	2,5
M2	2,5
H_20	16
ADN	4

Termociclador (temperatura en °C/tiempo):

94x5min

94x1min 55x1min 72x2min (35-40 ciclos)

72x7min

Gag-Pol anidada (Grego et al., 2007)

Mastermix	25
M1	2,5
M2	2,5
H_20	16
ADN	4

Termociclador (temperatura en °C/tiempo):

94x5min

94x1min 55x1min 72x2min (35-40 ciclos)

72x7min

Mastermix	25
M3	2,5
M4	2,5
H_20	16
ADN	4

Termociclador (temperatura en °C/tiempo):

94x5min

94x1min 55x1min 72x2min (35-40 ciclos)

72x7min

Wie5FW (Reina et al., 2006)

Mastermix	25
$Wie5_{fw}$	2,5
$Wie5_{rv}$	2,5
H_20	18
ADN	2

Termociclador (temperatura en °C/tiempo):

94x5min

94x30s 52x30s 72x40s (45 ciclos)

72x7min

Se obtuvieron resultados positivos en muestras congeladas con los protocolos de LTR descritos por Ryan et al. (2000), Rosati et al. (1999) y Grego et al. (2007). En bloques de parafina, la positividad se observó únicamente utilizado primers de las regiones LTR (de entre 200 y 300 pb), mientras que se obtuvieron resultados negativos utilizando primers de la región *gag* (de 744 pb).

Debido a la ausencia de resultados positivos o resultados positivos débiles en muchas muestras cuya positividad se había demostrado mediante IHQ, se realizó un estudio detallado de las secuencias víricas conocidas de nuestra región y se diseñó un nuevo primer, que se probó con el protocolo que se describe a continuación:

LTR Preziuso

Mastermix	25
LTR_{Prez-f}	2,5
LTR_{Prez-r}	2,5
H_20	18
ADN	2

Termociclador (temperatura en °C/tiempo):

94x5min

94x30s 52x30s 72x40s (40-45 ciclos)

72x7min

LTR Preziuso (Anidada con LTRe-Ryan)

Mastermix	25
LTR_{e-f}	2,5
LTR_{e-r}	2,5
H_20	18
ADN	2

Termociclador (temperatura en °C/tiempo):

94x5min

94x30s 55x1min 72x1min (40 ciclos)

72x7min

Mastermix	25
LTR_{Prez-f}	2,5
LTR_{Prez-r}	2,5
H_20	18
ADN	2

Termociclador (temperatura en °C/tiempo):

94x5min

94x30s 52x30s 72x40s (40-45 ciclos)

72x7min

Se observó que con los nuevos *primers* rediseñados con la doctora Preziuso se obtuvieron resultados positivos en todos los controles positivos y resultados negativos en los controles negativos (determinados por IHQ). Así mismo, la positividad fue más limpia, disminuyendo notablemente las colas de *primers* presentes con los protocolos descritos anteriormente, al permitir una unión más específica a las secuencias de interés.

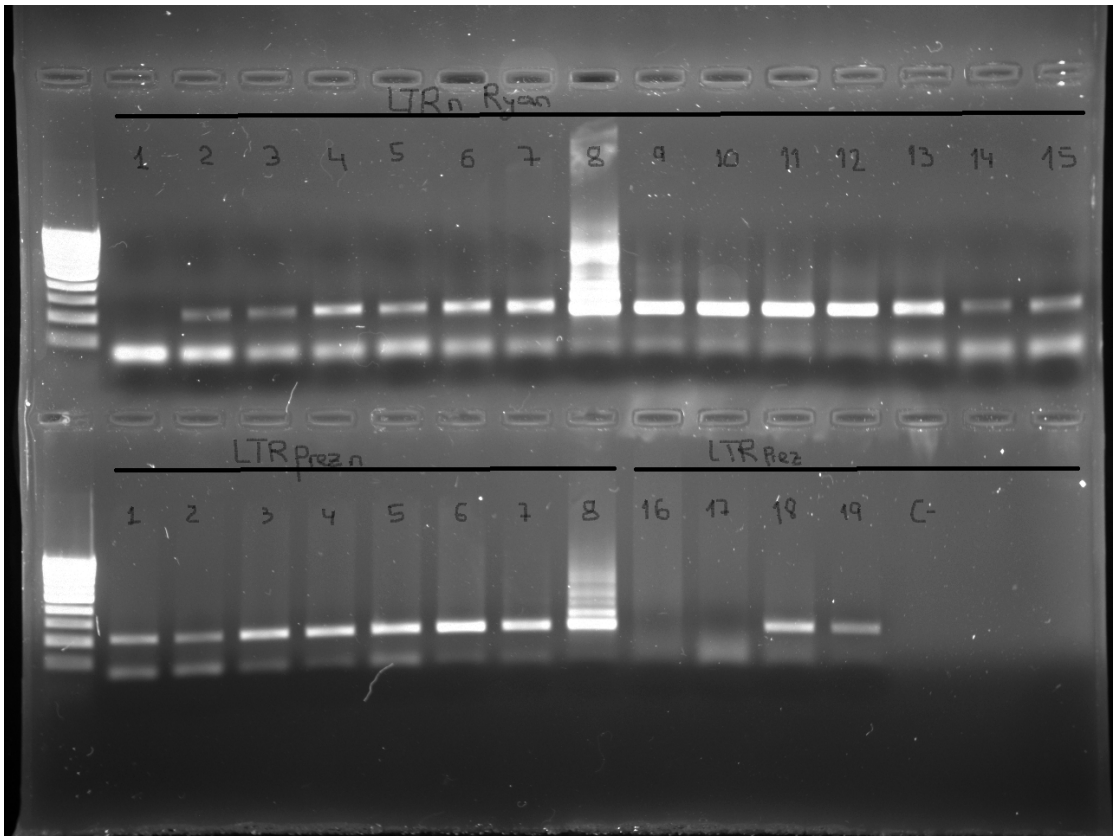

***Fig.7**: PCR con protocolos LTR anidada (Ryan et al., 2000) y LTR anidada y clásica (nuevos primers diseñados con la doctora Preziuso). Las muestras 8-12 se corresponden a tejido congelado mientras que el resto a cortes de bloques de parafina.*

4.3 TÉCNICA DE CITOLOGÍA EN LECHE

Para la optimización de la técnica de citología en muestras de leche se consideraron tres variables: la velocidad de centrifugación, el tiempo de centrifugación, el volumen de las muestras y los tiempos de secado y fijación.

Se utilizaron muestras de leche de tanque (n=10) procedentes de la granja de la Universidad de León. Se recogieron en tubos Falcon de 12 ml que fueron centrifugados (Orto® and p-Selecta®) probando los tiempos/velocidades que se indican en la tabla 1.

Tiempo de centrifugación (min)	**Velocidad de centrifugación (rpm)**
20	2000
15	2000
10	2000
5	2000
20	1500
15	1500
10	**1500**

Tabla 1: *combinaciones de tiempo y velocidad de centrifugación. Marcada la combinación óptima*

Posteriormente se recogieron muestras de 500 µl en un vial de 1 ml de tres localizaciones para localizar la región con más concentración de células y menos glóbulos grasos. Estas tres muestras se dividieron en diferentes volúmenes (150, 100, 80, 50 and 30 µl) y se citocentrifugaron (Cytospin 3®) utilizando diferentes tiempos de centrifugación a 1000 rpm siguiente las instrucciones del fabricante (Tabla 2).

Volumen (µl) de la parte superior del tubo	**Tiempo de citocentrifugación (min)**	**Velocidad de citocentrifugación (rpm)**
150	4	1000
150	3	1000
100	4	1000
100	3	1000
80	**4**	**1000**

80	3	1000
50	**4**	**1000**
50	3	1000
30	4	1000
30	3	1000
Volumen (µl) del medio del tubo	**Tiempo de citocentrifugación (min)**	**Velocidad de citocentrifugación (rpm)**
150	4	1000
150	3	1000
100	4	1000
100	3	1000
80	4	1000
80	3	1000
50	4	1000
50	3	1000
30	4	1000
30	3	1000
Volumen (µl) del fondo del tubo Falcon	**Tiempo de citocentrifugación (min)**	**Velocidad de citocentrifugación (rpm)**
150	4	1000
150	3	1000
100	4	1000
100	3	1000
80	**4**	**1000**
80	3	1000
50	**4**	**1000**
50	3	1000
30	4	1000
30	3	1000

***Tabla 2**: volúmenes de leche recogidos de las diferentes partes del tubo Falcon. Marcadas las combinaciones óptimas.*

Se probaron diferentes tiempos de secado después de la citocentrifugación : 10, 15, 20, 30, 50 and 60 minutos, así como diferentes tiempos de fijación con metanol: 10, 20, 30, 40, 50 y 60 minutos, ambos a temperatura ambiente. Se probaron el lavado con peróxido de hidrógeno al 3% en agua destilada después de la fijación durante 10 y 30 minutos para eliminar los glóbulos grasos, así como diferentes sucesivos con PBS. Todas las muestras se

tiñeron con hematoxilina y eosina (HE) y con May-Grunwald-Giemsa y se examinaron mediante microscopio. Se probó la dilución de 400 µl de leche de las tres localizaciones testadas, centrifugando, eliminando el sobrenadante y añadiendo 10 µl de PBS al 1%. La citocentrifugación se realizó utilizando el mismo protocolo descrito previamente en muestras de leche. También se probó a hacer preparaciones sin citocentrifugación con diferentes volúmenes (100, 80, 50, 35, 25 and 10 µl) utilizando micropipeta. Las muestras se fijaron con metanol tanto para estudios citológicos como inmunohistoquímicos.

4.4 TÉCNICA DE INMUNOCITOQUÍMICA EN MUESTRAS DE LECHE

La técnica de inmunocitoquímica en muestras de leche se realizó utilizando el mismo protocolo que en muestras de tejido a partir de preparaciones citocentrifugadas fijadas en metanol como se describe anteriormente.

V. Primer trabajo

PRIMER TRABAJO

Serological ELISA results are conditioned by individual immune response in ovine maedi visna. *Small Rumin Res* 2017, Vol 157, 27–31

E. Gayo[a,*], L. Polledo[a], S. Preziuso[b], G. Rossi[b], A. Balseiro[c], C. Pérez Martínez[a], M.J. García Iglesias[a], J.F. García Marín[a]

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Research Paper

Serological ELISA results are conditioned by individual immune response in ovine maedi visna

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ABSTRACT

The marked differences in sensitivity existing among maedi visna ELISA tests is a striking issue in the control of the disease, which so far have mainly been related to the different circulating viral strain or to the particular test used. The aim of this work is to discern whether or not the ELISA results could also be associated to different histological lesion patterns and therefore conditioned by individual immune response. Fifty infected animals and eight negative controls were used and histological, inmunohistochemical, PCR and serological studies were performed. Histological patterns were classified based on previous described immunophenotypical criteria: a lymphocytic pattern, characterized by a clear predominance of T cells, especially CD8+ T cells (n = 19), and a histiocytic pattern, with a high quantity of macrophages mixed with B cells (n= 23). A third mixed pattern characterized by a mixed inflammatory infiltration was observed (n = 8), predominantly in animals with minimal lesions with no clinical signs being observed (75%). An association between these lesion patterns and the ELISA optic density values exists ($p < 0.001$). Sheep with a histiocytic pattern (n =21) showed higher titers of antibodies compared to sheep with lymphocytic pattern (n =18), where values were much lower or even negative. Animals with histiocytic pattern are easily recognizable using the ELISA test, while sheep showing lymphocytic lesion pattern could go unnoticed in the flock as serological false negative animals, being a likely remaining source of infection. Animals with mixed pattern showed mixed values, and despite showing only minimal lesions, they are also carriers of the virus and can be easily underdiagnosed.

Keywords: Maedi Visna, ovine, serology, lesion pattern, immunopathology.

INTRODUCTION

Ovine Maedi Visna (MV) is a widespread disease caused by the lentivirus Visna/maedi virus (VMV) and causes direct loses in sheep production (Benavides *et al.*, 2013; Minguijón *et al.*, 2015). MV is characterized by a slow but progressive infection in sheep, resulting in a chronic inflammation of lung, mammary gland and central nervous system (CNS) as well as progressive weight loss (Cutlip *et al.*, 1988; Dawson, 1987). Histological changes mainly correspond to a chronic, interstitial inflammation of the lungs and mammary glands and to a non-suppurative encephalitis and demyelination of the CNS (Benavides *et al.*, 2009; Luján *et al.*, 1991; Minguijón *et al.*, 2015). Sub-clinical infections cause high viral spread among flocks and it is estimated that individual prevalence of VMV infection in Spanish Assaf dairy sheep kept in an intensive indoor farming system is could reach 77% (Leginagoikoa *et al.*, 2006).

No commercial vaccines are currently available and only adequate control programs can be used to limit the spreading of the virus or eradicate the disease (Polledo *et al.*, 2013). Early detection of infected animals from the flock by using antibody detection methods such as ELISA tests have been described as the most appropriate tool to use in MV control programs (de Andrés *et al.*, 2005; Minguijón *et al.*, 2015; Patel *et al.*, 2012). However, striking differences in sensitivity among MV ELISA tests has been detected up to now relating them to the different circulating viral strain or the particular test used, but not to the individual immunological response (de Andrés *et al.*, 2005; de Andrés *et al.*, 2013).

Individual immune response against VMV has been suggested to play a major role in the pathogenesis of the disease (Blacklaws, 2012; Polledo *et al.*, 2012a; Polledo *et al.*, 2012b; Torsteinsdóttir *et al.*, 2007; Torsteinsdóttir *et al.*, 1992). A possible link between the humoral response and the lesions has been suggested in neurological forms (Polledo *et al.*, 2012b) where two main lesion patterns were described with regard to the inflammatory infiltrate: a *lymphocytic pattern*, characterized by a clear predominance of T cells, especially CD8+ T cells,

and a *histiocytic pattern*, with a predominance of histiocytic cells mixed with B cells. The former could be related to a cytotoxic cellular immune response with a low antibody titer and the latter to a stronger humoral response with higher antibody titer. The aim of this study is to evaluate the correlation between individual serologic response against VMV and the histological lesion pattern. The results should be considered when diagnosing and controlling of MV disease, especially in infected but negative animals in serological tests.

MATERIAL AND METHODS

Animals

Fifty-eight adult Spanish Assaf sheep submitted to the Pathology Diagnostic Service of the School of Veterinary Medicine (León, Spain) for routine necropsies were used in this study. Fifty animals came from different intensive milk-producing flocks previously diagnosed with MV, while eight sheep came from non-infected flocks and were selected as negative controls. MV characteristic clinical signs were observed in 31 sheep, especially nervous and respiratory signs. The animals used in this study did not show macroscopic or histological lesions compatible with other pathologies such as bacterial, fungal or parasitic pneumonias or mastitis.

Sampling and Histopathology

Tissue samples from the 58 sheep were systematically taken for histopathology from diaphragmatic and apical lung lobes, glandular parenchyma and udder cisterns and CNS from 9 levels of the brain and 9 levels of the spinal cord following a previous description (Polledo *et al.*, 2012a) . All the samples were fixed in 10% neutral buffered formalin for 48 h at room

temperature. After fixation, samples were embedded in paraffin wax and sections (4 µm) were stained with hematoxylin and eosin (HE) and examined using light microscopy.

On microscopic examination, histiocytic and lymphocytic patterns were considered as previously described (Polledo *et al.*, 2012b). A mixed inflammatory infiltrate composed of macrophages and lymphocytes with no clear predominance of any of the cellular populations was also taken into account. Lesion patterns were studied in lung, mammary gland and CNS of all the animals included in this study, taking into account the predominant lesion pattern in the three target organs of each individual sheep.

Immunohistochemistry

After fixation, lung, mammary gland and CNS samples from the 58 animals studied were dehydrated through graded alcohol and embedded in paraffin wax and 4 µm sections were prepared. The following antibodies were used: polyclonal anti-CD3 for T cells (Dako, Denmark); monoclonal anti-CD79 for B cells (Dako, Denmark); monoclonal anti-CD68 for macrophages (Dako, Denmark), and monoclonal anti-p28 of CAEV/VMV (VMRD, USA) for viral detection. EnVision+ system (EnVision+ System Labelled Polymer-HRP anti-mouse or anti-rabbit; Dako, Denmark) and diaminobezidine solution (DAB) (Vector Laboratories, Burlingame, California, USA) were used for anti-CD3, CD79, and CD68 antibodies. An avidin-biotin-peroxidase complex (ABC) technique (Vectastain Elite, ABC Kit; Vector Laboratories, USA) previously described was used for anti-p28 of CAEV/VMV (Preziuso *et al.*, 2003b). The slides were counterstained with haematoxylin and mounted. The specificity of the technique was evaluated using positive and negative controls.

Polymerase Chain Reaction

PCR technique was performed as put forward by Ryan et al. (2000). Lung samples from 14 infected sheep were tested by PCR. Lung samples of 5 negative controls were also studied. Genomic DNA was extracted from paraffin-embedded tissue samples using QIAamp® DNA Mini Kit (QIAGEN).

Serology analysis

Serum samples were obtained from the 58 sheep to evaluate the presence of antibodies against VMV using a standardized comercial kit test (*Elitest*®, Hyphen BioMed, Neuville-Oise, France) following the manufacturer's instructions. Enzyme linked immunosorbent assay (ELISA) results were reported as positive or negative on the basis of the cut-off value calculated following the manufacturer's instructions. The cut-off point was established at 0.4±0.1. The optical density (OD) values were used as a semi-quantitative measure of anti-VMV antibody levels.

Statistical Analysis

One-way ANOVA was used to test whether data on the lesion patterns depended on their serological values or not. Newman-Keuls multiple comparison test was used to determine the OD values statistical differences between groups (histiocytic/lymphocytic pattern; histiocytic/mixed pattern; lymphocytic/mixed pattern). Data were expressed as mean values ±standard deviation (SD). Differences were considered statistically significant at $P < 0.05$. Analysis was carried out using IBM SPSS Statistics for Windows, Version 24.0 (Armonk, NY: IBM Corp).

RESULTS

Histopathology and Immunohistochemistry

The fifty animals selected from infected flocks were positive to VMV by IHC and showed characteristic MV lesions, while the 8 negative controls were negative and did not show any abnormalities within the target organs. All the MV infected animals showed MV lesions in lungs, 36 in CNS and 39 in mammary gland.

Three different patterns were observed: a *histiocytic pattern* in 23 animals (46%) (Fig.1A), a *lymphocytic pattern* in 19 sheep (38%) (Fig.1B) and a *mixed pattern* in 8 sheep (16%). The same lesion pattern was invariably observed in lung and CNS of every sheep studied. The mammary gland showed a low number of macrophages and a predominance of lymphocytes in the inflammatory infiltrate in all sheep. Lesions severity and extension varied between the different target organs in the same animal.

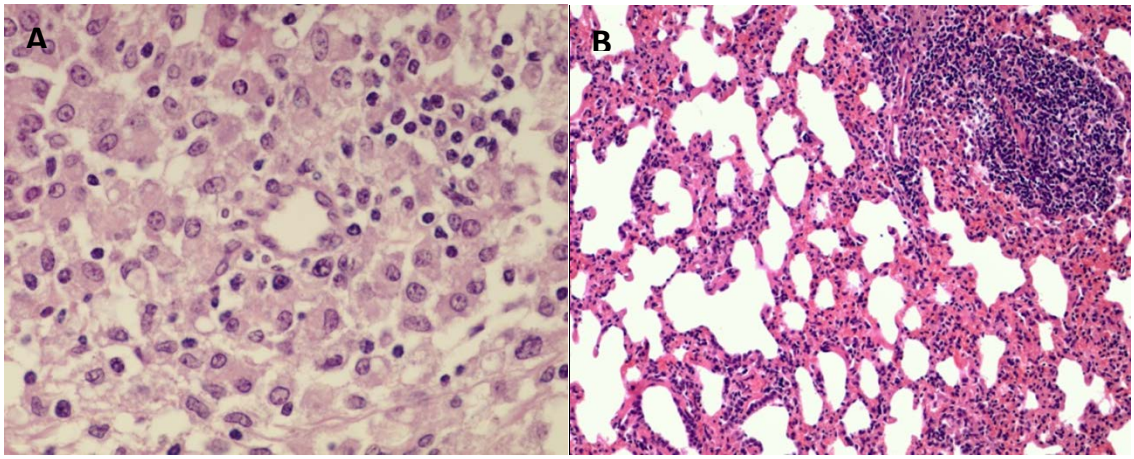

Fig. 1: *Maedi visna lesions patterns, HE.* ***A:*** *Histiocytic lesion pattern in a brain sample. Predominance of macrophages within the inflammatory infiltrate. 40X.* ***B:*** *Lymphocytic lesion pattern in a lung sample. Predominance of lymphocytes within the inflammatory infiltrate. Presence of a lymphoid follicle. 20X.*

Viral antigen immunolabelling appeared as a fine brownish deposit in the cytoplasm of macrophage-like cells in all the affected organs, with a subjectively more abundance in histiocytic lesion pattern than in lymphocytic pattern (Fig.2). The positive sign was always associated with lesions, and no labelling was detected in histologically unaffected areas. No immunolabelled cells were detected in sections used as negative controls but labelling was invariably seen in positive control sections.

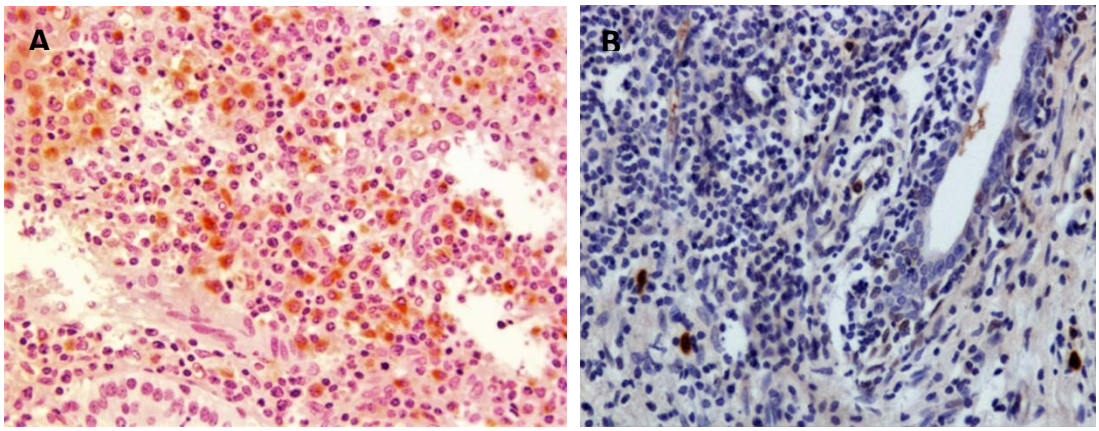

Fig.2: *Inmunohistochemistry against p28-MVV.* ***A:*** *Histiocytic lesion pattern in a lung sample. High amount of positive macrophage-like cells within the inflammatory infiltrate. 20X.* ***B:*** *Lymphocytic lesion pattern in a mammary gland sample. Scattered positive cells within the lymphocytic infiltrate. 20X.*

Polymerase Chain Reaction

The 14 animals from the infected flocks were positive to PCR. Seven of these sheep showed minimal lesions, two showed moderate lesions, while five showed severe lesions. Three positive sheep showed *histiocytic pattern*, ten *lymphocytic pattern* and one *mixed pattern* (Table 1). The 5 negative controls were negative to PCR.

Correlation between histopahological and serological findings

Fourty-five of the fifty infected animals showed positive optical density (OD) results, three of them presented doubtful results (0.4 ± 0.1), and two infected sheep showed negative OD results (Table 1).

Pattern	**OD**	**IHC**	**PCR**
H1	1.14	+	
H2	1.35	+	
H3	1.39	+	
H4	1.5	+	+
H5	1.5	+	
H6	1.6	+	
H7	1.68	+	+
H8	1.8	+	
H9	1.88	+	
H10	1.95	+	
H11	2.14	+	
H12	2.19	+	
H13	2.27	+	
H14	2.3	+	
H15	2.4	+	
H16	2.4	+	
H17	2.47	+	+
H18	2.5	+	
H19	2.5	+	
H20	2.5	+	
H21	2.65	+	
H22	2.77	+	
H23	2.99	+	
L1	0.09	+	+
L2	0.25	+	+
L3	0.42	+	+
L4	0.5	+	+
L5	0.53	+	+
L6	0.73	+	
L7	0.75	+	+
L8	0.75	+	
L9	0.79	+	
L10	0.81	+	
L11	0.91	+	+
L12	1	+	+
L13	1.07	+	

L14	1.11	+	
L15	1.12	+	
L16	1.47	+	
L17	1.61	+	
L18	1.65	+	+
L19	1.81	+	+
M1	0.73	+	
M2	0.77	+	
M3	1.12	+	
M4	1.18	+	
M5	1.37	+	
M6	1.39	+	
M7	1.44	+	+
M8	1.45	+	

Table 1: *ELISA optical densities, IHC and PCR results of every sheep studied. H: histiocytic lesion pattern; L: lymphocytic lesion pattern; M: mixed lesion pattern.*

Significant differences ($p<0.001$) in the values of the OD between histiocytic, lymphocytic and mixed patterns were observed in this study. All sheep with *histiocytic pattern* (n=23) were positive to VMV showing high OD values between of 1.14 and 2.99 (average: 2.08, SD: 0.51). Animals with a *lymphocytic pattern* (n=19) showed lower OD values, even with 2 negative and 3 doubtful values (min: 0.09 max: 1.81; average: 0.91; SD: 0.47). Sheep with a *mixed pattern* (n=8) showed mixed values between 0.77 and 2.09 (average: 1.35 SD: 0.37) (Fig.3).

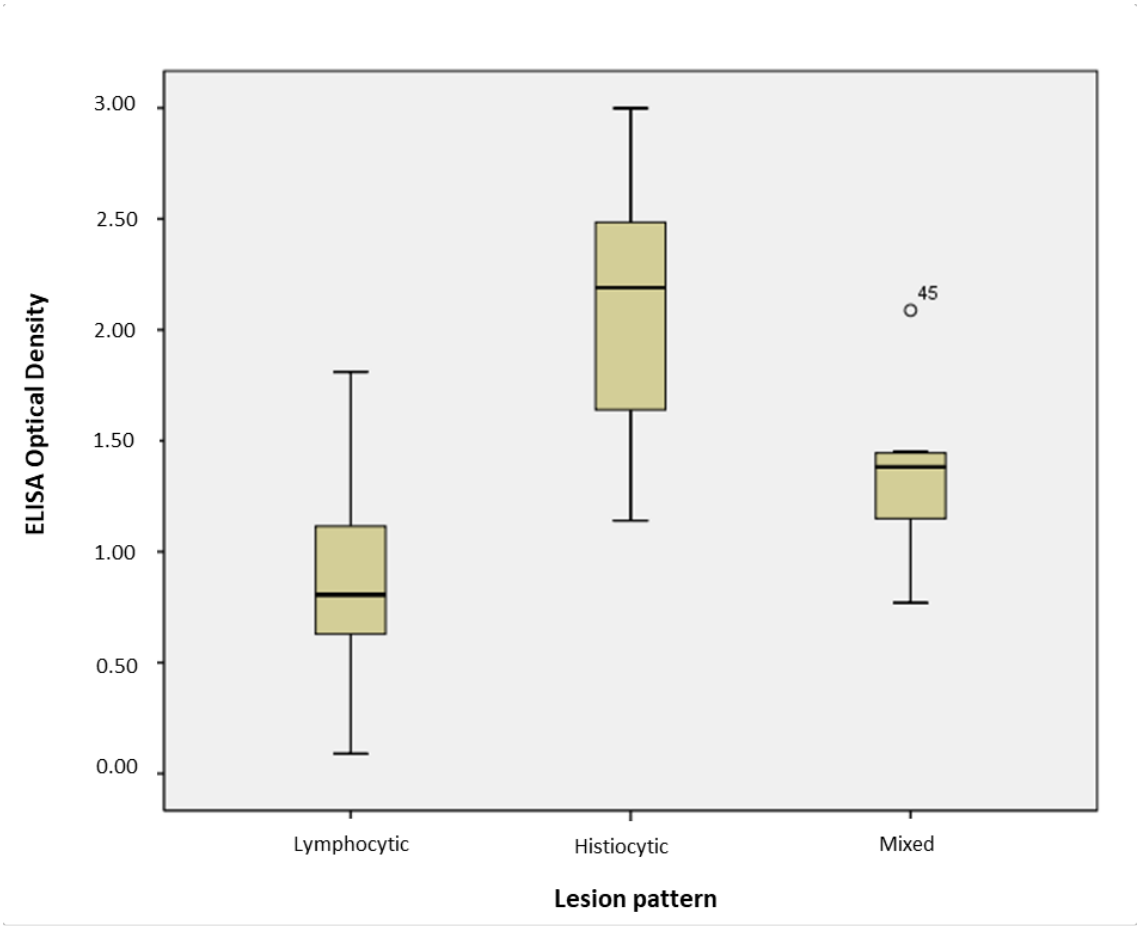

Fig. 3: *serological values distribution in lymphocytic, histiocytic and mixed lesion patterns. Cut-off point: 0.4 ± 0.1.*

DISCUSSION

This study describe for the first time an association between three histological lesion patterns (histiocytic, lymphocytic, and mixed) studied in lung, mammary gland and CNS and ELISA results on the basis of differences in OD values. Animals with a *histiocytic lesion pattern* showed high OD values (average: 2.08, max: 2.99 min: 1.14 s: 0.51). However, sheep with *lymphocytic pattern* showed significant ($p<0.001$) lower values with 2 seronegative and 3 doubtful sheep (average: 0.91, max: 1.81 min: 0.09 s: 0.37). Therefore, high titers of antibodies are related to histiocytic patterns while low OD values or negative results are related to

lymphocytic types. This is an important factor to consider for ELISA testing, in addition to the viral strain or the particular test used as have been previously reported (de Andrés *et al.*, 2005; de Andrés *et al.*, 2013). Similar results were previously reported in animals with only lymphoid follicles in lung and mammary gland which were seronegative in the AGID test, although at that time it was associated to the low sensitivity of the test used or to an initial stage of the disease with no antibody response (Luján *et al.*, 1991). Our results support the hypothesis of that these animals with lymphocytic pattern and minimal lesions have a very low serological response which could not be detected using the AGID test with lower sensibility than ELISA test.

Given the difference in antibody titers and in the cellular population forming the three histological patterns, sheep can show different individual humoral response. The *histiocytic lesion pattern,* usually with severe lesions, can represent a strong but non-efficient humoral response. The *lymphocytic pattern,* with mild or moderate lesions despite the low OD values, seem to represent an efficient immune response against the viral replication, very likely cellular, mediated by CD4+ and CD8+ T cells (Polledo *et al.*, 2012b). This would agree with previous results reported in HIV or feline immunodeficiency virus (FIV) (Freel *et al.*, 2011).

Most of the animals with minimal lesions showed positive ELISA results but low OD values. Seroconversion occurs from 15 days to 4 months after infection as described by (Lacerenza *et al.*, 2006; Pépin *et al.*, 1998) and typical MV-like lesions would then appear. Animals with minimal lesions and *mixed lesion pattern* could represent an initial or latent infection with a non-defined immune response against the virus and with variable antibody secretion which could develop lymphocytic or histiocytic types of immune response with the evolution of the infection. This could mean that the tissue infection, the beginning of developing lesions and seroconversion occurs very early and almost concurrently. However, further experimental studies should be performed to proof this hypothesis. The few

seronegative animals in this group could be sheep with minimal lesions and infection, though not significant enough to produce a peripheral or serological response detectable using an ELISA test. The presence of virus by IHC was associated exclusively to macrophage-like cells, even in minimal lesions, being subjectively more abundant in histiocytic lesions compared to lymphocytic ones, as has been previously suggested (Polledo *et al.*, 2012a).

To sum up, sheep with histiocytic type of lesions and easily recognizable using ELISA test could play a major role in the spreading of the infection compared animals with lymphocytic or mixed forms. However, animals with a *lymphocytic lesion pattern* could easily be misdiagnosed using ELISA methods due to the lower antibody production, going unnoticed in the flock as false negative animals in the control programs and staying on as carriers and source of infection. This fact should be considered in flocks with a low MV seroprevalence where these unobserved sheep could be the main source of MV infection. In conclusion, ELISA results are strongly associated to the type of histological lesion pattern, which are very likely conditioned by the individual immune response and therefore, seronegative infected carriers should be considered in control programs.

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REFERENCES

Benavides, J., Fuertes, M., García-Pariente, C., Otaola, J., Delgado, L., Giraldez, J., García Marín, J.F., Carmen Ferreras, M., Pérez, V., 2013. Impact of maedi-visna in intensively managed dairy sheep. Veterinary Journal 197, 607-612.

Benavides, J., García-Pariente, C., Fuertes, M., Ferreras, M.C., García-Marín, J.F., Juste, R.A., Pérez, V., 2009. Maedi-Visna: the Meningoencephalitis in Naturally Occurring Cases. Journal of comparative pathology 140, 1-11.

Blacklaws, B.A., 2012. Small ruminant lentiviruses: Immunopathogenesis of visna-maedi and caprine arthritis and encephalitis virus. Comparative Immunology, Microbiology and Infectious Diseases 35, 259-269.

Cutlip, R.C., Lehmkuhl, H.D., Schmerr, M.J.F., Brogden, K.A., 1988. Ovine progressive pneumonia (maedi-visna) in sheep. Veterinary microbiology 17, 237-250.

Dawson, M., 1987. Pathogenesis of maedi-visna. Veterinary Record 120, 451-454.

de Andrés, D., Klein, D., Watt, N.J., Berriatua, E., Torsteinsdottir, S., Blacklaws, B.A., Harkiss, G.D., 2005. Diagnostic tests for small ruminant lentiviruses. Veterinary microbiology 107, 49-62.

de Andrés, X., Ramírez, H., Bertolotti, L., San Román, B., Glaria, I., Crespo, H., Jáuregui, P., Minguijón, E., Juste, R., Leginagoikoa, I., Pérez, M., Luján, L., Badiola, J.J., Polledo, L., García-Marín, J.F., Riezu, J.I., Borrás-Cuesta, F., de Andrés, D., Rosati, S., Reina, R., Amorena, B., 2013. An insight into a combination of ELISA strategies to diagnose small ruminant lentivirus infections. Veterinary Immunology and Immunopathology 152, 277-288.

Freel, S.A., Saunders, K.O., Tomaras, G.D., 2011. CD8 +T-cell-mediated control of HIV-1 and SIV infection. Immunologic Research 49, 135-146.

Lacerenza, D., Giammarioli, M., Grego, E., Marini, C., Profiti, M., Rutili, D., Rosati, S., 2006. Antibody response in sheep experimentally infected with different small ruminant lentivirus genotypes. Veterinary Immunology and Immunopathology 112, 264-271.

Leginagoikoa, I., Daltabuit-Test, M., Álvarez, V., Arranz, J., Juste, R.A., Amorena, B., de Andrés, D., Luján, L.L., Badiola, J.J., Berriatua, E., 2006. Horizontal Maedi-Visna virus (MVV) infection in adult dairy-sheep raised under varying MVV-infection pressures investigated by ELISA and PCR. Research in Veterinary Science 80, 235-241.

Luján, L., García Marin, J.F., Fernández de Luco, D., Vargas, A., Badiola, J.J., 1991. Pathological changes in the lungs and mammary glands of sheep and their relationship with maedi-visna infection. Veterinary Record 129, 51-54.

Minguijón, E., Reina, R., Pérez, M., Polledo, L., Villoria, M., Ramírez, H., Leginagoikoa, I., Badiola, J.J., García-Marín, J.F., de Andrés, D., Luján, L., Amorena, B., Juste, R.A., 2015. Small ruminant lentivirus infections and diseases. Veterinary Microbiology 181, 75-89.

Patel, J.R., Heldens, J.G.M., Bakonyi, T., Rusvai, M., 2012. Important mammalian veterinary viral immunodiseases and their control. Vaccine 30, 1767-1781.

Polledo, L., Gonzalez, J., Benavides, J., Martinez-Fernandez, B., Carmen Ferreras, M., Garcia Marin, J.F., 2012a. Perivascular inflammatory cells in ovine Visna/maedi encephalitis and their possible role in virus infection and lesion progression. Journal of neurovirology 18, 532-537.

Polledo, L., Gonzalez, J., Fernandez, C., Miguelez, J., Martinez-Fernandez, B., Morales, S., Ferreras, M.C., Garcia Marin, J.F., 2013. Simple control strategy to reduce the level of Maedi-Visna infection in sheep flocks with high prevalence values (< 90%). Small Ruminant Research 112, 224-229.

Polledo, L., González, J., Benavides, J., Morales, S., Martínez-Fernández, B., Delgado, L., Reina, R., Glaria, I., Pérez, V., Ferreras, M.C., García Marín, J.F., 2012b. Patterns of Lesion and

Local Host Cellular Immune Response in Natural Cases of Ovine Maedi-Visna. Journal of comparative pathology 147, 1-10.

Preziuso, S., Taccini, E., Rossi, G., Renzoni, G., Braca, G., 2003. Experimental Maedi Visna Virus Infection in sheep: A morphological, immunohistochemical and PCR study after three years of infection. European Journal of Histochemistry 47, 373-378.

Pépin, M., Vitu, C., Russo, P., Mornex, J.F., Peterhans, E., 1998. Maedi-visna virus infection in sheep: A review. Veterinary Research 29, 341-367.

Torsteinsdóttir, S., Carlsdóttir, H.M., Svansson, V., Matthíasdóttir, S., Martin, A.H., Pétursson, G., 2007. Vaccination of sheep with Maedi-visna virus gag gene and protein, beneficial or harmful? Vaccine 25, 6713-6720.

Torsteinsdóttir, S., Georgsson, G., Gísladóttir, E., Rafnar, B., Pálsson, P.A., Pétursson, G., 1992. Pathogenesis of central nervous system lesions in visna: Cell-mediated immunity and lymphocyte subsets in blood, brain and cerebrospinal fluid. Journal of Neuroimmunology 41, 149-158.

VI. Segundo trabajo

SEGUNDO TRABAJO

Inflammatory Lesion Patterns in Target Organs of Visna/Maedi in Sheep and their Significance in the Pathogenesis and Diagnosis of the Infection *J. Comp. Path*. 2018, Vol. 159, 49-56.

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INFECTIOUS DISEASE

Inflammatory Lesion Patterns in Target Organs of Visna/Maedi in Sheep and their Significance in the Pathogenesis and Diagnosis of the Infection

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ABSTRACT

Ovine visna/maedi (VM) infection is characterized by the development of chronic inflammatory lesions in different organs, mainly in the lung, mammary gland and central nervous system (CNS), with either histiocytic or lymphocytic pattern predominance being described in the CNS. To help to understand the role of host immune response in the development of these patterns, 50 naturally-infected sheep and eight non-infected sheep from intensive milk-producing flocks were studied. The histological lesion patterns in the three main target organs in each sheep were characterized. Lesion severity was determined, including minimal lesions. A histiocytic pattern was observed in 23 sheep (46%), a lymphocytic inflammatory pattern in 19 sheep (38%) and a mixed inflammatory pattern in eight sheep (16%). Forty animals showed moderate or severe lesions (80%), while 10 had minimal lesions (20%). Moderate or severe lesions affected only one target organ in 20 sheep (50%), two organs in 14 sheep (35%) and all three target organs in six sheep (15%). Infection was confirmed by immunohistochemistry (IHC) using an antibody specific for p28 of VM virus/caprine arthritis and encephalitis virus and by polymerase chain reaction (PCR) in all sheep. Minimal inflammatory lesions associated with positive IHC and PCR were observed. The results suggest that the development of a predominant inflammatory pattern in different organs within the same animal may be related to the host immune response. Minimal and focal lesions, not considered previously, should be taken into account when formulating a differential diagnosis in affected sheep.

Keywords: immunopathology, lesion pattern, sheep, Visna/maedi.

INTRODUCTION

Ovine Visna/maedi (VM) is a widespread disease caused by a retrovirus of the genus lentivirus and is related to caprine arthritis encephalitis virus (CAEV) and human immunodeficiency virus (Thormar, 2005). The disease has a significant economic impact, which is especially noted in flocks in northwest Spain where an intensive indoor farming system is widespread and seroprevalence reaches up to 96.8% (Sotelo, 1998; Peterhans *et al.*, 2004; Leginagoikoa *et al*., 2006). VM is characterized by chronic inflammation of the lung, mammary gland and central nervous system (CNS), and rarely by arthritis. Microscopically, the changes in affected organs are interstitial pneumonia, mastitis and non-suppurative necrotizing encephalomyelitis, choroiditis and demyelination of the CNS (Cutlip *et al.*, 1979; Luján *et al.*, 1991; Benavides *et al.*, 2009; Minguijón *et al.*, 2015).

The importance of cell-mediated immunity with regard to the severity of the lesions has been described for the CNS lesions (Torsteinsdóttir *et al.*, 1992; Polledo *et al.*, 2012a) and an individual immune response against VM virus (VMV) has been suggested to play a major role in the pathogenesis of the disease (Torsteinsdóttir *et al.*, 1992, 2007; Blacklaws, 2012; Polledo *et al.*, 2012a, b). In previous studies carried out on sheep with spontaneously arising neurological forms of VM, two main patterns of lesion were described with regard to the predominant inflammatory cells in the CNS infiltrates. The lymphocytic pattern is characterized by a predominance of T lymphoyctes, particularly $CD8^+$ T cells, while the histiocytic pattern involves infiltration of macrophages and B lymphocytes (Polledo *et al.*, 2012b). Similar inflammatory patterns have been described in the lung and mammary gland (Gayo *et al.*, 2017).

In previous studies, moderate to severe VM lesions occurred in 35.5% of mammary glands and 32.3% of lungs in randomly selected sheep. These percentages increased to 58.1%

and 54%, respectively, in seropositive sheep (Luján *et al.*, 1991). In the latter study, 25.6% of VM-seropositive sheep had no lesions in any organ, 20.2% had lesions in the mammary gland only, 16.2% had lesions in the lung only and 37.8% had lesions in both organs (Luján *et al.*, 1991). In subsequent studies an increase in these percentages was observed when mild lesions were also considered, with percentages of affected mammary glands rising to 46.7% (Benavides *et al.*, 2013). Minimal infiltrates of cells were not included in these investigations and were considered as normal, but the presence of virus in those lesions was not evaluated.

The aim of the present study was to determine whether the same inflammatory pattern is present in all of the target organs in individual infected sheep.

MATERIALS AND METHODS

Animals

Fifty-eight Spanish Assaf sheep (1–3 years of age) from different intensive milk-producing flocks located in the northwest of Spain were selected from animals submitted to the Pathology Diagnostic Service, School of Veterinary Medicine, León, Spain, for routine necropsy examination. Fifty sheep were naturally infected and came from six VM-seropositive flocks subjected to VM control as described elsewhere (Polledo *et al.*, 2013). The seroprevalence in these flocks ranged between 62% and 97%. The remaining eight animals were selected as negative controls from three VMV-free flocks and showed no clinical signs. Sheep from affected flocks were culled because of loss of milk production and/or weight or because of the presence of neurological (e.g. hindlimb weakness, ataxia, hypermetria or paralysis usually leading to recumbency) or respiratory signs (e.g. dyspnoea). Animals in this study did not have gross or microscopical lesions compatible with other pathologies such as bacterial, fungal or parasitic pneumonias or mastitis.

Sampling, Histopathology and Immunohistochemistry

Tissue samples were collected systematically from the diaphragmatic and apical lung lobes, glandular parenchyma and udder cisterns and CNS from nine levels of the brain and nine levels of the spinal cord (Polledo *et al.*, 2012a). Mammary samples from six sheep and spinal cord samples from four sheep were not included in the histopathological studies as the tissues were not in good condition. Samples were fixed in 10% neutral buffered formalin for 48 h at room temperature. After fixation, samples were dehydrated through graded alcohols and embedded in paraffin wax. Sections (4 μm) were stained with hematoxylin and eosin (HE) and examined using light microscopy.

Additional sections (4 μm) were used for immunohistochemistry (IHC). The following primary antibodies were used: polyclonal anti-CD3 for T cells (Dako, Glostrup, Denmark); monoclonal anti-CD79 for B cells (Dako); monoclonal anti-CD68 for macrophages (Dako) and monoclonal anti-p28 of VMV/CAEV (VMRD, Pullman, Washington, USA) for viral detection. Subsequently, the sections were incubated for 30 min using the EnVision+ system (EnVision+ System Labelled Polymer-HRP anti-mouse or anti-rabbit; Dako) and labelling was 'visualized' using 3, 3' diaminobenzidine as chromogen (Vector Laboratories, Burlingame, California, USA) for anti-CD3, CD79, and CD68 antibodies. An avidin–biotin–peroxidase complex (ABC) technique (Vectastain Elite, ABC Kit; Vector Laboratories) was used for detection of p28 of VMV/CAEV (Preziuso *et al.*, 2003). The slides were counterstained with haematoxylin and mounted. The specificity of the technique was evaluated using positive (previously confirmed tissues) and negative controls (previously confirmed tissues and non-immune serum replacing primary antibody).

Three lesion patterns were considered based on the characteristics of the inflammatory infiltrate. Histiocytic and lymphocytic patterns were considered as previously described (Polledo *et al.*, 2012b). Lymphocytic lesions were characterized by a predominance of $CD3^+$ cells within the inflammatory infiltrate, while in histiocytic lesions $CD68^+$ cells were the

most abundant, together with some scattered $CD3^+$ and $CD79^+$ cells. A mixed inflammatory infiltrate was composed of macrophages and lymphocytes with no predominance of any of the cellular populations (Gayo *et al.,* 2017).

Evaluation and description of the characteristics and severity of the lesions in the mammary gland, lung and CNS were based on previous reports (Luján *et al.*, 1991; Benavides *et al.*, 2013) with some specific modifications. 'No lesion' was defined when no inflammatory cells were observed within the CNS, lung or mammary gland. 'Minimal lesions' (+) consisted of a few small clusters (5–20) to multifocal small groups of inflammatory cells with the presence of a few small lymphoid aggregates and/or one small lymphoid follicle. 'Moderate lesions' (++) were characterized by multifocal to diffuse interstitial non-suppurative inflammation, and/or the presence of two to 15 lymphoid aggregates/follicles. 'Severe lesions' (+++) consisted of a marked diffuse interstitial pneumonia, mastitis or encephalitis and/or the presence of >15 lymphoid aggregates/follicles. When differences between the severity of interstitial inflammatory infiltrates and the presence of lymphoid follicles were observed in the same organ of an animal, the most severe lesion was considered as the score for the lesion in that target organ. Lesion pattern in sheep with minimal lesions in one target organ and moderate or severe lesions in other organs were defined by considering the most severe pathology. Lesion pattern in sheep with only minimal lesions in the three target organs studies were defined on the basis of the predominant cell population observed.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed as described by Ryan *et al.* (2000) on CNS samples from 40 infected sheep, lung and CNS samples from six infected animals and lung and mammary gland samples from four infected sheep. Lung, mammary gland and CNS samples from the eight negative control animals were also studied. Genomic DNA was

extracted from paraffin wax-embedded tissue samples using QIAamp® DNA Mini Kit (Qiagen, Valencia, California, USA). For CNS PCR studies a pool of spinal cord, medulla oblongata, pons and peduncles and mesencephalon was used.

Serology

Serum samples were obtained from the 58 sheep to evaluate the presence of antibodies against VMV using a standardized commercial test kit (Elitest®, Hyphen BioMed, Neuville-Oise, France) as described by Gayo *et al*. (2017).

RESULTS

Histopathology and Immunohistochemistry

The 50 animals from the six infected flocks were positive for VMV by IHC and PCR and showed characteristic lesions of VM, while the eight negative control sheep were negative for virus detection and did not show abnormalities within the target organs. All of the VM-infected animals had lesions in the lungs, 36 infected animals had lesions in the CNS and 39 infected sheep had lesions in the mammary gland. Forty of these animals had moderate or severe lesions in one or more organs and displayed clinical signs of VM (80%). The lesions affected a single organ in 20 animals (50%), two organs in 14 animals (35%) and three organs in six animals (15%) (Table 1). Ten sheep showed only minimal lesions in the CNS, lung and/or mammary gland (20%).

	Moderate/severe lesions	N° Sheep	Total
	CNS	8	
1 Organ	Lung	3	**19**
	Mammary gland	8	
	CNS/Lung	4	
2 Organs	CNS/Mammary gland	5	**14**
	Lung/Mammary gland	5	
3 Organs	CNS/Lung/Mammary gland	6	**6**
Total			**39**

Table 1: *Moderate/severe Maedi Visna lesions distribution in the target organs.*

A histiocytic pattern was observed in 23 animals (46%), a lymphocytic pattern in 19 sheep (38%) and a mixed pattern in eight sheep (16%). Severe lesions (+++) were more common in animals with a histiocytic pattern (*n* = 16; 69.6% of the histiocytic lesions) and they were observed to a lesser extent in sheep with a lymphocytic pattern (*n* = 5; 26.3% of the lymphocytic lesions). Sheep with the histiocytic pattern had moderate or severe lesions in at least one target organ. Fifteen sheep with the lymphocytic pattern showed moderate or severe lesions in at least one target organ (78.9%), while four animals had minimal lesions with no clinical signs affecting the three target organs studied (21.1%). Sheep with a mixed pattern had predominantly minimal lesions in two or three target organs and no clinical signs (75%),

except for two animals with moderate lesions in the lung and mammary gland. The mixed inflammatory infiltrate in six sheep with only minimal lesions consisted of similar numbers of $CD3^+$ and $CD68^+$ cells. The other four sheep with only minimal lesions showed small groups of $CD3^+$ cells with only one or two macrophages within the interstitial inflammatory infiltrate, together with some small lymphocytic aggregates ($CD3^+$ cells) or one small lymphoid follicle. These were considered as having a lymphocytic pattern. $CD79^+$ cells were only observed in two of these animals with minimal lesions (one or two positive cells in each sheep within the inflammatory infiltrate). Minimal lesions in one organ of sheep with a global histiocytic pattern were always mixed, while minimal lesions in animals with severe lymphocytic lesions in other target organs were lymphocytic or mixed.

The lung and CNS always showed the same lesion pattern in the same animal. The inflammatory infiltrate in the mammary gland often consisted of a predominance of $CD3^+$ cells with a variable number of $CD68^+$ macrophages and some scattered $CD79^+$ B cells. Even if $CD3^+$ cells were predominant within the inflammatory infiltrate, more $CD68^+$ cells were usually observed in animals with a histiocytic pattern in the other target organs.

Mammary gland lesions are shown in Figs. 1A, 1B and 1C. Minimal (+) interstitial mastitis was observed in 15 sheep (37.5 %), moderate (++) in 18 sheep (45%) and severe (+++) in seven sheep (17.5%) (Table 2), with variable distribution in the severity of the inflammatory infiltrate and lymphoid hyperplasia among the mammary lobules. All infected animals had a predominance of $CD3^+$ cells within the inflammatory infiltrate of this target organ, with some scattered $CD79^+$ cells and a variable number of $CD68^+$ cells. No lesions were observed in the eight negative controls.

Lungs lesions are shown in Figs. 1D, 1E and 1F. Minimal lesions (+) were observed in 32 sheep (64%), moderate lesions (++) in nine sheep (18%), severe lesions (+++) in nine animals (18%) and no lesions were observed in the eight negative controls (Table 2). No connection

was observed between the severity of lymphoid hyperplasia and interstitial pneumonia. Some animals with >15 follicles showed a minimal interstitial pneumonia and vice versa. The histiocytic pattern was observed in 23 animals with respiratory lesions (46%), a lymphocytic pattern in 19 animals (38%) and a mixed pattern in eight animals (16%).

CNS lesions are shown in Figs. 1G, 1H and 1I. The choroid plexus was the most affected tissue (*n* = 32), followed by the brain (*n* = 21) and the spinal cord (*n* = 16). Minimal lesions (+) were observed in 13 animals (36.1%), moderate lesions (++) in six (16.7%) and severe lesions (+++) in 17 (47.2%) (Table 2). Histiocytic lesions were observed in 22 animals (61.1%), a lymphocytic pattern in 11 animals (30.6%) and a mixed pattern in three sheep (8.3%).

Lymphocytic, histiocytic and mixed patterns, as well as different lesion severity and extent, in the three main target organs of the disease, were observed in sheep from all six infected flocks studied.

Viral antigen immunolabelling appeared as a fine brown deposit in the cytoplasm of macrophage-like cells in all the affected organs, while epithelial cells showed no positivity (Fig.2). Labelled cells were observed within the interstitial inflammatory infiltrate and surrounding blood vessels. Positive labelling was always associated with lesions, even minimal lesions, and no labelling was detected in histologically unaffected areas. In the mammary gland, positive cells were located within the interstitial inflammatory infiltrates or within in the acinar lumina. In the lung, positive cells were observed occasionally in the bronchial and alveolar spaces, sometimes forming syncytia, and within the inflammatory infiltrates (Figs. 2B, 2C). Positive labelling was more abundant in histiocytic lesions than in lymphocytic lesions. No labelled cells were detected in sections used as negative controls, but labelling was invariably seen in positive control sections.

Polymerase Chain Reaction

All fifty infected animals had a positive PCR from at least one target organ. Thirty six sheep were positive for all samples tested while five sheep were negative with CNS samples, three were negative with lung samples, two were negative with udder samples, two were negative with udder and CNS samples and two were negative with lung and CNS tissues. Negative results were observed for organs with no lesions in six cases, with minimal lesions in ten cases and with moderate lesions in two cases. The eight negative controls were negative for all target organs.

Serology

Forty-five of the 50 infected animals were seropositive, three had inconclusive results (optical density of 0.4 ± 0.1) and two infected sheep were negative. The three sheep with inconclusive results showed lymphocytic lesions. The first animal had minimal lesions in the lung, mammary gland and brain, the second had minimal lesions in the lung and mammary gland and moderate lesions in choroid plexus, and the third had severe lesions in the lung and spinal cord and minimal lesions in the mammary gland. The two seronegative sheep had lymphocytic lesions. The first of these had minimal lesions in the lung and moderate lesions in the mammary gland and the second had severe lesions in the lung and mammary gland.

DISCUSSION

This study describes in detail for the first time the histological inflammatory patterns of VM (i.e. histiocytic, lymphocytic and mixed) in the three main target organs of the disease in the same animals. The presence of the same inflammatory lesion pattern in the main target

organs of VM in the same sheep and the higher number of $CD68^+$ cells in the mammary glands of sheep with the histiocytic pattern suggests that the host immune response plays a role in the development of the inflammatory infiltrate. The predominance of T lymphocytes observed in mammary glands with a variable number of macrophages may be related to a different local immunological response in this target organ; perhaps reflecting a higher T helper (Th) 1-mediated response. This may be due to the mucosal nature of the mammary gland, which has an outward-facing surface with continuous antigen stimulation (Butler *et al.*, 2015), especially in this type of dairy sheep with long lactation periods and intensive mechanical milking methods.

Histiocytic and lymphocytic patterns previously described in the CNS (Polledo *et al.*, 2012b) and the mixed pattern with both histiocytic and lymphocytic characteristics (Gayo *et al.*, 2017) are described in the three main target organs of the disease. The mixed pattern could represent an initial or latent infection with a non-defined immunopathological response that would evolve to become a lymphocytic or histiocytic lesions with progression of the infection. This might explain why in previous studies (Polledo *et al.*, 2012b), where only animals with moderate and severe CNS lesions were included, this lesion pattern was not observed. The fact that minimal lesions in one organ of sheep with a global histiocytic pattern were mixed, while minimal lesions in animals with severe lymphocytic lesions in other target organs were lymphocytic or mixed, would support the hypothesis of an evolution of mixed forms to a lymphocytic or histiocytic pattern. Minimal lymphocytic lesions were never observed together with a predominance of $CD68^+$ cells in moderate or severe lesions in other target organs of the same sheep. This suggests that the beginning of an infection or the latent stages of a mixed pattern could evolve to become either lymphocytic or histiocytic patterns; however, it would be unlikely that lymphocytic lesions become histiocytic or vice versa, as has been demonstrated in natural and experimental mycobacterial infections (Juste *et al.,* 1994;

Delgado *et al.,* 2013). It should be noted however, that this is a field study which shows the pathological features of a group of animals at the moment of the death and further detailed experimental studies would be necessary to confirm this hypothesis.

The association between the lesion pattern and the stage of VM and the amount of viral antigen as determined by IHC has been described previously for CNS lesions (Polledo *et al.*, 2012b). It was proposed that lymphocytic lesions in the CNS form of VM represent some type of natural resistance to viral replication, with an initial recruitment of lymphocytes into the CNS as the first response to viral infection and with scarce presence of viral antigen in tissue, as was observed in the present study in animals with the lymphocytic lesion pattern. The severe lymphocytic lesions observed, even when located simultaneously in the CNS, lung and mammary gland, could be related to local releasing of pro-inflammatory cytokines, contributing to a chronic stage of inflammation and injury of the affected tissue. However, histiocytic lesions have been associated with a poor individual response or greater virulence of the viral strain, with a higher amount of virus as detected by IHC (Polledo *et al.*, 2012b) as observed in the present study. The cellular infiltrate in other lentivirus infections, such as with simian immunodeficiency virus or human immunodeficiency virus, has similar features with macrophages comprising of 90 –95% of the infiltrating cells and $CD8^+$ T cells comprising the remainder of the population (Lackner *et al.*, 1991; Kim *et al.*, 2004).

Severe lesions were present only in one organ in 40% of the animals and in two organs in 28% (total 68%) of the sheep, regardless of the flock and with a predominance of CNS lesions. This may be related to the high prevalence of CNS cases in the study area (Benavides *et al.*, 2006), where a neurotropic strain of the virus is proposed to circulate (Glaria *et al.*, 2012). However, in the present study, all organs were affected with moderate or severe lesions, which we consider to be related to the host immune response more than the viral strain. All of the flocks studied were from the same geographical area and animals from the

same flock showed different lesion patterns, severity and extent, despite the possibility that similar strains of virus would circulate in the same flock or area (Glaria *et al.*, 2012). The possible route of migration through vascular spaces and progression of the lesions (Dawson, 1987; Polledo *et al.*, 2012a) in terms of host immunity should also be considered. Genetic factors could play an important role in the development of this disease because there is a high inbreeding in Spanish Assaf sheep, which have increased from a few hundred animals in the 1970s to almost two million animals today.

VMV infection was confirmed by IHC in all of the affected organs including those having minimal lesions, and most of these organs also tested positive by PCR. The PCR-negativity of some individual organs with characteristic VM lesions in confirmed infected sheep might be due either to degradation of proviral DNA after formalin fixation (Srinivasan *et al.*, 2002) or to a low virus load in those particular organs. The latter has been suggested to be one of the main difficulties in VMV detection by PCR (de Andrés *et al.*, 2005). Most of the sheep were also seropositive to VMV. Animals with inconclusive or negative ELISA results showed a lymphocytic pattern as previously described (Gayo *et al.,* 2017), which has been related to a lesser antibody production in comparison with histiocytic lesions.

In two previous studies, the percentage of VM lesions in the lungs of randomly selected sheep was 32.3% and 79.3% in intensively-managed dairy sheep farms (Luján *et al.*, 1991; Benavides *et al.*, 2013), reaching 100% in our study. Likewise, in the present study, 80% of sheep had mammary lesions in contrast with previous studies showing mammary lesions in 35.6% of the sheep (considering only moderate and severe changes) (Luján *et al.*, 1991), or reaching up to 46.7% of animals, taking into account mild (but not minimal) cellular infiltrates (Benavides *et al.*, 2013).

IHC for VMV antigen is a sensitive means of detecting infection in the lung, CNS and mammary gland. Viral antigen was associated exclusively with macrophage-like cells, even

within minimal lesions, and no antigen was observed in areas without lesions. The presence of positive cells mainly in perivascular spaces supports the hypothesis of viral invasion via infiltrating monocytes/macrophages (Peluso *et al.*, 1985; Polledo *et al.*, 2012a).

In conclusion, histiocytic, lymphocytic or mixed inflammatory infiltrates may occur in the lung, CNS and mammary gland of sheep with VM. The inflammatory pattern in the lung and CNS was consistent in every infected sheep, a finding that suggests a similar host immune response in each organ. In contrast, in affected udders the inflammatory infiltrate was always predominantly lymphocytic, with variable numbers of macrophages, and this may be related to a tissue-specific immune response. Minimal inflammatory lesions not previously considered were detected and these were associated with viral antigen. Such lesions should be taken into account not only for the diagnosis of VM, but also in assessing risks of viral transmission. The results of this study extend understanding of the pathology and pathogenesis of VM and establish a pathological model for further experimental studies.

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REFERENCES

Benavides J, Fuertes M, García-Pariente C, Otaola J, Delgado L *et al.* (2013) Impact of maedi-visna in intensively managed dairy sheep. *Veterinary Journal*, **197**, 607-612.

Benavides J, García-Pariente C, Fuertes M, Ferreras MC, García-Marín J.F *et al.* (2009) Maedi-visna: the meningoencephalitis in naturally occurring cases. *Journal of Comparative Pathology*, **140**, 1-11.

Benavides J, Gómez N, Gelmetti D, Ferreras MC, García-Pariente C *et al.* (2006) Diagnosis of the nervous form of maedi-visna infection with a high frequency in sheep in Castilla y León, Spain. *Veterinary Record*, **158**, 230-235.

Blacklaws BA (2012) Small ruminant lentiviruses: immunopathogenesis of visna-maedi and caprine arthritis and encephalitis virus. *Comparative Immunology, Microbiology and Infectious Diseases*, **35**, 259-269.

Butler JE, Rainard P, Lippolis J, Salmon H, Kacskovics I (2015) The mammary gland in mucosal and regional immunity. In: Mucosal Immunology, 4th Edit, Vol. 2e2, J Mestecky, W Strober, M Russell, B Kelsall, H Cheroutre et al., Eds., Academic Press, Boston, pp. 2269-2306.

Cutlip RC, Jackson TA, Lehmkuhl HD (1979) Lesions of ovine progressive pneumonia: interstitial pneumonitis and encephalitis. *American Journal of Veterinary Research*, **40**, 1370-1374.

Dawson M (1987) Pathogenesis of maedi-visna. *Veterinary Record*, **120,** 451-454.

de Andrés D, Klein D, Watt NJ, Berriatua E, Torsteinsdottir S *et al.* (2005) Diagnostic tests for small ruminant lentiviruses. *Veterinary Microbiology*, **107**, 49-62.

Delgado L, García Marín JF, Muñoz M, Benavides J, Juste RA *et al.* (2013) Pathological findings in young and adult sheep following experimental infection with 2 different doses of *Mycobacterium avium* subspecies *paratuberculosis*. *Veterinary Pathology*, **50**, 857-866.

Gayo E, Polledo L, Preziuso S, Rossi G, Balseiro A *et al.* (2017) Serological ELISA results are conditioned by individual immune response in ovine maedi visna. *Small Ruminant Research*, **157**, 27-31.

Glaria I, Reina R, Ramírez H, de Andrés X, Crespo H *et al.* (2012) Visna/maedi virus genetic characterization and serological diagnosis of infection in sheep from a neurological outbreak. *Veterinary Microbiology*, **155**, 137-146.

Juste RA, García Marín JF, Peris B, Saez de Ocariz C, Badiola JJ (1994) Experimental infection with *Mycobacterium paratuberculosis* in vaccinated and non-vaccinated lambs. *Journal of Comparative Pathology*, **110**, 185-194.

Kim WK, Corey S, Chesney G, Knight H, Klumpp S *et al.* (2004) Identification of T lymphocytes in simian immunodeficiency virus encephalitis: distribution of $CD8^+$ T cells in association with central nervous system vessels and virus. *Journal of Neurovirology*, **10**, 315-325.

Lackner AA, Smith MO, Munn RJ, Martfeld DJ, Gardner MB *et al.* (1991) Localization of simian immunodeficiency virus in the central nervous system of rhesus monkeys. *American Journal of Pathology*, **139**, 609-621.

Leginagoikoa I, Daltabuit-Test M, Álvarez V, Arranz J, Juste RA *et al.*

(2006) Horizontal maedi-visna virus (MVV) infection in adult dairy-sheep raised under varying MVV-infection pressures investigated by ELISA and PCR. *Reserch in Veterinary Science*, **80**, 235–241.

Luján L, García Marin JF, Fernández de Luco D, Vargas A, Badiola JJ (1991) Pathological changes in the lungs and mammary glands of sheep and their relationship with maedi-visna infection. *Veterinary Record*, **129**, 51-54.

Minguijón E, Reina R, Pérez M, Polledo L, Villoria M *et al.* (2015) Small ruminant lentivirus infections and diseases. *Veterinary Microbiology*, **181**, 75-89.

Peluso R, Haase A, Stowring L, Edwards M, Ventura P (1985) A Trojan horse mechanism for the spread of visna virus in monocytes. *Virology*, **147**, 231-236.

Peterhans E, Greenland T, Badiola J, Harkiss G, Bertoni G *et al.* (2004) Routes of transmission and consequences of small ruminant lentiviruses (SRLVs) infection and eradication schemes. *Veterinary Research*, **35**, 257-274.

Polledo L, Gonzalez J, Benavides J, Martinez-Fernandez B, Carmen Ferreras M *et al.* (2012a) Perivascular inflammatory cells in ovine Visna/maedi encephalitis and their possible role in virus infection and lesion progression. *Journal of Neurovirology*, **18**, 532-537.

Polledo L, González J, Benavides J, Morales S, Martínez-Fernández B *et al.* (2012b) Patterns of lesion and local host cellular immune response in natural cases of ovine maedi-visna. *Journal of Comparative Pathology*, **147**, 1-10.

Polledo L, Gonzalez J, Fernandez C, Miguelez J, Martinez-Fernandez B *et al.* (2013) Simple control strategy to reduce the level of maedi-visna infection in sheep flocks with high prevalence values (>90%). *Small Ruminant Research*, **112**, 224-229.

Preziuso S, Taccini E, Rossi G, Renzoni G, Braca G (2003) Experimental maedi visna virus infection in sheep: a morphological, immunohistochemical and PCR study after three years of infection. *European Journal of Histochemistry*, **47**, 373-378.

Ryan S, Tiley L, McConnell I, Blacklaws B (2000) Infection of dendritic cells by the maedi-visna lentivirus. *Journal of Virology*, **74**, 10096-10103.

Sotelo MJ (1998) La infección por lentivirus en ovinos y caprinos de la provincia de León: aspectos epidemiológicos y valoración de un plan de control. University of León, PhD Thesis.

Srinivasan M, Sedmak D, Jewell S (2002) Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *American Journal of Pathology*, **161**, 1961-1971.

Thormar H (2005) Maedi-visna virus and its relationship to human immunodeficiency virus. *AIDS Reviews*, **7**, 233-245.

Torsteinsdóttir S, Carlsdóttir HM, Svansson V, Matthíasdóttir S, Martin AH *et al.* (2007) Vaccination of sheep with maedi-visna virus gag gene and protein, beneficial or harmful? *Vaccine*, **25**, 6713-6720.

Torsteinsdóttir S, Georgsson G, Gísladóttir E, Rafnar B, Pálsson PA *et al.* (1992) Pathogenesis of central nervous system lesions in visna: cell-mediated immunity and lymphocyte

subsets in blood, brain and cerebrospinal fluid. *Journal of Neuroimmunology*, **41**, 149-158.

VII. Tercer trabajo

TERCER TRABAJO

Characterization of minimal lesion related to viral infection in mammary gland and milk in dairy sheep Visna/maedi. *Enviado a BMC Veterinary Research*

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ABSTRACT

Background

In order to characterize the whole range of lesions, especially minimal, affecting mammary gland and viral antigen distribution and target cells by IHC in naturally Visna/maedi (VM) 84 infected sheep were studied, 44 from flocks with clinical cases (A) and 35 randomly sampled from two abattoirs (B), together with five negative controls (C). An immunocytochemistry technic was developed and further milk samples (n=39) were used to study viral excretion, carrier cells and the role of milk and colostrum in the transmission of the disease.

Results

All sheep from group C and three sheep from group B were negative to VM in tissue sections by histopathology, immunohistochemistry and PCR, and also in serum ELISA test. Several degrees of CD3+lymphocytic interstitial mastitis were observed in groups A and B depending on the severity of the interstitial cellular infiltrate and the degree of lymphoid hyperplasia: minimal (+) n=26 sheep; moderate (++), n=32 and severe (+++), n=12. No differences in lesion distribution were observed between groups A and B. Viral presence was confirmed by immunohistochemistry using two different antibodies and/or PCR in every tissue with lesions while serology was negative in six sheep with lesions. Two tank milk samples from two flocks of group A and fourteen milk samples from 29 infected sheep of group B were positive to VM (most of them from animals with moderate and severe lesions and high number of positive cells in tissue). Positivity was only found in macrophage-like cells, even in focal and minimal lesions, while no sign was observed in epithelial or any other cells in both tissue and milk samples.

Conclusions

Minimal lesions increased the prevalence of VM lesions in mammary gland up to 90.9% and VM should be considered as a differential diagnosis when minimal interstitial lesions are detected. A high prevalence of VM is observed in intensive milk-producing sheep, while serology does not detect all infected animals, histology, IHC or PCR show higher sensibility. Cytological technique developed was very useful for milk-cells study by HE and immunocytochemistry. Viral detection in milk samples (16/39) confirms potential but limited role of milk/colostrum in viral transmission.

Keywords: histopathology, lentivirus, mammary gland, milk, minimal lesions, sheep, visna maedi.

BACKGROUND

Ovine Visna Maedi (VM) is caused by Visna/maedi virus (VMV), which together with caprine arthritis encephalitis virus belong to small ruminant lentiviruses (SRLV) (Thormar, 2005). Clinically, it is a slow disease mainly characterized by a progressive loss of body condition due to a multi-organic chronic inflammation (Georgsson and Pálsson, 1971; Oliver *et al.*, 1981; Pépin *et al.*, 1998) consisting of interstitial pneumonia, interstitial mastitis, non-purulent meningoencephalitis with demyelination of the CNS, also affecting spinal cord and rarely arthritis (Georgsson and Pálsson, 1971; Luján *et al.*, 1991; Sigurdsson *et al.*, 1957). The mammary syndrome is difficult to detect and mainly consists of mammary gland induration and milk production loss and deficient lamb growth (Minguijón *et al.*, 2015). Flock seroprevalence in Spanish Assaf dairy sheep kept under indoor intensive farming system is estimated at 77%, up to 98% in northern Spain (Leginagoikoa *et al.*, 2006; MJ, 1998), which is the most frequent management system in this study area, causing important economic loses (Benavides *et al.*, 2013). To date, adequate monitoring programs have proved to be the only tool to control the infection as no treatments or vaccine are available (Polledo *et al.*, 2013).

Monocytes/macrophages and dendritic cells are the main target cells of SRLV. These target cells could migrate to regional lymph nodes from where viral systemic dissemination could occur (Bird *et al.*, 1993; Blacklaws *et al.*, 1995b; Ryan *et al.*, 2000), or could reach the bone narrow infecting myeloid precursor cells and reaching the bloodstream (Blacklaws, 2012; Gendelman *et al.*, 1985), carrying the viral DNA into blood with minimum transcription until the maturation of monocytes into macrophages in the tissue

Viral presence in macrophages from mammary gland and milk or colostrum from infected sheep has been reported (Lee and Outteridge, 1981; Preziuso *et al.*, 2004; Preziuso *et al.*, 2003b; Sihvonen, 1980), and thus the possibility of infection of new-born lambs by ingestion of infected calostrum (Preziuso *et al.*, 2004). This fact has also been considered in

CAEV infected animals, where colostrum has been proposed as one of the most important routes of transmission (Ellis *et al.*, 1983; Ellis *et al.*, 1986). However, the importance of milk and colostrum in the spread of the disease in sheep under natural conditions is a continuous source of debate: lactogenic route of transmission has been described as important in enzootic infections (Blacklaws, 2012; Houwers *et al.*, 1989) while other studies suggest that its contribution to the spread of the disease seems to be low compared to horizontal/respiratory transmission (Broughton-Neiswanger *et al.*, 2010; Pérez *et al.*, 2010; Álvarez *et al.*, 2005).

SRLV presence in the nuclei of cells resembling mammary glandular epithelial, endothelial and fibroblast-like cells using immunohistochemistry (IHC) (Bolea *et al.*, 2006) or using *in situ* hybridization (Bolea *et al.*, 2006; Carrozza *et al.*, 2003) have been described.

Thus, the aims of this study are (1) to examine the cell types infected and the distribution of VMV antigen in healthy and injured areas of infected mammary gland, especially in minimal lesions and its relationship with blood vessels in order to better understand the pathogenesis of the disease in this target organ, both in animals from flocks with clinical signs or randomly selected from abattoirs, and the possible role in the transmission of the disease; (2) to develop a simple and suitable immunocytochemistry (ICC) technique to identify VMV antigen in different somatic milk cells and (3) to study viral excretion by PCR and cells involved in milk by ICC with the aim of contributing to clarifying the role of milk and colostrum in the transmission of the infection and it possible application in the diagnosis and control of the disease. (4) The last aim of this work is the evaluation of diagnosis techniques and virus identification in tissues and milk.

MATERIAL AND METHODS

Animals

A total of 84 udder samples from adult Spanish Assaf sheep from a region of Spain (Castilla y León) were studied (Supplementary S1).Forty-four sheep (Group A) were previously described (Gayo *et al.*, 2018). They were selected from those submitted to the Pathology Diagnostic Service of the Veterinary School (León, Spain) from seropositive flocks with clinical signs and diagnosis of the disease. These animals were 1-3 years of age and came from six different SRLV infected intensive milk-producing flocks of 300-1200 sheep subjected to SRLV control and with highly qualified staff (Gayo *et al.*, 2018; Gayo *et al.*, 2017). Additionally, 35 adult sheep (Group B) with unknown clinical history were randomly sampled from two abattoirs during regular slaughtering. Further samples of five sheep (Group C) from a free-SRLV flock were used as negative controls. None of sheep selected for this study showed gross or histological mastitis findings of bacterial or fungal origin.

Tissue Sampling, Histopathology.

Tissue samples from all 84 sheep were systematically taken from glandular parenchyma and udder cisterns. Samples were fixed in 10% neutral buffered formalin for 48 h at room temperature and were then embedded in paraffin wax. Sections (4 µm) were stained with hematoxylin and eosin (HE) and examined using light microscopy. Lesions were classified, without having previous knowledge of other result, as minimal (+), moderate (++) and severe (+++) as previously described (Gayo *et al.*, 2018; Gayo *et al.*, 2017).

Milk Sampling

Cistern milk samples from 2 flocks of group A (n=10) with 1267 x10^3 and 591 x 10^3 somatic cells/ml somatic cells/ml and 60% and 63% VM seroprevalence respectively and individual milk samples from group B (n=29) and C (n=5) were taken to tune up cytological, immunocitochemical (ICC) and PCR studies.

Citology optimization in milk samples

In order to optimize the procedure, the following variable on the protocol were considered: centrifugation speed, centrifugation time, volume of the samples, drying and fixation times. Milk tank samples (n=10) of 12 ml were taken in Falcon tubes from two seropositive flocks of group A. After milk centrifugation (Orto® and p-Selecta®) the largest number of cells was identified at 1500 rpm. Approximately three 500 µl samples were taken in 1 ml vial from three different locations (top, middle and bottom of the Falcon tube) in order to locate the region with more cell concentration and less fat globules. These three samples were subdivided in different volumes and cytocentrifuged (Cytospin 3®) using different centrifugation times at 1000 rpm following the manufacturer's instructions. Smears obtained from the top of the Falcon tube and cytocentrifugation at 1000 rpm for four minutes was established as the best combination. Different drying times after cytocentrifugation and fixation times with methanol at room temperature were tested and were established in 20 minutes each.

This technique was carried out in milk samples from the 29 sheep from group B and in further ten tank milk samples from the two infected flocks of group A after optimization. Samples were stained with hematoxylin and eosin (HE) and with May-Grunwald-Giemsa and were examined using light microscopy.

Immunohistochemistry and Immunocitochemistry

Immunohistochemistry in tissue samples: Further sections (4 µm) were prepared for IHC. The following antibodies were used as previously described (Gayo *et al.*, 2018): polyclonal anti-CD3 for T cells (Dako, Denmark); monoclonal anti-CD79 for B cells (Dako, Denmark); monoclonal anti-CD68 for macrophages (Dako, Denmark), and monoclonal anti-p28 of VMV/CAEV (VMRD, USA) for viral detection. Further policlonal anti-gp135 of SRLV elaborated by our research group and diluted 1:30000 was used in all samples. In order to obtain rabbit anti-serum against the VMV, a New Zealand white rabbit was immunized with gp135 protein supplied by Pourquier (1.6 g) emulsified with 1 ml of complete Freund's adjuvant on days 1, 14, 21 and 28. The last boost containing the gp135 protein in the absence of Freund's adjuvant was administered intravenously on day 35. The serum on day 38 and the pre-immune serum from the same rabbit were used for immunohistochemical studies. Sensibility and specificity of the technique were tested in ten known VM positive and negative samples, respectively.

Immunocitochemistry in milk samples. The optimized smear preparation was used for ICC in milk samples from the 29 sheep from group B and in ten tank milk samples from the two infected flocks of group A. Anti-p28 and anti-gp135 for SRLV antibodies described above were used (Gayo *et al.*, 2018).

Polymerase Chain Reaction in tissue and milk samples

Tissue samples from all sheep from group B were frozen for PCR studies. DNA was extracted using Genomic DNA Isolation Kit and eluting the DNA in 100 µl final volume (NORGEN, Biotek Corporation). A nested PCR protocol for amplifying a sequence of the LTR gene of VMV (Ryan *et al.*, 2000) and a PCR protocol to exclude *Mycoplasma spp* infection (Baird *et al.*, 1999) were used in both groups A (DNA previously extracted from paraffin embedded

tissue samples, (Gayo *et al.*, 2018) and B. Mammary gland DNA samples from sheep infected with VMV Pi130 and DNA from *Mycoplasma agassizii* were used as positive controls in PCR, while water instead of DNA was used as negative control in each PCR run.

White milk cells were extracted (centrifugation of 15 ml for 10 minutes at 2000 rpm at 10°C and fat removing with micropipette x3; centrifugation of 1,5 ml for 10 minutes at 1400 rpm at 10°C and washing with PBS x3) and pellets were frozen for PCR studies.

Serology

ELISA test (ELITEST®) for detection of SRLV antibodies was performed in animals from group A (n=44), group B (n=19) and group C (n=5) as previously described (Gayo *et al.*, 2017).

RESULTS

Three sheep from group B (3/35, 8.6%) and all animals from group C showed no lesions and were negative to IHC and PCR in tissue and milk samples and thus were considered as being not infected (Fig. 1A), while viral infection was confirmed using IHC (Figs. 1B-D), PCR and/or ELISA in the rest of the animals which were considered VM infected.

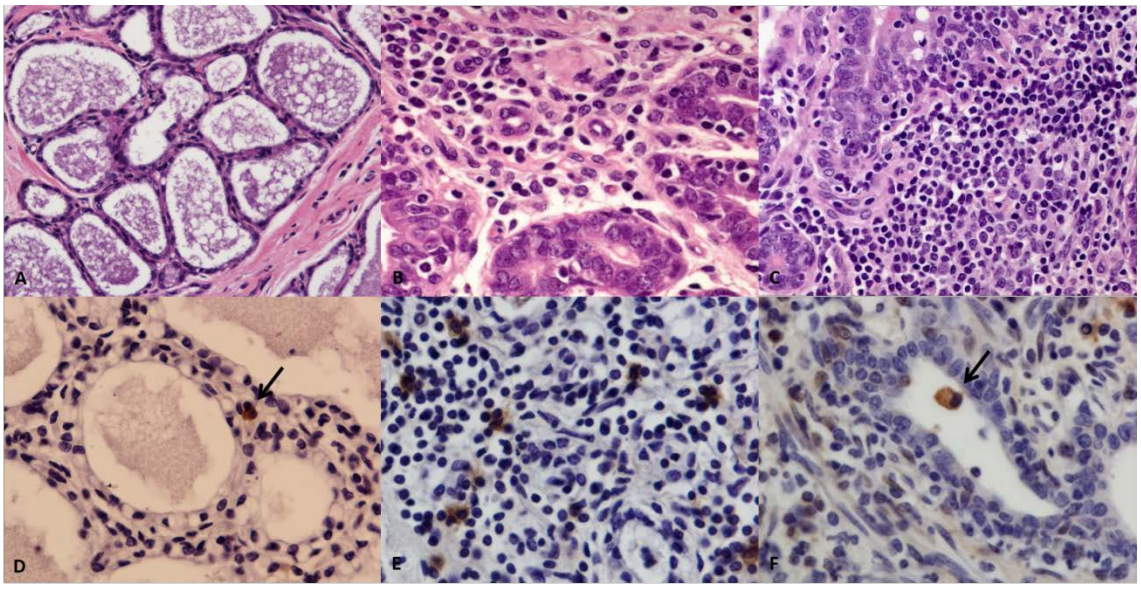

***Fig. 1**: Visna maedi (VM) lesions in udder sections. **A:** healthy mammary gland. No inflammatory infiltrate is observed. HE. 20X **B:** moderate lesions (++). Presence of inflammatory cells in mammary interstitium and within epithelial cells.40X **C:** severe lesions (+++) with high quantity of lymphocytes and macrophages producing acini destruction. 40X **D:** anti-gp135 immunohistochemistry in udder with minimal lesions. Black arrow indicates a positive macrophage-like cell within the intertitium with a few inflammatory cells in the surroundings. 40X. **E:** anti-p28 of CAEV/VMV immunohistochemistry in udder samples with severe lesion (+++). 40X. **F:** positive cell in the acinar lumen. No positivity is observed in epithelial cells.*

Histopathology

Interstitial mastitis was observed in 40 samples of group A (90.91%) (Gayo *et al.*, 2018) and in 30 samples from group B (85.7%). Minimal lesions (+) were observed in 26 infected animals (26/76, 34.2%), moderate lesions (++) were observed in 32 infected sheep (32/76, 42.1%) and severe lesions (+++) were observed in 12 infected sheep (12/76, 15.8%), with no large differences in severity lesion distribution between groups A and B (Table 1). Inflammatory infiltrates were always located in the interstitium between acini in every type of

lesion. A difference in the severity of the inflammatory infiltrate and lymphoid hyperplasia was observed in different mammary lobules. These differences were even observed between adjacent acinis, especially in minimal lesions, with most of them being healthy. No lesions were observed in six infected sheep (6/76, 7.9%) which showed no inflammatory cells in the interstitium and only a few and focal small lymphocyte aggregates were found in association with canaliculi.

Grade of lesions	**Group A**	**(%)**	**Group B**	**(%)**	**Total**	**(%)**
Minimal (+)	15	(34.1)	11	(31.4)	26	(32.9)
Moderate (++)	18	(40.9)	14	(40)	32	(40.5)
Severe (+++)	7	(15.9)	5	(14.3)	12	(15.2)
None (-)	4	(9.1)	5	(14.3)	9	(11.4)
Total	44		35		79	

Table 1*: Occurrence of lesions due to VM in terms of intensity of lesions and comparison between groups A and B.*

Immunohistochemistry

The predominant cell type within the interstitial inflammatory infiltrate was CD3+ cell with some scattered CD79+ and CD68+ cells, regardless of the severity and extension of the lesions. Macrophages were observed in different quantities independently of the predominance of CD3+ cells and were found not only in the interstitium but also between epithelial cells or in the acinar lumen.

All sheep from group A and 32 sheep from group B were positive to SRLV using IHC with both antibodies while the eight sheep considered not infected were negative (Table 2). Positivity against SRLV was only found in macrophage-like cells of mammary tissue, even in focal and minimal lesions, while no sign was observed in healthy mammary tissues in the same animals and in sheep with no lesions. SRLV antigen was found surrounding blood vessels in minimal lesions while in moderate and severe lesions was present in perivascular cuffs, within the epithelium and even in cells located in the acinar lumen in eight of sheep (Fig. 1). Positive cells were also observed isolated within the interstitium, associated with minimal or moderate lesions showing no IHC sign in closely areas and in intralobular and interlobular connective tissue. No positive sign was present in epithelial cells or other cell types using both monoclonal anti p28 and policlonal anti gp135 antibodies. No immunolabelled cells were detected in sections used as negative controls but labelling was invariably seen in positive control sections.

Polymerase Chain Reaction in tissue samples

Forty animals from group A and 32 from group B were positive to PCR against VMV (Table 2). Three of the 4 negative animals from group A showed minimal lesions and the remaining sheep showed moderate lesions. The three negative animals from group B were the sheep considered as being not infected. No positivity was observed in PCR against *Mycoplasma spp*.

Serology

Thirty-nine animal from group A were seropositive (88.6%), three had inconclusive results (OD of 0.4±0.1), and two were negative (Gayo *et al.*, 2018). Fourteen infected sheep from group B were seropositive (77.8%) while four were negative: two showed minimal lesions

and one moderate lesions (Table 3). (Supplementary S1).

Cytological studies in milk

HE and May-Grunwald Giemsa staining permitted a fine identification of the different milk cell populations. Macrophages and neutrophils were easily identified by HE staining, while May-Grunwald Giemsa was better for the identification of epithelial cells.

After milk cytocentrifugation cells appeared distributed within the slide in three concentric cellular rings: neutrophils were the predominant cell population in the centre, epithelial cells were the most abundant cells in the periphery, and macrophages were distributed homogeneously within the slide.

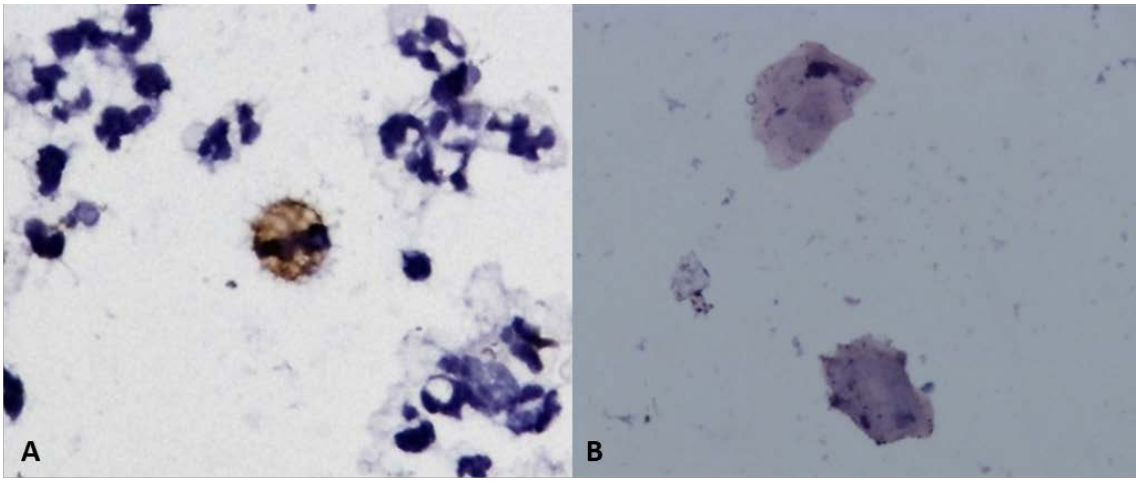

Fig. 2*: Anti-p28 of VMV/CAEV immunocytochemistry in milk samples. 60X.* ***A:*** *positive macrophage-like cell surrounded by some neutrophils and lymphocytes with no IHC sign.* ***B:*** *negative epithelial cells.*

Immunocytochemistry in milk samples

Positive macrophage-like cells were observed by ICC in milk from the two cistern milk samples and in seven individual milk samples (26.9%) (Table 2) (Fig 2A). Three of them were

from sheep with severe lesions and four from sheep with moderate lesions (Supplementary S1). This labelling appeared as a brownish deposit in the cytoplasm of macrophages-like cells and no positivity was observed in epithelial-like cells (Fig 2).

Polymerase Chain Reaction in milk samples

Milk samples from 13 sheep were positive to VMV by PCR (50%). Four of them showed severe lesions, seven showed moderate lesions and two minimal lesions. Six of them were also positive in ICC. (Table 2) (Supplementary S1).

Diagnosis techniques comparison

IHC was the most sensitive technique with 100% positivity in groups A and B. Histopathology detected 92.1% infected animals, with 90.9% detection in group A and 93.8% in group B. ELISA showed 88.6% and 77.8% positive values in groups A and B, respectively (85.5% infected animals). PCR was more sensitive than ELISA in VM viral detection with values increasing to 90.9% in group A and 100% in group B (94.7% infected animals). PCR was also more sensitive than ICC in milk samples, with values increasing from 25% to 50% of viral detection, respectively (Table 2).

Group	HE (%)	IHC (u) (%)	PCR (u) (%)	ICC(m) (%)	PCR(m) (%)	ELISA (%)
Infected	70/76 (92.1)	76/76 (100)	72/76 (94.7)	*9/36 (25)	13/26 (50)	53/62 (85.5)
Not infected	0/8 (0)	0/8 (0)	0/8 (0)	0/8 (0)	0/8 (0)	0/8 (0)

Table 2: *histopathology (HE), IHC and PCR results udder (u) and milk (m) samples and ELISA results in infected and non-infected sheep. *2/10 samples were from milk tank and 7/26 were from individual sheep.*

DISCUSSION

Detailed characterization of udder lesions and VMV antigen distribution and target cells, especially in minimal lesions, contribute to clarify the pathogenesis of VM infection in this target organ in naturally infected milk-producing sheep. VM has been reported as the major cause of sheep culling in two intensively-managed dairy sheep flocks in the same study area, having an important economic impact (Benavides *et al.*, 2013). Here, the percentages of lesions of different severity were similar in sheep from known infected flocks and randomly selected from two different abattoirs. Fifty-seven per cent of animals from infected flocks and 54% of sheep from abattoir showed moderate or severe lesions which confirm the importance of the mammary form of the disease in dairy sheep culling and it relationship with production loss. Serological results also highlights the high prevalence of VM infection in intensive milk-producing sheep in Castilla y León (Spain), with 91.4% sheep randomly selected from abattoirs being positive to PCR and IHC, similar to prevalence reported by serology many years ago (MJ, 1998). Serology did not detect any infection in six sheep with positive histopathology, IHC and

PCR results while diagnosis of all seropositive sheep was confirmed using histopathology, IHC and/or PCR. IHC and PCR clearly increased VM prevalence in infected sheep reaching up 100% and 94.7 positivity respectively in comparison with 85.5% seropositivity with ELISA test. The lower sensitivity obtained with PCR in comparison with IHC could be related with DNA degradation after formalin fixation of the samples from animals of group A or to low viral presence (de Andrés *et al.*, 2005). However, a PCR sensitivity higher than 90% in these animals point towards a high conservation of proviral VMV DNA in formalin-fixed and paraffin-embedded samples, which are the only available material in many retrospective studies. These results agree with previous studies where VMV positivity was described by PCR in formalin fixed and paraffin-embedded samples even after 30 days of fixation (Benavides *et al.*, 2006b).

On the other hand, the higher sensitivity of PCR (50%) in comparison with ICC (25%) in milk samples could be due to the difficulty of macrophages detection in the cytological smear, especially those positive to VMV. The existence of one milk sample positive to VMV by ICC but negative by PCR could be related to a scarce number of infected macrophages or a low viral load in this particular sample, which has been described as one of the main problems of VMV detection by PCR (de Andrés *et al.*, 2005). Viral detection by this technique even in animals with minimal lesions would confirm it usefulness for VMV detection.

More than one diagnosis technique should be performed in order to detect all infected animals. The IHC in tissues would be used as a “gold standard” technique for the disease detection, being the only one detecting all VM infected sheep. Using only the ELISA test for VM diagnosis as frequently happens in field conditions involves the risk of obtaining false negative animal (Gayo *et al.*, 2017). As has also been observed in this work, this feature is very important in flocks submitted to control programs where VM prevalence is always lower and detection of every single infected sheep, even with minimal lesions, is of great interest for eradication of the disease.

Two infected sheep with minimal lesions and one with no lesions showed negative ELISA values and positive IHC and PCR results agree with hypothesis of minimal lesions would be related to initial or latent stages of the disease where the number of inflammatory cells would not be enough to develop a local immune response and antibody production (Gayo *et al.*, 2017). Thus, after VM infection viral presence in tissue detectable using PCR and IHC could be first and serological response could be measurable after lesion and local immune response development. Negative ELISA results in animals with moderate or severe lesions could be related to the development of a lymphocytic pattern (Gayo *et al.*, 2018; Polledo *et al.*, 2012b) more frequent in mammary gland, as has been recently published (Gayo *et al.*, 2018).

Detection of minimal lesions clearly increased the incidence of VM lesions in mammary gland confirming previously published results.(Gayo *et al.*, 2018) Minimal inflammatory infiltrates and subepithelial lymphoid aggregates have been described in healthy udders on the limit between teat duct and teat cistern and have been related to protection in the early stages of bacterial invasion (Mavrogianni *et al.*, 2007) but were not found in non-infected animals or in non-affected tissue of infected sheep in this study and an early VM mastitis should be also considered. This lack of inflammatory infiltration could be explained by the selection and the constant microbiological and somatic cells controls performed in intensive milk-producing flocks which is the management system used for all dairy flocks of Spanish Assaf breed in the region studied. The negative result to the presence on *Mycoplama spp.* could confirm this hypothesis.

The predominance of a CD3+ lymphocytic response in animals from group B agree with our previous description in group A (Gayo *et al.*, 2018). However, it contrasts with findings in CNS and lung where two lesion pattern (histiocytic and lymphocytic) were described regarding the predominant inflammatory cell population (Gayo *et al.*, 2018; Polledo *et al.*, 2012b), which could confirm a different local immunological response in this target organ. Nevertheless, the presence of more macrophages within the inflammatory infiltrate in the mammary gland of

some animals may be related to the development of a histiocytic or lymphocytic pattern with the previously mentioned differences.

The presence of IHC-positive sign to p28 and gp135 of SRLV macrophage-like cells in mammary gland and milk agree with previous results in different VM target organs (Minguijón *et al.*, 2015). Presence of surface *env*-encoded protein gp135 together with nucleocapsid *gag*-enconded protein p28 indicates not only viral presence but also active infection. In most cases viral presence was associated to inflammatory cells and p28 and gp135 proteins of SRLV were not observed in non-affected areas of infected tissues. This fact together with the viral distribution surrounding blood vessels in minimal lesions and spreading to interstitial mammary tissue in moderate and severe lesions would agree with the hypothesis of viral invasion via monocytes/macrophages (Gayo *et al.*, 2018; Peluso *et al.*, 1985; Polledo *et al.*, 2012a). Infection would begin in very focally concrete locations of the organ and then spread to the rest of the tissue and increase the lesion in other areas facilitated by possible concurrent VM infections which could reach different blood vessels. VM positive IHC sign in isolated cells located in areas with no lesions or in connective tissue in some samples could be related with small blood vessels not observed in these particular histological sections or with free virus presence within the tissue.

SRLV immunoreactivity were not observed in epithelial cells using both antibodies against gp135 and p28 proteins in contrast to previous descriptions (Bolea *et al.*, 2006) and to the hypothesis of the possible SRLV replication in epithelial cells ''in vivo'' as has been described ''in vitro' (Bolea *et al.*, 2006; Lerondelle *et al.*, 1999). Furthermore, *in situ* hybridization or PCR as previously reported in epithelial cells of mammary gland (Bolea *et al.*, 2006; Carrozza *et al.*, 2003) show the presence of viral nucleic acids but not the presence of viral proteins or active viral replication. Nevertheless, we show in this study that active infection is happening by using two different antibodies against surface and nucleocapsid

proteins of VM as previously mentioned. However, further studies of viral replication in mammary gland should be performed.

The presence of SRLV antigen in all mammary glands with lesions including cells in acinar lumen in some cases and in milk cells confirm the potential transmission of the disease by lactation. However, the small positive number of cells and few positive milk samples in IHC and PCR could point out the limited potential of transmission, as has also been proposed by others authors, (Berriatua *et al.*, 2003) leading to a higher VM transmission risk by continuous lactation and not only by colostrum ingestion. Most positive milk samples came from sheep with moderate or severe lesions in udder (85.7%) and higher number of positive cells in tissue by IHC as well as positive PCR results. These findings would support that reaching canaliculi is easier for infected macrophages when lesions are more severe and widespread than when they are focal and limited to perivascular cuffs.

The ICC technique developed in milk in this study allows for the obtaining of well-preserved monolayer cell smears and optimal identification of cell populations and VMV antigen in somatic milk cells. After the first centrifugation, cell distribution within the Falcon tube are very likely conditioned by their density as happens in blood vessels during inflammation (V *et al.*, 2005). The fact that cells from the top seemed better preserved than the ones from the bottom could be due to a protecting role of fat during centrifugation. The optimum drying time of the cytology after cytocentrifugation was 20 minutes, much shorter than the three hours used in previous studies (Luján *et al.*, 1994). HE staining is very useful to identify leukocytes while epithelial cells were better identified with May-Grenwald Giemsa staining (Cowell, 2008). This latter staining helps to differentiate these cells from artefacts, bringing a different pink colour compared with artefacts or other cells populations. The epithelial cells show normal morphology without abnormalities, suggesting lack of mammary lesions absence of hyperplasia or neoplasia in the mammary gland epithelia of the sheep

studied (Cowell, 2008). ICC slides showed no fat globules unlike cytology slides stained with HE or May-Grenwald Giemsa, which we attribute to the successive washings in PBS and the blocking of the endogenous peroxidase activity with hydrogen peroxide 3% in distilled water for 30 minutes.

CONCLUSIONS

Detection of minimal lesions using histopathology increased the incidence of VMV in mammary gland and viral antigen distribution from a few cells in perivascular cuffs to the rest of tissue in moderate and severe lesions would confirm viral progression from blood monocytes to macrophages in this target organ. SRLV immunoreactivity was observed only in macrophages-like cells and not in epithelial cells. The histopathology with IHC in tissues would be used as a "gold standard" technique for the disease detection, being the only one detecting all VM infected sheep. Viral detection in milk samples using ICC and PCR confirms potential but limited role of milk/colostrum in viral transmission point toward a higher importance routes.

SUPPLEMENTARY MATERIALS

Sheep	Histology	OD	IHC (U)	ICC (M)	PCR (U)	PCR (M)
A1	+++	0.09	+	NS	+	NS
A2	++	0.25	+	NS	+	NS
A3	+	0.42	+	NS	+	NS
A4	+++	0.5	+	NS	+	NS
A5	++	0.53	+	NS	+	NS
A6	++	0.73	+	NS	+	NS
A7	+	0.75	+	NS	+	NS
A8	+	0.77	+	NS	+	NS
A9	++	0.79	+	NS	+	NS
A10	+	0.81	+	NS	-	NS
A11	+	0.91	+	NS	+	NS

A12	+++	1	+	NS	+	NS
A13	++	1.07	+	NS	+	NS
A14	++	1.11	+	NS	+	NS
A15	++	1.12	+	NS	-	NS
A16	-	1.12	+	NS	+	NS
A17	+	1.18	+	NS	-	NS
A18	++	1.35	+	NS	+	NS
A19	++	1.37	+	NS	+	NS
A20	-	1.39	+	NS	+	NS
A21	+	1.39	+	NS	+	NS
A22	++	1.44	+	NS	+	NS
A23	+	1.45	+	NS	+	NS
A24	+	1.47	+	NS	+	NS
A25	++	1.5	+	NS	+	NS
A26	++	1.61	+	NS	+	NS
A27	+++	1.65	+	NS	+	NS
A28	++	1.68	+	NS	+	NS
A29	+	1.8	+	NS	+	NS
A30	++	1.81	+	NS	+	NS
A31	+	1.88	+	NS	+	NS
A32	+	1.95	+	NS	+	NS
A33	+	2.09	+	NS	+	NS
A34	+	2.14	+	NS	-	NS
A35	++	2.19	+	NS	+	NS
A36	+++	2.27	+	NS	+	NS
A37	+++	2.4	+	NS	+	NS
A38	+++	2.4	+	NS	+	NS
A39	++	2.47	+	NS	+	NS
A40	-	2.5	+	NS	+	NS
A41	++	2.5	+	NS	+	NS
A42	++	2.5	+	NS	+	NS
A43	+	2.65	+	NS	+	NS
A44	-	2.77	+	NS	+	NS
B1	+	0.01	+	-	+	-
B2	-	0.01	-	-	-	-
B3	-	0.01	+	-	+	-
B4	+	0.06	+	-	+	+
B5	++	0.17	+	-	+	-
B6	++	1.05	+	+	+	+
B7	++	1.25	+	+	+	-
B8	+++	1.41	+	+	+	+
B9	++	1.43	+	-	+	+
B10	++	1.56	+	-	+	+
B11	+	1.65	+	-	+	-

B12	+++	1.7	+	-	+	+
B13	++	1.8	+	+	+	+
B14	++	1.97	+	-	+	-
B15	+	2.11	+	-	+	-
B16	++	2.21	+	-	+	+
B17	+	2.22	+	-	+	+
B18	++	2.31	+	+	+	+
B19	++	2.41	+	-	+	+
B20	-	NS	+	-	+	-
B21	-	NS	-	-	-	-
B22	+	NS	+	-	+	-
B23	+	NS	+	-	+	-
B24	+	NS	+	-	+	-
B25	+++	NS	+	+	+	+
B26	-	NS	-	-	-	-
B27	+++	NS	+	-	+	-
B28	++	NS	+	-	+	-
B29	+++	NS	+	+	+	+
B30	++	NS	+	NS	+	NS
B31	++	NS	+	NS	+	NS
B32	+	NS	+	NS	+	NS
B33	++	NS	+	NS	+	NS
B34	+	NS	+	NS	+	NS
B35	+	NS	+	NS	+	NS

Supplementary S1: *histology, ELISA (OD), IHC and PCR results in udder (U) and milk (M) samples. NS: no sample available. Animals highlighted in red were considered not infected.*

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DECLARATIONS

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Availability of data and materials

All the data supporting these findings are present within the manuscript. Detailed data of all sheep studied are showed in Supplementary S1 table.

Authors' contributions

EG is the main performer of experiments, the manuscript writer and the creator of tables and figures. JGM is the director of the study. LP, AM, CPM, MJGI and AB collaborated in gp135 immunohistochemical and cytological techniques. SP and GR collaborated in p28 immunohitochemical technique and PCR studies. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

REFERENCES

1. Thormar H: **Maedi-visna virus and its relationship to human immunodeficiency virus**. *AIDS Reviews* 2005, **7**(4):233-245.
2. Pépin M, Vitu C, Russo P, Mornex JF, Peterhans E: **Maedi-visna virus infection in sheep: A review**. *Veterinary Research* 1998, **29**(3-4):341-367.

3. Oliver RE, Gorham JR, Parish SF, Hadlow WJ, Narayan O: **Ovine progressive pneumonia: pathologic and virologic studies on the naturally occurring disease**. *American Journal of Veterinary Research* 1981, **42**(9):1554-1559.

4. Georgsson G, Pálsson PA: **The histopathology of maedi a slow, viral pneumonia of sheep**. *Veterinary pathology* 1971, **8**(1):63-80.

5. Sigurdsson B, Palsson P, Grimsson H: **Visna, a demyelinating transmissible disease of sheep**. *Journal of neuropathology and experimental neurology* 1957, **16**(3):389-403.

6. Luján L, García Marin JF, Fernández de Luco D, Vargas A, Badiola JJ: **Pathological changes in the lungs and mammary glands of sheep and their relationship with maedi-visna infection**. *Veterinary Record* 1991, **129**(3):51-54.

7. Minguijón E, Reina R, Pérez M, Polledo L, Villoria M, Ramírez H, Leginagoikoa I, Badiola JJ, García-Marín JF, de Andrés D *et al*: **Small ruminant lentivirus infections and diseases**. *Veterinary Microbiology* 2015, **181**(1–2):75-89.

8. Leginagoikoa I, Daltabuit-Test M, Álvarez V, Arranz J, Juste RA, Amorena B, de Andrés D, Luján LL, Badiola JJ, Berriatua E: **Horizontal Maedi-Visna virus (MVV) infection in adult dairy-sheep raised under varying MVV-infection pressures investigated by ELISA and PCR**. *Research in Veterinary Science* 2006, **80**(2):235-241.

9. Sotelo MJ: **La infección por lentivirus en ovinos y caprinos de la provincia de León: aspectos epidemiológicos y valoración deun plan de control.** In. PhD Thesis: University of León; 1998.

10. Benavides J, Fuertes M, García-Pariente C, Otaola J, Delgado L, Giraldez J, García Marín JF, Carmen Ferreras M, Pérez V: **Impact of maedi-visna in intensively managed dairy sheep**. *Veterinary Journal* 2013, **197**(3):607-612.

11. Polledo L, Gonzalez J, Fernandez C, Miguelez J, Martinez-Fernandez B, Morales S, Ferreras MC, Garcia Marin JF: **Simple control strategy to reduce the level of Maedi-**

Visna infection in sheep flocks with high prevalence values (> 90%). *Small Ruminant Research* 2013, **112**(1-3):224-229.

12. Ryan S, Tiley L, McConnell I, Blacklaws B: **Infection of dendritic cells by the Maedi-visna lentivirus**. *Journal of Virology* 2000, **74**(21):10096-10103.
13. Bird P, Blacklaws B, Reyburn HT, Allen D, Hopkins J, Sargan D, McConnell I: **Early events in immune evasion by the lentivirus maedi-visna occurring within infected lymphoid tissue**. *Journal of Virology* 1993, **67**(9):5187-5197.
14. Blacklaws BA, Bird P, Allen D, Roy DJ, Maclennan ICM, Hopkins J, Sargan DR, McConnell I: **Initial lentivirus-host interactions within lymph nodes: A study of maedi-visna virus infection in sheep**. *Journal of Virology* 1995, **69**(3):1400-1407.
15. Gendelman HE, Narayan O, Molineaux S, Clements JE, Ghotbi Z: **Slow, persistent replication of lentiviruses: Role of tissue macrophages and macrophage precursors in bone marrow**. *Proceedings of the National Academy of Sciences of the United States of America* 1985, **82**(20):7086-7090.
16. Blacklaws BA: **Small ruminant lentiviruses: Immunopathogenesis of visna-maedi and caprine arthritis and encephalitis virus**. *Comparative Immunology, Microbiology and Infectious Diseases* 2012, **35**(3):259-269.
17. Lee CS, Outteridge PM: **Leucocytes of sheep colostrum, milk and involution secretion, with particular reference to ultrastructure and lymphocyte sub-populations**. *Journal of Dairy Research* 1981, **48**(2):225-237.
18. Sihvonen L: **Studies on transmission of maedi virus to lambs**. *Acta veterinaria Scandinavica* 1980, **21**(4):689-698.
19. Preziuso S, Taccini E, Rossi G, Renzoni G, Braca G: **Experimental Maedi Visna Virus Infection in sheep: A morphological, immunohistochemical and PCR study after three years of infection**. *European Journal of Histochemistry* 2003, **47**(4):373-378.

20. Preziuso S, Renzoni G, Allen TE, Taccini E, Rossi G, Demartini JC, Braca G: **Colostral transmission of maedi visna virus: Sites of viral entry in lambs born from experimentally infected ewes**. *Veterinary microbiology* 2004, **104**(3-4):157-164.

21. Ellis T, Robinson W, Wilcox G: **Effect of colostrum deprivation of goat kids on the natural transmission of caprine retrovirus infection**. *Australian Veterinary Journal* 1983, **60**(11):326-329.

22. Ellis TM, Carman H, Robinson WF, Wilcox GE: **The effect of colostrum-derived antibody on neo-natal transmission of caprine arthritis-encephalitis virus infection**. *Australian Veterinary Journal* 1986, **63**(8):242-245.

23. Houwers DJ, Visscher AH, Defize PR: **Importance of ewe/lamb relationship and breed in the epidemiology of maedi-visna virus infections**. *Research in veterinary science* 1989, **46**(1):5-8.

24. Álvarez V, Arranz J, Daltabuit-Test M, Leginagoikoa I, Juste RA, Amorena B, de Andrés D, Luján LL, Badiola JJ, Berriatua E: **Relative contribution of colostrum from Maedi-Visna virus (MVV) infected ewes to MVV-seroprevalence in lambs**. *Research in veterinary science* 2005, **78**(3):237-243.

25. Pérez M, Biescas E, de Andrés X, Leginagoikoa I, Salazar E, Berriatua E, Reina R, Bolea R, de Andrés D, Juste RA *et al*: **Visna/maedi virus serology in sheep: Survey, risk factors and implementation of a successful control programme in Aragón (Spain)**. *The Veterinary Journal* 2010, **186**(2):221-225.

26. Broughton-Neiswanger LE, White SN, Knowles DP, Mousel MR, Lewis GS, Herndon DR, Herrmann-Hoesing LM: **Non-maternal transmission is the major mode of ovine lentivirus transmission in a ewe flock: A molecular epidemiology study**. *Infection, Genetics and Evolution* 2010, **10**(7):998-1007.

27. Bolea R, Monleón E, Carrasco L, Vargas A, De Andrés D, Amorena B, Badiola JJ, Luján L: **Maedi-visna virus infection of ovine mammary epithelial cells**. *Veterinary Research* 2006, **37**(1):133-144.

28. Carrozza ML, Mazzei M, Bandecchi P, Arispici M, Tolari F: **In situ PCR-associated immunohistochemistry identifies cell types harbouring the Maedi-Visna virus genome in tissue sections of sheep infected naturally**. *Journal of Virological Methods* 2003, **107**(2):121-127.

29. Gayo E, Polledo L, Balseiro A, Martínez CP, García Iglesias MJ, Preziuso S, Rossi G, García Marín JF: **Inflammatory Lesion Patterns in Target Organs of Visna/Maedi in Sheep and their Significance in the Pathogenesis and Diagnosis of the Infection**. *Journal of Comparative Pathology* 2018, **159**:49-56.

30. Gayo E, Polledo L, Preziuso S, Rossi G, Balseiro A, Pérez Martínez C, García Iglesias MJ, García Marín JF: **Serological ELISA results are conditioned by individual immune response in ovine maedi visna**. *Small Ruminant Research* 2017, **157**:27-31.

31. Baird SC, Carman J, Dinsmore RP, Walker RL, Collins JK: **Detection and identification of Mycoplasma from bovine mastitis infections using a nested polymerase chain reaction**. *Journal of Veterinary Diagnostic Investigation* 1999, **11**(5):432-435.

32. de Andrés D, Klein D, Watt NJ, Berriatua E, Torsteinsdottir S, Blacklaws BA, Harkiss GD: **Diagnostic tests for small ruminant lentiviruses**. *Veterinary microbiology* 2005, **107**(1–2):49-62.

33. Benavides J, García-Pariente C, Gelmetti D, Fuertes M, Ferreras MC, García-Marín JF, Pérez V: **Effects of fixative type and fixation time on the detection of Maedi Visna virus by PCR and immunohistochemistry in paraffin-embedded ovine lung samples**. *Journal of virological methods* 2006, **137**(2):317-324.

34. Polledo L, González J, Benavides J, Morales S, Martínez-Fernández B, Delgado L, Reina R, Glaria I, Pérez V, Ferreras MC *et al*: **Patterns of Lesion and Local Host Cellular**

Immune Response in Natural Cases of Ovine Maedi-Visna. *Journal of comparative pathology* 2012, **147**(1):1-10.

35. Mavrogianni VS, Cripps PJ, Brooks H, Taitzoglou IA, Fthenakis GC: **Presence of subepithelial lymphoid nodules in the teat of ewes**. *Anat Histol Embryol* 2007, **36**(3):168-171.

36. Peluso R, Haase A, Stowring L, Edwards M, Ventura P: **A Trojan Horse mechanism for the spread of visna virus in monocytes**. *Virology* 1985, **147**(1):231-236.

37. Polledo L, Gonzalez J, Benavides J, Martinez-Fernandez B, Carmen Ferreras M, Garcia Marin JF: **Perivascular inflammatory cells in ovine Visna/maedi encephalitis and their possible role in virus infection and lesion progression**. *Journal of neurovirology* 2012, **18**(6):532-537.

38. Lerondelle C, Godet M, Mornex JF: **Infection of primary cultures of mammary epithelial cells by small ruminant lentiviruses**. *Veterinary Research* 1999, **30**(5-6):467-474.

39. Berriatua E, Álvarez V, Extramiana B, González L, Daltabuit M, Juste R: **Transmission and control implications of seroconversion to Maedi-Visna virus in Basque dairy-sheep flocks**. *Preventive veterinary medicine* 2003, **60**(4):265-279.

40. V K, AK A, N F: **Inflamación aguda y crónica**. In: *En: Kumar V, Abbas AK, Fausto N Patología estructural y funcional, 7ª edición.* Madrid: Elsevier; 2005: 48.

41. Luján L, Begara I, Collie D, Watt NJ: **Ovine lentivirus (maedi-visna virus) protein expression in sheep alveolar macrophages**. *Veterinary Pathology* 1994, **31**(6):695-703.

42. Cowell RL: **Diagnostic cytology and hematology of the dog and cat**. St. Louis, Missouri: Mosby Elsevier; 2008.

VIII. Cuarto trabajo

CUARTO TRABAJO

Genetic Characterization and Phylogenetic Analysis of Small Ruminant Lentiviruses Detected in Spanish Assaf Sheep with Different Mammary Lesions *Viruses* 2018, *10*(6), 315;

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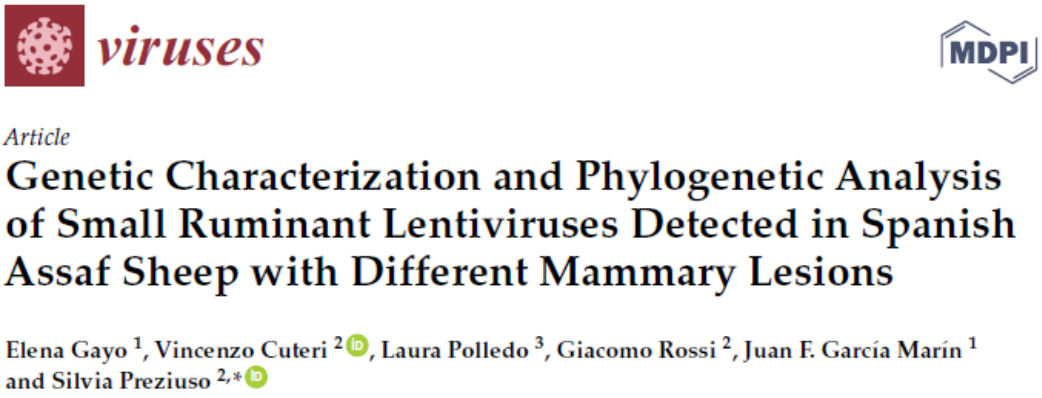

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ABSTRACT

Small Ruminant Lentiviruses (SRLVs) are widespread in many countries and cause economically relevant, slow, and persistent diseases in sheep and goats. Monitoring the genetic diversity of SRLVs is useful to improve the diagnostic tools used in the eradication programs. In this study, SRLVs detected in Spanish Assaf sheep with different grades of lymphoproliferative mastitis were sequenced. Genetic characterization showed that most samples belonged to type A and were closer to Spanish SRLV isolates previously classified as A2/A3. Four samples belonged to subtype B2 and showed higher homology with Italian B2 strains than with Spanish B2 isolates. Amino acid sequences of immuno-dominant epitopes in the gag region were very conserved while more alterations were found in the LTR sequences. No significant correlations were found between grades of mastitis and alterations in the sequences although samples with similar histological features were phylogenetically closer to each other. Broader genetic characterization surveys in samples with different grades of SRLV-lesions are required for evaluating potential correlations between SRLV sequences and the severity of diseases.

Keywords: sheep; Small Ruminant Lentivirus; genetic characterization; udder; histopathology

INTRODUCTION

Small Ruminant Lentiviruses (SRLVs) include Visna-maedi virus (VMV) and Caprine arthritis encephalitis virus (CAEV), which cause slow inflammatory diseases in sheep and goats named, respectively, Visna-Maedi (VM) and Caprine Arthritis Encephalitis (CAE). Viruses are transmitted mainly via the respiratory route and by colostrum intake [1,2] and cause persistent infections with a long incubation period. According to the current nomenclature based on gag–pol and pol sequences, SRLV can be subdivided into genotypes A–E with subtypes present in A, B, and E [3–5]. Typically, VMV was believed to infect specifically sheep and was included in genotype A while CAEV was considered goat-specific and was included in genotype B. However, several investigations showed that cross-infection may occur ([6] and other studies reviewed in [5]). Infected animals can develop neurological, pulmonary, arthritic, and/or mammary diseases that affect considerable animal welfare and production. Different patterns of inflammatory mononuclear cell accumulation are observed usually in the central nervous system, lung, joint, and/or udder and the predominant clinical manifestation depends on the severity and extension of the lesions reached in the affected organs [7].

Mammary lesions often consist of mononuclear cell infiltration with scattered hyperplastic lymphoid follicles [7,8]. Moderate lesions are described when diffused infiltration of lymphocytes within lobules with distortion of acini are observed and mild lesions are reported when occasional aggregates of lymphocytes in inter-acinar stroma are present [9]. Recently, minimal lesions consisting of a few small clusters to multifocal small groups of inflammatory cells with the presence of a few small lymphoid aggregates and/or one small lymphoid follicle have been reported in SRLV infected sheep [10]. One of the most important productive impacts of SRLV disease is due to the premature removal of diseased animals because of low milk production and quality with consequent economic losses in the milk-related and lamb/kids-related industry [11–13]. Due to the significant economic impact of

diseases, VM and CAE are included in the OIE List and specific control and eradication programs are carried out in many countries [14,15]. Availability of sensitive and specific diagnostic tests is of great importance for a correct discrimination between infected and non-infected animals. Due to the high rate of genetic diversity, new genotypes and subtypes might escape the diagnostic detection with the possible consequence of invalidating any eradication program in place [1]. Therefore, genotype and subtype surveys of the circulating SRLVs should be encouraged. Many studies describe phylogenetic analysis of SRLVs found during epidemiological surveys or in outbreaks of diseases, but only a few of them describe the histopathological lesions observed in target organs [16–18]. For example, mild mammary lesions with a multifocal method to diffuse mononuclear inflammatory interstitial infiltrates have been observed in sheep with arthritis and infected by B2 SRLV [17]. Different histopathological scores have been reported in mammary glands but not in lungs, synovial membranes of joints, or the chorioid plexus of five goats infected by A4 SRLV [16]. A 13–14 nucleotide deletion in the R region of the LTR has been observed in sheep with a decreased pathology in the lung but not in the udder even though SRLV subgenotypes were not known [18]. To our knowledge, correlations among histopathological grading of mammary lesions and SRLV genotypes and subtypes are yet to be investigated.

The aim of this work was to carry out genetic characterization and phylogenetic analysis of SRLV detected in Spanish sheep showing different histopathological grades of mastitis.

MATERIAL AND METHODS

2.1. Samples

A total of 35 udder samples were collected randomly at the slaughterhouse in the region of Castilla y Leon, Northwestern Spain, from Assaf sheep (1–4 years of age) belonging to seropositive flocks between March 2017 and May 2017. Nineteen samples were collected at the slaughterhouse named M (samples M1–M19) and 16 were collected at the slaughterhouse named Q (samples Q1–Q16), which was about 45 Km far from the slaughterhouse M. A first aliquot of each sample was stored at -20°C and DNA was obtained from 25 mg of each sample by using the Genomic DNA isolation Kit (Norgen Biotek Corp., Thorold, ON, Canada) and following the manufacturer's instructions when eluting the DNA in 100 µL final volume. A second aliquot of samples was fixed in 10% neutral buffered formalin for 48 h at room temperature and embedded in paraffin wax (FFPE) for histopathology and immunohistochemistry (IHC). In addition, 2 FFPE mammary samples (N16-426 and N-17-44) of sheep (Assaf breed, 3 years old) with histological mastitis referable to SRLV disease were available for this study. DNA from these latter samples was obtained from 4 slides 10 µm thick of each sample by Recover All Total Nucleic Acid Isolation (Ambion, Waltham, MA, USA) following the instructions and eluting DNA with 60 µL of elution solution warmed up at 95 °C.

2.2. Histopathology and Immunohistochemistry

Slides 4 µm thick were obtained from FFPE samples and were stained with haematoxylin and eosin (HE) for histopathology. Grading of histopathological lesions of mammary glands was carried out by three independent pathologists, which was previously described [10]. Briefly, "no lesions" was defined when no inflammatory cells were observed and "minimal lesions" (+) consisted of a few small clusters.

Multifocal small groups of inflammatory cells with the presence of a few small lymphoid aggregates and/or one small lymphoid follicle known as 'moderate lesions' (++) were characterized by the multifocal method to diffuse interstitial non-supportive inflammation and/or the presence of two to 15 lymphoid aggregates/follicles and 'severe lesions' (+++) consisted of a marked diffuse interstitial mastitis and/or the presence of >15 lymphoid aggregates/follicles. When differences between the severity of interstitial inflammatory infiltrates and the presence of lymphoid follicles were observed in the same organ of an animal, the most severe lesion was considered as the score for the lesion in that target organ [10]. Serial sections (4 µm) were used for IHC, which was previously reported [19]. A monoclonal antibody to the SRLV core protein p28 (VMRD Inc., Pullman, WA, USA) diluted 1:1000 was used. A technique based on an avidin-biotin-peroxidase complex (VECTASTAIN® ELITE® ABC Kits, Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine as the chromogen (DAB Peroxidase substrate kit—Vector Laboratories, Burlingame, CA, USA) was used to stain the antigen.

2.3. PCR

A nested PCR was used to amplify about 800 bp long sequences of SRLV gag-pol genes, which was reported previously [4]. Primers GAG-F1 and POL-R1 were used in the first PCR. The product obtained was used as a template in a second PCR with primers GAG-F2 and POL-R2. The PCR mixture included 50 µL 2x Taq PCR Master Mix, 500 nM each primer, 4 µL DNA, and PCR grade water up to 100 µL final volume. PCR conditions were 94 °C for 5 min, 45 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension of 72 °C for 10 min. The second PCR was carried out with the same conditions but 5 µL of the first PCR products were used as the template and the annealing temperature was 60 °C instead of 55 °C [4]. LTR sequences (203 bp long) were amplified by nested PCR with primers described elsewhere [20].

The PCR reaction mix was described above, but 2 µL of DNA (first PCR) or 2 µL of the first PCR products (second PCR) were used as the template. PCR conditions were 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C (or 50 °C in the second PCR) for 30 s, 72 °C for 40 s, and a final extension of 72 °C for 7 min. PCR products were visualized in 1.5% agarose gel and positive samples were submitted to an external laboratory for sequencing (BMR Genomics, Padova, Italy). Both the sense and antisense strands were sequenced by performing two independent reactions for each PCR product. Nucleotide gag-pol sequences were deposited in GenBank (Accession numbers MH179145—MH179153 and MH179156—MH179159).

2.4. Sequence Analysis

Nucleotide sequences were manually checked and edited with the program BioEdit. A preliminary analysis by BLASTn was carried out to detect regions of similarity with sequences included in databases. Sequences of strains considered to be prototypes of different genotypes and SRLV sequences highly similar to those found in the samples were included in the study (Figure 1 and Figure 3). Sequences were aligned by MUSCLE [21] and phylogenetic trees were inferred with the program MEGA 7.0.21 [22]. The best-fitting nucleotide substitution models were estimated and the General Time Reversible model [23] with a gamma distribution with invariant sites (gag sequences) or a Kimura 2-parameter (LTR sequences) model [24] with gamma-distributed rates among sites were used with bootstrap values based on 1000 repetitions. Phylogeny was estimated by both the neighbor-joining algorithm (NJ) and the maximum likelihood (ML) method. Correlations among sequence alterations and histological features were evaluated by using the Fisher's exact test. Pairwise distances between sequences of samples and sequences of reference strains belonging to different genotypes were calculated by MEGA 7.0.21 with the p-distance model [22].

RESULTS

Histological examination and grading of mammary lesions (see Figure 1) resulted in five samples with severe lesions, 13 samples with moderate lesions, 11 samples with minimal lesions, and five samples without lesions (see Table 1). Grading of M5 sample was not possible due to a concomitant purulent mastitis. IHC results were used to distinguish SRLV infected from uninfected sheep. Three out of the five samples without lesions (M13, M18 and Q2) were negative by IHC and by both gag-pol and LTR PCR. Therefore, they were considered negative (see Table 1). The remaining 32 samples of groups M and Q were positive by IHC (see Figure 2). Sixteen out of the 32 M and Q samples and the 2 N samples were positive by gag-pol PCR, but good-quality sequences were obtained only from 15 samples. LTR PCR products were obtained in all but two IHC-positive samples.

Sample	Grade of mastitis	IHC	LTR PCR	gag-pol PCR	genotype
M1	moderate	+	+	+	B2
M2	minimal	+	+	-	-
M3	severe	+	+	+	B2
M4	moderate	+	+	-	-
M5	not classified	+	+	+	A2/A3
M6	moderate	+	+	-	-
M7	moderate	+	+	+	-
M8	moderate	+	+	+	-
M9	severe	+	+	+	-
M10	minimal	+	+	-	-
M11	moderate	+	+	+	-
M12	minimal	+	+	+	A2/A3
M13	No	-	-	-	-
M14	moderate	+	+	-	-
M15	minimal	+	+	+	A2/A3
M16	minimal	+	+	-	-

M17	moderate	+	+	+	B2
M18	no	-	-	-	-
M19	moderate	+	+	+	A2/A3
Q1	no	+	+	+	B2
Q2	no	-	-	-	-
Q3	Minimal	+	-	-	-
Q4	Minimal	+	+	-	-
Q5	Minimal	+	+	-	-
Q6	Severe	+	+	-	-
Q7	No	+	+	+	A2/A3
Q8	Severe	+	+	+	A2/A3
Q9	Moderate	+	+	-	-
Q10	Severe	+	+	+	A2/A3
Q11	Moderate	+	+	-	-
Q12	Moderate	+	+	+	-
Q13	Minimal	+	-	-	-
Q14	Moderate	+	+	-	-
Q15	Minimal	+	+	-	-
Q16	Minimal	+	+	-	-
N16-426	Severe	+	+	+	A2/A3
N17-44	Moderate	+	+	+	A2/A3

Table 1: *List of ovine mammary gland samples collected for this study. Samples are classified on the basis of the grade of mastitis observed by histopathology. Lesions in sample M5 were not classified due to concomitant purulent mastitis was present. "+": positive result, "-": negative result.*

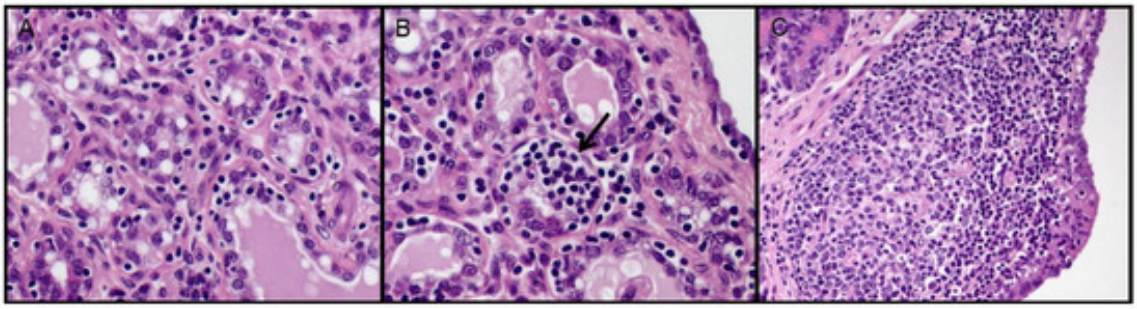

Figure 1: *SRLV lesions in mammary gland of sheep. (**A**) Minimal lesion (+) with small focal lymphocyte aggregates within the mammary interstitium. 40×. (**B**) Black arrow indicates moderate (++) focal inflammatory lesion surrounded by minimal lymphocytic infiltrates. 40×. (**C**) Large lymphoid follicle in a severe lesion (+++). Hematoxylin and eosin (HE) staining. 20×.*

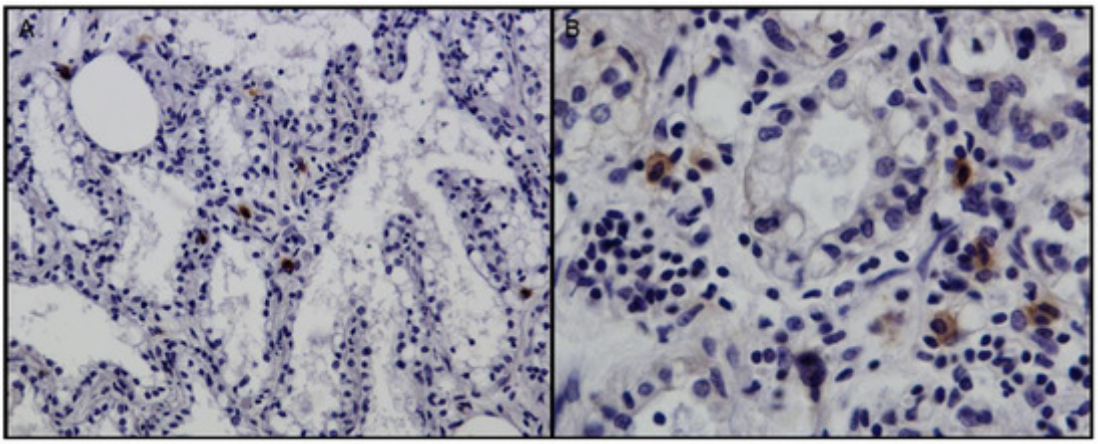

Figure 2. *IHC against p28 of CAEV/VMV in mammary gland. (**A**) Scattered positive cells within minimal inflammatory lesions (+). 20×. (**B**) Positive macrophage-like cell in moderate lesion (++). 40×.*

3.1. Analysis of Gag Sequences

Genotyping was carried out by phylogenetic analysis of partial *gag* gene sequences, according to the taxonomic classification proposed by Shah et al. [3]. All sequences were different from each other and nine samples were type A and four samples were type B. In particular, samples M5, M12, M15, M19, Q7, Q8, Q10, N16-426M, and N17-44M clustered within genotype A were more closely related with strains 292, 160, 166, and 697, which were

previously detected in the same Spanish region (see Figure 3). Only strain 697 had been fully sequenced. Since similar values located with this isolate intermingled between A2 and A3, the isolate 697 had been assigned to the A2/A3 subtype [25]. Samples M1, M3, M17, and Q1 resulted of genotype B and subtype B2 (see Figure 3). Additionally, phylogenic and BLAST analysis showed that they were more related to B2 viruses detected in Italy than in Spain (Ov496). These results were confirmed by the pairwise distances comparison (see Table 2). Samples M12, M15, M19, Q7, Q8, Q10, N16-426, and N17-44 were more closely related to the A2/A3 Spanish strain HQ848062.1 (0.105–0.142). Moreover, samples M1, M3, M17, and Q1 were more closely related to the B2 strains FJ195346.1 and EU010126.1. In particular, they were more closely related to the Italian strain EU010126.1 (0.064–0.081) than to the Spanish strain FJ195346.1 (0.092–0.102).

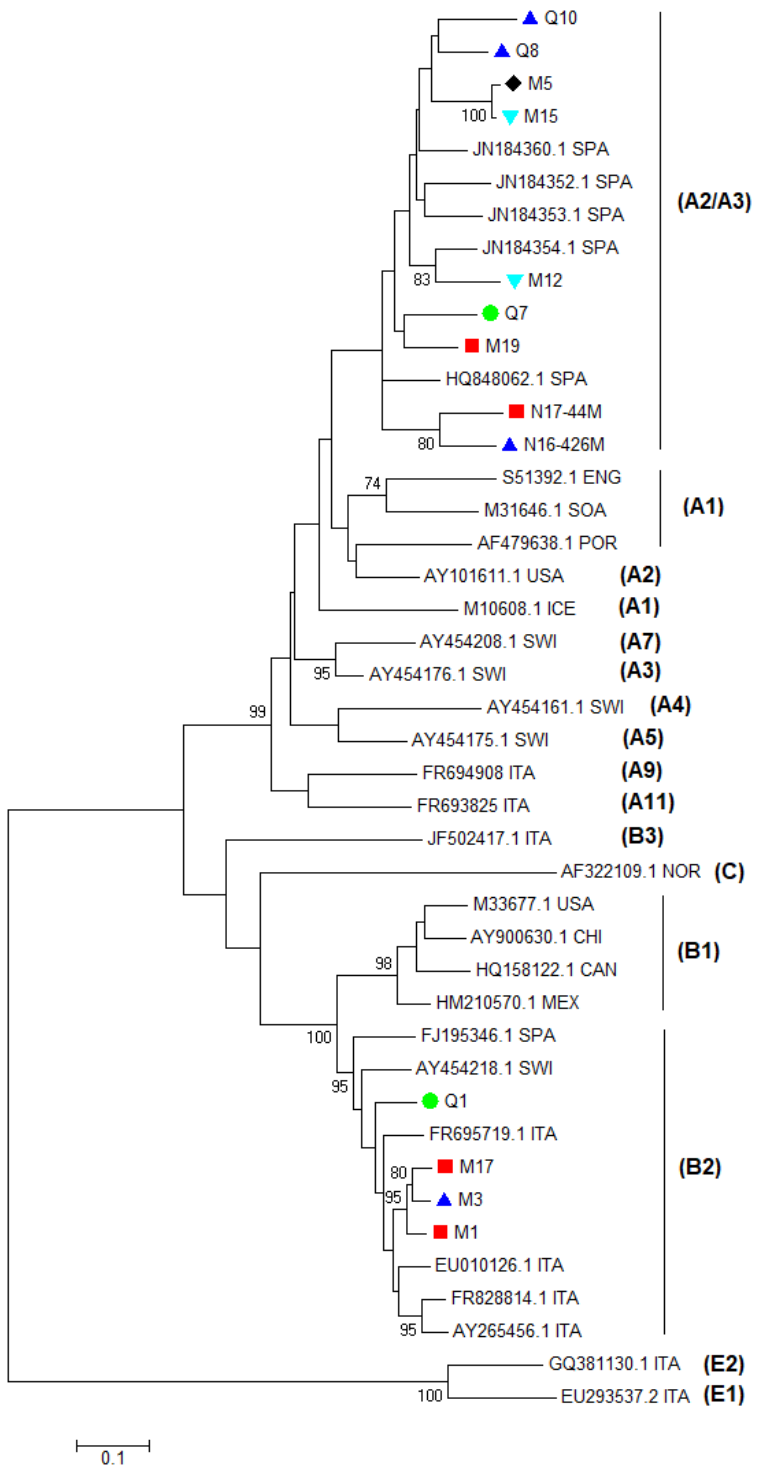

Figure 3. *Phylogenetic analysis of the SRLV partial gag-pol region. Sequences of different SRLV genotypes and subtypes available in GenBank were used as reference isolates. Reference sequences are indicated with their accession number and country of origin (CAN: Canada; CHI: China; ENG: England; ICE: Iceland; ITA: Italy; MEX: Mexico; NOR: Norway; POR: Portugal; SOA: South Africa; SPA: Spain; SWI: Switzerland;*

USA: the U.S.A.). Samples are indicated with their codes and are labeled on the basis of the score of the mammary lesions observed (▲ severe, ■ moderate, ▼ minimal, ● no lesions, ♦ not available). The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model with a gamma distribution with invariant sites and with bootstrap values based on 1000 repetitions. The tree is unrooted.

Sample	genotype	M5	M12	M15	M19	Q7	Q8	Q10	N16-426	N17-44	M1	M3	M17	Q1
M12		0.112	-											
M15		0.013	0.013	-										
M19		0.013	0.122	0.104	-									
Q7		0.130	0.133	0.129	0.109	-								
Q8		0.100	0.115	0.097	0.122	0.122	-							
Q10		0.109	0.119	0.105	0.129	0.127	0.105	-						
N16-426		0.127	0.138	0.129	0.115	0.137	0.127	0.135	-					
N17-44		0.152	0.155	0.152	0.138	0.163	0.157	0.152	0.094	-				
M1		0.208	0.213	0.208	0.213	0.216	0.209	0.216	0.217	0.216	-			
M3		0.216	0.222	0.211	0.221	0.231	0.211	0.221	0.224	0.217	0.040	-		
M17		0.214	0.221	0.209	0.226	0.232	0.217	0.217	0.231	0.227	0.048	0.041	-	
Q1		0.209	0.216	0.203	0.217	0.222	0.221	0.219	0.216	0.216	0.081	0.086	0.076	-
M10608.1	**A1**	0.171	0.160	0.168	0.137	0.157	0.166	0.145	0.175	0.175	0.214	0.208	0.229	0.224
S51392	**A1**	0.173	0.165	0.168	0.173	0.181	0.171	0.163	0.176	0.168	0.242	0.247	0.245	0.244
AY101611.1	**A2**	0.155	0.135	0.153	0.150	0.147	0.153	0.152	0.165	0.165	0.186	0.188	0.191	0.201
HQ848062.1	**A2/A3**	0.117	0.135	0.120	0.105	0.122	0.127	0.135	0.142	0.124	0.206	0.209	0.219	0.213
AY454176.1	**A3**	0.130	0.138	0.130	0.130	0.137	0.133	0.145	0.145	0.152	0.178	0.191	0.193	0.198
AY454161.1	**A4**	0.138	0.166	0.135	0.153	0.185	0.143	0.150	0.173	0.171	0.211	0.216	0.214	0.214
AY454175.1	**A5**	0.153	0.145	0.155	0.148	0.171	0.160	0.147	0.176	0.165	0.209	0.203	0.208	0.206
AY454208.1	**A7**	0.176	0.157	0.170	0.145	0.158	0.158	0.166	0.161	0.140	0.198	0.209	0.201	0.209
FR694908	**A9**	0.166	0.168	0.170	0.166	0.178	0.180	0.171	0.181	0.161	0.213	0.224	0.224	0.231
FR693825	**A11**	0.183	0.171	0.180	0.152	0.157	0.176	0.183	0.189	0.178	0.213	0.217	0.231	0.232
M33677	**B1**	0.204	0.214	0.201	0.211	0.224	0.211	0.211	0.222	0.211	0.120	0.119	0.120	0.129
FJ195346.1	**B2**	0.226	0.231	0.221	0.214	0.232	0.226	0.237	0.221	0.217	0.092	0.092	0.102	0.100
EU010126.1	**B2**	0.208	0.222	0.206	0.226	0.232	0.219	0.227	0.224	0.216	0.064	0.068	0.068	0.081
JF502417.1	**B3**	0.213	0.221	0.214	0.209	0.217	0.219	0.232	0.213	0.224	0.185	0.186	0.196	0.178
AF322109.1	**C**	0.262	0.252	0.257	0.250	0.252	0.245	0.255	0.244	0.236	0.213	0.214	0.214	0.216
EU293537.2	**E1**	0.292	0.295	0.292	0.297	0.301	0.293	0.300	0.293	0.290	0.293	0.285	0.290	0.303
GQ381130.1	**E2**	0.290	0.301	0.293	0.290	0.313	0.293	0.298	0.288	0.290	0.290	0.290	0.297	0.293

Table 2. *Pairwise nucleotidic genetic distances (p-distance model) of the partial gag-pol region of some SRLV reference strains and SRLV strains sequenced in this study.*

Nucleotide sequences were translated into amino acid sequences and the results of the alignment and comparison with the most representative sequences are reported in Figure 4a,b. The set of primers used in this study amplifies a partial sequence of the *gag* gene codifying for the majority of the capsid protein (CA). Comparing amino acid sequence alterations of the partial gag protein obtained, the "GG" motifs of the four type B sequences were glycine-glycine (GG) like type B reference SRLVs while those of the 11 type A samples were asparagine-valine (NV) like other type A reference SRLVs (see Figure 4a). In type A samples, sequences of epitopes 2 and 3 of reference isolates and of most samples were conserved (see Figure 4a). Only arginine (R) replaced lysine (K) in samples N16-426 and N17-44 and serine (S) replaced asparagine (N) in sample Q7. Type B samples had highly conserved epitope 3 sequences since there are only two alterations in M1 (isoleucine (I) instead of serine (S) and lysine (K) instead of glutamic acid (E). More alterations were found in epitope 2 where three out of four type B samples showed one alteration in comparison with type B reference isolates (see Figure 4a,b).

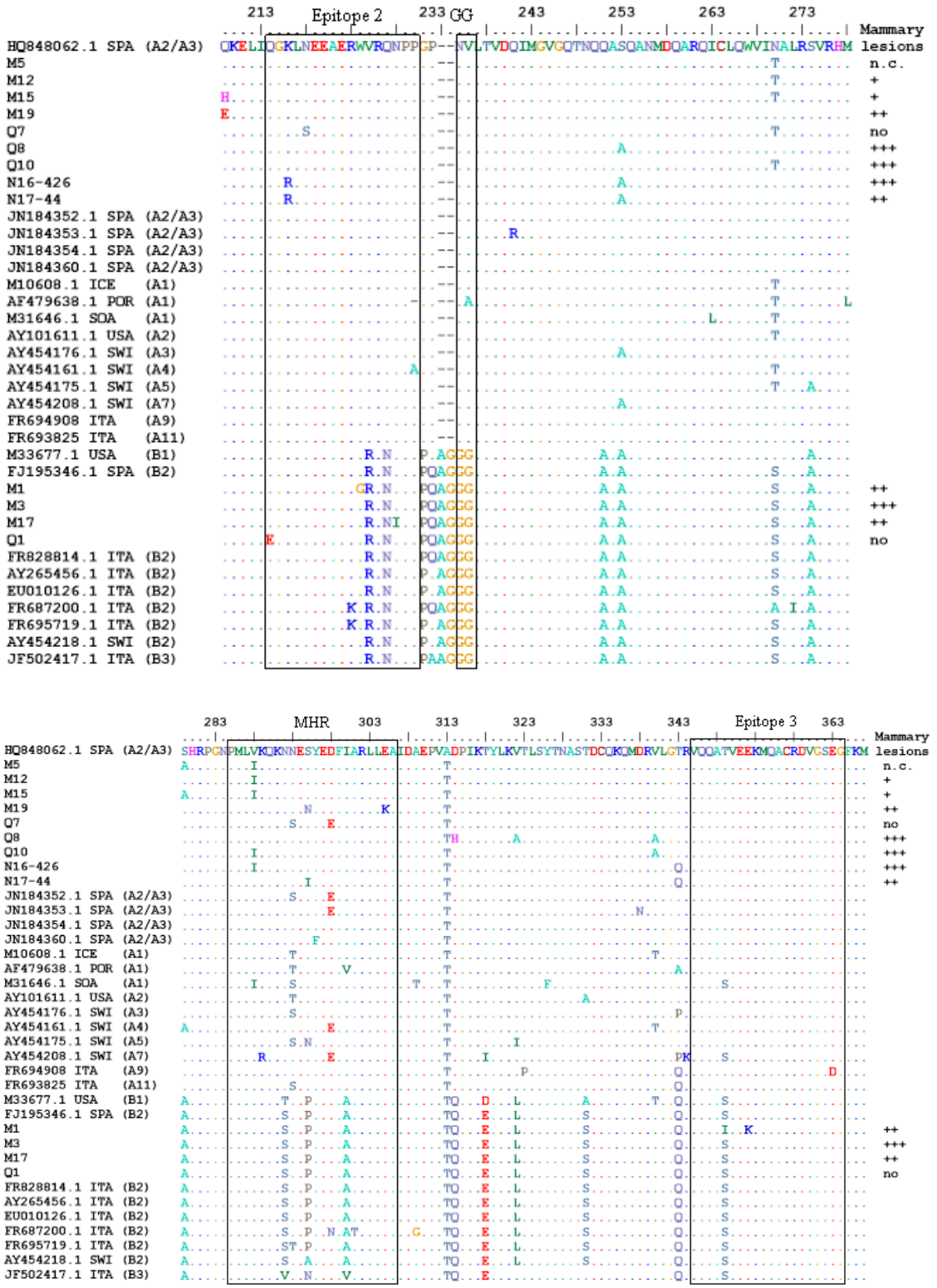

Figure 4: *Alignment (MUSCLE) of deduced amino acid sequences of partial gag-p25 of the SRLV sequences obtained and of some SRLV reference strains; (**a**) positions from 209 to 278, (**b**) posiions from 279 to 368. Two immuno-dominant epitopes of this capsid protein, the GG motif, and the Major*

Homology Region (MHR) are within squares. The score of mammary lesion of each sample is reported. Legend: (·) homology, (−) deletion, (+++) severe lesions, (++) moderate lesions, (+) minimal lesions, (no) no lesions, (n. c.) not classified, CAN: Canada; CHI: China; ENG: England; ICE: Iceland; ITA: Italy; MEX: Mexico; NOR: Norway; POR: Portugal; SOA: South Africa; SPA: Spain; SWI: Switzerland; USA.

In the Major Homology Region (MHR), which is usually a highly conserved sequence in the *gag* gene of all retroviruses, some alterations were present in type A samples. In particular, all but one type A samples showed one or two alterations comparing to the A2/A3 reference strain 697 (see Figure 4b). Samples M5, M12, M15, Q10, and N16-426 had isoleucine (I) instead of valine (V) at the fourth position as type A1 reference strain SA-OMVV. This latter had also a serine (S) instead of asparagine (N) at the ninth position. Sample Q7 had not only this alteration, but also glutamic acid (E) instead of aspartic acid (D) at the 14th position, which shows the same alterations found in the A2/A3 Spanish strain 160. Sample N17-44 had isoleucine (I) instead of serine (S) at the 11th position unlike the other samples and reference strains. Unusual alterations were found in sample M19 where asparagine (N) replaced serine (S) at the 11th position and lysine (K) substituted glutamic acid (E) at the 21st position. Type B samples had highly conserved MHR sequences and showed the same amino-acidic sequences of B2 reference isolates even though some single alterations were present in the nucleotide sequences (see Figure S1). A significant correlation among sequence alterations and severity of mastitis was not found ($p > 0.05$).

3.2. Analysis of LTR Sequences

The alignment and phylogenetic analysis of LTR nucleotide sequences showed that most samples were closer to the reference Spanish A2/A3 strain 697 (Figure 5 and Figure 6a,b). Comparing samples with the 697 reference strain, Q10 and Q11 showed a 23 nt deletion

(9133–9160 nt) in the R region, which appeared longer than the 13 nt deletion present in the reference strains 697, EV1, and in other Spanish strains (C3, 160, 292). Sample Q12 had similar deletions than 697 while other samples showed 2–8 nt deletions in the same tract. No significant differences were found among sequences of samples with a different grade of mastitis ($p > 0.05$). Similarly, as previously described, the TATAbox, the polyadenylation signal, and the AML (vis), which is a site possessing the consensus sequence for the AML/PEBP2/CBF transcriptional factor family [26], were conserved and only a substitution G with A was present in three samples at position 9065 of AML (vis).

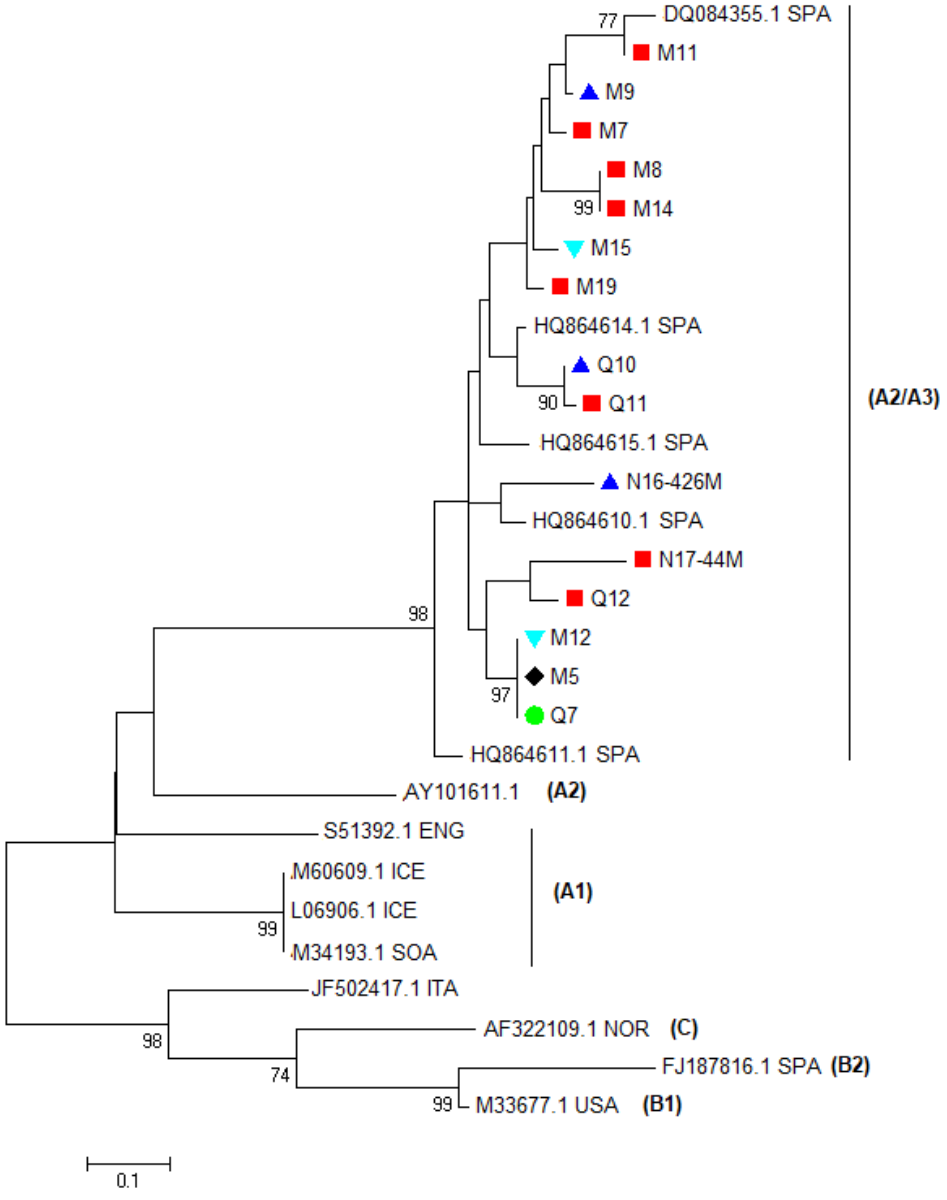

Figure 5: *Phylogenetic analysis of the SRLV partial LTR. Sequences of different SRLV genotypes and subtypes available in GenBank were used as reference isolates. Reference sequences are indicated with their accession number and country of origin (CAN: Canada; CHI: China; ENG: England; ICE: Iceland; ITA: Italy; MEX: Mexico; NOR: Norway; POR: Portugal; SOA: South Africa; SPA: Spain; SWI: Switzerland; USA). Samples are indicated with their codes and are labeled on the basis of the score of the mammary lesions observed (▲ severe, ■ moderate, ▼ minimal, ● no lesions, ♦ not available). The phylogenetic analysis was performed with a maximum likelihood (ML) method using the Kimura 2-parameter model with a gamma distribution and with bootstrap values based on 1000 repetitions. Sequences are not deposited in GenBank because they do not reach the minimum length required.*

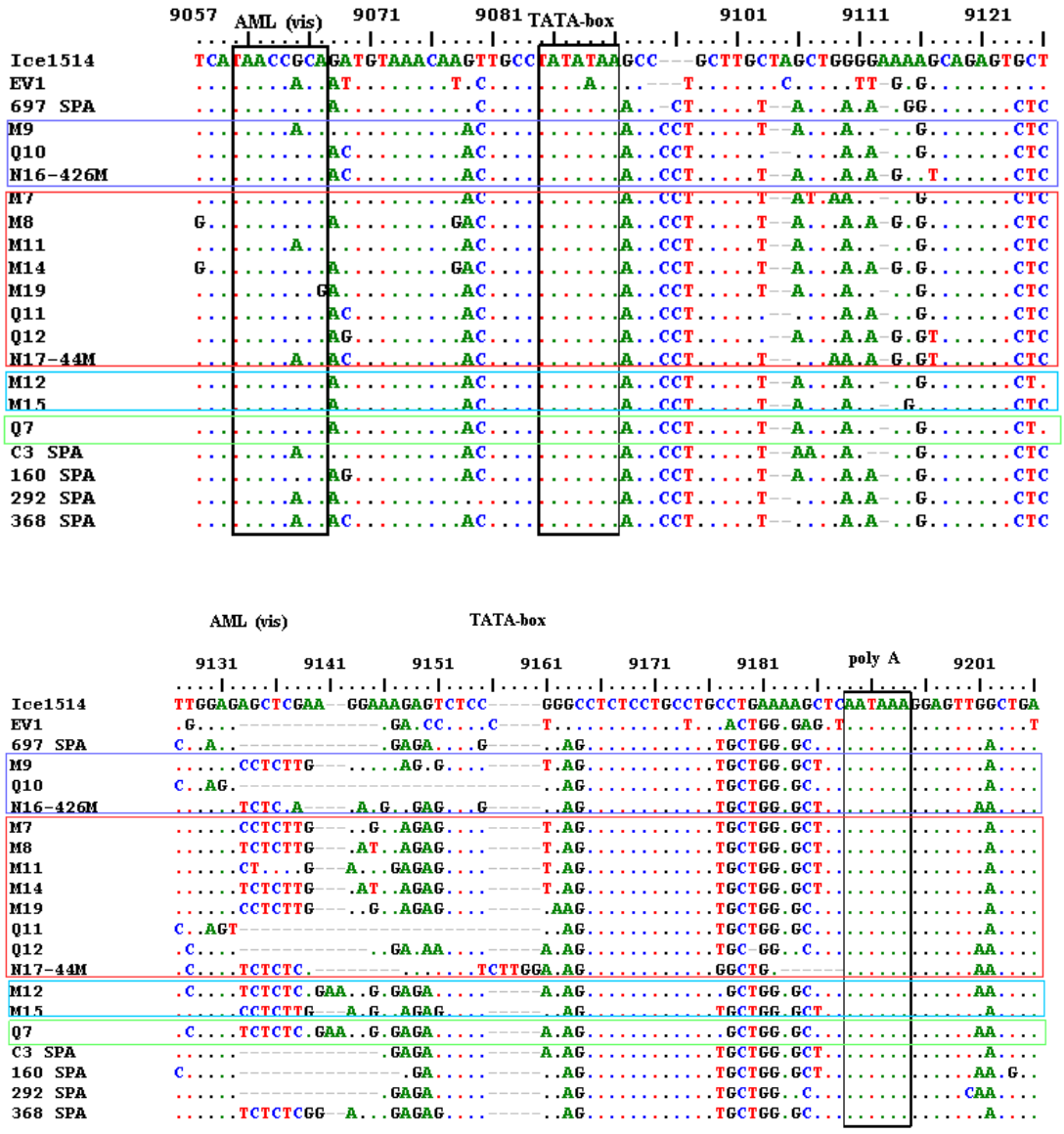

Figure 6: *Alignment (MUSCLE) of nucleotide sequences of the LTR region of the SRLV sequences obtained and of some SRLV reference strains. LTR sequence of the isolate Icelandic 1514 (M10609.1) was used as a reference; (**a**) positions from 9057 to 9126, (**b**) posiions from 9127 to 9206. Sequence of reference strains EV1 (S51392.1) was also used. Sequences previously found in Spain in goats (C3—DQ084355.1) and in sheep (697—HQ864615.1, 160—HQ864610.1, 292—HQ864611.1 and 368—HQ864614.1) and showing high homology with our samples were also included. Sequences of the AML (vis), the TATA box element, and the polyadenylation signal are within black squares. Sequences of samples with similar scores of mammary lesions are within colored squares. Legend: (·) homology, (−) deletion, (blue squares) severe lesions, (red squares) moderate lesions, (light blue squares) minimal lesions, and (green squares) no lesions.*

DISUSSION

This study describes for the first time *gag* and LTR sequences of SRLVs detected in Spanish Assaf sheep with different grades of histopathological mastitis and their phylogenetic relationships in the context of known SRLV sequences.

Although initially genotypes B viruses were thought to infect only goats, it is not unusual to find reports about infections by type B viruses in sheep and by type A viruses in goats [27,28,29,30]. Even in this study, both genotypes A and B SRLVs have been found in ovine samples. It was not known if the examined sheep had contact with goats, but infection with A genotypes in goats was not reared in contact with sheep, which was reported [31].

The *gag-pol* phylogenetic tree and the pairwise genetic distances comparison revealed that most sequences of the samples were closer to the Spanish A2/A3 isolate 697 while four sequences belonged to subtype B2. Isolate 697 has been previously detected in sheep with neurological diseases from the Spanish region of Castilla y León in Spain and has been classified as A2/A3 because differences between A2 and A3 are often not large enough to separate the two groups [3,25]. Partial sequences of viruses classified as A2/A3 have been detected further seven sheep with neurological signs in Spain [25]. These findings suggest that, in Northern Spain, subtype A2/A3 SRLVs is genetically related to SRLVs, which caused nervous diseases. However, in our cases, neurological signs were not reported. Samples M1, M3, M17, and Q1 were closer to Italian than to Spanish B2 isolates and mammary lesions were found from moderate to severe in three out of four samples while, in sample Q1, lesions were not found. B2 SRLV has been detected in Spain for the first time in SRLV-seropositive adult sheep of the Rasa Aragonesa breed, which shows clinical signs of arthritis [17,32]. Mammary histological lesions were present in 10 out of 13 animals with arthritis, which suggests that udders can be involved even if clinical signs might remain unrecognized until the losses of milk production are significant. B2 viruses have been detected in Italian small ruminants during

epidemiological surveys, but data about clinical signs or histological lesions are not reported [4,33].

In addition, good-quality sequences about 800 bp long were obtained from FFPE samples. Fixation in formalin and embedding in paraffin at high temperatures is thought to degrade DNA. Fragmentation of DNA molecules can interfere with their amplification by PCR and with consequent sequencing. In our case, good-quality DNA has been extracted and amplified by PCR from archival FFPE samples, which suggests that this method could be attempted for studying FFPE samples as well as for retrospective investigations.

Analysis of the genetic sequences is important not only for evaluating the spread of SRLV types and subtypes but also for monitoring antigenic variability. Actually, remarkable antigenic variation might be responsible for the misdiagnosis of highly divergent genotypes [34]. The *gag* gene encodes nucleocapsid, capsid, and matrix proteins. Indirect diagnostic assays usually use the capsid protein as the antigen, which helps monitor immuno-dominant epitopes of *gag*-encoded structural proteins. This is useful for detecting antigenic variability in the field and forevaluating and improving the sensitivity of indirect diagnostic tools. Alterations in the amino acid sequences of immuno-dominant epitope regions suggest altered antigenicity, which may affect the sensitivity of serological tests such as ELISA and AGID. The *gag-pol* set of primers used in this study allowed sequencing only of epitopes 2 and 3. Amino acid sequences of epitopes 2 and 3 of type A2/A3 viruses were quite conserved and limited alterations only in three and one samples, respectively. Epitope 2 of B2 isolates had more alterations, which shows single amino acid alterations in three out of four sequences. In addition, more variability was found in the MHR of A2/A3 viruses, which show all but two samples and at least one alteration in comparison with the reference A2/A3 strain 697. The MHR is usually conserved in many retroviruses and is essential for viral assembly [35]. Mutations in the MHR sequence of HIV-1 cause capsid assembly that reduces infectivity [36].

While some studies have been carried out on MHR of human retroviruses, the consequences of MHR mutations on infectivity of SRLVs should be better investigated. MHR of B2 viruses and GG motif of both A2/A3 and B2 viruses in the gag amino acid sequences, AML (vis) motif, TATA-box, and poly-A of both A2/A3 and B2 viruses in the LTR nucleotide sequences were highly conserved, which was previously reported in strains from different geographic areas [31,37,38].

Most LTR sequences showed higher homology with A2/A3 Spanish SRLV isolates. Samples Q10 and Q11 showed a 23 nt deletion in the R region, which appeared longer than the 13 nt deletion observed in type A2/3 reference isolate 697. A 13 nt deletion in this region has been found in sequences of clinically affected sheep and a correlation among this deletion and the appearance of clinical signs has been suggested [32]. On the contrary, a similar deletion has been found in SRLVs infecting asymptomatic sheep and the lungs of animals infected with viruses carrying the deletion were significantly less affected than sheep infected with viruses without deletion [18]. In the present study, significant correlations among deletions in the R region of the LTR and severity of mammary lesions were not found ($p > 0.05$). Samples with deletions were from sheep with more severe mammary lesions and sheep with moderate to severe mastitis did not show this deletion (see Figure 6b).

Although the severity of mammary lesions was not significantly related to the viral genotype, SRLV sequences from samples with similar grades of lesions (no/minimal and moderate/severe) were most closely related to each other (see Figure 3 and Figure 5 and Table 2). Considering the high economic impact of SRLV diseases, some countries aim to eradicate the diseases by identifying and prematurely culling infected animals. Selecting animals on the basis of serological results could determine the selection of SRLV variants with significant alterations in the antigen sequences. Permanent and extensive surveys should be encouraged in different countries to evaluate the antigenic variability of SRLV and to monitor

the sensitivity and specificity of diagnostic tests in detecting these variants. In particular, seronegative animals should be investigated for infections by new viral genotypes not detected by traditional serological tests. Histological screening of different target organs at the slaughterhouse could be a useful tool for selecting samples with lesions, which suggests an SRLV disease in seronegative animals.

CONCLUSIONS

In conclusion, this is the first study investigating the association between the SRLV sequence analysis and histopathological grading of mammary lesions in sheep. Circulation of SRLVs of types A2/A3 and B2 in Spanish Assaf sheep was confirmed and new viral variants have not been found, but moderate alterations were present in some immuno-dominant epitopes and in the MHR tract.

No significant correlation was found among histological features and alterations in the sequences. Although some sequences obtained from samples with similar grades of mammary lesions appeared closer to each other, more extensive and interdisciplinary studies are required for establishing the existence of viral clusters with a higher or lower pathogenicity for specific target organs.

SUPPLEMENTARY MATERIALS

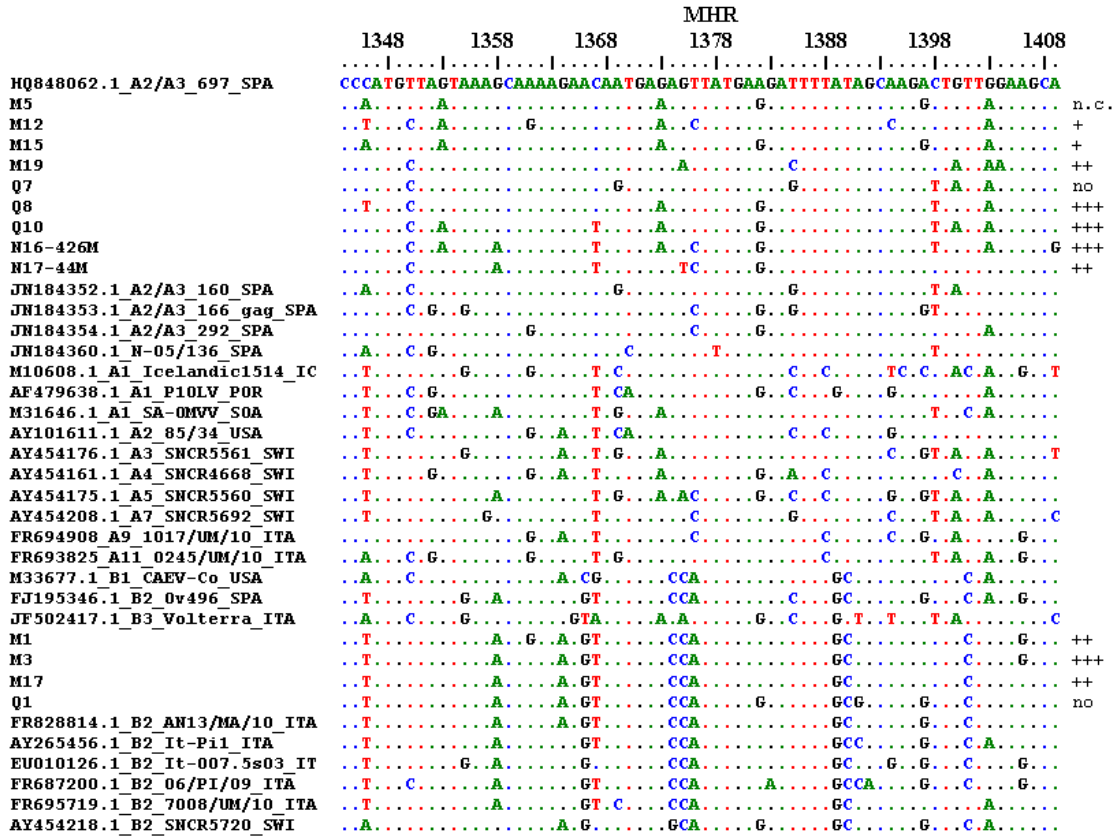

Figure S1: *Nucleotide alignment of MHR sequences in the gag gene of the samples and of some reference strains.*

AUTHOR CONTRIBUTIONS

S.P. and E.G. conceived and designed the experiments. E.G. performed many experiments. S.P., G.R., L.P., J.F.G.M., and V.C. performed some experiments. E.G. and S.P. analyzed the data and wrote the paper. All authors read and approved the paper.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Peterhans, E.; Greenland, T.; Badiola, J.; Harkiss, G.; Bertoni, G.; Amorena, B.; Eliaszewicz, M.; Juste, R.A.; Krassnig, R.; Lafont, J.P.; et al. Routes of transmission and consequences of small ruminant lentiviruses (SRLVs) infection and eradication schemes. *Vet. Res.* **2004**, *35*, 257–274.

Preziuso, S.; Renzoni, G.; Allen, T.E.; Taccini, E.; Rossi, G.; DeMartini, J.C.; Braca, G. Colostral transmission of maedi visna virus: Sites of viral entry in lambs born from experimentally infected ewes. *Vet. Microbiol.* **2004**, *104*, 157–164.

Shah, C.; Böni, J.; Huder, J.B.; Vogt, H.R.; Mühlherr, J.; Zanoni, R.; Miserez, R.; Lutz, H.; Schüpbach, J. Phylogenetic analysis and reclassification of caprine and ovine lentiviruses based on 104 new isolates: Evidence for regular sheep-to-goat transmission and worldwide propagation through livestock trade. *Virology***2004**, *319*, 12–26.

Grego, E.; Bertolotti, L.; Quasso, A.; Profiti, M.; Lacerenza, D.; Muz, D.; Rosati, S. Genetic characterization of small ruminant lentivirus in Italian mixed flocks: Evidence for a novel genotype circulating in a local goat population. *J. Gen. Virol.* **2007**, *88*, 3423–3427.

Ramírez, H.; Reina, R.; Amorena, B.; de Andrés, D.; Martínez, H.A. Small ruminant lentiviruses: Genetic variability; tropism and diagnosis. *Viruses* **2013**, *5*, 1175–1207.

Leroux, C.; Chastang, J.; Greenland, T.; Mornex, J.F. Genomic heterogeneity of small ruminant lentiviruses: Existence of heterogeneous populations in sheep and of the same lentiviral genotypes in sheep and goats. *Arch. Virol.* **1997**, *142*, 1125–1137.

Minguijón, E.; Reina, R.; Pérez, M.; Polledo, L.; Villoria, M.; Ramírez, H.; Leginagoikoa, I.; Badiola, J.J.; García-Marín, J.F.; de Andrés, D.; et al. Small ruminant lentivirus infections and diseases. *Vet. Microbiol.* **2015**, *181*, 75–89.

Lujan, L.; García Marin, J.F.; Fernandez de Luco, D.; Vargas, A.; Badiola, J.J. Pathological changes in the lungs and mammary glands of sheep and their relationship with maedi-visna infection. *Vet. Rec.* **1991**, *129*, 51–54.

Benavides, J.; Fuertes, M.; García-Pariente, C.; Otaola, J.; Delgado, L.; Giraldez, J.; García Marín, J.F.; Carmen Ferreras, M.; Pérez, V. Impact of maedi-visna in intensively managed dairy sheep. *Vet. J.* **2013**, *197*, 607–612.

Gayo, E.; Polledo, L.; Balseiro, A.; Martínez, C.P.; García Iglesias, M.J.; Preziuso, S.; Rossi, G.; García Marín, J.F. Inflammatory Lesion Patterns in Target Organs of Visna/Maedi in Sheep and their Significance in the Pathogenesis and Diagnosis of the Infection. *J. Comp. Pathol.* **2018**, *159*, 49–56.

Anderson, B.C.; Bulgin, M.S.; Adams, S.; Duelke, B. Firm udder in periparturient ewes with lymphocytic accumulations; retrovirus infection; and milk unavailable at the teat. *J. Am. Vet. Med. Assoc.* **1985**, *186*, 391–393.

Ryan, D.P.; Greenwood, P.L.; Nicholls, P.J. Effect of caprine arthritis-encephalitis virus infection on milk cell count and *N*-acetyl-beta-glucosaminidase activity in dairy goats. *J. Dairy Res.* **1993**, *60*, 299–306.

Martinez-Navalon, B.; Peris, C.; Gomez, E.A.; Peris, B.; Roche, M.L.; Caballero, C.; Goyena, E.; Berriatua, E. Quantitative estimation of the impact of caprine arthritis encephalitis virus infection on milk production by dairy goats. *Vet. J.***2013**, *197*, 311–317.

Sihvonen, L.; Nuotio, L.; Rikula, U.; Hirvelä-Koski, V.; Kokkonen, U. Preventing the spread of maedi-visna in sheep through a voluntary control programme in Finland. *Prev. Vet. Med.* **2000**, *47*, 213–220.

Tavella, A.; Bettini, A.; Ceol, M.; Zambotto, P.; Stifter, E.; Kusstatscher, N.; Lombardi, R.; Nardeli, S.; Beato, M.S.; Capello, K.; et al. Achievements of an eradication programme against caprine arthritis encephalitis virus in South Tyrol; Italy. *Vet. Rec.* **2018**, *182*, 51.

Deubelbeiss, M.; Blatti-Cardinaux, L.; Zahno, M.L.; Zanoni, R.; Vogt, H.R.; Posthaus, H.; Bertoni, G. Characterization of small ruminant lentivirus A4 subtype isolates and assessment of their pathogenic potential in naturally infected goats. *Virol. J.* **2014**, *11*, 65.

Pérez, M.; Biescas, E.; Reina, R.; Glaria, I.; Marín, B.; Marquina, A.; Salazar, E.; Álvarez, N.; de Andrés, D.; Fantova, E.; et al. Small ruminant lentivirus-induced arthritis: Clinicopathologic findings in sheep infected by a highly replicative SRLV B2 genotype. *Vet. Pathol.* **2015**, *52*, 132–139

Angelopoulou, K.; Poutahidis, T.; Brellou, G.D.; Greenland, T.; Vlemmas, I. A deletion in the R region of long terminal repeats in small ruminant lentiviruses is associated with decreased pathology in the lung. *Vet. J.* **2008**, *175*, 346–355.

Preziuso, S.; Taccini, E.; Rossi, G.; Renzoni, G.; Braca, G. Experimental Maedi Visna Virus Infection in sheep: A morphological; immunohistochemical and PCR study after three years of infection. *Eur. J. Histochem.* **2003**, *47*, 373–378.

Ryan, S.; Tiley, L.; McConnell, I.; Blacklaws, B. Infection of dendritic cells by the Maedi-Visna lentivirus. *J. Virol.* **2000**, *74*, 10096–10103.

Edgar, R.C. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **2004**, *32*, 1792–1797.

Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874.

Nei, M.; Kumar, S. *Molecular Evolution and Phylogenetics*; Oxford University Press: New York, NY, USA, 2000.

Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.***1980**, *16*, 111–120.

Glaria, I.; Reina, R.; Ramírez, H.; de Andrés, X.; Crespo, H.; Jauregui, P.; Salazar, E.; Luján, L.; Pérez, M.M.; Benavides, J.; et al. Visna/Maedi virus genetic characterization and serological diagnosis of infection in sheep from a neurological outbreak. *Vet. Microbiol.* **2012**, *155*, 137–146.

Sutton, K.A.; Lin, C.T.; Harkiss, G.D.; McConnell, I.; Sargan, D.R. Regulation of the long terminal repeat in visna virus by a transcription factor related to the AML/PEBP2/CBF superfamily. *Virology* **1997**, *229*, 240–250.

Kuhar, U.; Barlic-Maganja, D.; Grom, J. Phylogenetic analysis of small ruminant lentiviruses detected in Slovenia. *Vet. Microbiol.* **2012**, *162*, 201–206.

Olech, M.; Rachid, A.; Croise, B.; Kuźmak, J.; Valas, S. Genetic and antigenic characterization of small ruminant lentiviruses circulating in Poland. *Virus Res.***2012**, *163*, 528–536.

Fras, M.; Leboeuf, A.; Labrie, F.M.; Laurin, M.A.; Singh Sohal, J.; L'Homme, Y. Phylogenetic analysis of small ruminant lentiviruses in mixed flocks: Multiple evidence of dual infection and natural transmission of types A2 and B1 between sheep and goats. *Infect. Genet. Evol.* **2013**, *19*, 97–104.

Santry, L.A.; de Jong, J.; Gold, A.C.; Walsh, S.R.; Menzies, P.I.; Wootton, S.K. Genetic characterization of small ruminant lentiviruses circulating in naturally infected sheep and goats in Ontario, Canada. *Virus Res.* **2013**, *175*, 30–44.

Reina, R.; Mora, M.I.; Glaria, I.; García, I.; Solano, C.; Luján, L.; Badiola, J.J.; Contreras, A.; Berriatua, E.; Juste, R.; et al. Molecular characterization and phylogenetic study of Maedi Visna and Caprine Arthritis Encephalitis viral sequences in sheep and goats from Spain. *Virus Res.* **2006**, *121*, 189–198.

Glaria, I.; Reina, R.; Crespo, H.; de Andrés, X.; Ramírez, H.; Biescas, E.; Pérez, M.M.; Badiola, J.; Luján, L.; Amorena, B.; et al. Phylogenetic analysis of SRLV sequences from an arthritic sheep outbreak demonstrates the introduction of CAEV-like viruses among Spanish sheep. *Vet. Microbiol.* **2009**, *138*, 156–162.

Giammarioli, M.; Bazzucchi, M.; Puggioni, G.; Brajon, G.; Dei Giudici, S.; Taccori, F.; Feliziani, F.; De Mia, G.M. Phylogenetic analysis of small ruminant lentivirus (SRLV) in Italian flocks reveals the existence of novel genetic subtypes. *Virus Genes* **2011**, *43*, 380–384.

Cardinaux, L.; Zahno, M.L.; Deubelbeiss, M.; Zanoni, R.; Vogt, H.R.; Bertoni, G. Virological and phylogenetic characterization of attenuated small ruminant lentivirus isolates eluding efficient serological detection. *Vet. Microbiol.* **2013**, *162*, 572–581.

Purdy, J.G.; Flanagan, J.M.; Ropson, I.J.; Rennoll-Bankert, K.E.; Craven, R.C. Critical role of conserved hydrophobic residues within the major homology region in mature retroviral capsid assembly. *J. Virol.* **2008**, *82*, 5951–5961.

Chu, H.H.; Chang, Y.F.; Wang, C.T. Mutations in the alpha-helix directly C-terminal to the major homology region of human immunodeficiency virus type 1 capsid protein disrupt Gag multimerization and markedly impair virus particle production. *J. Biomed. Sci.* **2006**, *13*, 645–656.

Angelopoulou, K.; Brellou, G.D.; Greenland, T.; Vlemmas, I. A novel deletion in the LTR region of a Greek small ruminant lentivirus may be associated with low pathogenicity. *Virus Res.* **2006**, *118*, 178–184.

Kokawa, S.; Oba, M.; Hirata, T.; Tamaki, S.; Omura, M.; Tsuchiaka, S.; Nagai, M.; Omatsu, T.; Mizutani, T. Molecular characteristics and prevalence of small ruminant lentiviruses in goats in Japan. *Arch. Virol.* **2017**, *162*, 3007–3015.

IX. Discusión global

El Maedi-Visna es una enfermedad crónica que sigue suponiendo importantes pérdidas económicas en el ganado ovino intensivo de leche en nuestro país, y en especial, en Castilla y León, y cuya erradicación es difícil debido a la ausencia de tratamientos o de vacunas y a la elevada prevalencia de la infección. Por ese motivo, el objetivo de esta tesis doctoral es ampliar el conocimiento sobre la patogenia y la respuesta inmune individual frente a la infección y sus posibles implicaciones en el diagnóstico y control de la enfermedad. En primer lugar, se describen los diferentes patrones de lesiones en los tres principales órganos diana y su relación con los resultados en diferentes test diagnósticos y por otro lado, se estudian las cepas víricas circulantes en esta región y si ciertas variaciones genéticas del hospedador pueden influir en la resistencia a la infección o en el desarrollo de lesiones.

Los dos primeros trabajos de esta tesis doctoral describen de forma detallada la respuesta inmunológica frente al MV que muestran animales naturalmente infectados en pulmón, glándula mamaria y SNC, así como su relación con la respuesta serológica, mientras que el tercer trabajo se centra en las lesiones en la glándula mamaria y su detección mediante diferentes técnicas de diagnóstico, así como de la puesta a punto de una técnica de citología e inmunocitoquímica en muestras de leche para el estudio de las células portadoras del VMV en la misma y de la excreción vírica por vía lactógena.

El estudio mediante histopatología y técnicas de inmunohistoquímica de las poblaciones celulares que forman el infiltrado inflamatorio y de la respuesta serológica mediante test ELISA permiten la descripción de tres patrones de lesión o tipos de respuesta inmune del hospedador: histiocítico, linfocítico y mixto. Los patrones linfocítico e histiocítico se describieron previamente en SNC (Polledo *et al.*, 2012b), donde se introdujo la posible relación entre la respuesta inmune individual y el desarrollo de lesiones de ambos tipos. Esta posibilidad de que la respuesta inmune del hospedador juegue un papel importante en la

patogenia de la enfermedad también se ha descrito en otros estudios (Torsteinsdóttir *et al.*, 1992; Torsteinsdottir *et al.*, 2007a; Blacklaws, 2012; Polledo *et al.*, 2012a).

La presencia de un mismo patrón de lesión en pulmón y SNC de un mismo animal y la mayor cantidad de células CD68+ en mamas de animales con patrón histiocítico sugieren que la respuesta inmune individual sí juega un papel importante en el desarrollo del tipo de infiltrado inflamatorio. El hecho de que la glándula mamaria no siga exactamente el mismo patrón que el SNC o el pulmón, si no que presente un predominio de células CD3+ en el infiltrado inflamatorio aunque también con un número variable de macrófagos, puede ser debido al desarrollo de una respuesta inmune local diferente en este órgano diana, de naturaleza mucosa, y con continua estimulación antigénica (Butler *et al.*, 2015), especialmente en esta raza de alta producción con lactaciones prolongadas en las que se utilizan métodos mecánicos de ordeño. En este trabajo demostramos que se puede asignar un patrón de lesión individual a cada animal estudiando la respuesta inmunológica en los principales órganos diana del MV: pulmón, glándula mamaria y SNC. Dichos patrones se denominan histiocítico, linfocítico y mixto atendiendo a la población celular predominante del infiltrado inflamatorio y estarían relacionados con la respuesta inmunológica del hospedador a la infección por MV, hecho no considerado hasta el momento y que, como se demuestra también en esta tesis doctoral, tiene una gran importancia a la hora de establecer relaciones con el diagnóstico o con las cepas víricas.

Se describe por primera vez la asociación entre el patrón de lesión y la respuesta serológica en SNC, pulmón y glándula mamaria. Los animales con patrón histiocítico presentaron densidades ópticas en el test ELISA (ELITEST®), que entendemos como título de anticuerpos, significativamente más elevados que los animales con patrón linfocítico, habiendo incluso animales con patrón linfocítico con resultados dudosos (n=3) o negativos (n=2). En estudios anteriores se describieron resultados que podrían considerarse similares, donde

animales que presentaban únicamente folículos linfoides en pulmón o glándula mamaria resultaban negativos al test IGDA, aunque en ese momento se asoció exclusivamente a la baja sensibilidad del test utilizado y no a que pudiera ser una forma linfocítica o inicial de la enfermedad, con escasa producción de anticuerpos (Luján *et al.*, 1991). Sin embargo, los resultados de nuestros estudios reforzarían la hipótesis de que estos animales con un patrón linfocítico y lesiones mínimas no producirían suficiente respuesta inmune de tipo humoral como para ser detectada con un test IGDA, que tiene menor sensibilidad que el test ELISA. Estos resultados tienen gran interés ya que la respuesta inmune del hospedador debería considerarse cuando se hace un estudio serológico, además del tipo de test utilizado o la cepa vírica circulante (de Andrés *et al.*, 2005; de Andrés *et al.*, 2013).

La respuesta de tipo histiocítico, caracterizada por un predominio de células CD68+, por la presencia de lesiones graves en la mayoría de las ocasiones y asociada a mayor cantidad de virus en tejido mediante técnicas de IHQ, representaría el desarrollo de una respuesta inmune humoral intensa pero no eficiente en el control de la infección, como se ha propuesto en estudios previos en SNC (Polledo *et al.*, 2012b), donde incluso se ha relacionado con la posibilidad de cepas víricas más virulentas. Este tipo de infiltrado inflamatorio se ha descrito en otras infecciones por lentivirus como en el HIV o en el SIV, donde se habla de un 90-95% de macrófagos formando el infiltrado inflamatorio, completado por células CD8+ (Kim *et al.*, 2004; Lackner *et al.*, 1991). La respuesta de tipo linfocítica, a menudo acompañada de lesiones mínimas o moderadas, se asociaría a una respuesta inmune de tipo celular, mediada por células T CD4+ y CD8+, más eficientes en el control de la replicación vírica, también descrito en el HIV o la FIV (Freel *et al.*, 2011). La presencia de lesiones graves de tipo linfocítico en varios animales de esta tesis doctoral, incluso localizadas simultáneamente en SNC, pulmón y glándula mamaria, podrían relacionarse con la producción continuada de citoquinas pro-inflamatorias a nivel local, que contribuyen a la cronificación de la inflamación y a la lesión tisular. En estudios previos realizados en SNC se ha propuesto que las formas linfocíticas

podrían representar cierto tipo de resistencia natural a la replicación vírica, con un reclutamiento inicial de linfocitos como primera respuesta a la infección, efectiva en el control de la misma, y con escasa presencia de antígeno vírico en tejido (Polledo *et al.*, 2012b). Dicha hipótesis se confirmaría en nuestros estudios con la menor cantidad de virus observado en tejido de forma subjetiva mediante técnicas de IHQ en ovejas con patrón linfocítico, en comparación con animales con patrón histiocítico, donde la positividad mediante IHQ fue mayor. Por otro lado, el predominio de la respuesta inmune de tipo celular en el patrón linfocítico, con escasa producción de anticuerpos, concuerda con los valores de densidad óptica más bajos observados en estos animales, incluso con 3 ovejas con valores dudosos y 2 con resultados negativos.

El patrón mixto, caracterizado por una respuesta inmune sin predominio de ninguna de las poblaciones celulares y con resultados en el test ELISA intermedios a los de los patrones previamente descritos se correspondía en la mayoría de los casos con lesiones mínimas. Podría representar formas iniciales o latentes de la infección, con una respuesta inmunopatológica no bien definida que evolucionaría a histiocítica o linfocítica con la progresión de la lesión. La no descripción de un patrón mixto en estudios anteriores (Polledo *et al.*, 2012b) podría deberse a la inclusión de animales con únicamente lesiones moderadas o graves, donde la respuesta inmune individual ya se encontraría bien definida, y a la ausencia de animales con infecciones mínimas o recientes, donde podría encontrarse este patrón mixto. El hecho de que animales con lesiones graves de tipo linfocítico en uno o dos de los órganos diana estudiados presentaran lesiones mínimas de tipo linfocítico o mixto en otro órgano diana o de que animales con lesiones graves de tipo histiocítico presentaran únicamente lesiones mínimas de tipo mixto en otro órgano diana apoyaría la hipótesis de patrón mixto como forma inicial o latente y respuesta inmune no bien definida que podría evolucionar a un patrón linfocítico o histiocítico con la progresión de la lesión. Así mismo, el que nunca se observaran lesiones mínimas de tipo linfocítico en animales con predominio de células CD68+ en lesiones

moderadas o graves en otros órganos hace pensar que mientras que un patrón mixto puede evolucionar a linfocítico o histiocítico, es poco probable que lesiones mínimas de tipo linfocítico evolucionen a un patrón histiocítico en estadios tardíos de la enfermedad o que haya cambios en el tipo de patrón una vez que se hayan desarrollado lesiones moderadas o graves y por tanto una respuesta inmunológica bien definida, como se ha descrito en otras enfermedades crónicas en ovino (Juste *et al.*, 1994; Delgado *et al.*, 2013).

Los animales con patrón mixto presentaron valores en el test ELISA variables, intermedios entre los de los patrones linfocítico e histiocítico. La mayoría eran animales con lesiones mínimas, con resultados positivos en el test ELISA pero valores de densidad óptica bajos, concordando con la hipótesis de que los ovinos con patrón mixto y lesiones mínimas representarían formas iniciales o latentes de infección, donde aún no se han producido títulos elevados de anticuerpos. Se ha descrito que la seroconversión ocurre entre el día 15 y los cuatro meses post-infección y que la aparición de lesiones se produce posteriormente (Pépin *et al.*, 1998; Lacerenza *et al.*, 2006). Sin embargo, nuestros hallazgos hacen pensar que la infección, la aparición de lesiones y la seroconversión ocurren de forma muy temprana y casi contemporáneamente, y que en cualquier caso, debe de existir un mínimo de lesión y de células inflamatorias que produzcan una respuesta inmune local para que pueda existir seropositividad.

La descripción de un patrón de lesión predominante en cada individuo y de diferencias significativas entre las respuestas serológicas de animales con distinto patrón de lesión supone un avance en el conocimiento de la respuesta individual de animales naturalmente infectados a la enfermedad. En resumen, los animales con patrón histiocítico, fácilmente reconocibles mediante test ELISA, presentarían una mayor carga viral y jugarían un papel importante en la difusión de la infección. Sin embargo, los ovinos con patrón linfocítico podrían no ser detectados mediante estos test diagnósticos utilizados habitualmente debido a su baja carga

viral y escasa producción de anticuerpos, pasando desapercibidos en el rebaño como falsos negativos pero contribuyendo igualmente como portadores y fuente de infección. Dichos animales cobrarían especial importancia en rebaños con baja prevalencia de MV, a lo que se tiende en el futuro con los actuales programas de control de la enfermedad, ya que incluso rebaños considerados libres de infección utilizando los test serológicos habituales podrían presentar algún animal de este tipo, cuya detección sería fundamental para la erradicación del MV en la explotación. En estos casos, el empleo de una sola técnica de diagnóstico, normalmente test ELISA, sería insuficiente y la toma de biopsias de glándula mamaria para el estudio histopatológico e IHQ, así como la realización de la técnica de PCR y inmunocitoquímica en leche podrían ser de interés como se discutirá más adelante, aunque de muy difícil realización en condiciones de campo. Sin embargo, sería muy interesante profundizar en estos estudios en ovinos desechados por otras causas o enviados a matadero para tener una visión más amplia de la situación real de infección por MV en una explotación.

La caracterización pormenorizada de las lesiones producidas por MV en pulmón, glándula mamaria y SNC en ovinos naturalmente infectados y su clasificación en lesiones mínimas (+), moderadas (++) o graves (+++) aporta un modelo útil y sencillo para la clasificación de lesiones producidas por MV aplicable a los tres principales órganos diana de la enfermedad en ovinos. Las lesiones graves se observaron solo en un órgano diana en el 40% de los animales y en dos órganos en el 28% (total 68%), independientemente del rebaño de procedencia y con predominio de lesiones en SNC, hecho que podría relacionarse con la elevada prevalencia de casos de la forma nerviosa de MV en el área de estudio (Benavides *et al.*, 2006a) donde se ha descrito la circulación de una cepa neurotrópica de LVPR (Glaria *et al.*, 2012). Sin embargo, en los estudios realizados en esta tesis doctoral se observaron lesiones moderadas y graves no solo en SNC, sino también en pulmón y en glándula mamaria, lo que relacionamos más con el tipo de respuesta individual que con la cepa vírica. Así, en el estudio filogenético realizado en

muestras de glándula mamaria con diferentes grados de lesión, no se observaron diferencias significativas entre la cepa vírica y la gravedad de la lesión.

En el caso concreto de la glándula mamaria los porcentajes de lesiones de diferentes grados de gravedad fueron similares en animales con infección conocida procedentes de diferentes explotaciones seropositivas con clínica que los tomados al azar en dos mataderos, todos ellos localizados en Castilla y León. El 57% de los animales del primer grupo y el 54% de los del segundo grupo presentaron lesiones moderadas o graves, lo que pone de manifiesto la importancia de la forma mamaria de la enfermedad en el desecho de ovejas de raza Assaf Española de producción intensiva (Benavides *et al.*, 2013), con las pérdidas económicas que conlleva en esta y otras razas de producción lechera (Peterhans *et al*., 2004). La obtención de un 91,4% de infectados por MV utilizando diferentes técnicas de diagnóstico en los animales tomados aleatoriamente en matadero, también destacan la importancia del MV en esta Comunidad Autónoma en ovino intensivo de leche.

La detección de las lesiones mínimas, supuso el incremento de la prevalencia de lesiones producidas por MV en pulmón desde un 32,3% en animales seleccionados al azar (Luján *et al.*, 1991) o un 79,3% en ovinos de explotaciones intensivas de leche (Benavides *et al.*, 2013) hasta el 100% en nuestros estudios. De la misma manera, la detección de lesiones mínimas supuso un incremento de la prevalencia de lesiones producidas por MV en la glándula mamaria hasta el 80%, en comparación con el 35,6% descrito considerando solo lesiones moderadas y graves (Luján *et al.*, 1991), o el 46,7% considerando también lesiones leves, pero no mínimas (Benavides *et al.*, 2013). A pesar de que en algunos estudios se ha descrito como normal un cierto grado de infiltrado linfoide subepitelial en la glándula mamaria, que se ha relacionado con protección frente a las primeras etapas de infecciones bacterianas (Mavrogianni *et al.*, 2007), no hemos observado la presencia de células inflamatorias en el intersticio en nuestras preparaciones de animales no infectados o en zonas de tejido sano de animales con infección.

La ausencia de infiltrado inflamatorio en las muestras utilizadas en nuestros estudios podría deberse a la selección genética y a los continuos controles microbiológicos y de células somáticas que se llevan actualmente a cabo en ovino intensivo de leche, sistema de explotación utilizado en el área de estudio en la raza Assaf Española. En cualquier caso, el MV se debería considerar como diagnóstico diferencial en mamas con presencia de infiltrado inflamatorio intersticial, especialmente en esta zona geográfica donde la prevalencia de la enfermedad es tan elevada.

En cuanto a las diferentes técnicas de diagnóstico de MV, en el tercer estudio de esta tesis doctoral se compara la sensibilidad de las más utilizadas en el diagnóstico de la enfermedad: histopatología, ELISA (ELITEST®), IHQ y PCR, las dos últimas también en muestras de leche. Mientras que la serología detectó un 85,5% de los animales infectados, la IHQ y la PCR fueron más sensibles con sensibilidades del 100% y 94,7% respectivamente en muestras de tejido mamario procedente de animales infectados. La menor sensibilidad de la PCR con respecto a la IHQ coincide con descripciones anteriores en MV (Benavides *et al.*, 2006b). En los estudios realizados en la presente tesis doctoral, la sensibilidad en el grupo de animales de matadero, donde la PCR se realizó a partir de tejido congelado, fue del 100% mientras que en las muestras de animales de las diferentes explotaciones en las que se realizaron necropsias de forma rutinaria, con fijación de muestras en formol durante 48 horas, fue del 90,9%. Estos resultados nos indicarían una posible degradación del ADN proviral tras la fijación del tejido en formol (Srinivasan *et al.*, 2002). También podría guardar relación con la presencia de menor carga viral en las muestras analizadas, lo que se ha sugerido como una de las principales dificultades del diagnóstico del VMV mediante PCR (de Andrés *et al.*, 2005). Sin embargo, la obtención de una positividad de más del 90% en muestras fijadas en formol y embebidas en parafina en nuestros estudios junto con la obtención de secuencias de 800 pb de buena calidad a partir de las mismas para la realización de estudios filogenéticos destaca la posible utilización de este tipo de muestras, a menudo las únicas disponibles, para la realización de

estudios retrospectivos. Estos resultados concuerdan con los obtenidos en estudios previos, donde se ha descrito positividad frente a MV en PCR con tiempos de fijación de las muestras en formol de hasta 30 días (Benavides *et al.*, 2006b).

En muestras de leche, la mayor sensibilidad de la PCR (50%) con respecto a la ICQ (25%) podría relacionarse con la dificultad de detectar macrófagos en la preparación citológica utilizando la segunda técnica, y especialmente aquellos macrófagos positivos a VMV. La existencia de una muestra de leche positiva a ICQ y negativa a PCR, podría deberse a una baja carga viral en dicha muestra, como discutido en el párrafo anterior (de Andrés *et al.*, 2005). La detección de virus en leche mediante PCR incluso en muestras procedentes de animales con lesiones mínimas en glándula mamaria confirma su utilidad en la detección del VMV.

La única utilización de test serológicos para el diagnóstico del MV, como ocurre frecuentemente en los programas de control de la enfermedad, presenta el riesgo de obtención de falsos negativos, los cuales podrían relacionarse como discutido con anterioridad con una respuesta inmunológica de tipo linfocítico del hospedador o con la presencia de lesiones mínimas, o bien con variaciones antigénicas de determinadas cepas de LVPR que pueden pasar desapercibidos utilizando determinados test de diagnóstico como se discutirá más adelante. Los test ELISA, rápidos y fáciles de realizar en condiciones de campo, económicos y poco cruentos serían la técnica de elección en explotaciones de elevada seroprevalencia (Polledo *et al.*, 2013), donde los animales con patrón histiocítico, relacionados con mayor carga viral y excreción vírica, serían fácilmente detectados y los primeros en ser eliminados en las sucesivas reposiciones. Sin embargo, en granjas con bajas prevalencias, la detección de los animales con patrón linfocítico y lesiones mínimas cobra una mayor importancia, pues aunque en menor medida también podrían excretar virus, como se demuestra en muestras de leche por ICQ y PCR en el tercer estudio de esta tesis, y que en cualquier caso deberían ser eliminados para una completa erradicación de la enfermedad.

La descripción de las poblaciones de células positivas mediante IHC en los diferentes órganos diana y en la leche, así como su localización, especialmente en las lesiones mínimas, son de gran interés en el conocimiento de la patogenia de la enfermedad y la posible evolución de la infección y transmisión de la enfermedad. Se confirmó mediante IHQ la presencia de antígeno vírico del VMV en tejido de los tres órganos diana de todos los animales estudiados, incluyendo aquellos con lesiones mínimas. La presencia de antígeno vírico se relacionó en los tres tipos de tejido con células morfológicamente compatibles con macrófagos, como se ha descrito en numerosos estudios (Minguijón *et al.*, 2015), pero nunca en células epiteliales, a pesar de haber sido propuestas como posible diana del virus del VMV y de replicación vírica, especialmente en estudios en glándula mamaria (Lerondelle *et al.*, 1999; Bolea *et al.*, 2006). En este órgano, la utilización de un segundo anticuerpo obtenido en nuestro laboratorio frente a la proteína de envoltura gp135 codificada por el gen *env*, confirma los resultados obtenidos con el anticuerpo comercial frente a la proteína de la nucleocápside p28 codificada por el gen *gag*, confirmando la presencia de VMV en células compatibles con macrófagos. Además, a diferencia de lo que ocurre con las técnicas de PCR o hibridación *in situ,* con las que se ha descrito positividad en glándula mamaria en células epiteliales (Carrozza *et al.*, 2003; Bolea *et al.*, 2006), la IHQ demuestra la existencia no solo de virus si no de infección activa al detectarse la presencia de proteínas tanto de la nucleocápside como de la envoltura.

La distribución de las células inflamatorias y del antígeno vírico en glándula mamaria mediante IHQ utilizando los dos anticuerpos descritos previamente se corresponde con la hipótesis de invasión vírica con el paso de monocitos infectados desde la sangre a macrófagos en los tejidos conocido como mecanismo de ‘’Caballo de Troya’’ (Peluso *et al.*, 1985; Polledo *et al.*, 2012a). Así, la infección comenzaría en zonas focales y muy concretas del órgano, próximas a vasos sanguíneos y formando manguitos perivasculares, y continuaría diseminándose al resto del mismo facilitado por la posibilidad de infecciones concurrentes que podrían alcanzar diferentes vasos sanguíneos.

La presencia de macrófagos positivos a VMV mediante IHQ en la luz acinar en algunas muestras de mama junto con la positividad en leche mediante técnicas de IHC y PCR confirman la posible transmisión de la enfermedad por vía lactogénica (Preziuso *et al.*, 2004; Álvarez *et al.*, 2005; Álvarez *et al.*, 2006). Sin embargo el bajo número de muestras positivas en leche mediante ambas técnicas confirmaría el limitado potencial de transmisión vírica por esta vía, como se ha propuesto por otros autores (Berriatua *et al.*, 2003). No obstante, el riesgo de contagio podría ser superior por lactación continua y vía respiratoria que por ingestión puntual del calostro.

La mayoría de las muestras de leche positivas procedían de animales con lesiones moderadas o graves en la glándula mamaria (85,7%) y con elevado número de células positivas a VMV mediante IHQ así como resultados positivos a PCR. Estos resultados sugieren que es más fácil para los macrófagos infectados alcanzar los espacios acinares cuando las lesiones son más graves y difusas que cuando son focales y limitadas a los espacios perivasculares.

En cuanto a la técnica de citología que se desarrolla en el tercer trabajo de esta tesis doctoral, resulta de gran utilidad para la obtención de preparaciones bien conservadas con células dispuestas en monocapa en las que se pueden identificar fácilmente las distintas poblaciones celulares y la presencia de antígeno del VMV mediante técnicas de inmunocitoquímica. Tras la primera centrifugación, es muy probable que la distribución de las células en el tubo Falcon se relacione con la densidad de las mismas como ocurre en los vasos sanguíneos durante la inflamación (Kumar *et al.*, 2005). El hecho de que las células de la parte superior del tubo estuvieran mejor conservadas que las del fondo podría ser debido al papel protector de la nata acumulada en la superficie de la muestra durante la centrifugación mientras que la distribución de los distintos tipos celulares formando diferentes halos concéntricos después de la citocentrifugación se relacionaría con la diferente densidad de los distintos tipos celulares. La fijación del tiempo óptimo de secado de la citología después de la

citocentrifugación en 20 minutos resulta mucho más corto que el descrito en estudios previos (Luján *et al.*, 1994). La tinción de las citología mediante HE resultó útil para la detección de leucocitos mientras que con la tinción de May-Grenwald Giemsa se identificaban mejor las células epiteliales (Cowell, 2008), permitiendo además una mejor diferenciación de las mismas de artefactos. La morfología normal de las células epiteliales en las muestras de leche estudiadas sugiere además la ausencia de hiperplasia o neoplasia en el epitelio de las glándulas mamarias estudiadas (Cowell, 2008). La ausencia de glóbulos grasos en las preparaciones de ICC, a diferencia de lo que ocurría en las tinciones citológicas, se puede atribuir a los sucesivos lavados con PBS y al bloqueo de la peroxidasa endógena en peróxido de hidrógeno al 3% en agua destilada.

En el cuarto trabajo, ante la necesidad de explicar los falsos negativos o positivos débiles que se venían obteniendo con diferentes técnicas de PCR, se plantea estudiar las cepas víricas circulantes en el área de estudio en ovinos de raza Assaf Española con lesiones en la glándula mamaria, y su relación con la gravedad de las lesiones desarrolladas. En este sentido se describe la circulación de LVPR de tipo A2/A3 y B2 en animales con diferente gravedad de lesión, así como alteraciones moderadas en algunos epitopos inmunodominantes de la *Major Homology Region* (MHR), que podrían relacionarse con alteraciones en el ensamblaje vírico y en su capacidad de infección, pudiendo interferir en la sensibilidad de algunas pruebas de diagnóstico como el test ELISA y la PCR, por lo que sería necesario elaborar *primers* específicos para cada cepa.

Se describen por primera vez las secuencias *gag* y LTR observadas en ovejas de raza Assaf Española con diferentes grados histopatológicos de mamitis causada por MV, así como su relación filogenética con secuencias conocidas de LVPR. A pesar de que inicialmente se pensaba que el genotipo A infectaba a ovinos y que el genotipo B se asociaba a infección en caprino, actualmente es bien sabido que ambos genotipos pueden infectar a las dos especies.

Así, el ganado ovino puede presentar cepas tanto del genotipo A como del B, como ocurre en nuestro estudio (Olech *et al.*, 2012; Fras *et al.*, 2013; Kuhar *et al.*, 2013; Santry *et al.*, 2013). A pesar de no poder confirmar que los ovinos incluidos en este estudio, cuyas muestras se tomaron en matadero, no estuvieran en contacto con cabras, podría ser posible que la presencia de un genotipo B en estos animales no se asociara a haber mantenido contacto con esa especie, como se ha demostrado en el caso de caprinos con genotipo A y sin contacto con ovinos (Reina *et al.*, 2006). Además, es muy infrecuente que las explotaciones de raza Assaf Española en Castilla y León, sometidas a régimen de manejo intensivo, sean mixtas.

El árbol filogenético de las secuencias *gag-pol* y la comparación de las distancias filogenéticas reveló que mayoría de las muestras se encontraban próximas al genotipo del aislado 697 (A2/A3), mientras que cuatro muestras pertenecían al subtipo B2. El aislado ovino 697, descrito también en Castilla y León, se clasificó como A2/A3 debido a que las diferencias entre ambos subtipos son a menudo tan pequeñas que es difícil separar ambos grupos (Glaria *et al.*, 2012; Shah *et al.*, 2004a). Este aislado se relacionó con la forma nerviosa de la enfermedad, al igual que otras siete secuencias parciales procedentes de ovinos con clínica neurológica, también clasificados como A2/A3 (Glaria *et al.*, 2012), sugiriendo que esta cepa vírica neurológica circula por el norte de España, a pesar de que en nuestros animales no se observaron siempre clínica o lesiones en el SNC. En cuanto a los animales que presentaron subtipo B2, las secuencias resultaron ser más próximas a los aislados descritos en Italia (Grego *et al.*, 2007; Giammarioli *et al.*, 2011) que a los de tipo B2 españoles. Los últimos se han relacionado con lesiones artríticas en ovinos de raza Rasa Aragonesa (Glaria *et al.*, 2009; Pérez *et al.*, 2015), donde se observaron lesiones mamarias en 10 de 13 animales con artritis, sugiriendo que la mama puede estar a menudo afectada aunque no se observen signos clínicos hasta que la pérdida de producción láctea no resulte evidente. No se tiene conocimiento sin embargo de la clínica o lesiones histológicas que presentaban los animales de los que se

tomaron los aislados italianos, lo que hace más interesante la descripción de las lesiones mamarias asociadas a dicho subtipo en el presente estudio.

El análisis de las secuencias genéticas resulta de gran interés no solo para evaluar la difusión de los distintos tipos y subtipos de LVPR entre diferentes áreas geográficas, sino también para monitorizar la variabilidad genética, la cual podría ser causante de errores de diagnóstico y obtención de falsos negativos en animales con genotipos muy divergentes (Cardinaux *et al.*, 2013). El gen *gag* codifica proteínas de la nucleocápside, cápside y matriz proteica. Los ensayos de diagnóstico indirectos utilizan a menudo como antígeno proteínas de la cápside, por lo que el estudio de las variaciones genéticas en los epítopos dominantes de las proteínas estructurales codificadas por el gen *gag* es muy interesante para evaluar y mejorar la sensibilidad de las técnicas de diagnóstico. Así, las alteraciones en aminoácidos de estas regiones, que sugieren una antigenicidad alterada, pueden afectar al principal test de diagnóstico empleado en el MV (Polledo *et al.*, 2013). Los primers *gag-pol* empleados en nuestro estudio permitieron el análisis de los epitopos 2 y 3, que estaban bastante conservados en los animales con genotipo A2/A3, en los que solo hubo pequeñas alteraciones en tres muestras en el epitopo 2 y en una en el epitopo 3. Sin embargo, en el genotipo B2 las diferencias fueron mayores, habiendo alteraciones en un aminoácido en 3 de las 4 muestras estudiadas. Sorprendentemente en los animales con genotipo A2/A3 se observó una mayor variabilidad en el MHR, esencial para el ensamblaje vírico y normalmente muy conservado entre los retrovirus (Purdy *et al.*, 2008), mostrando todas las muestras menos dos al menos una alteración en comparación con el aislado de referencia 697, cepa A2/A3. Las mutaciones en la secuencia del MHR en el HIV-1 humano se han asociado a una reducción de la infectividad del virus debida a modificaciones en el ensamblaje de la cápside (Chu *et al.*, 2006). A pesar de que se han realizado algunos estudios sobre el MHR de los retrovirus humanos, las consecuencias de las variaciones en el MHR de los LVPR no han sido suficientemente estudiadas.

Por otro lado aparecieron bien conservadas las secuencias de los aminoácidos *gag* de las regiones MHR de los animales con genotipo B2 y el GG *motif* de todas las muestras con ambos genotipos, así como las secuencias de los nucleóticos LTR del AML (vis) *motif*, TATA-box y poly-A de todos los animales, en concordancia con descripciones previas en diferentes cepas localizadas en diferentes zonas geográficas (Angelopoulou *et al.*, 2006a; Reina *et al.*, 2006; Kokawa *et al.*, 2017). La mayoría de las secuencias LTR mostraron gran homología con los aislados españoles A2/A3 (Glaria *et al.*, 2012). Sin embargo, dos animales incluidos en el presente estudio mostraron una delección de 23 nucleótidos en la región R, más larga que la delección de 13 nucleótidos que se observó en animales con sintomatología clínica de la cepa de referencia 697, la cual se asoció por lo tanto con la aparición de signos clínicos (Glaria *et al.*, 2012). Sin embargo, en otro estudio previo se describió una delección similar en esta región en animales asintomáticos, estando los pulmones de los animales portadores de virus con dicha delección significativamente menos afectados que los de animales del mismo estudio que no la presentaban (Angelopoulou *et al.*, 2008). En el presente estudio, no se encontraron diferencias significativas ($p>0,05$) entre la delección en la región R de la LTR y la gravedad de lesiones en glándula mamaria, ya que se observó en animales con lesiones más graves, pero otros ovinos con lesiones moderadas o graves no presentaron la delección. Estos hechos redundarían en la observación manifestada anteriormente de la posible ausencia de relación significativa entre la cepa vírica y la gravedad de las lesiones producidas, destacando más la respuesta inmunopatológica individual en el desarrollo de la enfermedad.

Considerando el elevado impacto económico del MV y en general de los LVPR, muchos países intentan erradicar la enfermedad basándose en la identificación y el desecho prematuro de los animales infectados. La selección de dichos animales mediante la utilización de técnicas serológicas puede suponer la selección de variantes de LVPR con alteraciones significativas en sus secuencias antigénicas, así como la no detección de animales con respuesta inmunológica de tipo linfocítico o en estadios tempranos de la enfermedad, como se ha discutido con

anterioridad. Se deberían realizar estudios más amplios de la variabilidad genética de los LVPR en diferentes países para tener una visión más amplia de la situación actual y de las cepas circulantes y así poder diseñar test más sensibles y específicos para las nuevas variantes. El estudio histopatológico en muestras de matadero puede ser de gran interés para detectar animales que, a pesar de presentar resultados serológicos negativos, pueden presentar lesiones compatibles con LVPR, probablemente infectados por nuevas variedades genéticas no detectadas mediante los test ELISA tradicionales o que han desarrollado una respuesta inmune individual que les permite pasar desapercibidos utilizando únicamente esta herramienta de diagnóstico.

X. Resumen

El Maedi-Visna (MV) es una enfermedad muy difundida a nivel mundial y en nuestro país, que tiene especial importancia en Castilla y León, donde causa importantes pérdidas económicas en ovino intensivo de leche de raza Assaf Española. La enfermedad, de curso crónico, pasa a menudo desapercibida hasta estados avanzados de la misma, especialmente la forma mamaria, dando lugar a una pérdida progresiva de la producción lechera. La falta de vacunas o tratamientos efectivos hacen que sea fundamental el contar con técnicas de diagnóstico sensibles que permitan conocer la situación epidemiológica de los rebaños y la detección precoz de los animales infectados para su eliminación en las sucesivas reposiciones. El objetivo general de esta tesis doctoral es contribuir al conocimiento de la patogenia del MV, describiendo la respuesta inmune del hospedador frente a la infección, así como la valoración de diferentes métodos de diagnóstico para la detección precoz y el estudio de las cepas circulantes.

Se describen tres patrones de lesión en pulmón, glándula mamaria y SNC en función de la población celular predominante en el infiltrado inflamatorio: histiocítico, linfocítico y mixto. El patrón histiocítico se caracterizó por un predominio de macrófagos formando el infiltrado inflamatorio junto con algunos linfocitos B, por una respuesta serológica más intensa, caracterizada por altos títulos de anticuerpos, y por una mayor cantidad de virus en tejido mediante valoración subjetiva en IHQ. Al contrario, los animales con patrón linfocítico presentaron lesiones con un predominio de linfocitos T, títulos bajos de anticuerpos en test ELISA y una menor cantidad de virus en tejido. Por lo tanto, los animales con patrón histiocítico serían fácilmente detectables mediante test serológicos, mientras que aquellos con patrón linfocítico, incluso con valores negativos, pueden pasar desapercibidos en el rebaño. El patrón mixto, descrito por primera vez en esta tesis doctoral, se caracterizó por un infiltrado inflamatorio mixto formado por células CD68+y CD3+ en cantidades similares, así como por una respuesta serológica variable. Dicho patrón apareció principalmente en animales con lesiones mínimas, que podrían ser iniciales o latentes, y que evolucionarían a formas

linfocíticas o histiocíticas con la progresión de la infección. Se observa que el patrón de lesión es el mismo en pulmón y SNC de un mismo animal, mientras que la glándula mamaria presenta un predominio de lesiones linfocíticas, aunque con un número superior de macrófagos en el tipo histiocítico. Estas diferencias podrían deberse a que se trata de un órgano de naturaleza mucosa, en continua estimulación antigénica, que podría desarrollar una respuesta inmune de tipo Th1 más marcada.

Se hace una clasificación detallada de las lesiones producidas por el MV en los tres principales órganos diana de la enfermedad, clasificándose como mínimas (+), moderadas (++) o graves (++). Las lesiones mínimas, con presencia de VMV confirmada mediante IHQ, supusieron un claro aumento de la prevalencia de la enfermedad con respecto a estudios anteriores, llegando a la detección de lesiones en prácticamente la totalidad de los animales infectados. Así, la histopatología resulta una herramienta de gran utilidad en la detección de la enfermedad, que debe complementarse con otras técnicas de diagnóstico, especialmente en lesiones mínimas. Mediante la valoración de las principales técnicas utilizadas en la actualidad para el diagnóstico del MV, se observó que la más sensible es la IHQ, que llegó a detectar el 100% de los animales positivos, seguida por la PCR y por la serología. El test ELISA, económico y sencillo de realizar en condiciones de campo, mostró una sensibilidad del 85,5%. Estos resultados suponen que una parte de animales infectados están pasando desapercibidos con los actuales sistemas de control, y que debería utilizarse más de una técnica para el diagnóstico. El ‘’gold standard’’ según los resultados obtenidos en nuestros estudios, sería complementar la serología con la IHQ y la histopatología, que aunque no es viable en condiciones de campo, podría realizarse en muestras de matadero para conocer la situación real de las explotaciones. La obtención de falsos negativos mediante test ELISA podría relacionarse con la existencia de cepas con variedades antigénicas no detectadas por un determinado test, así como a la existencia de un patrón de lesión de tipo linfocítico con baja producción de anticuerpos o a formas iniciales, donde se pueden detectar lesiones mínimas y

virus en tejido mediante IHQ, pero aún no se ha desarrollado una respuesta inmune periférica detectable mediante test ELISA.

La única célula diana del MV mediante técnicas de IHQ tantos en muestras de tejido de los tres órganos diana como en leche fue el macrófago, no observándose positividad en células epiteliales o en otros tipos celulares. La distribución de las lesiones y del antígeno vírico formando manguitos perivasculares en lesiones mínimas y entre el infiltrado inflamatorio más difuso en lesiones moderadas y graves, y el hecho de que no se observara positividad en zonas sin lesión hace pensar en una difusión de la infección por vía saguínea, como se ha propuesto en estudios previos por el paso de monocitos infectados de sangre a macrófagos.

La presencia de virus en leche tanto mediante ICC como PCR confirma la posible transmisión del virus por esta vía, aunque la detección del mismo únicamente en la mitad de las muestras hace pensar en un mayor peso de la vía respiratoria en la transmisión de madre a cordero. La mayoría de animales positivos a MV en muestras de leche presentaban lesiones moderadas o graves en la glándula mamaria, siendo más fácil la llegada de macrófagos infectados a las luces acinares en el caso de infecciones difusas que en lesiones mínimas.

Esta tesis doctoral también contribuye al conocimiento de las cepas víricas circulantes en Castilla y León, detectándose los genotipos A2/A3, similar al previamente descrito en la región en la forma nerviosa de la enfermedad y B2, más próximo a cepas italianas que a las artríticas descritas en España. La secuenciación y el conocimiento de las cepas circulantes tiene gran importancia para el diagnóstico y el control de la enfermedad, ya que permite desarrollar test serológicos que empleen proteínas específicas para determinadas variedades genéticas o bien el diseño de primers específicos para la detección mediante PCR. Además se describen diferentes variaciones en la región MHR y otros genes relacionados con la patogenia del MV, contribuyendo al conocimiento de los factores genéticos que pueden influir en la resistencia del hospedador a la infección o al desarrollo de lesiones. No se observaron diferencias entre la

cepa vírica y la gravedad de las lesiones en glándula mamaria, haciendo pensar que el tipo y la gravedad de la lesión desarrollada se relacionan más con la respuesta inmune del hospedador que con factores virales.

En conclusión, los resultados de esta tesis doctoral muestran que la respuesta inmune del hospedador parece jugar un papel fundamental en el desarrollo de las lesiones producidas por el MV, determinando el tipo de infiltrado inflamatorio predominante y la respuesta serológica, y relacionándose con la cantidad de virus presente en los tejidos y su posibilidad de excreción y transmisión, así como con la facilidad para detectar la infección mediante diferentes técnicas de diagnóstico. La detección precoz, incluso de animales con lesiones mínimas, es de gran importancia especialmente en rebaños en los que se hayan obtenido bajas prevalencias de MV mediante los métodos de control tradicionales, para poder eliminar todos los animales infectados y conseguir la erradicación de la enfermedad.

XI. Conclusiones

1. Se puede asignar un patrón de lesión individual a cada animal estudiando la respuesta inmunológica en los principales órganos diana del MV: pulmón, glándula mamaria y SNC. Dichos patrones se denominan histiocítico, linfocítico y mixto atendiendo a la población celular predominante del infiltrado inflamatorio y estarían relacionados con la respuesta inmunológica del hospedador a la infección por MV.
2. Se describen tres tipos de lesiones en cuanto a su extensión y gravedad: mínimas (+), moderadas (++) y graves (+++). Las lesiones mínimas fueron de tipo linfocítico o mixto y aparecieron en los tres órganos diana en forma de pocas células inflamatorias perivasculares junto con la presencia de antígeno vírico. Este hecho, así como que las lesiones moderadas y graves fueran generalizadas, confirmaría que la lesión inicial se correspondería con el comienzo de la infección y que habría una progresión posterior de la misma a través de los espacios perivasculares.
3. La detección de 'lesiones mínimas' incrementa notablemente la prevalencia de lesiones en animales infectados y la detección de MV mediante histopatología. La combinación de esta última técnica con la inmunohistoquímica detecta la totalidad de animales infectados y se propone como 'gold standard' para el diagnóstico de la enfermedad.
4. Existe una asociación entre patrón de lesión y respuesta serológica. Los animales con patrón histiocítico presentan una respuesta más intensa que los de patrón linfocítico, en los que en ocasiones se dieron incluso resultados negativos. Es por ello que los ovinos que presentan patrón histiocítico, con elevada presencia vírica y respuesta serológica más intensa, se asociarían con una mayor capacidad de diseminación de la infección.
5. Se confirma mediante inmunohistoquímica la infección de macrófagos de la glándula mamaria y de la leche, no obteniéndose positividad en células epiteliales con los anticuerpos empleados.

6. La detección de virus de MV en leche mediante inmunocitoquímica y PCR confirma la posibilidad de transmisión mediante esta vía. No obstante, la escasa presencia de antígeno vírico tanto en leche como en acinis mamarios podría indicar un papel limitado de la leche en la transmisión viral y contagio de corderos.
7. Se confirma la circulación de las cepas A2/A3 y se describe la de la cepa B2 en ovinos Assaf Española, esta última similar a las cepas italianas. Se describen variaciones moderadas en algunos epítopos inmunodominantes y en el tracto MHR que podrían relacionarse con alteraciones en el ensamblaje vírico y en su capacidad de infección, y que podrían interferir en la sensibilidad de algunos test diagnóstico como el test ELISA, por lo que sería necesario elaborar *primers* específicos para cada cepa.
8. No se observan diferencias significativas entre la cepa vírica y la gravedad de las lesiones producidas en la glándula mamaria, destacando la respuesta inmunopatológica individual en el desarrollo de la enfermedad.

XII. Summary

Ovine Maedi Visna (MV) is a disease that exists the world over, with special importance in Spain, in the Castile y León region, where it causes severe economic losses in Spanish Assaf high-intensive milk-producing flocks. MV has a chronic progression and often goes unnoticed until the advanced stage of the disease, especially the mammary form, producing progressive milk production loss. The absence of vaccines or efficient therapies make the existence of sensitive diagnostic techniques crucial for knowing the epidemiological status of flocks and for early detection of infected sheep for culling. The general aim of this PhD is to increase the knowledge on MV pathogenesis, describing the host's immune response against MV infection, evaluating different diagnosis techniques for early detection and characterizing circulating viral strains in the study area.

Three lesion patterns are described in the lungs, mammary glands and in the CNS depending on the predominant cell population composition of the inflammatory infiltrate: histiocytic, lymphocytic and mixed. The histiocytic pattern is characterized by a predominance of macrophages within the inflammatory infiltrate together with a small amount B lymphocytes, a more intensive serological response characterized by higher antibody titer and a higher amount of virus in tissue by IHC carrying out a subjective valuation. By contrast, animals with the lymphocytic lesion pattern showed lesions with predominance of T cells, lower antibody titer as detected by ELISA (suprimí test porque está en las siglas) and a lower amount of virus in tissue by IHC. Thus, animals with the histiocytic pattern are easily detected by serological test, while those with the lymphocytic pattern, even with negative results, could go unnoticed in the flock. The mixed pattern, described for the first time in this PhD thesis was characterized by a mixed inflammatory infiltrate composed by a similar number of CD68+ and CD3+ cells and a variable serological response. This pattern was mainly observed in sheep with minimal lesions, which could be initial or latent, and would evolve to lymphocytic or histiocytic forms with the progression of the infection.

The same lesion pattern was observed in the lung and the CNS of the same animal, while the mammary glands showed a predominance of lymphocytic lesions, although a higher number of macrophages were observed in sheep with the histiocytic pattern. These differences could be due to the mucosal nature of this target organ, which has an outward-facing surface with continuous antigen stimulation perhaps reflecting a higher Th1-mediated response.

A detailed classification of MV lesions was established in the three main target organs of the disease. Lesions were classified as minimal (+), moderate (++) or severe (+++). Minimal lesions diagnosis, with VMV presence confirmed by IHC, clearly increased MV prevalence in comparison with previous studies and supposed the presence of MV lesions in almost all infected animals. Thus, histopathology turned out to be a very useful diagnostic tool in MV detection which has to be complemented with other techniques, especially in sheep with minimal lesions. After evaluation of the main MV diagnostic tools available nowadays, IHC was the most sensitive, detecting 100% of infected animals, followed by PCR and serology. ELISA, economic and convenient in field conditions, showed a sensitivity of 85.5%. These results presume that a part of infected animals are going unnoticed with actual control systems and that more than one diagnostic tool should be performed. The gold standard according to our results would be complementing serology with IHC and histopathology which, even if it is not viable in field conditions could be performed in abattoir to know the real flock status. False negatives obtained with ELISA could be related to the existence of viral strains characterized by antigen variations not detected by a certain test, to the existence of a lymphocytic pattern with low antibody production, or to initial infections where minimal lesions and viral presence in tissue can be detected by IHC but a peripheral immune response detectable by ELISA has not been developed yet.

Macrophages were the only MV target cell observed by IHC in the three main target organs and in milk samples studied, with no positivity being observed in epithelial or other cell types. Lesion and viral antigen distribution forming perivascular cuffs in minimal lesions and within the more dispersed inflammatory infiltrate in moderate and severe lesions together with the fact that no positivity was observed in healthy areas point towards viral dissemination from infected monocytes in blood to macrophages in tissue as previously reported.

Viral presence in milk by ICC and PCR confirms possible viral transmission by this route, even if viral detection in only 50% of the samples studied considers the respiratory tract more important in MV transmission to lambs. Most of sheep with positive results in milk showed moderate or severe lesions in mammary gland, with the macrophages arrival to the acinar lumen being easier in widespread than in minimal lesions.

This PhD Thesis also contributes to the Knowledge of the viral strains circulating in the Castile y León region, where A2/A3 and B2 genotypes were detected. The former was similar to the previously described strains in the nervous disease while the later was closer to Italian strains than to arthritic forms described in Spain. Sequencing and knowledge of circulating strains is very revealing for the diagnosis and control of the disease, as allowed by the developing of specific serological test or specific primers for PCR detection. Furthermore, different variations in MHR region and other genes related to MV pathogenesis are described, contributing to knowledge of genetic factors which may influence host resistance to infection or lesions development. No differences were observed between viral strain and lesion severity in mammary glands, which agrees with the hypothesis that type and severity of lesions would be more related with the host's immune response than with viral factors.

In conclusion, the results of this PhD Thesis show that the host's immune response plays a major role in the development of lesions caused by MV. It determines the predominant type of inflammatory infiltrate and the resulting serological response developed which is in relation with the amount of VMV present in tissue and its possible transmission as well as with viral detection by using different techniques. Early detection, even in animals with minimal lesions, is of great interest especially in flocks where low MV prevalence has been obtained with traditional control programs in order to be able to eradicate the disease by the culling of every infected sheep.

XIII. Conclusions

1. An individual lesion pattern can be assigned to each animal on the basis of the immunological response in the three main MV target organs: the lung, the mammary glands and the CNS. These lesion patterns are called histiocytic, lymphocytic and mixed regarding the predominant cell population of the inflammatory infiltrate and would be related with the host's immune response to MV infection.
2. Three types of lesions are described according to their extension and severity: minimal (+), moderate (++) and severe (+++). Minimal lesions were lymphocytic or mixed and were found in the three target organs like a few perivascular inflammatory cells associated with viral antigen presence. This fact, together with the spreading of moderate and severe lesions confirms the initial lesion corresponds to the beginning of the infection and a later spreading would occur through vascular spaces.
3. Minimal lesion detection clearly increases the prevalence of lesions in infected animals and MV detection by histopathology. Combination of this technique with immunohistochemistry detects all infected animals and is proposed as the 'gold standard' for diagnosis of the disease.
4. An association between lesion pattern and serological response is established. Animals with the histiocytic pattern showed higher serological response than animals with the lymphocytic pattern, with some of the later showing even negative results. Thus, animals with the histiocytic pattern with higher viral presence and stronger ELISA results are associated with a higher potential to spread the infection.
5. Macrophages are confirmed as the only MV target cells by immunohistochemistry in mammary gland and milk samples, with epithelial cells being negative with the antibodies used.
6. Viral detection in milk by immunocytochemistry and PCR confirms the possibility of viral transmission in this way. However, the scarce viral antigen present in milk and

mammary acini indicates the limited role of milk in viral transmission and the infection of lambs.

7. The circulation of the A2/A3 strain and the B2 strain similar to those found in Italian specimens has been confirmed in Spanish Assaf sheep. Moderate alterations are described in some immune-dominant epitopes and in the MHR tract which could be related with viral assembly modifications and infectivity. This could interfere with some diagnostic test sensitivity such as ELISA and thus new specific primers should be developed for each strain.
8. No significant correlation was found among the viral strain and the lesion severity in mammary glands, highlighting the importance of the host immune response in the developing of the disease.

XIV. Bibliografía

Adebayo, I. A., Awoniyi, T. A. M. and Olaleye, O. D. (2008). Affinity (tropism) of caprine arthritis encephalitis virus for brain cells. *African Journal of Biotechnology,* **7,** 3980-3983.

Agrimi, P., Legrottaglie, R. and Tolari, F. (1984). Maedi Visna. I. Indagine sieroepidemiologica in greggiovine e caprini di alcune province Toscane. II. Presenza di anticorpi precipitanti in sieri ovini e caprini d´importazione, Vol. 38, *Atti della Società Italiana delle Scienze Veterinaire*, pp. 720-725.

Alba, A., Allepuz, A., Serrano, E. and Casal, J. (2008). Seroprevalence and spatial distribution of maedi-visna virus and pestiviruses in Catalonia (Spain). *Small Ruminant Research,* **78,** 80-86.

Ali Al Ahmad, M. Z., Chebloune, Y., Chatagnon, G., Pellerin, J. L. and Fieni, F. (2012). Is caprine arthritis encephalitis virus (CAEV) transmitted vertically to early embryo development stages (morulae or blastocyst) via in vitro infected frozen semen? *Theriogenology,* **77,** 1673-1678.

Alshanbari, F. A., Mousel, M. R., Reynolds, J. O., Herrmann-Hoesing, L. M., Highland, M. A., Lewis, G. S. and White, S. N. (2014). Mutations in Ovis aries TMEM154 are associated with lower small ruminant lentivirus proviral concentration in one sheep flock. *Animal Genetics,* **45,** 565-571.

Alter, G., Martin, M. P., Teigen, N., Carr, W. H., Suscovich, T. J., Schneidewind, A., Streeck, H., Waring, M., Meier, A., Brander, C., Lifson, J. D., Allen, T. M., Carrington, M. and Altfeld, M. (2007). Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. *Journal of Experimental Medicine,* **204,** 3027-3036.

Álvarez, V., Arranz, J., Daltabuit-Test, M., Leginagoikoa, I., Juste, R. A., Amorena, B., de Andrés, D., Luján, L. L., Badiola, J. J. and Berriatua, E. (2005). Relative contribution of colostrum from Maedi-Visna virus (MVV) infected ewes to MVV-seroprevalence in lambs. *Research in veterinary science,* **78,** 237-243.

Álvarez, V., Daltabuit-Test, M., Arranz, J., Leginagoikoa, I., Juste, R. A., Amorena, B., de Andrés, D., Luján, L. L., Badiola, J. J. and Berriatua, E. (2006). PCR detection of colostrum-associated Maedi-Visna virus (MVV) infection and relationship with ELISA-antibody status in lambs. *Research in Veterinary Science,* **80,** 226-234.

Ameztoy, M., J., Santamaría, C. and Muñoz, P. (1998). Seroprevalencia del maedi-visna en explotaciones ovinas del centro y sur de Navarra. *Medicina Veterinaria*, **15**, 28-31.

An, P., Johnson, R., Phair, J., Kirk, G. D., Yu, X. F., Donfield, S., Buchbinder, S., Goedert, J. J. and Winkler, C. A. (2009). APOBEC3B deletion and risk of HIV-1 acquisition. *Journal of Infectious Diseases,* **200,** 1054-1058.

Andrésdóttir, V. (2003). Evidence for recombination in the envelope gene of maedi-visna virus. *Virus Genes,* **27,** 5-9.

Andrésdóttir, V., Skraban, R., Matthíasdóttir, S., Lutley, R., Agnarsdóttir, G. and Thorsteinsdóttir, H. (2002). Selection of antigenic variants in maedi-visna virus infection. *Journal of General Virology,* **83,** 2543-2551.

Angelopoulou, K., Brellou, G. D., Greenland, T. and Vlemmas, I. (2006a). A novel deletion in the LTR region of a Greek small ruminant lentivirus may be associated with low pathogenicity. *Virus Res,* **118,** 178-184.

Angelopoulou, K., Brellou, G. D. and Vlemmas, I. (2006b). Detection of Maedi-Visna Virus in the Kidneys of Naturally Infected Sheep. *Journal of comparative pathology,* **134,** 329-335.

Angelopoulou, K., Poutahidis, T., Brellou, G. D., Greenland, T. and Vlemmas, I. (2008). A deletion in the R region of long terminal repeats in small ruminant lentiviruses is associated with decreased pathology in the lung. *Veterinary Journnal,* **175,** 346-355.

Arnarson, H., Pálsson, A., Gudnadóttir, M. and Andrésdóttir, V. (2017). Maedi-visna virus persistence: Antigenic variation and latency. *Comparative Immunology, Microbiology and Infectious Diseases,* **55,** 6-12.

Arnaud, F., Black, S. G., Murphy, L., Griffiths, D. J., Neil, S. J., Spencer, T. E. and Palmarini, M. (2010). Interplay between ovine bone marrow stromal cell antigen 2/tetherin and endogenous retroviruses. *Journal of Virology,* **84,** 4415-4425.

Artigas, C., Vendrell, J. P. and Mejías, J. M. (1999). Estudio seroepidemiológico frente a Chlamydiosis, Visna-Maedi y Toxoplasmosis en 64 rebaños de la sierra del norte de Sevilla, *XXIV Jornadas Científicas, III Internacionales de la Sociedad Española de Ovinotecnia y Caprinotecnia* . (SEOC), Soria.

Badiola, J. J., and González, L. (1990). Maedi-Visna, OVIS.

Badiola, J. J., González, L. and Gelabert, J. L. (1983). Demonstration of Maedi in Spain., EEC W*orkshop "Slow viruses in sheep, goats and cattle"*, Edimburg.

Badiola, J. J., Lujan, L., García Marín, J. F. and Simón, M. A. (1990). Incidence of pathological changes in sheep seropositive to Maedi-Visna. *Schweiz. Arch. Tierheilk*, **132**, 409-413.

Baird, S. C., Carman, J., Dinsmore, R. P., Walker, R. L. and Collins, J. K. (1999). Detection and identification of Mycoplasma from bovine mastitis infections using a nested polymerase chain reaction. *Journal of Veterinary Diagnostic Investigation,* **11,** 432-435.

Begara, I., Lujan, L., Hopkins, J., Collie, D. D. S., Miller, H. R. P. and Watt, N. J. (1995). A study on lymphocyte activation in maedi-visna virus induced pneumonia. *Veterinary Immunology and Immunopathology,* **45,** 197-210.

Begara, I., Luján, L., Collie, D. D. S., Miller, H. R. P. and Watt, N. J. (1996). Early pulmonary cell response during experimental maedi-visna virus infection. *Veterinary immunology and immunopathology,* **55,** 115-126.

Benavides, J., Fuertes, M., García-Pariente, C., Ferreras, M. C., Marín, J. F. G. and Pérez, V. (2006a). Natural Cases of Visna in Sheep with Myelitis as the Sole Lesion in the Central Nervous System. *Journal of comparative pathology,* **134,** 219-230.

Benavides, J., Fuertes, M., García-Pariente, C., Otaola, J., Delgado, L., Giraldez, J., García Marín, J. F., Carmen Ferreras, M. and Pérez, V. (2013). Impact of maedi-visna in intensively managed dairy sheep. *Veterinary Journal,* **197,** 607-612.

Benavides, J., García-Pariente, C., Carmen Ferreras, M., Fuertes, M., Francisco García-Marín, J. and Pérez, V. (2007a). Diagnosis of clinical cases of the nervous form of Maedi-Visna in 4- and 6-month-old lambs. *Veterinary Journal,* **174,** 655-658.

Benavides, J., García-Pariente, C., Carmen Ferreras, M., Fuertes, M., Francisco García-Marín, J. and Pérez, V. (2007b). Diagnosis of clinical cases of the nervous form of Maedi-Visna in 4- and 6-month-old lambs. *The Veterinary Journal,* **174,** 655-658.

Benavides, J., García-Pariente, C., Fuertes, M., Ferreras, M. C., García-Marín, J. F., Juste, R. A. and Pérez, V. (2009). Maedi-Visna: the Meningoencephalitis in Naturally Occurring Cases. *Journal of comparative pathology,* **140,** 1-11.

Benavides, J., García-Pariente, C., Gelmetti, D., Fuertes, M., Ferreras, M. C., García-Marín, J. F. and Pérez, V. (2006b). Effects of fixative type and fixation time on the detection of Maedi Visna virus by PCR and immunohistochemistry in paraffin-embedded ovine lung samples. *Journal of virological methods,* **137,** 317-324.

Benavides, J., Gomez, N., Gelmetti, D., Ferreras, M. C., Garcia-Pariente, C., Fuertes, M., Garcia-Marin, J. F. and Perez, V. (2006c). Diagnosis of the nervous form of Maedi-Visna infection with a high frequency in sheep in Castilla y Leon, Spain. *Veterinary Record,* **158,** 230-235.

Berriatua, E., Álvarez, V., Extramiana, B., González, L., Daltabuit, M. and Juste, R. (2003). Transmission and control implications of seroconversion to Maedi-Visna virus in Basque dairy-sheep flocks. *Preventive veterinary medicine,* **60,** 265-279.

Bertoni, G., Hertig, C., Zahno, M. L., Vogt, H. R., Dufour, S., Cordano, P., Peterhans, E., Cheevers, W. P., Sonigo, P. and Pancino, G. (2000). B-cell epitopes of the envelope

glycoprotein of caprine arthritis-encephalitis virus and antibody response in infected goats. *Journal of General Virology,* **81,** 2929-2940.

Bertoni, G., Zahno, M. L., Zanoni, R., Vogt, H. R., Peterhans, E., Ruff, G., Cheevers, W. P., Sonigo, P. and Pancino, G. (1994). Antibody reactivity to the immunodominant epitopes of the caprine arthritis-encephalitis virus gp38 transmembrane protein associates with the development of arthritis. *Journal of Virology,* **68,** 7139-7147.

Bird, P., Blacklaws, B., Reyburn, H. T., Allen, D., Hopkins, J., Sargan, D. and McConnell, I. (1993). Early events in immune evasion by the lentivirus maedi-visna occurring within infected lymphoid tissue. *Journal of Virology,* **67,** 5187-5197.

Blacklaws, B., Bird, P. and McConnell, I. (1995a). Early events in infection of lymphoid tissue by a lentivirus, maedi-visna. *Trends in Microbiology,* **3,** 434-440.

Blacklaws, B. A. (2012). Small ruminant lentiviruses: Immunopathogenesis of visna-maedi and caprine arthritis and encephalitis virus. *Comparative Immunology, Microbiology and Infectious Diseases,* **35,** 259-269.

Blacklaws, B. A., Berriatua, E., Torsteinsdottir, S., Watt, N. J., de Andres, D., Klein, D. and Harkiss, G. D. (2004). Transmission of small ruminant lentiviruses. *Veterinary microbiology,* **101,** 199-208.

Blacklaws, B. A., Bird, P., Allen, D. and McConnell, I. (1994). Circulating cytotoxic T lymphocyte precursors in maedi-visna virus-infected sheep. *Journal of General Virology,* **75,** 1589-1596.

Blacklaws, B. A., Bird, P., Allen, D., Roy, D. J., Maclennan, I. C. M., Hopkins, J., Sargan, D. R. and McConnell, I. (1995b). Initial lentivirus-host interactions within lymph nodes: A study of maedi-visna virus infection in sheep. *Journal of Virology,* **69,** 1400-1407.

Bolea, R., Monleón, E., Carrasco, L., Vargas, A., De Andrés, D., Amorena, B., Badiola, J. J. and Luján, L. (2006). Maedi-visna virus infection of ovine mammary epithelial cells. *Veterinary Research,* **37,** 133-144.

Bowles, D., Carson, A. and Isaac, P. (2014). Genetic distinctiveness of the Herdwick sheep breed and two other locally adapted hill breeds of the UK. *PLoS ONE,* **9**: e87823. https://doi.org/10.1371/journal.pone.0087823.

Brinkhof, J. and Van Maanen, C. (2007). Evaluation of five enzyme-linked immunosorbent assays and an agar gel immunodiffusion test for detection of antibodies to small ruminant lentiviruses. *Clinical and Vaccine Immunology,* **14,** 1210-1214.

Brinkhof, J. M. A., Houwers, D. J., Moll, L., Dercksen, D. and van Maanen, C. (2010). Diagnostic performance of ELISA and PCR in identifying SRLV-infected sheep and goats using serum, plasma and milk samples and in early detection of infection in dairy flocks through bulk milk testing. *Veterinary Microbiology,* **142,** 193-198.

Brinkhof, J. M. A., Houwers, D. J. and van Maanen, C. (2007). Development of a sample pooling strategy for the serodiagnosis of small ruminant lentiviral infections using the ELITEST-MVV ELISA. *Small Ruminant Research,* **70,** 194-199.

Brodie, S. J., De La Concha-Bermejillo, A., Snowder, G. D. and DeMartini, J. C. (1998). Current concepts in the epizootiology, diagnosis, and economic importance of ovine progressive pneumonia in North America: A review. *Small Ruminant Research,* **27,** 1-17.

Brodie, S. J., Pearson, L. D., Zink, M. C., Bickle, H. M., Anderson, B. C., Marcom, K. A. and DeMartini, J. C. (1995). Ovine lentivirus expression and disease. Virus replication, but not entry, is restricted to macrophages of specific tissues. *American Journal of Pathology,* **146,** 250-263.

Broughton-Neiswanger, L. E., White, S. N., Knowles, D. P., Mousel, M. R., Lewis, G. S., Herndon, D. R. and Herrmann-Hoesing, L. M. (2010). Non-maternal transmission is the major mode of ovine lentivirus transmission in a ewe flock: A molecular epidemiology study. *Infection, Genetics and Evolution,* **10,** 998-1007.

Buitrago, D., Rocha, A., Tena-Tomás, C., Vigo, M., Agüero, M. and Jiménez-Clavero, M. A. (2012). Real-time fluorogenic reverse transcription polymerase chain reaction assay for the specific detection of Bagaza virus. *Journal of Veterinary Diagnostic Investigation,* **24,** 959-963.

Butler, J. E., Rainard, P., Lippolis, J., Salmon, H. and Kacskovics, I. (2015). The Mammary Gland in Mucosal and Regional Immunity. In: *Mucosal Immunology: Fourth Edition*, Vol. 2-2, 2269-2306.

Cadoré, J. L., Greenland, T., Cordier, G., Guiguen, F. and Mornex, J. F. (1996). Histogenesis of the pulmonary lesions in the course of visna maedi virus-induced pneumonia. *Veterinary Research,* **27,** 419-426.

Cadoré, J. L., Guiguen, F., Cordier, G., Loire, R., Lyon, M., Chastang, J., Greenland, T., Court-Fortune, I., Revel, D. and Mornex, J. F. (1993). Early events in the experimental interstitial lung disease induced in sheep by the Visna-maedi virus. *Special Issue: Beer-Sheva - Lyon Symposium on Immunology,* **39,** 39-43.

Caporale, V. P., Lelli, R. and Rutili, D. (1985). Isolation of visna-maedi virus from the choroid plexus of an apparently healthy sheep in Italy. *Veterinary research communications,* **9,** 115-122.

Capucchio, M. T., Sanna, E., Sanna, M. P., Farigu, S., Minelli, R. and Guarda, F. (2003). Maedi-visna virus detection in ovine third eyelids. *Journal of comparative pathology,* **129,** 37-43.

Cardinaux, L., Zahno, M. L., Deubelbeiss, M., Zanoni, R., Vogt, H. R. and Bertoni, G. (2013). Virological and phylogenetic characterization of attenuated small ruminant lentivirus isolates eluding efficient serological detection. *Veterinary Microbiology,* **162,** 572-581.

Carey, N. and Dalziel, R. G. (1993). The biology of maedi-visna virus-An overview. *British Veterinary Journal,* **149,** 437-454.

Carey, N. and Dalziel, R. G. (1994). Sequence variation in the gp135 gene of Maedi visna virus strain EV1. *Virus Genes,* **8,** 115-123.

Carrozza, M. L., Mazzei, M., Bandecchi, P., Arispici, M. and Tolari, F. (2003). In situ PCR-associated immunohistochemistry identifies cell types harbouring the Maedi-Visna virus genome in tissue sections of sheep infected naturally. *Journal of Virological Methods,* **107,** 121-127.

Celer Jr, V., Celer, V., Němcová, H., Zanoni, R. G. and Peterhans, E. (1998). Serologic Diagnosis of Ovine Lentiviruses by Whole Virus ELISA and AGID Test. *Journal of Veterinary Medicine, Series B,* **45,** 183-188.

Cheevers, W. P., Beyer, J. C. and Hötzel, I. (2001). Plasmid DNA encoding caprine interferon gamma inhibits antibody response to caprine arthritis-encephalitis virus (CAEV) surface protein encoded by a co-administered plasmid expressing CAEV env and tat genes. *Vaccine,* **19,** 3209-3215.

Cheevers, W. P., Cordery-Cotter, R., McGuire, T. C. and DeMartini, J. C. (1999). Neutralizing Antibody Responses and Evolution of Antigenic Variants in Monozygotic Twin Lambs Infected with Phenotypically Distinct Ovine Lentiviruses. *Virology,* **258,** 382-388.

Cheevers, W. P., Hötzel, I., Beyer, J. C. and Kumpula-Mcwhirter, N. (2000). Immune response to caprine arthritis-encephalitis virus surface protein induced by coimmunization with recombinant vaccinia viruses expressing the caprine arthritis-encephalitis virus envelope gene and caprine interleukin-12. *Vaccine,* **18,** 2494-2503.

Christodoulopoulos, G. (2006). Maedi-Visna: Clinical review and short reference on the disease status in Mediterranean countries. *Small Ruminant Research,* **62,** 47-53.

Chu, H. H., Chang, Y. F. and Wang, C. T. (2006). Mutations in the alpha-helix directly C-terminal to the major homology region of human immunodeficiency virus type 1 capsid protein disrupt Gag multimerization and markedly impair virus particle production. *Journal of Biomedical Sciences,* **13,** 645-656.

Clawson, M. L., Redden, R., Schuller, G., Heaton, M. P., Workman, A., Chitko-Mckown, C. G., Smith, T. P. and Leymaster, K. A. (2015). Genetic subgroup of small ruminant lentiviruses that infects sheep homozygous for TMEM154 frameshift deletion mutation A4Δ53. *Veterinary Research,* **46**, Doi: 1.1186/s13567-015-0162-7.

Clements, J.E. and Gabuzda, D. H. (1989). Cellular regulation of visna virus gene expression and its role in pathogenesis. D*evelopments in Biological Standardization,* **72**, 207-2012.

Clements, J. E. and Wong-Staal, F. (1992). Molecular biology of lentiviruses, Vol. Semin Virol. 3., Johns Hopkins University. EEUU, 137-146.

Clements, J. E. Z., M. C., Narayan, O. and Gabuzda, D. H. (1994). Lentivirus infection of macrophages. *Immunology Series*, **60**, 589-600.

Coffin, J. M. (1996). Retrovirus restriction revealed. *Nature,* **382,** 762-763.

Coffin, J. M., Essex, M., Gallo, R. C., Graf, T. M., Hinuma, Y., Hunter, E., Jaenisch, R., Orosalan, S., Svoboda, J., Teich, N., Toyoshima, K. and Varmus, H. E. (1995). Family Retroviridae. , I*CTV VI Report*, 193-204.

Cork, L. C., Hadlow, and W.J., C., T.B., Gorham, J.R. and Piper, R.C. (1974). Infectious leukoencephalomyelitis of young goats. *The Journal of Infectous Diseases,* **129**, 134–141.

Cortez Romero, C., Fieni, F., Roux, C., Russo, P., Guibert, J. M., Guiguen, F., Chebloune, Y., Pépin, M. and Pellerin, J. L. (2006). Detection of ovine lentivirus in the cumulus cells, but not in the oocytes or follicular fluid, of naturally infected sheep. *Theriogenology,* **66,** 1131-1139.

Cortez-Romero, C., Fieni, F., Russo, P., Pepin, M., Roux, C. and Pellerin, J. L. (2011). Presence of Maedi Visna Virus (MVV)-Proviral DNA in the Genital Tissues of Naturally Infected Ewes. *Reproduction in Domestic Animals,* **46,** e1-e6.

Cortez-Romero, C., Pellerin, J. L., Ali-Al-Ahmad, M. Z., Chebloune, Y., Gallegos-Sánchez, J., Lamara, A., Pépin, M. and Fieni, F. (2013). The risk of small ruminant lentivirus (SRLV)

transmission with reproductive biotechnologies: State-of-the-art review. *Theriogenology,* **79,** 1-9.

Cowell, R. L. (2008). *Diagnostic cytology and hematology of the dog and cat*. Mosby Elsevier, St. Louis, Missouri, 475.

Crane, S. E., Buzy, J. and Clements, J. E. (1991). Identification of cell membrane proteins that bind visna virus. *Journal of Virology,* **65,** 6137-6143.

Crawford, T. B., Adams, D. S., Cheevers, W. P. and Cork, L. C. (1980). Chronic arthritis in goats caused by a retrovirus. *Science,* **207,** 997-999.

Crespo, H., Bertolotti, L., Proffiti, M., Cascio, P., Cerruti, F., Acutis, P. L., de Andrés, D., Reina, R. and Rosati, S. (2016). Low proviral small ruminant lentivirus load as biomarker of natural restriction in goats. *Veterinary Microbiology,* **192,** 152-162.

Cutlip, R. C., Jackson, T. A. and Lehmkuhl, H. D. (1979). Lesions of ovine progressive pneumonia: interstitial pneumonitis and encephalitis. *American Journal of Veterinary Research,* **40,** 1370-1374.

Cutlip, R. C. and Lehmkuhl, H. D. (1986). Eradication of ovine progressive pneumonia from sheep flocks. *Journal of the American Veterinary Medical Association,* **188,** 1026-1027.

Cutlip, R. C., Lehmkuhl, H. D., Brogden, K. A. and Bolin, S. R. (1985a). Mastitis associated with ovine progressive pneumonia virus infection in sheep. *American Journal of Veterinary Research,* **46,** 326-328.

Cutlip, R. C., Lehmkuhl, H. D., Schmerr, M. J. F. and Brogden, K. A. (1988). Ovine progressive pneumonia (maedi-visna) in sheep. *Veterinary microbiology,* **17,** 237-250.

Cutlip, R. C., Lehmkuhl, H. D., Wood, R. L. and Brogden, K. A. (1985b). Arthritis associated with ovine progressive pneumonia. *American Journal of Veterinary Research,* **46,** 65-68.

Dawson, M. (1980). Maedi/visna: a review. *Veterinary Record,* **106,** 212-216.

Dawson, M. (1987). Pathogenesis of maedi-visna. *Veterinary Record,* **120,** 451-454.

de Andrés, D., Klein, D., Watt, N. J., Berriatua, E., Torsteinsdottir, S., Blacklaws, B. A. and Harkiss, G. D. (2005). Diagnostic tests for small ruminant lentiviruses. *Veterinary microbiology,* **107,** 49-62.

de Andrés, X., Ramírez, H., Bertolotti, L., San Román, B., Glaria, I., Crespo, H., Jáuregui, P., Minguijón, E., Juste, R., Leginagoikoa, I., Pérez, M., Luján, L., Badiola, J. J., Polledo, L., García-Marín, J. F., Riezu, J. I., Borrás-Cuesta, F., de Andrés, D., Rosati, S., Reina, R. and Amorena, B. (2013). An insight into a combination of ELISA strategies to diagnose small ruminant lentivirus infections. *Veterinary Immunology and Immunopathology,* **152,** 277-288.

de Andrés, X., Reina, R., Ciriza, J., Crespo, H., Glaria, I., Ramírez, H., Grilló, M. J., Pérez, M. M., Andrésdóttir, V., Rosati, S., Suzan-Monti, M., Luján, L., Blacklaws, B. A., Harkiss, G. D., de Andrés, D. and Amorena, B. (2009). Use of B7 costimulatory molecules as adjuvants in a prime-boost vaccination against Visna/Maedi ovine lentivirus. *Vaccine,* **27,** 4591-4600.

De Boer, G. F., Terpstra, C., Houwers, D. J. and Hendriks, J. (1979). Studies in epidemiology of maedi/visna in sheep. *Research in Veterinary Science,* **26,** 202-208.

de la Concha-Bermejillo, A. (1997). Maedi-Visna and ovine progressive pneumonia. *The Veterinary clinics of North America. Food animal practice,* **13,** 13-33.

De Pablo-Maiso, L., Glaria, I., Crespo, H., Nistal-Villán, E., Andrésdóttir, V., De Andrés, D., Amorena, B. and Reina, R. (2017). Characterization of ovine A3Z1 restriction properties against small ruminant lentiviruses (SRLVs). *Viruses,* **9**. doi: 10.3390/v9110345.

Delgado, L., Marín, J. F., Muñoz, M., Benavides, J., Juste, R. A., García-Pariente, C., Fuertes, M., González, J., Ferreras, M. C. and Pérez, V. (2013). Pathological findings in young and adult sheep following experimental infection with 2 different doses of Mycobacterium avium subspecies paratuberculosis. *Vet Pathol,* **50,** 857-866.

Dualde, D. 1964. Estudios sobre la adenomatosis pulmonar ovina en España. Tesis Doctoral. Facultad de Veterinaria. Universidad de Zaragoza.

Eigen, M. (1971). Selforganization of matter and the evolution of biological macromolecules. *Die Naturwissenschaften,* **58,** 465-523.

Ellis, J. A. and DeMartini, J. C. (1985). Ovine interleukin-2: partial purification and assay in normal sheep and sheep with ovine progressive pneumonia. *Veterinary Immunology and Immunopathology*, **8**, 15-25.

Ellis, T., Robinson, W. and Wilcox, G. (1983). Effect of colostrum deprivation of goat kids on the natural transmission of caprine retrovirus infection. *Australian Veterinary Journal,* **60,** 326-329.

Ellis, T. M., Carman, H., Robinson, W. F. and Wilcox, G. E. (1986). The effect of colostrum-derived antibody on neo-natal transmission of caprine arthritis-encephalitis virus infection. *Australian Veterinary Journal,* **63,** 242-245.

Epizootias., O. O. i. d. (2008). Artritis/Encefalitis caprina y Maedi-Visna. In: *Manual of Standard for diagnostic test and vaccines for terrestrial animals.*, Vol. Cap. 2.7.3/4, OIE, París.

Erhouma, E., Guiguen, F., Chebloune, Y., Gauthier, D., Lakhal, L. M., Greenland, T., Mornex, J. F., Leroux, C. and Alogninouwa, T. (2008). Small ruminant lentivirus proviral sequences from wild ibexes in contact with domestic goats. *Journal of General Virology,* **89,** 1478-1484.

Espí, A., Prieto, J. M. and Álvarez, M. (2001). Situación sanitaria del ovino en Asturias: seroprevalencia de la enfermedad de la frontera, Maedi Visna, Aborto enzoótico, Agalaxia contagiosa, Fiebre Q y Leptospirosis, XXVI Jornadas Científicas de la Sociedad Española de Ovinotecnia y Caprinotecnia. (SEOC). , Sevilla.

Extramiana, A. B., González, L., Cortabarría, N., García, M. and Juste, R. A. (2002). Evaluation of a PCR technique for the detection of Maedi-Visna proviral DNA in blood, milk and tissue samples of naturally infected sheep. *Small Ruminant Research,* **44,** 109-118.

Fieni, F., Rowe, J., Van Hoosear, K., Burucoa, C., Oppenheim, S., Anderson, G., Murray, J. and BonDurant, R. (2002). Presence of caprine arthritis-encephalitis virus (CAEV) infected cells in flushing media following oviductal-stage embryo collection. *Theriogenology,* **57,** 931-940.

Fieni, F., Rowe, J., Van Hoosear, K., Burucoa, C., Oppenheim, S., Anderson, G., Murray, J. and BonDurant, R. (2003). Presence of caprine arthritis-encephalitis virus (CAEV) proviral DNA in genital tract tissues of superovulated dairy goat does. *Theriogenology,* **59,** 1515-1523.

Fluri, A., Nenci, C., Zahno, M. L., Vogt, H. R., Charan, S., Busato, A., Pancino, G., Peterhans, E., Obexer-Ruff, G. and Bertoni, G. (2006). The MHC-haplotype influences primary, but not memory, immune responses to an immunodominant peptide containing T- and B-cell epitopes of the caprine arthritis encephalitis virus Gag protein. *Vaccine,* **24,** 597-606.

Foley, B. T. (2000). An Overview of the Molecular Phylogeny of Lentiviruses. In: *In HIV Sequence Compedium; Kuiken, C., Foley, B., Freed, E., Hahn, B., Korber, B., Marx, P.A., McCutchan, F., Mellors, J.W., Mullins, J.I., Sodroski, J., Wolinksy, S., Eds.;* , Theoretical Biology and Biophysics Group, Los Alamos National Laboratory: Los Alamos, NM, USA,. 35-43.

Fras, M., Leboeuf, A., Labrie, F. M., Laurin, M. A., Singh Sohal, J. and L'Homme, Y. (2013). Phylogenetic analysis of small ruminant lentiviruses in mixed flocks: multiple evidence of dual infection and natural transmission of types A2 and B1 between sheep and goats. *Infect Genet Evol,* **19,** 97-104.

Freel, S. A., Saunders, K. O. and Tomaras, G. D. (2011). CD8 +T-cell-mediated control of HIV-1 and SIV infection. *Immunologic Research,* **49,** 135-146.

García Marín, J. F., Gómez García, N., González, J., García Fernández, R. A., Corpa, J. M., Gutiérrez, M. M., Pérez, V., Ferreras, M. C., García Iglesias, M. J., Pérez, C., Espinosa, J. and Escudero, A. (1998). Diagnóstico de enfermedades nerviosas en ganado ovino entre 1994 y 1998, en el Servicio de Diagnóstico Anatomopatológico de la Facultad de Veterinaria de León . *Producción Ovina y Caprina*, **23**, 355-358.

Gayo, E., Polledo, L., Balseiro, A., Martínez, C. P., García Iglesias, M. J., Preziuso, S., Rossi, G. and García Marín, J. F. (2018). Inflammatory Lesion Patterns in Target Organs of Visna/Maedi in Sheep and their Significance in the Pathogenesis and Diagnosis of the Infection. *Journal of Comparative Pathology,* **159,** 49-56.

Gayo, E., Polledo, L., Preziuso, S., Rossi, G., Balseiro, A., Pérez Martínez, C., García Iglesias, M. J. and García Marín, J. F. (2017). Serological ELISA results are conditioned by individual immune response in ovine maedi visna. *Small Ruminant Research,* **157,** 27-31.

Gelmetti, D., Gibelli, L., Brocchi, E. and Cammarata, G. (2000). Using a panel of monoclonal antibodies to detect Maedi virus (MV) in chronic pulmonary distress of sheep. *Journal of virological methods,* **88,** 9-14.

Gendelman, H. E., Narayan, O., Kennedy-Stoskopf, S., Kennedy, P. G., Ghotbi, Z., Clements, J. E., Stanley, J. and Pezeshkpour, G. (1986). Tropism of sheep lentiviruses for monocytes: susceptibility to infection and virus gene expression increase during maturation of monocytes to macrophages, *Journal of Virology*, **58**, 67-74.

Gendelman, H. E., Narayan, O., Molineaux, S., Clements, J. E. and Ghotbi, Z. (1985). Slow, persistent replication of lentiviruses: Role of tissue macrophages and macrophage precursors in bone marrow. *Proceedings of the National Academy of Sciences of the United States of America,* **82,** 7086-7090.

Georgsson, G. (1994). Neuropathologic Aspects of Lentiviral Infections. In: *Annals of the New York Academy of Sciences*, Vol. 724, pp. 50-67.

Georgsson, G. and Pálsson, P. A. (1971). The histopathology of maedi a slow, viral pneumonia of sheep. *Veterinary pathology,* **8,** 63-80.

Germain, K. and Valas, S. (2006). Distribution and heterogeneity of small ruminant lentivirus envelope subtypes in naturally infected French sheep. *Virus research,* **120,** 156-162.

Giammarioli, M., Bazzucchi, M., Puggioni, G., Brajon, G., Dei Giudici, S., Taccori, F., Feliziani, F. and De Mia, G. M. (2011). Phylogenetic analysis of small ruminant lentivirus (SRLV) in Italian flocks reveals the existence of novel genetic subtypes. *Virus Genes,* **43,** 380-384.

Gjerset, B., Rimstad, E., Teige, J., Soetaert, K. and Jonassen, C. M. (2009). Impact of natural sheep-goat transmission on detection and control of small ruminant lentivirus group C infections. *Veterinary Microbiology,* **135,** 231-238.

Glaria, I., Reina, R., Crespo, H., de Andrés, X., Ramírez, H., Biescas, E., Pérez, M. M., Badiola, J., Luján, L., Amorena, B. and de Andrés, D. (2009). Phylogenetic analysis of SRLV sequences from an arthritic sheep outbreak demonstrates the introduction of CAEV-like viruses among Spanish sheep. *Veterinary Microbiology,* **138,** 156-162.

Glaria, I., Reina, R., Ramírez, H., de Andrés, X., Crespo, H., Jauregui, P., Salazar, E., Luján, L., Pérez, M. M., Benavides, J., Pérez, V., Polledo, L., García-Marín, J. F., Riezu, J. I., Borrás, F., Amorena, B. and de Andrés, D. (2012). Visna/Maedi virus genetic characterization and serological diagnosis of infection in sheep from a neurological outbreak. *Veterinary microbiology,* **155,** 137-146.

Gogolewski, R. P., Adams, D. S., McGuire, T. C., Banks, K. L. and Cheevers, W. P. (1985). Antigenic cross-reactivity between caprine arthritis-encephalitis, visna and progressive pneumonia viruses involves all virion-associated proteins and glycoproteins. *Journal of General Virology,* **66,** 1233-1240.

González, L., Badiola, J. J. and Gelabert, J. L. (1984). Neumonía progresiva (MAEDI) en el ganado ovino del País Vasco, *Medicina Veterinaria*, **1**, 277-282.

González, L., Badiola, J. J. and Gelabert, J. L. (1985). Demonstration of Maedi in Spain: clinical, pathological and serological observations. In: *Sharp, J. M. y Hoff-Jorgensen, R. (ed). Slow viruses in sheep, goats and cattle. Ed. CEC Report EUR 8076 EN*, 271-278.

González, L., Anderson, I., Deane, D., Summers, C. and Buxton, D. (2001). Detection of immune system cells in paraffin wax-embedded ovine tissues. *Journal of Comparative Pathology,* **125,** 41-47.

Gorrell, M. D., Brandon, M. R., Sheffer, D., Adams, R. J. and Narayan, O. (1992). Ovine lentivirus is macrophagetropic and does not replicate productively in T lymphocytes. *Journal of Virology,* **66,** 2679-2688.

Grego, E., Bertolotti, L., Quasso, A., Profiti, M., Lacerenza, D., Muz, D. and Rosati, S. (2007). Genetic characterization of small ruminant lentivirus in Italian mixed flocks: Evidence for a novel genotype circulating in a local goat population. *Journal of General Virology,* **88,** 3423-3427.

Grego, E., Profiti, M., Giammarioli, M., Giannino, L., Rutili, D., Woodall, C. and Rosati, S. (2002). Genetic heterogeneity of small ruminant lentiviruses involves immunodominant epitope of capsid antigen and affects sensitivity of single-strain-based immunoassay. *Clinical and diagnostic laboratory immunology,* **9,** 828-832.

Gudmundsson, B., Bjarnadottir, H., Kristjansdottir, S. and Jonsson, J. J. (2003). Quantitative assays for maedi-visna virus genetic sequences and mRNA's based on RT-PCR with real-time FRET measurements. *Virology,* **307,** 135-142.

Gómez, N., González, J., Corpa, J. M., Pérez, V. and García Marín, J. F. (1999). Diagnóstico de la forma nerviosa de maedi-visna en ovinos de aptitud lechera. *Producción ovina y caprina*, **24**, 393-397.

Haase, A. T., Stowring, L., Narayan, O., Griffin, D. and Price, D. (1977). Slow persistent infection caused by visna virus: Role of host restriction. *Science,* **195,** 175-177.

Haflidadóttir, B. S., Matthíasdóttir, S., Agnarsdóttir, G., Torsteinsdóttir, S., Pétursson, G., Andrésson, Ó. S. and Andrésdóttir, V. (2008). Mutational analysis of a principal neutralization domain of visna/maedi virus envelope glycoprotein. *Journal of General Virology,* **89,** 716-721.

Harris, R. S. and Dudley, J. P. (2015). APOBECs and virus restriction. *Virology,* **479-480,** 131-145.

Heaton, M. P., Clawson, M. L., Chitko-Mckown, C. G., Leymaster, K. A., Smith, T. P. L., Harhay, G. P., White, S. N., Herrmann-Hoesing, L. M., Mousel, M. R., Lewis, G. S., Kalbfleisch, T. S., Keen, J. E. and Laegreid, W. W. (2012). Reduced lentivirus susceptibility in sheep with TMEM154 mutations. *PLoS Genetics,* **8**, **doi**:10.1371/journal.pone.0087823.

Henriques, A. M., Madeira, C., Fevereiro, M., Prazeres, D. M. F., Aires-Barros, M. R. and Monteiro, G. A. (2009). Effect of cationic liposomes/DNA charge ratio on gene expression and antibody response of a candidate DNA vaccine against Maedi Visna virus. *International Journal of Pharmaceutics,* **377,** 92-98.

Herrmann-Hoesing, L. M. (2010). Diagnostic assays used to control small ruminant lentiviruses. *Journal of Veterinary Diagnostic Investigation,* **22,** 843-855.

Hoff-Jorgensen, R. (1985). Control programme for lentivirus infections in danish sheep and goats. In: *Sharp JM y Hoff-Jorgensen R (ed). Slow viruses in sheep, goats and cattle. CEC Report EUR 8076 EN*, 271-278.

Houwers, D. J., König, C. D. W., de Boer, G. F. and Schaake Jr, J. (1983). Maedi-visna control in sheep I. Artificial rearing of colostrum-deprived lambs. *Veterinary microbiology,* **8,** 179-185.

Houwers, D. J., Pekelder, J. J., Akkermans, J. W., van der Molen, E. J. and Schreuder, B. E. (1988). Incidence of indurative lymphocytic mastitis in a flock of sheep infected with maedi-visna virus. *Veterinary Record,* **122,** 435-437.

Houwers, D. J., Schaake Jr, J. and de Boer, G. F. (1984). Maedi-visna control in sheep II. Half-yearly serological testing with culling of positive ewes and progeny. *Veterinary microbiology,* **9,** 445-451.

Houwers, D. J. and van der Molen, E. J. (1987). A five-year serological study of natural transmission of maedi-visna virus in a flock of sheep, completed with post mortem investigation. *Zentralblatt fur Veterinarmedizin.Reihe B.Journal of veterinary medicine.Series B,* **34,** 421-431.

Houwers, D. J., Visscher, A. H. and Defize, P. R. (1989). Importance of ewe/lamb relationship and breed in the epidemiology of maedi-visna virus infections. *Research in veterinary science,* **46,** 5-8.

Huang, J., Sun, Y., Liu, Y., Xiao, H. and Zhuang, S. (2012). Development of a loop-mediated isothermal amplification method for rapid detection of caprine arthritis-encephalitis virus proviral DNA. *Archives of Virology,* **157,** 1463-1469.

Jolly, P. E., Gangopadhyay, A., Chen, S., Reddy, P. G., Weiss, H. L. and Sapp, W. J. (1997). Changes in the leukocyte phenotype profile of goats infected with the caprine arthritis encephalitis virus. *Veterinary Immunology and Immunopathology,* **56,** 97-106.

Julian Motha, M. X. and Ralston, J. C. (1994). Evaluation of ELISA for detection of antibodies to CAEV in milk. *Veterinary Microbiology,* **38,** 359-367.

Juste, R. A., García Marín, J. F., Peris, B., Sáez de Ocáriz, C. S. and Badiola, J. J. (1994). Experimental infection of vaccinated and non-vaccinated lambs with Mycobacterium paratuberculosis. *J Comp Pathol,* **110,** 185-194.

Jáuregui, P., Crespo, H., Glaria, I., Luján, L., Contreras, A., Rosati, S., de Andrés, D., Amorena, B., Towers, G. J. and Reina, R. (2012). Ovine TRIM5α can restrict visna/maedi virus. *Journal of Virology,* **86,** 9504-9509.

Kaba, J., Winnicka, A., Zaleska, M., Nowicki, M. and Bagnicka, E. (2011). Influence of chronic caprine arthritis-encephalitis virus infection on the population of peripheral blood leukocytes. *Polish Journal of Veterinary Sciences,* **14,** 585-590.

Keen, J., Kwang, J., Littledike, E. T. and Hungerford, L. L. (1996). Ovine lentivirus antibody detection in serum, colostrum and milk using a recombinant transmembrane protein ELISA. *Veterinary Immunology and Immunopathology,* **51,** 253-275.

Kennedy-Stoskopf, S. and Narayan, O. (1986). Neutralizing antibodies to visna lentivirus: Mechanism of action and possible role in virus persistence. *Journal of Virology,* **59,** 37-44.

Kim, W. K., Corey, S., Chesney, G., Knight, H., Klumpp, S., Wüthrich, C., Letvin, N. L., Koralnik, I. J., Lackner, A., Veasey, R. S. and Williams, K. C. (2004). Identification of T lymphocytes in simian immunodeficiency virus encephalitis: Distribution of CD8+ T cells in association with central nervous system vessels and virus. *Journal of neurovirology,* **10,** 315-325.

Knowles Jr, D., Cheevers, W., McGuire, T., Stem, T. and Gorham, J. (1990). Severity of arthritis is predicted by antibody response to gp135 in chronic infection with caprine arthritis-encephalitis virus. *Journal of Virology,* **64,** 2396-2398.

Knowles Jr, D. P., Evermann, J. F., Shropshire, C., VanderSchalie, J., Bradway, D., Gezon, H. M. and Cheevers, W. P. (1994). Evaluation of agar gel immunodiffusion serology using caprine and ovine lentiviral antigens for detection of antibody to caprine arthritis-encephalitis virus. *Journal of Clinical Microbiology,* **32,** 243-245.

Kokawa, S., Oba, M., Hirata, T., Tamaki, S., Omura, M., Tsuchiaka, S., Nagai, M., Omatsu, T. and Mizutani, T. (2017). Molecular characteristics and prevalence of small ruminant lentiviruses in goats in Japan. *Arch Virol,* **162,** 3007-3015.

Korber, B., Gaschen, B., Yusim, K., Thakallapally, R., Kesmir, C. and Detours, V. (2001). Evolutionary and immunological implications of contemporary HIV-1 variation. *British Medical Bulletin,* **58,** 19-42.

Krosgrud, J. (1985). Control of Maedi in Norway. In: *Sharp JM y Hoff Jorgensen R (ed). Slow viruses in sheep, goats and cattle. CEC Report EUR 8076 EN*, 271-278.

Kuhar, U., Barlič-Maganja, D. and Grom, J. (2013). Phylogenetic analysis of small ruminant lentiviruses detected in Slovenia. *Vet Microbiol,* **162,** 201-206.

Kuniholm, M. H., Gao, X., Xue, X., Kovacs, A., Anastos, K., Marti, D., Greenblatt, R. M., Cohen, M. H., Minkoff, H., Gange, S. J., Fazzari, M., Young, M. A., Strickler, H. D. and Carrington, M. (2011). Human leukocyte antigen genotype and risk of HIV disease progression before and after initiation of antiretroviral therapy. *Journal of Virology,* **85,** 10826-10833.

Lacerenza, D., Genovese, F., Profiti, M., Nucera, D. and Rosati, S. (2008). Characterization of an immunodominant epitope of small ruminant lentivirus (SRLV) nucleoprotein. *Veterinary immunology and immunopathology,* **125,** 361-367.

Lacerenza, D., Giammarioli, M., Grego, E., Marini, C., Profiti, M., Rutili, D. and Rosati, S. (2006). Antibody response in sheep experimentally infected with different small ruminant lentivirus genotypes. *Veterinary Immunology and Immunopathology,* **112,** 264-271.

Lackner, A. A., Smith, M. O., Munn, R. J., Martfeld, D. J., Gardner, M. B., Marx, P. A. and Dandekar, S. (1991). Localization of simian immunodeficiency virus in the central nervous system of rhesus monkeys. *American Journal of Pathology,* **139,** 609-621.

Lago, N., López, C., Panadero, R., Cienfuegos, S., Pato, J., Prieto, A., Díaz, P., Mourazos, N. and Fernández, G. (2012). Seroprevalence and risk factors associated with Visna/Maedi virus in semi-intensive lamb-producing flocks in northwestern Spain. *Preventive veterinary medicine,* **103,** 163-169.

Lamara, A., Fieni, F., Chatagnon, G., Larrat, M., Dubreil, L. and Chebloune, Y. (2013). Caprine arthritis encephalitis virus (CAEV) replicates productively in cultured epididymal cells from goats. *Comparative Immunology, Microbiology and Infectious Diseases,* **36,** 397-404.

Lamara, A., Fieni, F., Mselli-Lakhal, L., Chatagnon, G., Bruyas, J. F., Tainturier, D., Battut, I., Fornazero, C. and Chebloune, Y. (2002a). Early embryonic cells from in vivo-produced goat embryos transmit the caprine arthritis - Encephalitis virus (CAEV). *Theriogenology,* **58,** 1153-1163.

Lamara, A., Fieni, F., Mselli-Lakhal, L., Tainturier, D. and Chebloune, Y. (2001). Efficient replication of caprine arthritis-encephalitis virus in goat granulosa cells. *Virus Research,* **79,** 165-172.

Lamara, A., Fieni, F., Mselli-Lakhal, L., Tainturier, D. and Chebloune, Y. (2002b). Epithelial cells from goat oviduct are highly permissive for productive infection with caprine arthritis-encephalitis virus (CAEV). *Virus Research,* **87,** 69-77.

Larruskain, A., Bernales, I., Luján, L., de Andrés, D., Amorena, B. and Jugo, B. M. (2013). Expression analysis of 13 ovine immune response candidate genes in Visna/Maedi disease progression. *Comparative Immunology, Microbiology and Infectious Diseases,* **36,** 405-413.

LaRue, R. S., Jónsson, S. R., Silverstein, K. A. T., Lajoie, M., Bertrand, D., El-Mabrouk, N., Hötzel, I., Andrésdóttir, V., Smith, T. P. L. and Harris, R. S. (2008). The artiodactyl APOBEC3 innate immune repertoire shows evidence for a multi-functional domain organization that existed in the ancestor of placental mammals. *BMC Molecular Biology,* **9**, doi.org/10.1186/1471-2199-**9**-104.

Lee, C. S. and Outteridge, P. M. (1981). Leucocytes of sheep colostrum, milk and involution secretion, with particular reference to ultrastructure and lymphocyte sub-populations. *Journal of Dairy Research,* **48,** 225-237.

Leginagoikoa, I., Daltabuit-Test, M., Álvarez, V., Arranz, J., Juste, R. A., Amorena, B., de Andrés, D., Luján, L. L., Badiola, J. J. and Berriatua, E. (2006). Horizontal Maedi-Visna virus (MVV) infection in adult dairy-sheep raised under varying MVV-infection pressures investigated by ELISA and PCR. *Research in Veterinary Science,* **80,** 235-241.

Leginagoikoa, I., Minguijón, E., Juste, R. A., Barandika, J., Amorena, B., de Andrés, D., Badiola, J. J., Luján, L. and Berriatua, E. (2010). Effects of housing on the incidence of visna/maedi virus infection in sheep flocks. *Research in veterinary science,* **88,** 415-421.

Lerondelle, C., Godet, M. and Mornex, J. F. (1999). Infection of primary cultures of mammary epithelial cells by small ruminant lentiviruses. *Veterinary Research,* **30,** 467-474.

Leroux, C., Cordier, G., Mercier, I., Chastang, J., Lyon, M., Quérat, G., Greenland, T., Vigne, R. and Mornex, J. F. (1995). Ovine aortic smooth muscle cells allow the replication of visna-maedi virus in vitro. *Archives of Virology,* **140,** 1-11.

Leroux, C., Cruz, J. C. and Mornex, J. F. (2010). SRLVs: a genetic continuum of lentiviral species in sheep and goats with cumulative evidence of cross species transmission, *Current HIV Research*, **8**, 94-100.

Leroux, C., Lerondelle, C., Chastang, J. and Mornex, J. F. (1997). RT-PCR detection of lentiviruses in milk or mammary secretions of sheep or goats from infected flocks. *Veterinary Research,* **28,** 115-121.

León, L. and Pats, J. (1996). Encuesta serológica de la infección por el virus Maedi/Visna en la población ovina de Moratella (Murcia), *XXI Jornadas Científicas de la Sociedad Española de Ovinotecnia y Caprinotecnia.* (SEOC), Logroño, 119-127.

Lichtensteiger, C. A., Cheevers, W. P. and Davis, W. C. (1993). CD8+ cytotoxic T lymphocytes against antigenic variants of caprine arthritis-encephalitis virus. *Journal of General Virology,* **74,** 2111-2116.

Lujan, L., Badiola, J. J., Marin, J. F. G., Moreno, B., Vargas, M. A., Deluco, D. F. and Perez, V. (1993). Seroprevalence of Maedi-Visna Infection in Sheep in the Northeast of Spain. *Preventive veterinary medicine,* **15,** 181-190.

Luján, L., Begara, I., Collie, D. and Watt, N. J. (1994). Ovine lentivirus (maedi-visna virus) protein expression in sheep alveolar macrophages. *Veterinary Pathology,* **31,** 695-703.

Luján, L., Begara, I., Collie, D. D. S. and Watt, N. J. (1995). CD8+ lymphocytes in bronchoalveolar lavage and blood: in vivo indicators of lung pathology caused by maedi-visna virus. *Veterinary immunology and immunopathology,* **49,** 89-100.

Luján, L., García Marin, J. F., Fernández de Luco, D., Vargas, A. and Badiola, J. J. (1991). Pathological changes in the lungs and mammary glands of sheep and their relationship with maedi-visna infection. *Veterinary Record,* **129,** 51-54.

Luján, L., Juste, R., Berriatua, J. and Badiola, J. (2001). Epidemiología y control. Maedi-Visna. *OVIS*, **72**, 81-93.

Luo, M., Sainsbury, J., Tuff, J., Lacap, P. A., Yuan, X. Y., Hirbod, T., Kimani, J., Wachihi, C., Ramdahin, S., Bielawny, T., Embree, J., Broliden, K., Ball, T. B. and Plummera, F. A. (2012). A genetic polymorphism of FREM1 is associated with resistance against HIV infection in the pumwani sex worker cohort. *Journal of Virology,* **86,** 11899-11905.

Malim, M. H. and Bieniasz, P. D. (2012). HIV restriction factors and mechanisms of evasion. *Cold Spring Harbor Perspectives in Medicine,* **2**, doi: 10.1101/cshperspect.a006940.

Mariotti, F., Preziuso, S., Rossi, G., Taccini, E., Braca, G. and Renzoni, G. (2007). CD4+CD25+ T cells in maedi visna infection: Preliminary immunohistochemical study in experimentally infected sheep. *Veterinary Research Communications,* **31,** 237-239.

Matsuura, K., Inoshima, Y., Kameyama, K. I. and Murakami, K. (2011). Establishment of a novel ovine kidney cell line for isolation and propagation of viruses infecting domestic cloven-hoofed animal species. *In Vitro Cellular and Developmental Biology - Animal,* **47,** 459-463.

Mavrogianni, V. S., Cripps, P. J., Brooks, H., Taitzoglou, I. A. and Fthenakis, G. C. (2007). Presence of subepithelial lymphoid nodules in the teat of ewes. *Anatomia Histologia Embryologia,* **36,** 168-171.

McNeilly, T. N., Tennant, P., Luján, L., Pérez, M. and Harkiss, G. D. (2007). Differential infection efficiencies of peripheral lung and tracheal tissues in sheep infected with Visnal/maedi virus via the respiratory tract. *Journal of General Virology,* **88,** 670-679.

Mikula, I., Bhide, M. and Pastorekova, S. (2010). Characterization of ovine TLR7 and TLR8 protein coding regions, detection of mutations and Maedi Visna virus infection. *Veterinary Immunology and Immunopathology,* **138,** 51-59.

Minguijón, E., Reina, R., Pérez, M., Polledo, L., Villoria, M., Ramírez, H., Leginagoikoa, I., Badiola, J. J., García-Marín, J. F., de Andrés, D., Luján, L., Amorena, B. and Juste, R. A. (2015). Small ruminant lentivirus infections and diseases. *Veterinary Microbiology,* **181,** 75-89.

Sotelo MJ. (1998). La infección por lentivirus en ovinos y caprinos de la provincia de León: aspectos epidemiológicos y valoración deun plan de control. Tesis Doctoral. Universidad de León.

Molitor, T. W., Schipper, I. A., Berryhill, D. L. and Light, M. R. (1979). Evaluation of the agar-gel immunodiffusion test for the detection of precipitating antibodies against progressive pneumonia virus of sheep. *Canadian Journal of Comparative Medicine,* **43,** 280-287.

Murphy, F. A., Gibbs, E. P. J., Horzinek, M. C. and Studdert, M. J. (1999). Veterinary Virology, 3rd ed. Academic Press., San Diego.

Narayan, O. (1983). Role of Macrophages in the Immunopathogenesis of Visna-Maedi of Sheep. *Progress in Brain Research,* **59**, 233-235.

Narayan, O. and Clements, J. E. (1989). Biology and pathogenesis of lentiviruses. *Journal of General Virology,* **70,** 1617-1639.

Narayan, O., Clements, J. E., Griffin, D. E. and Wolinsky, J. S. (1981). Neutralizing antibody spectrum determines the antigenic profiles of emerging mutants of visna virus. *Infection and immunity,* **32,** 1045-1050.

Narayan, O., Griffin, D. E. and Chase, J. (1977a). Antigenic shift of visna virus in persistently infected sheep. *Science*, **197**, 376-378.

Narayan, O., Griffin, D. E. and Silverstein, A. M. (1977b). Slow virus infection: Replication and mechanisms of persistence of visna virus in sheep. *Journal of Infectious Diseases,* **135,** 800-806.

Narayan, O., Kennedy-Stoskopf, S., Sheffer, D., Griffin, D. E. and Clements, J. E. (1983). Activation of caprine arthritis-encephalitis virus expression during maturation of monocytes to macrophages. *Infection and immunity,* **41,** 67-73.

Narayan, O., Sheffer, D., Griffin, D. E., Clements, J. and Hess, J. (1984). Lack of neutralizing antibodies to caprine arthritis-encephalitis lentivirus in persistently infected goats can be overcome by immunization with inactivated Mycobacterium tuberculosis. *Journal of virology,* **49,** 349-355.

Naruto, T., Gatanaga, H., Nelson, G., Sakai, K., Carrington, M., Oka, S. and Takiguchi, M. (2012). HLA class I-mediated control of HIV-1 in the Japanese population, in which the protective HLA-B*57 and HLA-B*27 alleles are absent. *Journal of Virology,* **86,** 10870-10872.

Nathason, N., Georgsson, G., Palsson, P. A., Najjar, J. A., Lutley, R. and Petursson, G. (1985). Experimental visna in Icelandic sheep: the prototype lentiviral infection. R*eviews of infectous diseases,* **7**, 75-82.

Niesalla, H., McNeilly, T. N., Ross, M., Rhind, S. M. and Harkiss, G. D. (2008). Experimental infection of sheep with visna/maedi virus via the conjunctival space. *Journal of General Virology,* **89,** 1329-1337.

Ojosnegros, S., Perales, C., Mas, A. and Domingo, E. (2011). Quasispecies as a matter of fact: Viruses and beyond. *Virus Research,* **162,** 203-215.

Olech, M., Rachid, A., Croisé, B., Kuźmak, J. and Valas, S. (2012). Genetic and antigenic characterization of small ruminant lentiviruses circulating in Poland. *Virus Res,* **163,** 528-536.

Oliver, R. E., Gorham, J. R., Parish, S. F., Hadlow, W. J. and Narayan, O. (1981). Ovine progressive pneumonia: pathologic and virologic studies on the naturally occurring disease. *American Journal of Veterinary Research,* **42,** 1554-1559.

Palsson, P. A. (1976). Maedi and visna in sheep. *Frontiers of Biology*, **44**, 17-43.

Pascual Canals, J. (1961). Estudios estadísticos y anatomopatológicos sobre granulomas pulmonares de los óvidos. Tesis Doctoral. Universidad de Zaragoza.

Pasick, J. (1998). Maedi-Visna Virus and Caprine Arthritis-Encephalitis Virus: Distinct Species or Quasispecies and its Implications for Laboratory Diagnosis. *Canadian Journal of Veterinary Research,* **62,** 241-244.

Patel, J. R., Heldens, J. G. M., Bakonyi, T. and Rusvai, M. (2012). Important mammalian veterinary viral immunodiseases and their control. *Vaccine,* **30,** 1767-1781.

Paula, N. R. d. O., Andrioli, A., Cardoso, J. d. F. S., Pinheiro, R. R., Sousa, F. M. L., Souza, K. C. d., Alves, F. S. F., Campello, C. C., Ricarte, A. R. F. and Teixeira, M. F. d. S. (2009). Profile of the Caprine arthritis-encephalitis virus (CAEV) in blood, semen from bucks naturally and experimentally infected in the semi-arid region of Brazil. *Small Ruminant Research,* **85,** 27-33.

Pekelder, J. J., Veenink, G. J., Akkermans, J. P., van Eldik, P., Elving, L. and Houwers, D. J. (1994). Ovine lentivirus induced indurative lymphocytic mastitis and its effect on the growth of lambs. *Veterinary Record,* **134,** 348-350.

Peluso, R., Haase, A., Stowring, L., Edwards, M. and Ventura, P. (1985). A Trojan Horse mechanism for the spread of visna virus in monocytes. *Virology,* **147,** 231-236.

Perry, L. L., Wilkerson, M. J., Hullinger, G. A. and Cheevers, W. P. (1995). Depressed cd4+T lymphocyte proliferative response and enhanced antibody response to viral antigen in chronic lentivirus-induced arthritis. *Journal of Infectious Diseases,* **171,** 328-334.

Peterhans, E., Greenland, T., Badiola, J., Harkiss, G., Bertoni, G., Amorena, B., Eliaszewicz, M., Juste, R. A., Kraßnig, R., Lafont, J. P., Lenihan, P., Pétursson, G., Pritchard, G., Thorley, J., Vitu, C., Mornex, J. F. and Pépin, M. (2004). Routes of transmission and consequences of small ruminant lentiviruses (SRLVs) infection and eradication schemes. *Veterinary research,* **35,** 257-274.

Peterson, K., Brinkhof, J., Houwers, D. J., Colenbrander, B. and Gadella, B. M. (2008). Presence of pro-lentiviral DNA in male sexual organs and ejaculates of small ruminants. *Theriogenology,* **69,** 433-442.

Petrovski, S., Fellay, J., Shianna, K. V., Carpenetti, N., Kumwenda, J., Kamanga, G., Kamwendo, D. D., Letvin, N. L., McMichael, A. J., Haynes, B. F., Cohen, M. S. and Goldstein, D. B. (2011). Common human genetic variants and HIV-1 susceptibility: A genome-wide survey in a homogeneous African population. *AIDS,* **25,** 513-518.

Petursson, G., Nathanson, N., Georgsson, G., Panitch, H. and Pálsson, P. A. (1976). Pathogenesis of visna. I. Sequential virologic, serologic, and pathologic studies. *Laboratory Investigation,* **35,** 402-412.

Pisoni, G., Bertoni, G., Puricelli, M., Maccalli, M. and Moroni, P. (2007). Demonstration of coinfection with and recombination by caprine arthritis-encephalitis virus and maedi-visna virus in naturally infected goats. *Journal of Virology,* **81,** 4948-4955.

Pisoni, G., Quasso, A. and Moroni, P. (2005). Phylogenetic analysis of small-ruminant lentivirus subtype B1 in mixed flocks: Evidence for natural transmission from goats to sheep. *Virology,* **339,** 147-152.

Plaza, M., Sánchez, A., Corrales, J. C., De la Fe, C. and Contreras, A. (2009). Caprine arthritis encephalitis virus diagnosed by ELISA in lactating goats using milk samples. *Small Ruminant Research,* **81,** 189-192.

Polledo, L., Gonzalez, J., Benavides, J., Martinez-Fernandez, B., Carmen Ferreras, M. and Garcia Marin, J. F. (2012a). Perivascular inflammatory cells in ovine Visna/maedi encephalitis and their possible role in virus infection and lesion progression. *Journal of neurovirology,* **18,** 532-537.

Polledo, L., González, J., Benavides, J., Morales, S., Martínez-Fernández, B., Delgado, L., Reina, R., Glaria, I., Pérez, V., Ferreras, M. C. and García Marín, J. F. (2012b). Patterns of Lesion and Local Host Cellular Immune Response in Natural Cases of Ovine Maedi-Visna. *Journal of comparative pathology,* **147,** 1-10.

Polledo, L., Gonzalez, J., Fernandez, C., Miguelez, J., Martinez-Fernandez, B., Morales, S., Ferreras, M. C. and Garcia Marin, J. F. (2013). Simple control strategy to reduce the level of Maedi-Visna infection in sheep flocks with high prevalence values (> 90%). *Small Ruminant Research,* **112,** 224-229.

Ponti, W., Paape, M., Bronzo, V., Pisoni, G., Pollera, C. and Moroni, P. (2008). Phenotypic alteration of blood and milk leukocytes in goats naturally infected with caprine arthritis-encephalitis virus (CAEV). *Small Ruminant Research,* **78,** 176-180.

Preziuso, S., Renzoni, G., Allen, T. E., Taccini, E., Rossi, G., Demartini, J. C. and Braca, G. (2004). Colostral transmission of maedi visna virus: Sites of viral entry in lambs born from experimentally infected ewes. *Veterinary microbiology,* **104,** 157-164.

Preziuso, S., Sanna, E., Sanna, M. P., Loddo, C., Cerri, D., Taccini, E., Mariotti, F., Braca, G., Rossi, G. and Renzoni, G. (2003a). Association of Maedi Visna Virus with Brucella ovis infection in rams. *European Journal of Histochemistry,* **47,** 151-157.

Preziuso, S., Taccini, E., Rossi, G., Renzoni, G. and Braca, G. (2003b). Experimental Maedi Visna Virus Infection in sheep: A morphological, immunohistochemical and PCR study after three years of infection. *European Journal of Histochemistry,* **47,** 373-378.

Purdy, J. G., Flanagan, J. M., Ropson, I. J., Rennoll-Bankert, K. E. and Craven, R. C. (2008). Critical role of conserved hydrophobic residues within the major homology region in mature retroviral capsid assembly. *J Virol,* **82,** 5951-5961.

Pálsson, P. A. (1990). Maedi-visna. History and clinical description. In: *Pétursson, G., Hoff-Jørgensen, R. (Eds.), Maedi-Visna and Related Diseases.* , Kluwer Academic Publishers, Boston, 3-17.

Pépin, M., Vitu, C., Russo, P., Mornex, J. F. and Peterhans, E. (1998). Maedi-visna virus infection in sheep: A review. *Veterinary Research,* **29,** 341-367.

Pérez, M., Biescas, E., de Andrés, X., Leginagoikoa, I., Salazar, E., Berriatua, E., Reina, R., Bolea, R., de Andrés, D., Juste, R. A., Cancer, J., Gracia, J., Amorena, B., Badiola, J. J. and Luján, L. (2010). Visna/maedi virus serology in sheep: Survey, risk factors and implementation of a successful control programme in Aragón (Spain). *The Veterinary Journal,* **186,** 221-225.

Pérez, M., Biescas, E., Reina, R., Glaria, I., Marín, B., Marquina, A., Salazar, E., Álvarez, N., de Andrés, D., Fantova, E., Badiola, J. J., Amorena, B. and Luján, L. (2015). Small ruminant lentivirus-induced arthritis: clinicopathologic findings in sheep infected by a highly replicative SRLV B2 genotype. *Veterinary Pathology,* **52,** 132-139

Pérez, M., Biescas, E., Salazar, E., Marín, B., Marquina, A., Pinczowski, P., Gimeno, M., Fantova, E., Reina, R. and Luján, L. (2012). Artritis ovina por Visna/maedi. *Sociedad Española de Ovinotecnia y Caprinotecnia*, Ciudad Real.

Pérez, M., Muñoz, J. A., Biescas, E., Salazar, E., Bolea, R., de Andrés, D., Amorena, B., Badiola, J. J., Reina, R. and Luján, L. (2013). Successful Visna/maedi control in a highly infected

ovine dairy flock using serologic segregation and management strategies. *Preventive Veterinary Medicine,* **112,** 423-427.

Pétursson, G. (1994). Experience with Visna Virus in Iceland. *Annals of the New York Academy of Sciences*, **724**, 43-49.

Pétursson, G., Andrésdóttir, V., Andrésson, Ó., Torsteinsdóttir, S., Georgsson, G. and Pálsson, P. A. (1991). Human and ovine lentiviral infections compared. *Comparative Immunology, Microbiology and Infectious Diseases,* **14,** 277-287.

Pétursson, G., Andrésdóttir, V., Andrésson, O.S., and Georgsson, G., Pálsson, P.A., Rafnar, B., Torsteinsdóttir, S. (1992). Lentivirus diseases of sheep and goats: maedi-visna and caprine arthritis-encephalitis, Vol. Progress in Sheep and Goat Research. CAB International, Speedy, A.W. (Ed.)., Progress in Sheep and Goat Research. CAB International Oxford, 107-129.

Pétursson, G., Matthíasdóttir, S., Svansson, V., Andrésdóttir, V., Georgsson, G., Martin, A. H., Agnarsdóttir, G., Gísladóttir, E., Árnadóttir, S., Högnadóttir, S., Jónsson, S. R., Andrésson, O. S. and Torsteinsdóttir, S. (2005). Mucosal vaccination with an attenuated maedi-visna virus clone. *Vaccine,* **23,** 3223-3228.

Rachid, A., Croisé, B., Russo, P., Vignoni, M., Lacerenza, D., Rosati, S., Kuźmak, J. and Valas, S. (2013). Diverse host-virus interactions following caprine arthritis-encephalitis virus infection in sheep and goats. *Journal of General Virology,* **94,** 634-642.

Ramírez, H., Reina, R., Amorena, B., de Andrés, D. and Martínez, H. A. (2013). Small ruminant Lentiviruses: Genetic variability, tropism and diagnosis. *Viruses,* **5,** 1175-1207.

Ramírez, H., Reina, R., Bertolotti, L., Cenoz, A., Hernández, M. M., San Román, B., Glaria, I., de Andrés, X., Crespo, H., Jáuregui, P., Benavides, J., Polledo, L., Pérez, V., García-Marín, J. F., Rosati, S., Amorena, B. and de Andrés, D. (2012). Study of compartmentalization in the visna clinical form of small ruminant lentivirus infection in sheep. *BMC Veterinary Research,* **8**, doi: 10.1186/1746-6148-8-8.

Ramírez, H., Román, B. S., Glaria, I., Reina, R., Hernández, M. M., de Andrés, X., Crespo, H., Hichou, B., Cianca, S., Goñi, C., Grandas, A., García-Pastor, L., Vijil, L. E., Quintín, F., Grilló, M. J., de Andrés, D. and Amorena, B. (2009). Antibody-based diagnosis of small ruminant lentivirus infection in seminal fluid. *Theriogenology,* **72,** 1085-1096.

Reina, R., Barbezange, C., Niesalla, H., de Andrés, X., Arnarson, H., Biescas, E., Mazzei, M., Fraisier, C., McNeilly, T. N., Liu, C., Perez, M., Carrozza, M. L., Bandecchi, P., Solano, C., Crespo, H., Glaria, I., Huard, C., Shaw, D. J., de Blas, I., de Andrés, D., Tolari, F., Rosati, S., Suzan-Monti, M., Andrésdottir, V., Torsteinsdottir, S., Petursson, G., Lujan, L., Pepin, M., Amorena, B., Blacklaws, B. and Harkiss, G. D. (2008). Mucosal immunization against ovine lentivirus using PEI-DNA complexes and modified vaccinia Ankara encoding the gag and/or env genes. *Vaccine,* **26,** 4494-4505.

Reina, R., Berriatua, E., Luján, L., Juste, R., Sánchez, A., de Andrés, D. and Amorena, B. (2009a). Prevention strategies against small ruminant lentiviruses: An update. *The Veterinary Journal,* **182,** 31-37.

Reina, R., Bertolotti, L., Dei Giudici, S., Puggioni, G., Ponti, N., Profiti, M., Patta, C. and Rosati, S. (2010). Small ruminant lentivirus genotype E is widespread in Sarda goat. *Veterinary Microbiology,* **144,** 24-31.

Reina, R., Glaria, I., Benavides, J., De Andrés, X., Crespo, H., Solano, C., Pérez, V., Luján, L., Pérez, M. M., De La Lastra, J. M. P., Rosati, S., Blacklaws, B., Harkiss, G., De Andrés, D. and Amorena, B. (2007). Association of CD80 and CD86 expression levels with disease status of Visna/Maedi virus infected sheep. *Viral Immunology,* **20,** 609-622.

Reina, R., Grego, E., Profiti, M., Glaria, I., Robino, P., Quasso, A., Amorena, B. and Rosati, S. (2009b). Development of specific diagnostic test for small ruminant lentivirus genotype E. *Veterinary Microbiology,* **138,** 251-257.

Reina, R., Mora, M. I., Glaria, I., García, I., Solano, C., Luján, L., Badiola, J. J., Contreras, A., Berriatua, E., Juste, R., Mamoun, R. Z., Rolland, M., Amorena, B. and de Andrés, D.

(2006). Molecular characterization and phylogenetic study of Maedi Visna and Caprine Arthritis Encephalitis viral sequences in sheep and goats from Spain. *Virus Research,* **121,** 189-198.

Reviriego, J.F. (1998). Seroprevalencia del maedi-visna y arthritis encephalitis caprina. Estudio realizado en la provincial de Ávila. , Vol. 21, *Albéitar*, pp. 15.

Roberts, J. D., Bebenek, K. and Kunkel, T. A. (1988). The accuracy of reverse transcriptase from HIV-1. *Science,* **242,** 1171-1173.

Romero, C. C., Pellerin, J. L., Poulin, N., Cognié, Y., Chebloune, Y., Pépin, M. and Fieni, F. (2010). Maedi-Visna virus was detected in association with virally exposed IVF-produced early ewes embryos. *Theriogenology,* **74,** 682-690.

Rosati, S., Kwang, J., Tolari, F. and Keen, J. (1994). A comparison of whole virus and recombinant transmembrane ELISA and immunodiffusion for detection of ovine lentivirus antibodies in Italian sheep flocks. *Veterinary research communications,* **18,** 73-80.

Russo, P., Remond, M., Sarrazin, C., Laserre, M., Larenaudie, B., Giauffret, A. (1980). Recherché èpidemiologiues sur la maladie Maedi-Visna. Mise en evidence de foyers confirmés, Vol. 53, *Bulletin de l'Académie Vétérinaire de France*, 129-138.

Ryan, S., Tiley, L., McConnell, I. and Blacklaws, B. (2000). Infection of dendritic cells by the Maedi-visna lentivirus. *Journal of Virology,* **74,** 10096-10103.

Saman, E., Van Eynde, G., Lujan, L., Extramiana, B., Harkiss, G., Tolari, F., Gonzàlez, L., Amorena, B., Watt, N. and Badiola, J. (1999). A new sensitive serological assay for detection of lentivirus infections in small ruminants. *Clinical and diagnostic laboratory immunology,* **6,** 734-740.

Santry, L. A., de Jong, J., Gold, A. C., Walsh, S. R., Menzies, P. I. and Wootton, S. K. (2013). Genetic characterization of small ruminant lentiviruses circulating in naturally infected sheep and goats in Ontario, Canada. *Virus Research,* **175,** 30-44.

Sarafidou, T., Stamatis, C., Kalozoumi, G., Spyrou, V., Fthenakis, G. C., Billinis, C. and Mamuris, Z. (2013). Toll Like Receptor 9 (TLR9) Polymorphism G520R in Sheep Is Associated with Seropositivity for Small Ruminant Lentivirus. *PLoS ONE,* **8**, doi.org/10.1371/journal.pone.0063901.

Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B. J., Ghrayeb, J., Forman, M. A., Montefiori, D. C., Peter Rieber, E., Letvin, N. L. and Reimann, K. A. (1999). Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science,* **283,** 857-860.

Shah, C., Böni, J., Huder, J. B., Vogt, H. R., Mühlherr, J., Zanoni, R., Miserez, R., Lutz, H. and Schüpbach, J. (2004a). Phylogenetic analysis and reclassification of caprine and ovine lentiviruses based on 104 new isolates: Evidence for regular sheep-to-goat transmission and worldwide propagation through livestock trade. *Virology,* **319,** 12-26.

Shah, C., Huder, J. B., Böni, J., Schönmann, M., Mühlherr, J., Lutz, H. and Schüpbach, J. (2004b). Direct evidence for natural transmission of small-ruminant lentiviruses of subtype A4 from goats to sheep and vice versa. *Journal of Virology,* **78,** 7518-7522.

Sigurdardottir, B. and Thormar, H. (1964). ISOLATION OF A VIRAL AGENT FROM THE LUNGS OF SHEEP AFFECTED WITH MAEDI. *The Journal of infectious diseases,* **114,** 55-60.

Sigurdsson, B. (1954a). Maedi, a slow pregressive pneumonia of sheep: an epizoological and pathological study. *British Veterinary Journal*, **110**, 225-270.

Sigurdsson, B. (1954b). Rida, a chronic encephalitis of sheep. B*ritish Veterinary Journal,* **110**, 341-354.

Sigurdsson, B., Grimsson, H. and Palsson, P. A. (1952). Maedi, a chronic, progressive infection of sheep's lungs. *The Journal of infectious diseases,* **90,** 233-241.

Sigurdsson, B., Palsson, P. and Grimsson, H. (1957). Visna, a demyelinating transmissible disease of sheep. *Journal of neuropathology and experimental neurology,* **16,** 389-403.

Sigurdsson, B. and Palsson, P. A. (1958). Visna of sheep; a slow, demyelinating infection. *British journal of experimental pathology,* **39,** 519-528.

Sihvonen, L. (1980). Studies on transmission of maedi virus to lambs. *Acta veterinaria Scandinavica,* **21,** 689-698.

Singh, I., McConnell, I., Dalziel, R. and Blacklaws, B. A. (2006). Serum containing ovine IgG2 antibody specific for maedi visna virus envelope glycoprotein mediates antibody dependent cellular cytotoxicity. *Veterinary Immunology and Immunopathology,* **113,** 357-366.

Smyth, R. P., Davenport, M. P. and Mak, J. (2012). The origin of genetic diversity in HIV-1. *Virus Research,* **169,** 415-429.

Souza, K. C. d., Pinheiro, R. R., Santos, D. O., Brito, R. L. L. d., Rodrigues, A. d. S., Sider, L. H., Paula, N. R. O., Avila, A. A., Cardoso, J. d. F. S. and Andrioli, A. (2013). Transmission of the caprine arthritis–encephalitis virus through artificial insemination. *Small Ruminant Research,* **109,** 193-198.

Srinivasan, M., Sedmak, D. and Jewell, S. (2002). Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *American Journal of Pathology,* **161,** 1961-1971.

Staskus, K. A., Couch, L., Bitterman, P., Retzel, E. F., Zupancic, M., List, J. and Haase, A. T. (1991). In situ amplification of visna virus DNA in tissue sections reveals a reservoir of latently infected cells. *Microbial Pathogenesis,* **11,** 67-76.

Stonos, N., Wootton, S. K. and Karrow, N. (2014). Immunogenetics of small ruminant lentiviral infections. *Viruses,* **6,** 3311-3333.

Synge, B. A. and Ritchie, C. M. (2010). Elimination of small ruminant lentivirus infection from sheep flocks and goat herds aided by health schemes in Great Britain. *Veterinary Record,* **167,** 739-743.

Thormar, H. (1961). Stability of visna virus in infectious tissue culture fluid. *Archiv für die gesamte Virusforschung,* **10,** 501-509.

Thormar, H. (1965). A comparison of visna and maedi viruses. I. physical, chemical and biological properties. *Research in veterinary science,* **6,** 117-129.

Thormar, H. (2005). Maedi-visna virus and its relationship to human immunodeficiency virus. *AIDS Reviews,* **7,** 233-245.

Thormar, H. (2013). The origin of lentivirus research: Maedi-visna virus. *Current HIV Research,* **11,** 2-9.

Tompkins, M. B. and Tompkins, W. A. (2008). Lentivirus-induced immune dysregulation. *Veterinary Immunology and Immunopathology,* **123,** 45-55.

Tong-Starksen, S. E., Sepp, T. and Santos Pagtakhan, A. (1996). Activation of caprine arthritis-encephalitis virus long terminal repeat by Gamma interferon. *Journal of Virology,* **70,** 595-599.

Torsteinsdottir, S., Andresdottir, V., Arnarson, H. and Petursson, G. (2007a). Immune response to maedi-visna virus. *Frontiers in Biosciences,* **12,** 1532-1543.

Torsteinsdottir, S., Carlsdottir, H. M., Svansson, V., Matthiasdottir, S., Martin, A. H. and Petursson, G. (2007b). Vaccination of sheep with Maedi-visna virus gag gene and protein, beneficial or harmful? *Vaccine,* **25,** 6713-6720.

Torsteinsdóttir, S., Georgsson, G., Gísladóttir, E., Rafnar, B., Pálsson, P. A. and Pétursson, G. (1992). Pathogenesis of central nervous system lesions in visna: Cell-mediated immunity and lymphocyte subsets in blood, brain and cerebrospinal fluid. *Journal of Neuroimmunology,* **41,** 149-158.

Torsteinsdóttir, S., Matthíasdóttir, S., Vidarsdóttir, N., Svansson, V. and Pétursson, G. (2003). Intratracheal inoculation as an efficient route of experimental infection with maedi-visna virus. *Research in Veterinary Science,* **75,** 245-247.

Trujillo, J. D., Hötzel, K. J., Snekvik, K. R. and Cheevers, W. P. (2004). Antibody response to the surface envelope of caprine arthritis-encephalitis lentivirus: Disease status is predicted by SU antibody isotype. *Virology,* **325,** 129-136.

Kumar V, Abbas AK, and Fausto N. (2005). Inflamación aguda y crónica. In: *En: Kumar V, Abbas AK, Fausto N. Patología estructural y funcional, 7ª edición.*, Elsevier, Madrid, pp. 48.

Valas, S., Le Ven, A., Croise, B., Maquigneau, M. and Perrin, C. (2011). Interference of vaccination against bluetongue virus serotypes 1 and 8 with serological diagnosis of small-ruminant lentivirus infection. *Clinical and Vaccine Immunology,* **18,** 513-517.

van der Molen, E. J. and Houwers, D. J. (1987). Indurative lymphocytic mastitis in sheep after experimental infection with maedivisna virus. *Veterinary Quarterly,* **9,** 193-202.

van der Molen, E. J., Vecht, U. and Houwers, D. J. (1985). A chronic indurative mastitis in sheep, associated with maedi/visna virus infection. *Veterinary Quarterly,* **7,** 112-119.

Varea, R., Monleón, E., Pacheco, C., Luján, L., Bolea, R., Vargas, M. A., Van Eynde, G., Saman, E., Dickson, L., Harkiss, G., Amorena, B. and Badiola, J. J. (2001). Early detection of maedi-visna (ovine progressive pneumonia) virus seroconversion in field sheep samples. *Journal of Veterinary Diagnostic Investigation,* **13,** 301-307.

Villoria, M., Leginagoikoa, I., Luján, L., Pérez, M., Salazar, E., Berriatua, E., Juste, R. A. and Minguijón, E. (2013). Detection of Small Ruminant Lentivirus in environmental samples of air and water. *Small Ruminant Research,* **110,** 155-160.

Watt, N. J., King, T. J., Collie, D., McIntyre, N., Sargan, D. and McConnell, I. (1992a). Clinicopathological investigation of primary, uncomplicated maedi-visna virus infection. *Veterinary Record,* **131,** 455-461.

Watt, N. J., MacIntyre, N., Collie, D., Sargan, D. and McConnell, I. (1992b). Phenotypic analysis of lymphocyte populations in the lungs and regional lymphoid tissue of sheep naturally infected with maedi visna virus. *Clinical and Experimental Immunology,* **90,** 204-208.

Watt, N. J., Roy, D. J., McConnell, I. and King, T. J. (1990). A case of visna in the United Kingdom. *Veterinary Record,* **126,** 600-601.

White, S. N., Mousel, M. R., Herrmann-Hoesing, L. M., Reynolds, J. O., Leymaster, K. A., Neibergs, H. L., Lewis, G. S. and Knowles, D. P. (2012). Genome-Wide Association Identifies Multiple Genomic Regions Associated with Susceptibility to and Control of Ovine Lentivirus. *PLoS ONE,* **7**, doi.org/10.1371/journal.pone.

White, S. N., Mousel, M. R., Reynolds, J. O., Lewis, G. S. and Herrmann-Hoesing, L. M. (2009). Common promoter deletion is associated with 3.9-fold differential transcription of ovine CCR5 and reduced proviral level of ovine progressive pneumonia virus. *Animal Genetics,* **40,** 583-589.

Winward, L. D., Leendertsen, L. and Shen, D. T. (1979). Microimmunodiffusion test for diagnosis of ovine progressive pneumonia. *American Journal of Veterinary Research,* **40,** 564-566.

Wood, P. R. and Seow, H. F. (1996). T cell cytokines and disease prevention. *Veterinary Immunology and Immunopathology,* **54,** 33-44.

Woodall, C. J., Maclaren, L. J. and Watt, N. J. (1997). Differential Levels of mRNAs for Cytokines, the Interleukin-2 Receptor and Class II DR/DQ Genes in Ovine Interstitial Pneumonia Induced by Maedi Visna Virus Infection. *Veterinary Pathology,* **34,** 204-211.

Zanoni, R. G., Vogt, H.-R., Pohl, B., Böttcher, J., Bommeli, W. and Peterhans, E. (1994). An ELISA Based on Whole Virus for the Detection of Antibodies to Small-ruminant Lentiviruses. *Journal of Veterinary Medicine*, Series B, **41**, 662–669.

Zhang, Z., Watt, N. J., Hopkins, J., Harkiss, G. and Woodall, C. J. (2000). Quantitative analysis of maedi-visna virus DNA load in peripheral blood monocytes and alveolar macrophages. *Journal of Virological Methods,* **86,** 13-20.

Zink, M. C. and Johnson, L. K. (1994). Pathobiology of lentivirus infections of sheep and goats. *Virus research,* **32,** 139-154.

Zink, M. C., Narayan, O., Kennedy, P. G. E. and Clements, J. E. (1987). Pathogenesis of visna/maedi and caprine arthritis-encephalitis: New leads on the mechanism of restricted virus replication and persistent inflammation. *Veterinary Immunology and Immunopathology,* **15,** 167-180.

XV. Anexo I: Trabajos en formato original

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Research Paper

Serological ELISA results are conditioned by individual immune response in ovine maedi visna

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ABSTRACT

The marked differences in sensitivity existing among maedi visna ELISA tests is a striking issue in the control of the disease, which so far have mainly been related to the different circulating viral strain or to the particular test used. The aim of this work is to discern whether or not the ELISA results could also be associated to different histological lesion patterns and therefore conditioned by individual immune response. Fifty infected animals and eight negative controls were used and histological, inmunohistochemical, PCR and serological studies were performed. Histological patterns were classified based on previous described immunophenotypical criteria: a *lymphocytic pattern,* characterized by a clear predominance of T cells, especially CD8+ T cells (n = 19), and a *histiocytic pattern*, with a high quantity of macrophages mixed with B cells (n = 23). A third *mixed pattern* characterized by a mixed inflammatory infiltration was observed (n = 8), predominantly in animals with minimal lesions with no clinical signs being observed (75%). An association between these lesion patterns and the ELISA optic density values exists ($p < 0.001$). Sheep with a *histiocytic pattern* (n = 21) showed higher titers of antibodies compared to sheep with *lymphocytic pattern* (n = 18), where values were much lower or even negative. Animals with *histiocytic pattern* are easily recognizable using the ELISA test, while sheep showing lymphocytic lesion pattern could go unnoticed in the flock as serological false negative animals, being a likely remaining source of infection. Animals with *mixed pattern* showed mixed values, and despite showing only minimal lesions, they are also carriers of the virus and can be easily underdiagnosed.

1. Introduction

Ovine maedi visna (MV) is a widespread disease caused by the lentivirus Visna/maedi virus (VMV) and causes direct loses in sheep production (Benavides et al., 2013; Minguijón et al., 2015). MV is characterized by a slow but progressive infection in sheep, resulting in a chronic inflammation of lung, mammary gland and central nervous system (CNS) as well as progressive weight loss (Dawson, 1987; Cutlip et al., 1988). Histological changes mainly correspond to a chronic, interstitial inflammation of the lungs and mammary glands and to a non-suppurative encephalitis and demyelination of the CNS (Luján et al., 1991; Benavides et al., 2009; Minguijón et al., 2015). Sub-clinical infections cause high viral spread among flocks and it is estimated that individual prevalence of VMV infection in Spanish Assaf dairy sheep kept in an intensive indoor farming system is could reach 77% (Leginagoikoa et al., 2006).

No commercial vaccines are currently available and only adequate control programs can be used to limit the spreading of the virus or eradicate the disease (Polledo et al., 2013). Early detection of infected animals from the flock by using antibody detection methods such as ELISA tests have been described as the most appropriate tool to use in MV control programs (de Andrés et al., 2005; Patel et al., 2012; Minguijón et al., 2015). However, striking differences in sensitivity among MV ELISA tests has been detected up to now relating them to the different circulating viral strain or the particular test used, but not to the individual immunological response (de Andrés et al., 2005; de Andrés et al., 2013).

Individual immune response against VMV has been suggested to play a major role in the pathogenesis of the disease (Torsteinsdóttir et al., 1992, 2007; Blacklaws, 2012; Polledo et al., 2012a,b). A possible link between the humoral response and the lesions has been suggested in neurological forms (Polledo et al., 2012b) where two main lesion patterns were described with regard to the inflammatory infiltrate: a *lymphocytic pattern*, characterized by a clear predominance of T cells,

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especially CD8+ T cells, and a *histiocytic pattern*, with a predominance of histiocytic cells mixed with B cells. The former could be related to a cytotoxic cellular immune response with a low antibody titer and the latter to a stronger humoral response with higher antibody titer. The aim of this study is to evaluate the correlation between individual serologic response against VMV and the histological lesion pattern. The results should be considered when diagnosing and controlling of MV disease, especially in infected but negative animals in serological tests.

2. Material and methods

2.1. Animals

Fifty-eight adult Spanish Assaf sheep submitted to the Pathology Diagnostic Service of the School of Veterinary Medicine (León, Spain) for routine necropsies were used in this study. Fifty animals came from different intensive milk-producing flocks previously diagnosed with MV, while eight sheep came from non-infected flocks and were selected as negative controls. MV characteristic clinical signs were observed in 31 sheep, especially nervous and respiratory signs. The animals used in this study did not show macroscopic or histological lesions compatible with other pathologies such as bacterial, fungal or parasitic pneumonias or mastitis.

2.2. Sampling and histopathology

Tissue samples from the 58 sheep were systematically taken for histopathology from diaphragmatic and apical lung lobes, glandular parenchyma and udder cisterns and CNS from 9 levels of the brain and 9 levels of the spinal cord following a previous description (Polledo et al., 2012a). All the samples were fixed in 10% neutral buffered formalin for 48 h at room temperature. After fixation, samples were embedded in paraffin wax and sections (4 μm) were stained with hematoxylin and eosin (HE) and examined using light microscopy.

On microscopic examination, histiocytic and lymphocytic patterns were considered as previously described (Polledo et al., 2012b). A mixed inflammatory infiltrate composed of macrophages and lymphocytes with no clear predominance of any of the cellular populations was also taken into account. Lesion patterns were studied in lung, mammary gland and CNS of all the animals included in this study, taking into account the predominant lesion pattern in the three target organs of each individual sheep.

2.3. Immunohistochemistry

After fixation, lung, mammary gland and CNS samples from the 58 animals studied were dehydrated through graded alcohol and embedded in paraffin wax and 4 μm sections were prepared. The following antibodies were used: polyclonal anti-CD3 for T cells (Dako, Denmark); monoclonal anti-CD79 for B cells (Dako, Denmark); monoclonal anti-CD68 for macrophages (Dako, Denmark), and monoclonal anti-p28 of CAEV/VMV (VMRD, USA) for viral detection. EnVision+ system (EnVision+ System Labelled Polymer-HRP anti-mouse or anti-rabbit; Dako, Denmark) and diaminobezidine solution (DAB) (Vector Laboratories, Burlingame, California, USA) were used for anti-CD3, CD79, and CD68 antibodies. An avidin-biotin-peroxidase complex (ABC) technique (Vectastain Elite, ABC Kit; Vector Laboratories, USA) previously described was used for anti-p28 of CAEV/VMV (Preziuso et al., 2003). The slides were counterstained with haematoxylin and mounted. The specificity of the technique was evaluated using positive and negative controls.

2.4. Polymerase chain reaction

PCR technique was performed as put forward by Ryan et al. (2000). Lung samples from 14 infected sheep were tested by PCR. Lung samples of 5 negative controls were also studied. Genomic DNA was extracted from paraffin-embedded tissue samples using QIAamp® DNA Mini Kit (QIAGEN).

2.5. Serology analysis

Serum samples were obtained from the 58 sheep to evaluate the presence of antibodies against VMV using a standardized comercial kit test (*Elitest*®, Hyphen BioMed, Neuville-Oise, France) following the manufacturer's instructions. Enzyme linked immunosorbent assay (ELISA) results were reported as positive or negative on the basis of the cut-off value calculated following the manufacturer's instructions. The cut-off point was established at 0.4 ± 0.1. The optical density (OD) values were used as a semi-quantitative measure of anti-VMV antibody levels.

2.6. Statistical analysis

One-way ANOVA was used to test whether data on the lesion patterns depended on their serological values or not. Newman-Keuls multiple comparison test was used to determine the OD values statistical differences between groups (histiocytic/lymphocytic pattern; histiocytic/mixed pattern; lymphocytic/mixed pattern). Data were expressed as mean values ± standard deviation (SD). Differences were considered statistically significant at $P < 0.05$. Analysis was carried out using IBM SPSS Statistics for Windows, Version 24.0 (Armonk, NY: IBM Corp).

3. Results

3.1. Histopathology and immunohistochemistry

The fifty animals selected from infected flocks were positive to VMV by IHC and showed characteristic MV lesions, while the 8 negative controls were negative and did not show any abnormalities within the target organs. All the MV infected animals showed MV lesions in lungs, 36 in CNS and 39 in mammary gland.

Three different patterns were observed: a *histiocytic pattern* in 23 animals (46%) (Fig. 1a), a *lymphocytic pattern* in 19 sheep (38%) (Fig. 1b) and a *mixed pattern* in 8 sheep (16%). The same lesion pattern was invariably observed in lung and CNS of every sheep studied. The mammary gland showed a low number of macrophages and a predominance of lymphocytes in the inflammatory infiltrate in all sheep. Lesions severity and extension varied between the different target organs in the same animal.

Viral antigen immunolabelling appeared as a fine brownish deposit in the cytoplasm of macrophage-like cells in all the affected organs, with a subjectively more abundance in histiocytic lesion pattern than in lymphocytic pattern (Fig. 2). The positive sign was always associated with lesions, and no labelling was detected in histologically unaffected areas. No immunolabelled cells were detected in sections used as negative controls but labelling was invariably seen in positive control sections.

3.2. Polymerase chain reaction

The 14 animals from the infected flocks were positive to PCR. Seven of these sheep showed minimal lesions, two showed moderate lesions, while five showed severe lesions. Three positive sheep showed *histiocytic pattern*, ten *lymphocytic pattern* and one *mixed pattern* (Table 1). The 5 negative controls were negative to PCR.

3.3. Correlation between histopahological and serological findings

Fourty-five of the fifty infected animals showed positive optical density (OD) results, three of them presented doubtful results

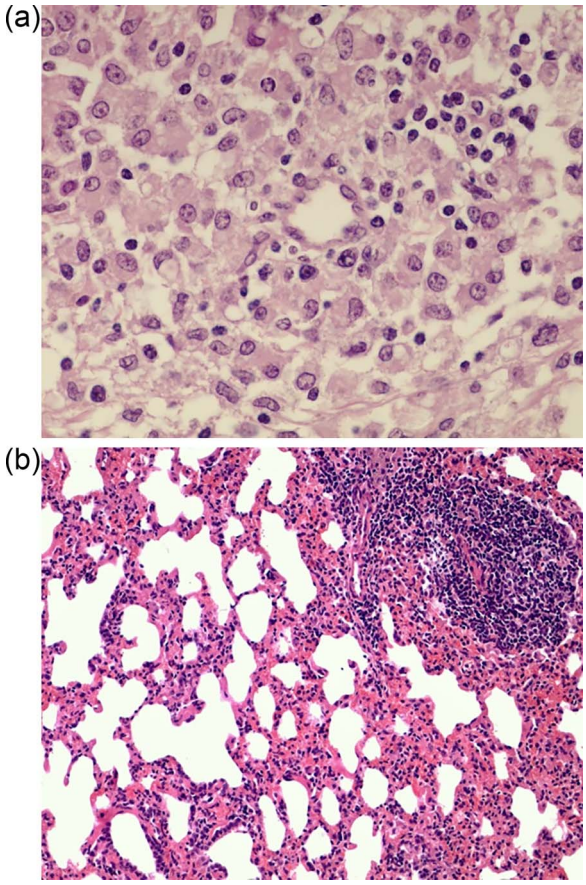

Fig. 1. Maedi visna lesions patterns, HE. (a): Histiocytic lesion pattern in a brain sample. Predominance of macrophages within the inflammatory infiltrate. 40X. (b): Lymphocytic lesion pattern in a lung sample. Predominance of lymphocytes within the inflammatory infiltrate. Presence of a lymphoid follicle. 20X.

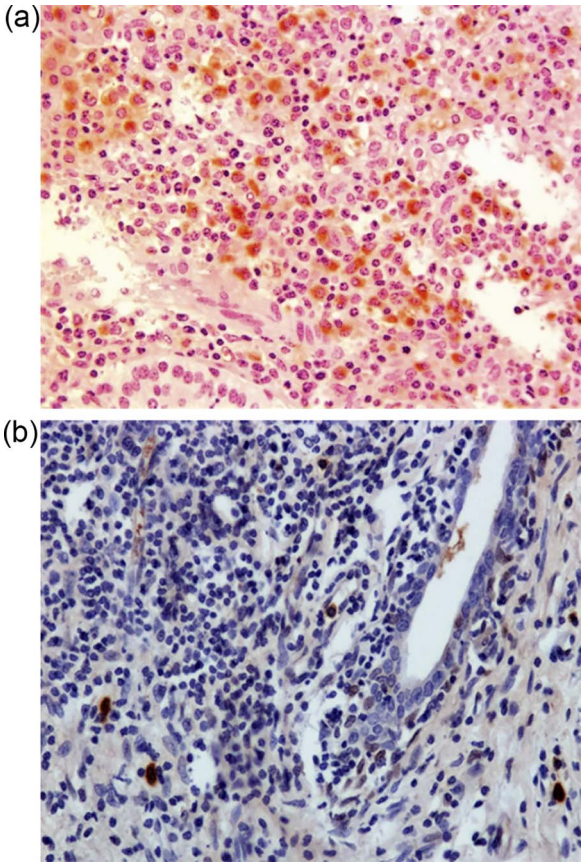

Fig. 2. Inmunohistochemistry against p28-MVV. (a): Histiocytic lesion pattern in a lung sample. High amount of positive macrophage-like cells within the inflammatory infiltrate. 20X. (b): Lymphocytic lesion pattern in a mammary gland sample. Scattered positive cells within the lymphocytic infiltrate. 20X.

(0.4 ± 0.1), and two infected sheep showed negative OD results (Table 1).

Significant differences ($p < 0.001$) in the values of the OD between histiocytic, lymphocytic and mixed patterns were observed in this study. All sheep with *histiocytic pattern* (n = 23) were positive to VMV showing high OD values between of 1.14 and 2.99 (average: 2.08, SD: 0.51). Animals with a *lymphocytic pattern* (n = 19) showed lower OD values, even with 2 negative and 3 doubtful values (min: 0.09 max: 1.81; average: 0.91; SD: 0.47). Sheep with a *mixed pattern* (n = 8) showed mixed values between 0.77 and 2.09 (average: 1.35 SD: 0.37) (Fig. 3).

4. Discussion

This study describe for the first time an association between three histological lesion patterns (histiocytic, lymphocytic, and mixed) studied in lung, mammary gland and CNS and ELISA results on the basis of differences in OD values. Animals with a *histiocytic lesion pattern* showed high OD values (average: 2.08, max: 2.99 min: 1.14 s: 0.51). However, sheep with *lymphocytic pattern* showed significant ($p < 0.001$) lower values with 2 seronegative and 3 doubtful sheep (average: 0.91, max: 1.81 min: 0.09 s: 0.37). Therefore, high titers of antibodies are related to histiocytic patterns while low OD values or negative results are related to lymphocytic types. This is an important factor to consider for ELISA testing, in addition to the viral strain or the particular test used as have been previously reported (de Andrés et al., 2005; de Andrés et al., 2013). Similar results were previously reported in animals with only lymphoid follicles in lung and mammary gland which were seronegative in the AGID test, although at that time it was associated to the low sensitivity of the test used or to an initial stage of the disease with no antibody response (Luján et al., 1991). Our results support the hypothesis of that these animals with lymphocytic pattern and minimal lesions have a very low serological response which could not be detected using the AGID test with lower sensibility than ELISA test.

Given the difference in antibody titers and in the cellular population forming the three histological patterns, sheep can show different individual humoral response. The *histiocytic lesion pattern,* usually with severe lesions, can represent a strong but non-efficient humoral response. The *lymphocytic pattern,* with mild or moderate lesions despite the low OD values, seem to represent an efficient immune response against the viral replication, very likely cellular, mediated by CD4+ and CD8+ T cells (Polledo et al., 2012b). This would agree with previous results reported in HIV or feline immunodeficiency virus (FIV) (Freel et al., 2011).

Most of the animals with minimal lesions showed positive ELISA results but low OD values. Seroconversion occurs from 15 days to 4 months after infection as described by (Pépin et al., 1998; Lacerenza et al., 2006) and typical MV-like lesions would then appear. Animals with minimal lesions and *mixed lesion pattern* could represent an initial or latent infection with a non-defined immune response against the virus and with variable antibody secretion which could develop lymphocytic or histiocytic types of immune response with the evolution of the infection. This could mean that the tissue infection, the beginning of developing lesions and seroconversion occurs very early and almost concurrently. However, further experimental studies should be performed to proof this hypothesis. The few seronegative animals in this group could be sheep with minimal lesions and infection, though not significant enough to produce a peripheral or serological response detectable using an ELISA test. The presence of virus by IHC was

Table 1
ELISA optical densities, IHC and PCR results of every sheep studied. H: histiocytic lesion pattern; L: lymphocytic lesion pattern; M: mixed lesion pattern.

Pattern	OD	IHC	PCR
H1	1.14	+	
H2	1.35	+	
H3	1.39	+	
H4	1.5	+	+
H5	1.5	+	
H6	1.6	+	
H7	1.68	+	+
H8	1.8	+	
H9	1.88	+	
H10	1.95	+	
H11	2.14	+	
H12	2.19	+	
H13	2.27	+	
H14	2.3	+	
H15	2.4	+	
H16	2.4	+	
H17	2.47	+	+
H18	2.5	+	
H19	2.5	+	
H20	2.5	+	
H21	2.65	+	
H22	2.77	+	
H23	2.99	+	
L1	0.09	+	+
L2	0.25	+	+
L3	0.42	+	+
L4	0.5	+	+
L5	0.53	+	+
L6	0.73	+	
L7	0.75	+	+
L8	0.75	+	
L9	0.79	+	
L10	0.81	+	
L11	0.91	+	+
L12	1	+	+
L13	1.07	+	
L14	1.11	+	
L15	1.12	+	
L16	1.47	+	
L17	1.61	+	
L18	1.65	+	+
L19	1.81	+	+
M1	0.73	+	
M2	0.77	+	
M3	1.12	+	
M4	1.18	+	
M5	1.37	+	
M6	1.39	+	
M7	1.44	+	+
M8	1.45	+	

associated exclusively to macrophage-like cells, even in minimal lesions, being subjectively more abundant in histiocytic lesions compared to lymphocytic ones, as has been previously suggested (Polledo et al., 2012a).

To sum up, sheep with histiocytic type of lesions and easily recognizable using ELISA test could play a major role in the spreading of the infection compared animals with lymphocytic or mixed forms. However, animals with a *lymphocytic lesion pattern* could easily be misdiagnosed using ELISA methods due to the lower antibody production, going unnoticed in the flock as false negative animals in the control programs and staying on as carriers and source of infection. This fact should be considered in flocks with a low MV seroprevalence where these unobserved sheep could be the main source of MV infection. In conclusion, ELISA results are strongly associated to the type of histological lesion pattern, which are very likely conditioned by the individual immune response and therefore, seronegative infected carriers should be considered in control programs.

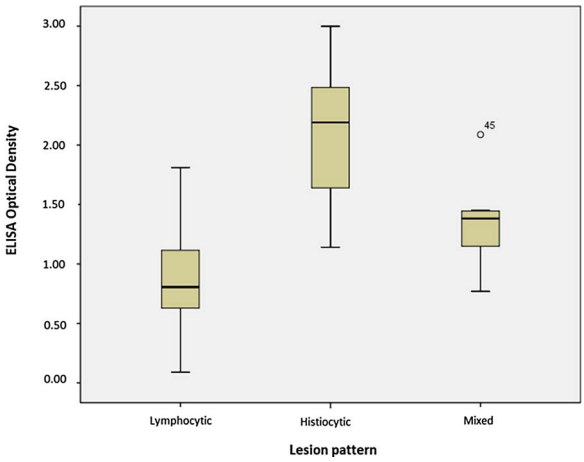

Fig. 3. serological values distribution in lymphocytic, histiocytic and mixed lesion patterns. Cut-off point: 0.4 ± 0.1.

Conflict of interest

None of the authors (EG, LP, AB, CM, MJGI, SP, GR, JFGM) have any financial and personal relationships that may pose any conflicts of interest with the results presented here.

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References

Benavides, J., García-Pariente, C., Fuertes, M., Ferreras, M.C., García-Marín, J.F., Juste, R.A., Pérez, V., 2009. Maedi-Visna: the meningoencephalitis in naturally occurring cases. J. Comp. Pathol. 140, 1–11.

Benavides, J., Fuertes, M., García-Pariente, C., Otaola, J., Delgado, L., Giraldez, J., García Marín, J.F., Carmen Ferreras, M., Pérez, V., 2013. Impact of maedi-visna in intensively managed dairy sheep. Vet. J. 197, 607–612.

Blacklaws, B.A., 2012. Small ruminant lentiviruses: immunopathogenesis of visna-maedi and caprine arthritis and encephalitis virus. Comp. Immunol. Microbiol. Infect. Dis. 35, 259–269.

Cutlip, R.C., Lehmkuhl, H.D., Schmerr, M.J.F., Brogden, K.A., 1988. Ovine progressive pneumonia (maedi-visna) in sheep. Vet. Microbiol. 17, 237–250.

Dawson, M., 1987. Pathogenesis of maedi-visna. Vet. Rec. 120, 451–454.

de Andrés, D., Klein, D., Watt, N.J., Berriatua, E., Torsteinsdottir, S., Blacklaws, B.A., Harkiss, G.D., 2005. Diagnostic tests for small ruminant lentiviruses. Vet. Microbiol. 107, 49–62.

de Andrés, X., Ramírez, H., Bertolotti, L., San Román, B., Glaria, I., Crespo, H., Jáuregui, P., Minguijón, E., Juste, R., Leginagoikoa, I., Pérez, M., Luján, L., Badiola, J.J., Polledo, L., García-Marín, J.F., Riezu, J.I., Borrás-Cuesta, F., de Andrés, D., Rosati, S., Reina, R., Amorena, B., 2013. An insight into a combination of ELISA strategies to diagnose small ruminant lentivirus infections. Vet. Immunol. Immunopathol. 152, 277–288.

Freel, S.A., Saunders, K.O., Tomaras, G.D., 2011. CD8 +T-cell-mediated control of HIV-1 and SIV infection. Immunol. Res. 49, 135–146.

Lacerenza, D., Giammarioli, M., Grego, E., Marini, C., Profiti, M., Rutili, D., Rosati, S., 2006. Antibody response in sheep experimentally infected with different small ruminant lentivirus genotypes. Vet. Immunol. Immunopathol. 112, 264–271.

Leginagoikoa, I., Daltabuit-Test, M., Álvarez, V., Arranz, J., Juste, R.A., Amorena, B., de Andrés, D., Luján, L.L., Badiola, J.J., Berriatua, E., 2006. Horizontal Maedi-Visna virus (MVV) infection in adult dairy-sheep raised under varying MVV-infection pressures investigated by ELISA and PCR. Res. Vet. Sci. 80, 235–241.

Luján, L., García Marin, J.F., Fernández de Luco, D., Vargas, A., Badiola, J.J., 1991. Pathological changes in the lungs and mammary glands of sheep and their relationship with maedi-visna infection. Vet. Rec. 129, 51–54.

Minguijón, E., Reina, R., Pérez, M., Polledo, L., Villoria, M., Ramírez, H., Leginagoikoa, I., Badiola, J.J., García-Marín, J.F., de Andrés, D., Luján, L., Amorena, B., Juste, R.A.,

2015. Small ruminant lentivirus infections and diseases. Vet. Microbiol. 181, 75–89.
Pépin, M., Vitu, C., Russo, P., Mornex, J.F., Peterhans, E., 1998. Maedi-visna virus infection in sheep: a review. Vet. Res. 29, 341–367.
Patel, J.R., Heldens, J.G.M., Bakonyi, T., Rusvai, M., 2012. Important mammalian veterinary viral immunodiseases and their control. Vaccine 30, 1767–1781.
Polledo, L., Gonzalez, J., Benavides, J., Martinez-Fernandez, B., Carmen Ferreras, M., Garcia Marin, J.F., 2012a. Perivascular inflammatory cells in ovine visna/maedi encephalitis and their possible role in virus infection and lesion progression. J. Neurovirol. 18, 532–537.
Polledo, L., González, J., Benavides, J., Morales, S., Martínez-Fernández, B., Delgado, L., Reina, R., Glaria, I., Pérez, V., Ferreras, M.C., García Marín, J.F., 2012b. Patterns of lesion and local host cellular immune response in natural cases of ovine maedi-visna. J. Comp. Pathol. 147, 1–10.
Polledo, L., Gonzalez, J., Fernandez, C., Miguelez, J., Martinez-Fernandez, B., Morales, S., Ferreras, M.C., Garcia Marin, J.F., 2013. Simple control strategy to reduce the level of maedi-visna infection in sheep flocks with high prevalence values (& gt; 90%). Small Ruminant Res. 112, 224–229.
Preziuso, S., Taccini, E., Rossi, G., Renzoni, G., Braca, G., 2003. Experimental maedi visna virus infection in sheep: a morphological, immunohistochemical and PCR study after three years of infection. Eur. J. Histochem. 47, 373–378.
Ryan, S., Tiley, L., McConnell, I., Blacklaws, B., 2000. Infection of dendritic cells by the Maedi-visna lentivirus. J. Virol. 74, 10096–11010.
Torsteinsdóttir, S., Georgsson, G., Gísladóttir, E., Rafnar, B., Pálsson, P.A., Pétursson, G., 1992. Pathogenesis of central nervous system lesions in visna: cell-mediated immunity and lymphocyte subsets in blood, brain and cerebrospinal fluid. J. Neuroimmunol. 41, 149–158.
Torsteinsdóttir, S., Carlsdóttir, H.M., Svansson, V., Matthíasdóttir, S., Martin, A.H., Pétursson, G., 2007. Vaccination of sheep with maedi-visna virus gag gene and protein, beneficial or harmful? Vaccine 25, 6713–6720.

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INFECTIOUS DISEASE

Inflammatory Lesion Patterns in Target Organs of Visna/Maedi in Sheep and their Significance in the Pathogenesis and Diagnosis of the Infection

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Summary

Ovine visna/maedi (VM) infection is characterized by the development of chronic inflammatory lesions in different organs, mainly in the lung, mammary gland and central nervous system (CNS), with either histiocytic or lymphocytic pattern predominance being described in the CNS. To help to understand the role of host immune response in the development of these patterns, 50 naturally-infected sheep and eight non-infected sheep from intensive milk-producing flocks were studied. The histological lesion patterns in the three main target organs in each sheep were characterized. Lesion severity was determined, including minimal lesions. A histiocytic pattern was observed in 23 sheep (46%), a lymphocytic inflammatory pattern in 19 sheep (38%) and a mixed inflammatory pattern in eight sheep (16%). Forty animals showed moderate or severe lesions (80%), while 10 had minimal lesions (20%). Moderate or severe lesions affected only one target organ in 20 sheep (50%), two organs in 14 sheep (35%) and all three target organs in six sheep (15%). Infection was confirmed by immunohistochemistry (IHC) using an antibody specific for p28 of VM virus/caprine arthritis and encephalitis virus and by polymerase chain reaction (PCR) in all sheep. Minimal inflammatory lesions associated with positive IHC and PCR were observed. The results suggest that the development of a predominant inflammatory pattern in different organs within the same animal may be related to the host immune response. Minimal and focal lesions, not considered previously, should be taken into account when formulating a differential diagnosis in affected sheep.

Keywords: immunopathology; lesion pattern; sheep; visna/maedi

Introduction

Ovine visna/maedi (VM) is a widespread disease caused by a retrovirus of the genus lentivirus and is related to caprine arthritis encephalitis virus (CAEV) and human immunodeficiency virus (Thormar, 2005). The disease has a significant economic impact, which is especially noted in flocks in northwest Spain where an intensive indoor farming system is widespread and seroprevalence reaches up to 96.8% (Sotelo, 1998; Peterhans *et al.*, 2004; Leginagoikoa *et al.*, 2006). VM is characterized by chronic inflammation of the lung, mammary gland and central nervous system (CNS) and rarely by arthritis. Microscopically, the changes in affected organs are interstitial pneumonia, mastitis and non-suppurative necrotizing encephalomyelitis, choroiditis and demyelination of the CNS (Cutlip *et al.*, 1979; Luján *et al.*, 1991; Benavides *et al.*, 2009; Minguijón *et al.*, 2015).

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The importance of cell-mediated immunity with regard to the severity of the lesions has been described for the CNS lesions (Torsteinsdóttir *et al.*, 1992; Polledo *et al.*, 2012a) and an individual immune response against VM virus (VMV) has been suggested to play a major role in the pathogenesis of the disease (Torsteinsdóttir *et al.*, 1992, 2007; Blacklaws, 2012; Polledo *et al.*, 2012a, b). In previous studies carried out on sheep with spontaneously arising neurological forms of VM, two main patterns of lesion were described with regard to the predominant inflammatory cells in the CNS infiltrates. The lymphocytic pattern is characterized by a predominance of T lymphocytes, particularly $CD8^+$ T cells, while the histiocytic pattern involves infiltration of macrophages and B lymphocytes (Polledo *et al.*, 2012b). Similar inflammatory patterns have been described in the lung and mammary gland (Gayo *et al.*, 2017).

In previous studies, moderate to severe VM lesions occurred in 35.5% of mammary glands and 32.3% of lungs in randomly selected sheep. These percentages increased to 58.1% and 54%, respectively, in seropositive sheep (Luján *et al.*, 1991). In the latter study, 25.6% of VM-seropositive sheep had no lesions in any organ, 20.2% had lesions in the mammary gland only, 16.2% had lesions in the lung only and 37.8% had lesions in both organs (Luján *et al.*, 1991). In subsequent studies an increase in these percentages was observed when mild lesions were also considered, with percentages of affected mammary glands rising to 46.7% (Benavides *et al.*, 2013). Minimal infiltrates of cells were not included in these investigations and were considered as normal, but the presence of virus in those lesions was not evaluated.

The aim of the present study was to determine whether the same inflammatory pattern is present in all of the target organs in individual infected sheep.

Materials and Methods

Animals

Fifty-eight Spanish Assaf sheep (1–3 years of age) from different intensive milk-producing flocks located in the northwest of Spain were selected from animals submitted to the Pathology Diagnostic Service, School of Veterinary Medicine, León, Spain, for routine necropsy examination. Fifty sheep were naturally infected and came from six VM-seropositive flocks subjected to VM control as described elsewhere (Polledo *et al.*, 2013). The seroprevalence in these flocks ranged between 62% and 97%. The remaining eight animals were selected as negative controls from three VMV-free flocks and showed no clinical signs. Sheep from affected flocks were culled because of loss of milk production and/or weight or because of the presence of neurological (e.g. hindlimb weakness, ataxia, hypermetria or paralysis usually leading to recumbency) or respiratory signs (e.g. dyspnoea). Animals in this study did not have gross or microscopical lesions compatible with other pathologies such as bacterial, fungal or parasitic pneumonias or mastitis.

Sampling, Histopathology and Immunohistochemistry

Tissue samples were collected systematically from the diaphragmatic and apical lung lobes, glandular parenchyma and udder cisterns and CNS from nine levels of the brain and nine levels of the spinal cord (Polledo *et al.*, 2012a). Mammary samples from six sheep and spinal cord samples from four sheep were not included in the histopathological studies as the tissues were not in good condition. Samples were fixed in 10% neutral buffered formalin for 48 h at room temperature. After fixation, samples were dehydrated through graded alcohols and embedded in paraffin wax. Sections (4 μm) were stained with haematoxylin and eosin (HE) and examined using light microscopy.

Additional sections (4 μm) were used for immunohistochemistry (IHC). The following primary antibodies were used: polyclonal anti-CD3 for T cells (Dako, Glostrup, Denmark); monoclonal anti-CD79 for B cells (Dako); monoclonal anti-CD68 for macrophages (Dako); and monoclonal anti-p28 of VMV/CAEV (VMRD, Pullman, Washington, USA) for viral detection. Subsequently, the sections were incubated for 30 min using the EnVision + system (EnVision + System Labelled Polymer-HRP anti-mouse or anti-rabbit; Dako) and labelling was 'visualized' using 3, 3′ diaminobenzidine as chromogen (Vector Laboratories, Burlingame, California, USA) for anti-CD3, CD79 and CD68 antibodies. An avidin–biotin–peroxidase complex (ABC) technique (Vectastain Elite, ABC Kit; Vector Laboratories) was used for detection of p28 of VMV/CAEV (Preziuso *et al.*, 2003). The slides were counterstained with haematoxylin and mounted. The specificity of the technique was evaluated using positive (previously confirmed tissues) and negative controls (previously confirmed tissues and non-immune serum replacing primary antibody).

Three lesion patterns were considered based on the characteristics of the inflammatory infiltrate. Histiocytic and lymphocytic patterns were considered as previously described (Polledo *et al.*, 2012b). Lymphocytic lesions were characterized by a predominance of $CD3^+$ cells within the inflammatory infiltrate, while in histiocytic lesions $CD68^+$ cells were the most abundant, together with some scattered $CD3^+$ and

$CD79^+$ cells. A mixed inflammatory infiltrate was composed of macrophages and lymphocytes with no predominance of any of the cellular populations (Gayo *et al.*, 2017).

Evaluation and description of the characteristics and severity of the lesions in the mammary gland, lung and CNS were based on previous reports (Luján *et al.*, 1991; Benavides *et al.*, 2013) with some specific modifications. 'No lesion' was defined when no inflammatory cells were observed within the CNS, lung or mammary gland. 'Minimal lesions' (+) consisted of a few small clusters (5–20) to multifocal small groups of inflammatory cells with the presence of a few small lymphoid aggregates and/or one small lymphoid follicle. 'Moderate lesions' (++) were characterized by multifocal to diffuse interstitial non-suppurative inflammation and/or the presence of two to 15 lymphoid aggregates/follicles. 'Severe lesions' (+++) consisted of a marked diffuse interstitial pneumonia, mastitis or encephalitis and/or the presence of >15 lymphoid aggregates/follicles. When differences between the severity of interstitial inflammatory infiltrates and the presence of lymphoid follicles were observed in the same organ of an animal, the most severe lesion was considered as the score for the lesion in that target organ. Lesion pattern in sheep with minimal lesions in one target organ and moderate or severe lesions in other organs were defined by considering the most severe pathology. Lesion patterns in sheep with only minimal lesions in the three target organs studies were defined on the basis of the predominant cell population observed.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed as described by Ryan *et al.* (2000) on CNS samples from 40 infected sheep, lung and CNS samples from six infected animals and lung and mammary gland samples from four infected sheep. Lung, mammary gland and CNS samples from the eight negative control animals were also studied. Genomic DNA was extracted from paraffin wax-embedded tissue samples using QIAamp® DNA Mini Kit (Qiagen, Valencia, California, USA). For CNS PCR studies a pool of spinal cord, medulla oblongata, pons and peduncles and mesencephalon was used.

Serology

Serum samples were obtained from the 58 sheep to evaluate the presence of antibodies against VMV using a standardized commercial test kit (Elitest®, Hyphen BioMed, Neuville-Oise, France) as described by Gayo *et al.* (2017).

Results

Histopathology and Immunohistochemistry

The 50 animals from the six infected flocks were positive for VMV by IHC and PCR and showed characteristic lesions of VM, while the eight negative control sheep were negative for virus detection and did not show abnormalities within the target organs. All of the VM-infected animals had lesions in the lungs, 36 infected animals had lesions in the CNS and 39 infected sheep had lesions in the mammary gland. Forty of these animals had moderate or severe lesions in one or more organs and displayed clinical signs of VM (80%). The lesions affected a single organ in 20 animals (50%), two organs in 14 animals (35%) and three organs in six animals (15%) (Table 1). Ten sheep showed only minimal lesions in the CNS, lung and/or mammary gland (20%).

A histiocytic pattern was observed in 23 animals (46%), a lymphocytic pattern in 19 sheep (38%) and a mixed pattern in eight sheep (16%). Severe lesions (+++) were more common in animals with a histiocytic pattern ($n = 16$; 69.6% of the histiocytic lesions) and they were observed to a lesser extent in sheep with a lymphocytic pattern ($n = 5$; 26.3% of the lymphocytic lesions). Sheep with the histiocytic pattern had moderate or severe lesions in at least one target organ. Fifteen sheep with the lymphocytic pattern showed moderate or severe lesions in at least one target organ (78.9%), while four animals had minimal lesions with no clinical signs affecting the three target organs studied (21.1%). Sheep with a mixed pattern had predominantly minimal lesions in two or three target organs and no clinical signs (75%), except for two animals with moderate lesions in the lung and mammary gland. The mixed inflammatory infiltrate in six sheep with only minimal lesions consisted of similar numbers of $CD3^+$ and $CD68^+$ cells. The other four sheep with only minimal lesions showed small groups of $CD3^+$ cells with only one or two macrophages within the interstitial

Table 1
Moderate/severe VM lesion distribution in the target organs

Moderate/severe lesions		Number of sheep	Total
1 Organ	CNS	8	
	Lung	3	20
	Mammary gland	9	
2 Organs	CNS/lung	4	
	CNS/mammary gland	5	14
	Lung/mammary gland	5	
3 Organs	CNS/lung/mammary gland	6	6
Total			40

inflammatory infiltrate, together with some small lymphocytic aggregates ($CD3^+$ cells) or one small lymphoid follicle. These were considered as having a lymphocytic pattern. $CD79^+$ cells were only observed in two of these animals with minimal lesions (one or two positive cells in each sheep within the inflammatory infiltrate). Minimal lesions in one organ of sheep with a global histiocytic pattern were always mixed, while minimal lesions in animals with severe lymphocytic lesions in other target organs were lymphocytic or mixed.

The lung and CNS always showed the same lesion pattern in the same animal. The inflammatory infiltrate in the mammary gland often consisted of a predominance of $CD3^+$ cells with a variable number of $CD68^+$ macrophages and some scattered $CD79^+$ B cells. Even if $CD3^+$ cells were predominant within the inflammatory infiltrate, more $CD68^+$ cells were usually observed in animals with a histiocytic pattern in the other target organs.

Mammary gland lesions are shown in Figs. 1A–C. Minimal (+) interstitial mastitis was observed in 15 sheep (37.5 %), moderate (++) in 18 sheep (45%) and severe (+++) in seven sheep (17.5%) (Table 2), with variable distribution in the severity of the inflammatory infiltrate and lymphoid hyperplasia among the mammary lobules. All infected animals had a predominance of $CD3^+$ cells within the inflammatory infiltrate of this target organ, with some scattered $CD79^+$ cells and a variable number of $CD68^+$ cells. No lesions were observed in the eight negative controls.

Lung lesions are shown in Figs. 1D–F. Minimal lesions (+) were observed in 32 sheep (64%), moderate lesions (++) in nine sheep (18%), severe lesions (+++) in nine animals (18%) and no lesions were observed in the eight negative controls (Table 2). No connection was observed between the severity of lymphoid hyperplasia and interstitial pneumonia. Some animals with >15 follicles showed a minimal

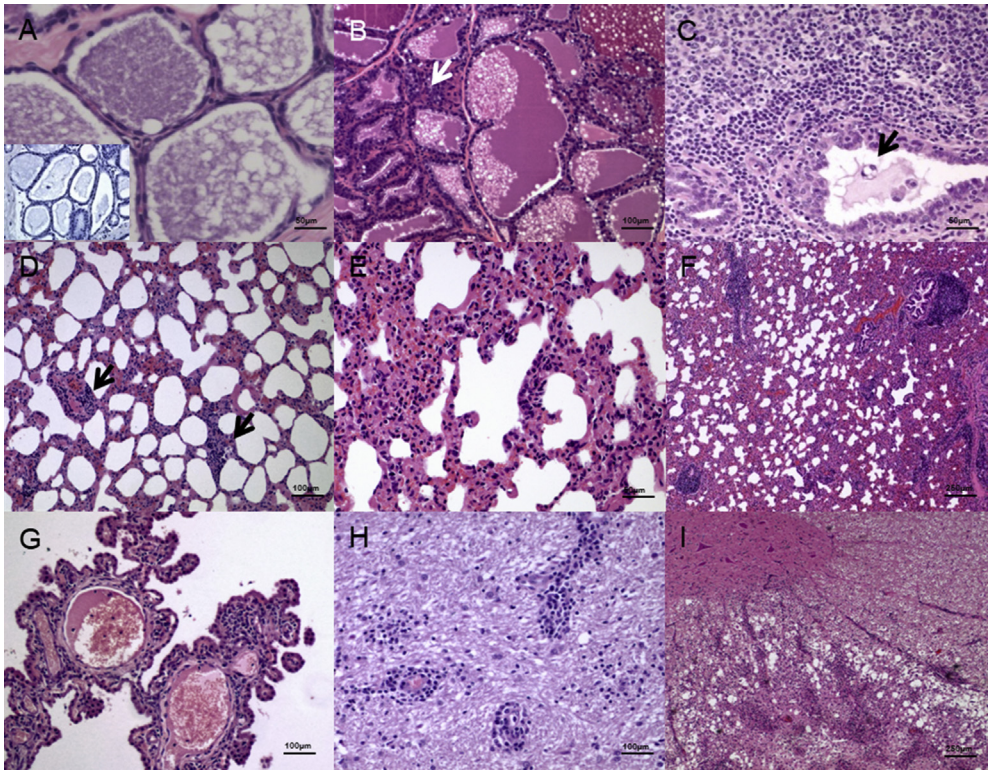

Fig. 1. (A–C) Visna/maedi (VM) lesions in the mammary gland. (A) Healthy mammary gland negative for p28 of VMV/CAEV by IHC. No inflammatory infiltrate is observed. (B) Minimal lesions (+). Presence of small focal inflammatory aggregates in the mammary interstitium, not affecting all lobules. (C) Severe lesions (+++) with a high number of lymphocytes and macrophages producing acinar destruction. Presence of macrophages in the acinar lumen (arrow). (D–F) VM lesions in the lung. (D) Minimal lesions (+) characterized by the presence of small and focal inflammatory aggregates (arrows). (E) Moderate lesions (++) characterized by a moderate interstitial pneumonia. (F) Severe lesions (+++) with lymphoid aggregates and follicles and thickening of the alveolar septa with collapse of alveolar spaces. (G–I) VM lesions in the central nervous system. (G) Minimal lesions in the choroid plexus (+) characterized by the presence of small and focal groups of inflammatory cells. (H) Moderate lesions in the brain (++). Presence of multilayered perivascular cuffs and mononuclear inflammation of the neuropil. (I) Severe lesions in the spinal cord (+++) with malacia and tissue destruction. HE.

Table 2
Occurrence of lesions due to VM in terms of interstitial infiltrate and hyperplasia of lymphoid follicles and relationship to the patterns of lesion observed

	Number with histiocytic lesions	Number with lymphocytic lesions	Number with mixed lesions	Total
CNS lesions				
+	4	6	3	13
+ +	2	4	–	6
+ + +	16	1	–	17
Total	**22**	**11**	**3**	**36**
Lung lesions				
+	11	14	7	32
+ +	7	1	1	9
+ + +	5	4	0	9
Total	**23**	**19**	**8**	**50**
Mammary gland lesions				
+	–	15	–	15
+ +	–	18	–	18
+ + +	–	7	–	7
Total	–	**40**	–	**40**

+, minimal lesions; + +, moderate lesions; + + +, severe lesions.

interstitial pneumonia and vice versa. The histiocytic pattern was observed in 23 animals with respiratory lesions (46%), a lymphocytic pattern in 19 animals (38%) and a mixed pattern in eight animals (16%).

CNS lesions are shown in Figs. 1G–I. The choroid plexus was the most affected tissue ($n = 32$), followed by the brain ($n = 21$) and the spinal cord ($n = 16$). Minimal lesions (+) were observed in 13 animals (36.1%), moderate lesions (+ +) in six (16.7%) and severe lesions (+ + +) in 17 (47.2%) (Table 2). Histiocytic lesions were observed in 22 animals (61.1%), a lymphocytic pattern in 11 animals (30.6%) and a mixed pattern in three sheep (8.3%).

Lymphocytic, histiocytic and mixed patterns, as well as different lesion severity and extent, in the three main target organs of the disease, were observed in sheep from all six infected flocks studied.

Viral antigen immunolabelling appeared as a fine brown deposit in the cytoplasm of macrophage-like cells in all the affected organs, while epithelial cells showed no positivity (Fig. 2). Labelled cells were observed within the interstitial inflammatory infiltrate and surrounding blood vessels. Positive labelling was always associated with lesions, even minimal lesions, and no labelling was detected in histologically unaffected areas. In the mammary gland, positive cells were located within the interstitial inflammatory infiltrates or within in the acinar lumina. In the lung, positive cells were observed occasionally in the bronchial and alveolar spaces, sometimes forming syncytia and within the inflammatory infiltrates (Figs. 2B, 2C). Positive labelling was more abundant in histiocytic lesions than in lymphocytic lesions. No labelled cells were detected in sections used as negative controls, but labelling was invariably seen in positive control sections.

Polymerase Chain Reaction

All fifty infected animals had a positive PCR from at least one target organ. Thirty six sheep were positive for all samples tested while five sheep were negative

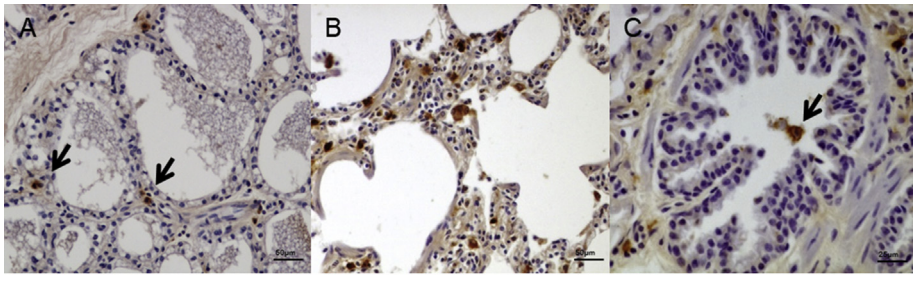

Fig. 2. (A) IHC for p28 of CAEV/VMV in the mammary gland. Scattered positive macrophages in minimal lesions (+) (arrows). (B) IHC for p28 of CAEV/VMV in the lung. Positive macrophages within the interstitial inflammatory infiltrate. (C) IHC for p28 of CAEV/VMV in the lung. Positive macrophages in the bronchial lumen (arrow) and in the peribronchial inflammatory aggregate.

with CNS samples, three were negative with lung samples, two were negative with udder samples, two were negative with udder and CNS samples and two were negative with lung and CNS tissues. Negative results were observed for organs with no lesions in six cases, with minimal lesions in ten cases and with moderate lesions in two cases. The eight negative controls were negative for all target organs.

Serology

Forty-five of the 50 infected animals were seropositive, three had inconclusive results (optical density of 0.4 ± 0.1) and two infected sheep were negative. The three sheep with inconclusive results showed lymphocytic lesions. The first animal had minimal lesions in the lung, mammary gland and brain, the second had minimal lesions in the lung and mammary gland and moderate lesions in choroid plexus, and the third had severe lesions in the lung and spinal cord and minimal lesions in the mammary gland. The two seronegative sheep had lymphocytic lesions. The first of these had minimal lesions in the lung and moderate lesions in the mammary gland and the second had severe lesions in the lung and mammary gland.

Discussion

This study describes in detail for the first time the histological inflammatory patterns of VM (i.e. histiocytic, lymphocytic and mixed) in the three main target organs of the disease in the same animals. The presence of the same inflammatory lesion pattern in the main target organs of VM in the same sheep and the higher number of $CD68^+$ cells in the mammary glands of sheep with the histiocytic pattern suggests that the host immune response plays a role in the development of the inflammatory infiltrate. The predominance of T lymphocytes observed in mammary glands with a variable number of macrophages may be related to a different local immunological response in this target organ, perhaps reflecting a higher T helper (Th) 1-mediated response. This may be due to the mucosal nature of the mammary gland, which has an outward-facing surface with continuous antigen stimulation (Butler *et al.*, 2015), especially in this type of dairy sheep with long lactation periods and intensive mechanical milking methods.

Histiocytic and lymphocytic patterns previously described in the CNS (Polledo *et al.*, 2012b) and the mixed pattern with both histiocytic and lymphocytic characteristics (Gayo *et al.*, 2017) are described in the three main target organs of the disease. The mixed pattern could represent an initial or latent infection with a non-defined immunopathological response that would evolve to become a lymphocytic or histiocytic lesion with progression of the infection. This might explain why in previous studies (Polledo *et al.*, 2012b), where only animals with moderate and severe CNS lesions were included, this lesion pattern was not observed. The fact that minimal lesions in one organ of sheep with a global histiocytic pattern were mixed, while minimal lesions in animals with severe lymphocytic lesions in other target organs were lymphocytic or mixed, would support the hypothesis of an evolution of mixed forms to a lymphocytic or histiocytic pattern. Minimal lymphocytic lesions were never observed together with a predominance of $CD68^+$ cells in moderate or severe lesions in other target organs of the same sheep. This suggests that the beginning of an infection or the latent stages of a mixed pattern could evolve to become either lymphocytic or histiocytic patterns; however, it would be unlikely that lymphocytic lesions become histiocytic or vice versa, as has been demonstrated in natural and experimental mycobacterial infections (Juste *et al.*, 1994; Delgado *et al.*, 2013). It should be noted, however, that this is a field study which shows the pathological features of a group of animals at the moment of death and further detailed experimental studies would be necessary to confirm this hypothesis.

The association between the lesion pattern and the stage of VM and the amount of viral antigen as determined by IHC has been described previously for CNS lesions (Polledo *et al.*, 2012b). It was proposed that lymphocytic lesions in the CNS form of VM represent some type of natural resistance to viral replication, with an initial recruitment of lymphocytes into the CNS as the first response to viral infection and with scarce presence of viral antigen in tissue, as was observed in the present study in animals with the lymphocytic lesion pattern. The severe lymphocytic lesions observed, even when located simultaneously in the CNS, lung and mammary gland, could be related to local releasing of pro-inflammatory cytokines, contributing to a chronic stage of inflammation and injury of the affected tissue. However, histiocytic lesions have been associated with a poor individual response or greater virulence of the viral strain, with a higher amount of virus detected by IHC (Polledo *et al.*, 2012b), as observed in the present study. The cellular infiltrate in other lentivirus infections, such as with simian immunodeficiency virus or human immunodeficiency virus, has similar features with macrophages comprising of 90–95% of the infiltrating cells and $CD8^+$ T cells comprising the remainder of the population (Lackner *et al.*, 1991; Kim *et al.*, 2004).

Severe lesions were present only in one organ in 40% of the animals and in two organs in 28% (total

68%) of the sheep, regardless of the flock and with a predominance of CNS lesions. This may be related to the high prevalence of CNS cases in the study area (Benavides *et al*., 2006), where a neurotropic strain of the virus is proposed to circulate (Glaria *et al*., 2012). However, in the present study, all organs were affected with moderate or severe lesions, which we consider to be related to the host immune response more than the viral strain. All of the flocks studied were from the same geographical area and animals from the same flock showed different lesion patterns, severity and extent, despite the possibility that similar strains of virus would circulate in the same flock or area (Glaria *et al*., 2012). The possible route of migration through vascular spaces and progression of the lesions (Dawson, 1987; Polledo *et al*., 2012a) in terms of host immunity should also be considered. Genetic factors could play an important role in the development of this disease because there is a high inbreeding in Spanish Assaf sheep, which have increased from a few hundred animals in the 1970s to almost two million animals today.

VMV infection was confirmed by IHC in all of the affected organs including those having minimal lesions, and most of these organs also tested positive by PCR. The PCR-negativity of some individual organs with characteristic VM lesions in confirmed infected sheep might be due either to degradation of proviral DNA after formalin fixation (Srinivasan *et al*., 2002) or to a low virus load in those particular organs. The latter has been suggested to be one of the main difficulties in VMV detection by PCR (de Andrés *et al*., 2005). Most of the sheep were also seropositive to VMV. Animals with inconclusive or negative ELISA results showed a lymphocytic pattern as previously described (Gayo *et al*., 2017), which has been related to a lesser antibody production in comparison with histiocytic lesions.

In two previous studies, the percentage of VM lesions in the lungs of randomly selected sheep was 32.3% and 79.3% in intensively-managed dairy sheep farms (Luján *et al*., 1991; Benavides *et al*., 2013), reaching 100% in our study. Likewise, in the present study, 80% of sheep had mammary lesions in contrast with previous studies showing mammary lesions in 35.6% of the sheep (considering only moderate and severe changes) (Luján *et al*., 1991) or reaching up to 46.7% of animals taking into account mild (but not minimal) cellular infiltrates (Benavides *et al*., 2013).

IHC for VMV antigen is a sensitive means of detecting infection in the lung, CNS and mammary gland. Viral antigen was associated exclusively with macrophage-like cells, even within minimal lesions and no antigen was observed in areas without lesions. The presence of positive cells mainly in perivascular spaces supports the hypothesis of viral invasion via infiltrating monocytes/macrophages (Peluso *et al*., 1985; Polledo *et al*., 2012a).

In conclusion, histiocytic, lymphocytic or mixed inflammatory infiltrates may occur in the lung, CNS and mammary gland of sheep with VM. The inflammatory pattern in the lung and CNS was consistent in every infected sheep, a finding that suggests a similar host immune response in each organ. In contrast, in affected udders the inflammatory infiltrate was always predominantly lymphocytic, with variable numbers of macrophages, and this may be related to a tissue-specific immune response. Minimal inflammatory lesions not previously considered were detected and these were associated with viral antigen. Such lesions should be taken into account not only for the diagnosis of VM, but also in assessing risks of viral transmission. The results of this study extend the understanding of the pathology and pathogenesis of VM and establish a pathological model for further experimental studies.

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Conflict of interest statement

None of the authors has any financial or personal relationships that may pose any conflict of interest with the present study.

References

Benavides J, Fuertes M, García-Pariente C, Otaola J, Delgado L *et al*. (2013) Impact of maedi-visna in intensively managed dairy sheep. *The Veterinary Journal*, **197**, 607–612.

Benavides J, García-Pariente C, Fuertes M, Ferreras MC, García-Marín JF *et al*. (2009) Maedi-visna: the meningoencephalitis in naturally occurring cases. *Journal of Comparative Pathology*, **140**, 1–11.

Benavides J, Gómez N, Gelmetti D, Ferreras MC, García-Pariente C *et al*. (2006) Diagnosis of the nervous form of maedi-visna infection with a high frequency in sheep in Castilla y León, Spain. *The Veterinary Record*, **158**, 230–235.

Blacklaws BA (2012) Small ruminant lentiviruses: immunopathogenesis of visna-maedi and caprine arthritis and encephalitis virus. *Comparative Immunology, Microbiology and Infectious Diseases*, **35**, 259–269.

Butler JE, Rainard P, Lippolis J, Salmon H, Kacskovics I (2015) The mammary gland in mucosal and regional

immunity. In: *Mucosal Immunology*, 4th Edit, Vol. 2–2, J Mestecky, W Strober, M Russell, B Kelsall, H Cheroutre *et al.*, Eds., Academic Press, Boston, pp. 2269–2306.

Cutlip RC, Jackson TA, Lehmkuhl HD (1979) Lesions of ovine progressive pneumonia: interstitial pneumonitis and encephalitis. *American Journal of Veterinary Research*, **40**, 1370–1374.

Dawson M (1987) Pathogenesis of maedi-visna. *Veterinary Record*, **120**, 451–454.

de Andrés D, Klein D, Watt NJ, Berriatua E, Torsteinsdottir S *et al.* (2005) Diagnostic tests for small ruminant lentiviruses. *Veterinary Microbiology*, **107**, 49–62.

Delgado L, García Marín JF, Muñoz M, Benavides J, Juste RA *et al.* (2013) Pathological findings in young and adult sheep following experimental infection with 2 different doses of *Mycobacterium avium* subspecies *paratuberculosis*. *Veterinary Pathology*, **50**, 857–866.

Gayo E, Polledo L, Preziuso S, Rossi G, Balseiro A *et al.* (2017) Serological ELISA results are conditioned by individual immune response in ovine maedi visna. *Small Ruminant Research*, **157**, 27–31.

Glaria I, Reina R, Ramírez H, de Andrés X, Crespo H *et al.* (2012) Visna/maedi virus genetic characterization and serological diagnosis of infection in sheep from a neurological outbreak. *Veterinary Microbiology*, **155**, 137–146.

Juste RA, García Marín JF, Peris B, Saez de Ocariz C, Badiola JJ (1994) Experimental infection with *Mycobacterium paratuberculosis* in vaccinated and non-vaccinated lambs. *Journal of Comparative Pathology*, **110**, 185–194.

Kim WK, Corey S, Chesney G, Knight H, Klumpp S *et al.* (2004) Identification of T lymphocytes in simian immunodeficiency virus encephalitis: distribution of $CD8^+$ T cells in association with central nervous system vessels and virus. *Journal of Neurovirology*, **10**, 315–325.

Lackner AA, Smith MO, Munn RJ, Martfeld DJ, Gardner MB *et al.* (1991) Localization of simian immunodeficiency virus in the central nervous system of rhesus monkeys. *American Journal of Pathology*, **139**, 609–621.

Leginagoikoa I, Daltabuit-Test M, Álvarez V, Arranz J, Juste RA *et al.* (2006) Horizontal maedi-visna virus (MVV) infection in adult dairy-sheep raised under varying MVV-infection pressures investigated by ELISA and PCR. *Research in Veterinary Science*, **80**, 235–241.

Luján L, García Marin JF, Fernández de Luco D, Vargas A, Badiola JJ (1991) Pathological changes in the lungs and mammary glands of sheep and their relationship with maedi-visna infection. *The Veterinary Record*, **129**, 51–54.

Minguijón E, Reina R, Pérez M, Polledo L, Villoria M *et al.* (2015) Small ruminant lentivirus infections and diseases. *Veterinary Microbiology*, **181**, 75–89.

Peluso R, Haase A, Stowring L, Edwards M, Ventura P (1985) A Trojan horse mechanism for the spread of visna virus in monocytes. *Virology*, **147**, 231–236.

Peterhans E, Greenland T, Badiola J, Harkiss G, Bertoni G *et al.* (2004) Routes of transmission and consequences of small ruminant lentiviruses (SRLVs) infection and eradication schemes. *Veterinary Research*, **35**, 257–274.

Polledo L, Gonzalez J, Benavides J, Martinez-Fernandez B, Carmen Ferreras M *et al.* (2012a) Perivascular inflammatory cells in ovine Visna/maedi encephalitis and their possible role in virus infection and lesion progression. *Journal of Neurovirology*, **18**, 532–537.

Polledo L, González J, Benavides J, Morales S, Martínez-Fernández B *et al.* (2012b) Patterns of lesion and local host cellular immune response in natural cases of ovine maedi-visna. *Journal of Comparative Pathology*, **147**, 1–10.

Polledo L, Gonzalez J, Fernandez C, Miguelez J, Martinez-Fernandez B *et al.* (2013) Simple control strategy to reduce the level of maedi-visna infection in sheep flocks with high prevalence values (>90%). *Small Ruminant Research*, **112**, 224–229.

Preziuso S, Taccini E, Rossi G, Renzoni G, Braca G (2003) Experimental maedi visna virus infection in sheep: a morphological, immunohistochemical and PCR study after three years of infection. *European Journal of Histochemistry*, **47**, 373–378.

Ryan S, Tiley L, McConnell I, Blacklaws B (2000) Infection of dendritic cells by the maedi-visna lentivirus. *Journal of Virology*, **74**, 10096–10103.

Sotelo MJ (1998) *La infección por lentivirus en ovinos y caprinos de la provincia de León: aspectos epidemiológicos y valoración de un plan de control*. PhD Thesis. University of León, Spain.

Srinivasan M, Sedmak D, Jewell S (2002) Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *American Journal of Pathology*, **161**, 1961–1971.

Thormar H (2005) Maedi-visna virus and its relationship to human immunodeficiency virus. *AIDS Reviews*, **7**, 233–245.

Torsteinsdóttir S, Carlsdóttir HM, Svansson V, Matthíasdóttir S, Martin AH *et al.* (2007) Vaccination of sheep with maedi-visna virus gag gene and protein, beneficial or harmful? *Vaccine*, **25**, 6713–6720.

Torsteinsdóttir S, Georgsson G, Gísladóttir E, Rafnar B, Pálsson PA *et al.* (1992) Pathogenesis of central nervous system lesions in visna: cell-mediated immunity and lymphocyte subsets in blood, brain and cerebrospinal fluid. *Journal of Neuroimmunology*, **41**, 149–158.

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Article

Genetic Characterization and Phylogenetic Analysis of Small Ruminant Lentiviruses Detected in Spanish Assaf Sheep with Different Mammary Lesions

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Abstract: Small Ruminant Lentiviruses (SRLVs) are widespread in many countries and cause economically relevant, slow, and persistent diseases in sheep and goats. Monitoring the genetic diversity of SRLVs is useful to improve the diagnostic tools used in the eradication programs. In this study, SRLVs detected in Spanish Assaf sheep with different grades of lymphoproliferative mastitis were sequenced. Genetic characterization showed that most samples belonged to type A and were closer to Spanish SRLV isolates previously classified as A2/A3. Four samples belonged to subtype B2 and showed higher homology with Italian B2 strains than with Spanish B2 isolates. Amino acid sequences of immuno-dominant epitopes in the gag region were very conserved while more alterations were found in the LTR sequences. No significant correlations were found between grades of mastitis and alterations in the sequences although samples with similar histological features were phylogenetically closer to each other. Broader genetic characterization surveys in samples with different grades of SRLV-lesions are required for evaluating potential correlations between SRLV sequences and the severity of diseases.

Keywords: sheep; Small Ruminant Lentivirus; genetic characterization; udder; histopathology

1. Introduction

Small Ruminant Lentiviruses (SRLVs) include *Visna-maedi virus* (VMV) and *Caprine arthritis encephalitis virus* (CAEV), which cause slow inflammatory diseases in sheep and goats named, respectively, Visna-Maedi (VM) and Caprine Arthritis Encephalitis (CAE). Viruses are transmitted mainly via the respiratory route and by colostrum intake [1,2] and cause persistent infections with a long incubation period. According to the current nomenclature based on *gag–pol* and *pol* sequences, SRLV can be subdivided into genotypes A–E with subtypes present in A, B, and E [3–5]. Typically, VMV was believed to infect specifically sheep and was included in genotype A while CAEV was considered goat-specific and was included in genotype B. However, several investigations showed that cross-infection may occur ([6] and other studies reviewed in [5]). Infected animals can develop neurological, pulmonary, arthritic, and/or mammary diseases that affect considerable animal welfare and production. Different patterns of inflammatory mononuclear cell accumulation are observed usually in the central nervous system, lung, joint, and/or udder and the predominant clinical

manifestation depends on the severity and extension of the lesions reached in the affected organs [7]. Mammary lesions often consist of mononuclear cell infiltration with scattered hyperplastic lymphoid follicles [7,8]. Moderate lesions are described when diffused infiltration of lymphocytes within lobules with distortion of acini are observed and mild lesions are reported when occasional aggregates of lymphocytes in inter-acinar stroma are present [9]. Recently, minimal lesions consisting of a few small clusters to multifocal small groups of inflammatory cells with the presence of a few small lymphoid aggregates and/or one small lymphoid follicle have been reported in SRLV infected sheep [10].

One of the most important productive impacts of SRLV disease is due to the premature removal of diseased animals because of low milk production and quality with consequent economic losses in the milk-related and lamb/kids-related industry [11–13]. Due to the significant economic impact of diseases, VM and CAE are included in the OIE List and specific control and eradication programs are carried out in many countries [14,15]. Availability of sensitive and specific diagnostic tests is of great importance for a correct discrimination between infected and non-infected animals. Due to the high rate of genetic diversity, new genotypes and subtypes might escape the diagnostic detection with the possible consequence of invalidating any eradication program in place [1]. Therefore, genotype and subtype surveys of the circulating SRLVs should be encouraged. Many studies describe phylogenetic analysis of SRLVs found during epidemiological surveys or in outbreaks of diseases, but only a few of them describe the histopathological lesions observed in target organs [16–18]. For example, mild mammary lesions with a multifocal method to diffuse mononuclear inflammatory interstitial infiltrates have been observed in sheep with arthritis and infected by B2 SRLV [17]. Different histopathological scores have been reported in mammary glands but not in lungs, synovial membranes of joints, or the chorioid plexus of five goats infected by A4 SRLV [16]. A 13–14 nucleotide deletion in the R region of the LTR has been observed in sheep with a decreased pathology in the lung but not in the udder even though SRLV subgenotypes were not known [18]. To our knowledge, correlations among histopathological grading of mammary lesions and SRLV genotypes and subtypes are yet to be investigated.

The aim of this work was to carry out genetic characterization and phylogenetic analysis of SRLV detected in Spanish sheep showing different histopathological grades of mastitis.

2. Material and Methods

2.1. Samples

A total of 35 udder samples were collected randomly at the slaughterhouse in the region of Castilla y Leon, Northwestern Spain, from Assaf sheep (1–4 years of age) belonging to seropositive flocks between March 2017 and May 2017. Nineteen samples were collected at the slaughterhouse named M (samples M1–M19) and 16 were collected at the slaughterhouse named Q (samples Q1–Q16), which was about 45 Km far from the slaughterhouse M. A first aliquot of each sample was stored at −20 °C and DNA was obtained from 25 mg of each sample by using the Genomic DNA isolation Kit (Norgen Biotek Corp., Thorold, ON, Canada) and following the manufacturer's instructions when eluting the DNA in 100 µL final volume. A second aliquot of samples was fixed in 10% neutral buffered formalin for 48 h at room temperature and embedded in paraffin wax (FFPE) for histopathology and immunohistochemistry (IHC). In addition, 2 FFPE mammary samples (N16-426 and N-17-44) of sheep (Assaf breed, 3 years old) with histological mastitis referable to SRLV disease were available for this study. DNA from these latter samples was obtained from 4 slides 10 µm thick of each sample by Recover All Total Nucleic Acid Isolation (Ambion, Waltham, MA, USA) following the instructions and eluting DNA with 60 µL of elution solution warmed up at 95 °C.

2.2. Histopathology and Immunohistochemistry

Slides 4 µm thick were obtained from FFPE samples and were stained with haematoxylin and eosin (HE) for histopathology. Grading of histopathological lesions of mammary glands was carried

out by three independent pathologists, which was previously described [10]. Briefly, "no lesions" was defined when no inflammatory cells were observed and "minimal lesions" (+) consisted of a few small clusters.

Multifocal small groups of inflammatory cells with the presence of a few small lymphoid aggregates and/or one small lymphoid follicle known as 'moderate lesions' (++) were characterized by the multifocal method to diffuse interstitial non-supportive inflammation and/or the presence of two to 15 lymphoid aggregates/follicles and 'severe lesions' (+++) consisted of a marked diffuse interstitial mastitis and/or the presence of >15 lymphoid aggregates/follicles. When differences between the severity of interstitial inflammatory infiltrates and the presence of lymphoid follicles were observed in the same organ of an animal, the most severe lesion was considered as the score for the lesion in that target organ [10].

Serial sections (4 µm) were used for IHC, which was previously reported [19]. A monoclonal antibody to the SRLV core protein p28 (VMRD Inc., Pullman, WA, USA) diluted 1:1000 was used. A technique based on an avidin-biotin-peroxidase complex (VECTASTAIN® ELITE® ABC Kits, Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine as the chromogen (DAB Peroxidase substrate kit—Vector Laboratories, Burlingame, CA, USA) was used to stain the antigen.

2.3. PCR

A nested PCR was used to amplify about 800 bp long sequences of SRLV *gag-pol* genes, which was reported previously [4]. Primers GAG-F1 and POL-R1 were used in the first PCR. The product obtained was used as a template in a second PCR with primers GAG-F2 and POL-R2. The PCR mixture included 50 µL 2× Taq PCR Master Mix, 500 nM each primer, 4 µL DNA, and PCR grade water up to 100 µL final volume. PCR conditions were 94 °C for 5 min, 45 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension of 72 °C for 10 min. The second PCR was carried out with the same conditions but 5 µL of the first PCR products were used as the template and the annealing temperature was 60 °C instead of 55 °C [4]. LTR sequences (203 bp long) were amplified by nested PCR with primers described elsewhere [20]. The PCR reaction mix was described above, but 2 µL of DNA (first PCR) or 2 µL of the first PCR products (second PCR) were used as the template. PCR conditions were 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C (or 50 °C in the second PCR) for 30 s, 72 °C for 40 s, and a final extension of 72 °C for 7 min. PCR products were visualized in 1.5% agarose gel and positive samples were submitted to an external laboratory for sequencing (BMR Genomics, Padova, Italy). Both the sense and antisense strands were sequenced by performing two independent reactions for each PCR product. Nucleotide *gag-pol* sequences were deposited in GenBank (Accession numbers MH179145—MH179153 and MH179156—MH179159).

2.4. Sequence Analysis

Nucleotide sequences were manually checked and edited with the program BioEdit. A preliminary analysis by BLASTn was carried out to detect regions of similarity with sequences included in databases. Sequences of strains considered to be prototypes of different genotypes and SRLV sequences highly similar to those found in the samples were included in the study (Figure 1 and Figure 3). Sequences were aligned by MUSCLE [21] and phylogenetic trees were inferred with the program MEGA 7.0.21 [22]. The best-fitting nucleotide substitution models were estimated and the General Time Reversible model [23] with a gamma distribution with invariant sites (*gag* sequences) or a Kimura 2-parameter (LTR sequences) model [24] with gamma-distributed rates among sites were used with bootstrap values based on 1000 repetitions. Phylogeny was estimated by both the neighbor-joining algorithm (NJ) and the maximum likelihood (ML) method. Correlations among sequence alterations and histological features were evaluated by using the Fisher's exact test. Pairwise distances between sequences of samples and sequences of reference strains belonging to different genotypes were calculated by MEGA 7.0.21 with the p-distance model [22].

3. Results

Histological examination and grading of mammary lesions (see Figure 1) resulted in five samples with severe lesions, 13 samples with moderate lesions, 11 samples with minimal lesions, and five samples without lesions (see Table 1). Grading of M5 sample was not possible due to a concomitant purulent mastitis.

IHC results were used to distinguish SRLV infected from uninfected sheep. Three out of the five samples without lesions (M13, M18 and Q2) were negative by IHC and by both *gag-pol* and LTR PCR. Therefore, they were considered negative (see Table 1). The remaining 32 samples of groups M and Q were positive by IHC (see Figure 2). Sixteen out of the 32 M and Q samples and the 2 N samples were positive by *gag-pol* PCR, but good-quality sequences were obtained only from 15 samples. LTR PCR products were obtained in all but two IHC-positive samples.

Table 1. List of ovine mammary gland samples collected for this study. Samples are classified on the basis of the grade of mastitis observed by histopathology. Lesions in sample M5 were not classified due to concomitant purulent mastitis was present. "+": positive result, "−": negative result.

Sample	Grade of Mastitis	IHC	LTR PCR	*Gag-pol* PCR	Genotype
M1	moderate	+	+	+	B2
M2	minimal	+	+	−	−
M3	severe	+	+	+	B2
M4	moderate	+	+	−	−
M5	not classified	+	+	+	A2/A3
M6	moderate	+	+	−	−
M7	moderate	+	+	+	−
M8	moderate	+	+	+	−
M9	severe	+	+	+	−
M10	minimal	+	+	−	−
M11	moderate	+	+	+	−
M12	minimal	+	+	+	A2/A3
M13	no	−	−	−	−
M14	moderate	+	+	−	−
M15	minimal	+	+	+	A2/A3
M16	minimal	+	+	−	−
M17	moderate	+	+	+	B2
M18	no	−	−	−	−
M19	moderate	+	+	+	A2/A3
Q1	no	+	+	+	B2
Q2	no	−	−	−	−
Q3	minimal	+	−	−	−
Q4	minimal	+	+	−	−
Q5	minimal	+	+	−	−
Q6	severe	+	+	−	−
Q7	no	+	+	+	A2/A3
Q8	severe	+	+	+	A2/A3
Q9	moderate	+	+	−	−
Q10	severe	+	+	+	A2/A3
Q11	moderate	+	+	−	−
Q12	moderate	+	+	+	−
Q13	minimal	+	−	−	−
Q14	moderate	+	+	−	−
Q15	minimal	+	+	−	−
Q16	minimal	+	+	−	−
N16-426	severe	+	+	+	A2/A3
N17-44	moderate	+	+	+	A2/A3

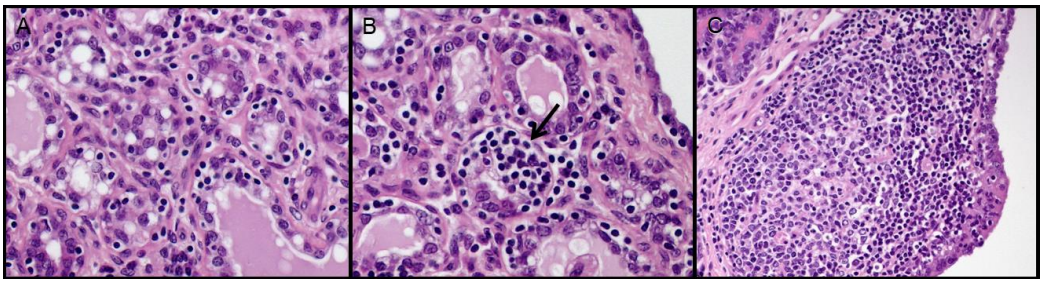


Figure 1. SRLV lesions in mammary gland of sheep. (**A**) Minimal lesion (+) with small focal lymphocyte aggregates within the mammary interstitium. 40×. (**B**) Black arrow indicates moderate (++) focal inflammatory lesion surrounded by minimal lymphocytic infiltrates. 40×. (**C**) Large lymphoid follicle in a severe lesion (+++). Hematoxylin and eosin (HE) staining. 20×.

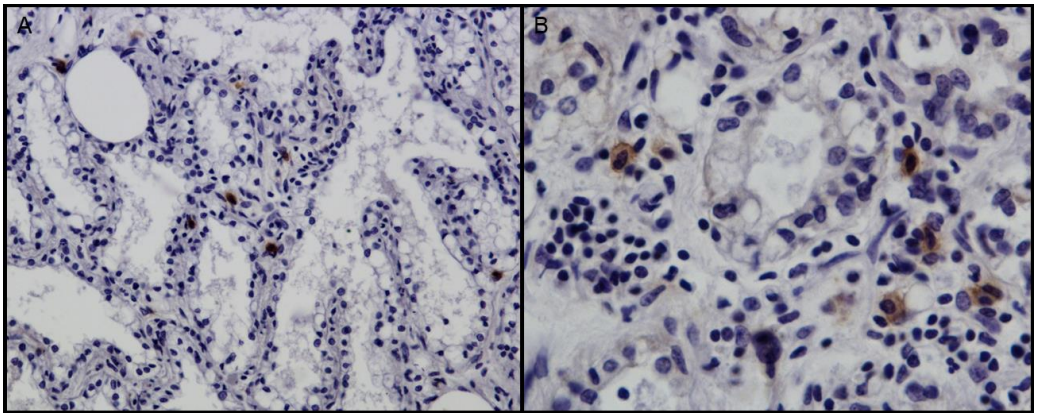


Figure 2. IHC against p28 of CAEV/VMV in mammary gland. (**A**) Scattered positive cells within minimal inflammatory lesions (+). 20×. (**B**) Positive macrophage-like cell in moderate lesion (++). 40×.

3.1. Analysis of Gag Sequences

Genotyping was carried out by phylogenetic analysis of partial *gag* gene sequences, according to the taxonomic classification proposed by Shah et al. [3]. All sequences were different from each other and nine samples were type A and four samples were type B. In particular, samples M5, M12, M15, M19, Q7, Q8, Q10, N16-426M, and N17-44M clustered within genotype A were more closely related with strains 292, 160, 166, and 697, which were previously detected in the same Spanish region (see Figure 3). Only strain 697 had been fully sequenced. Since similar values located with this isolate intermingled between A2 and A3, the isolate 697 had been assigned to the A2/A3 subtype [25]. Samples M1, M3, M17, and Q1 resulted of genotype B and subtype B2 (see Figure 3). Additionally, phylogenic and BLAST analysis showed that they were more related to B2 viruses detected in Italy than in Spain (Ov496). These results were confirmed by the pairwise distances comparison (see Table 2). Samples M12, M15, M19, Q7, Q8, Q10, N16-426, and N17-44 were more closely related to the A2/A3 Spanish strain HQ848062.1 (0.105–0.142). Moreover, samples M1, M3, M17, and Q1 were more closely related to the B2 strains FJ195346.1 and EU010126.1. In particular, they were more closely related to the Italian strain EU010126.1 (0.064–0.081) than to the Spanish strain FJ195346.1 (0.092–0.102).

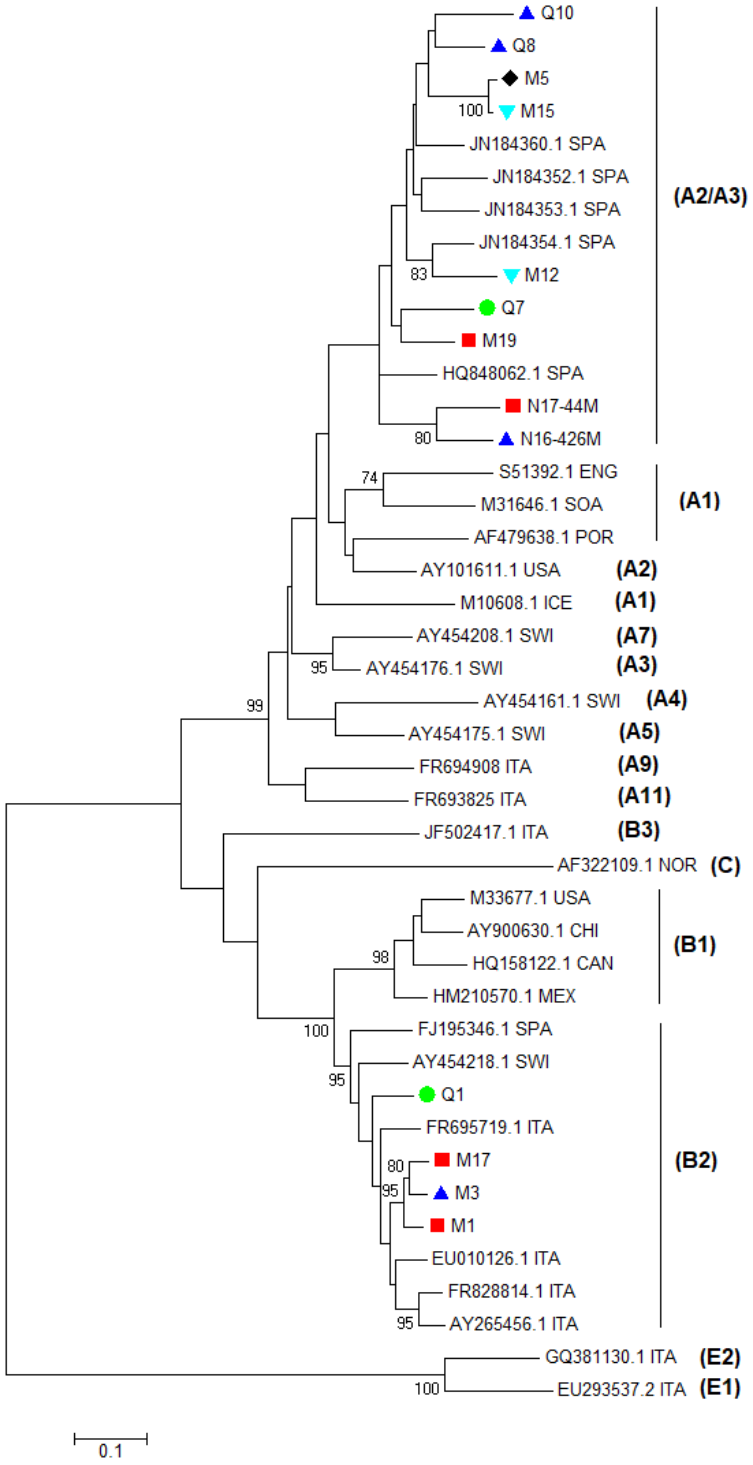


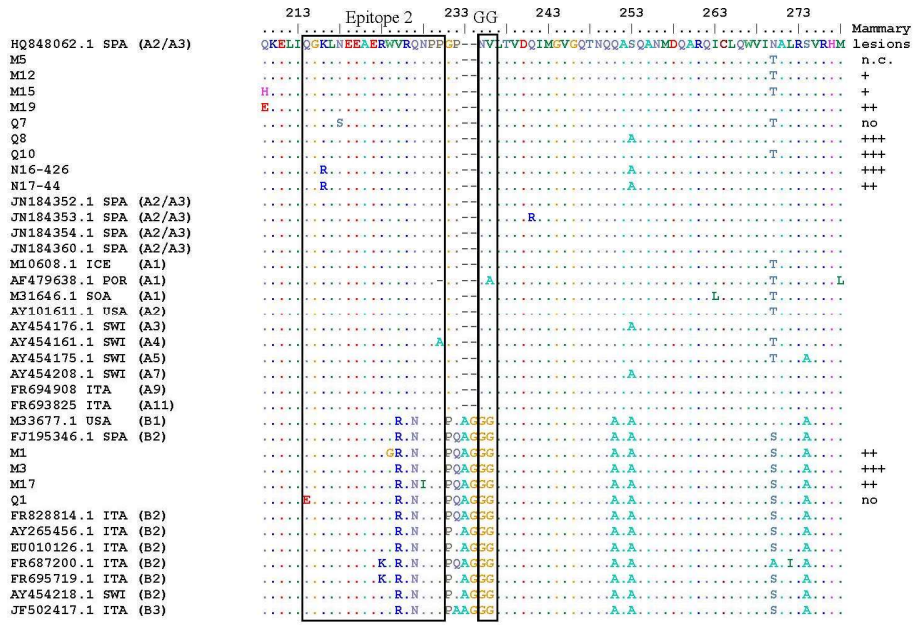
Figure 3. Phylogenetic analysis of the SRLV partial *gag-pol* region. Sequences of different SRLV genotypes and subtypes available in GenBank were used as reference isolates. Reference sequences are indicated with their accession number and country of origin (CAN: Canada; CHI: China; ENG: England; ICE: Iceland; ITA: Italy; MEX: Mexico; NOR: Norway; POR: Portugal; SOA: South Africa; SPA: Spain; SWI: Switzerland; USA: the U.S.A.). Samples are indicated with their codes and are labeled on the basis of the score of the mammary lesions observed (▲ severe, ■ moderate, ▼ minimal, ● no lesions, ♦ not available). The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model with a gamma distribution with invariant sites and with bootstrap values based on 1000 repetitions. The tree is unrooted.

Table 2. Pairwise nucleotidic genetic distances (p-distance model) of the partial *gag-pol* region of some SRLV reference strains and SRLV strains sequenced in this study.

Sample	Genotype	M5	M12	M15	M19	Q7	Q8	Q10	N16-426	N17-44	M1	M3	M17	Q1
M12		0.112	-											
M15		0.013	0.013	-										
M19		0.013	0.122	0.104	-									
Q7		0.130	0.133	0.129	0.109	-								
Q8		0.100	0.115	0.097	0.122	0.122	-							
Q10		0.109	0.119	0.105	0.129	0.127	0.105	-						
N16-426		0.127	0.138	0.129	0.115	0.137	0.127	0.135	-					
N17-44		0.152	0.155	0.152	0.138	0.163	0.157	0.152	0.094	-				
M1		0.208	0.213	0.208	0.213	0.216	0.209	0.216	0.217	0.216	-			
M3		0.216	0.222	0.211	0.221	0.231	0.211	0.221	0.224	0.217	0.040	-		
M17		0.214	0.221	0.209	0.226	0.232	0.217	0.217	0.231	0.227	0.048	0.041	-	
Q1		0.209	0.216	0.203	0.217	0.222	0.221	0.219	0.216	0.216	0.081	0.086	0.076	-
M10608.1	A1	0.171	0.160	0.168	0.137	0.157	0.166	0.145	0.175	0.175	0.214	0.208	0.229	0.224
S51392	A1	0.173	0.165	0.168	0.173	0.181	0.171	0.163	0.176	0.168	0.242	0.247	0.245	0.244
AY101611.1	A2	0.155	0.135	0.153	0.150	0.147	0.153	0.152	0.165	0.165	0.186	0.188	0.191	0.201
HQ848062.1	A2/A3	0.117	0.135	0.120	0.105	0.122	0.127	0.135	0.142	0.124	0.206	0.209	0.219	0.213
AY454176.1	A3	0.130	0.138	0.130	0.130	0.137	0.133	0.145	0.145	0.152	0.178	0.191	0.193	0.198
AY454161.1	A4	0.138	0.166	0.135	0.153	0.185	0.143	0.150	0.173	0.171	0.211	0.216	0.214	0.214
AY454175.1	A5	0.153	0.145	0.155	0.148	0.171	0.160	0.147	0.176	0.165	0.209	0.203	0.208	0.206
AY454208.1	A7	0.176	0.157	0.170	0.145	0.158	0.158	0.166	0.161	0.140	0.198	0.209	0.201	0.209
FR694908	A9	0.166	0.168	0.170	0.166	0.178	0.180	0.171	0.181	0.161	0.213	0.224	0.224	0.231
FR693825	A11	0.183	0.171	0.180	0.152	0.157	0.176	0.183	0.189	0.178	0.213	0.217	0.231	0.232
M33677	B1	0.204	0.214	0.201	0.211	0.224	0.211	0.211	0.222	0.211	0.120	0.119	0.120	0.129
FJ195346.1	B2	0.226	0.231	0.221	0.214	0.232	0.226	0.237	0.221	0.217	0.092	0.092	0.102	0.100
EU010126.1	B2	0.208	0.222	0.206	0.226	0.232	0.219	0.227	0.224	0.216	0.064	0.068	0.068	0.081
JF502417.1	B3	0.213	0.221	0.214	0.209	0.217	0.219	0.232	0.213	0.224	0.185	0.186	0.196	0.178
AF322109.1	C	0.262	0.252	0.257	0.250	0.252	0.245	0.255	0.244	0.236	0.213	0.214	0.214	0.216
EU293537.2	E1	0.292	0.295	0.292	0.297	0.301	0.293	0.300	0.293	0.290	0.293	0.285	0.290	0.303
GQ381130.1	E2	0.290	0.301	0.293	0.290	0.313	0.293	0.298	0.288	0.290	0.290	0.290	0.297	0.293

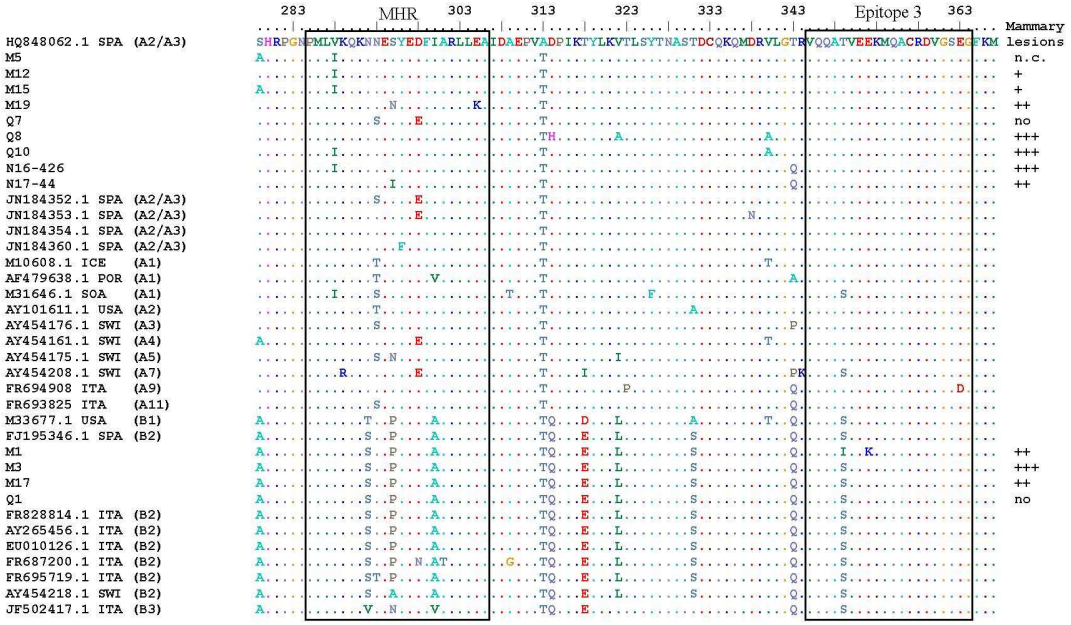
Nucleotide sequences were translated into amino acid sequences and the results of the alignment and comparison with the most representative sequences are reported in Figure 4a,b. The set of primers used in this study amplifies a partial sequence of the *gag* gene codifying for the majority of the capsid protein (CA). Comparing amino acid sequence alterations of the partial gag protein obtained, the "GG" motifs of the four type B sequences were glycine-glycine (GG) like type B reference SRLVs while those of the 11 type A samples were asparagine-valine (NV) like other type A reference SRLVs (see Figure 4a). In type A samples, sequences of epitopes 2 and 3 of reference isolates and of most samples were conserved (see Figure 4a). Only arginine (R) replaced lysine (K) in samples N16-426 and N17-44 and serine (S) replaced asparagine (N) in sample Q7. Type B samples had highly conserved epitope 3 sequences since there are only two alterations in M1 (isoleucine (I) instead of serine (S) and lysine (K) instead of glutamic acid (E). More alterations were found in epitope 2 where three out of four type B samples showed one alteration in comparison with type B reference isolates (see Figure 4a,b).

In the Major Homology Region (MHR), which is usually a highly conserved sequence in the *gag* gene of all retroviruses, some alterations were present in type A samples. In particular, all but one type A samples showed one or two alterations comparing to the A2/A3 reference strain 697 (see Figure 4b). Samples M5, M12, M15, Q10, and N16-426 had isoleucine (I) instead of valine (V) at the fourth position as type A1 reference strain SA-OMVV. This latter had also a serine (S) instead of asparagine (N) at the ninth position. Sample Q7 had not only this alteration, but also glutamic acid (E) instead of aspartic acid (D) at the 14th position, which shows the same alterations found in the A2/A3 Spanish strain 160. Sample N17-44 had isoleucine (I) instead of serine (S) at the 11th position unlike the other samples and reference strains. Unusual alterations were found in sample M19 where asparagine (N) replaced serine (S) at the 11th position and lysine (K) substituted glutamic acid (E) at the 21st position. Type B samples had highly conserved MHR sequences and showed the same amino-acidic sequences of B2 reference isolates even though some single alterations were present in the nucleotide sequences (see Figure S1). A significant correlation among sequence alterations and severity of mastitis was not found ($p > 0.05$).



(**a**)

Figure 4. *Cont.*



(**b**)

Figure 4. Alignment (MUSCLE) of deduced amino acid sequences of partial *gag*-p25 of the SRLV sequences obtained and of some SRLV reference strains; (**a**) positions from 209 to 278, (**b**) posiions from 279 to 368. Two immuno-dominant epitopes of this capsid protein, the GG motif, and the Major Homology Region (MHR) are within squares. The score of mammary lesion of each sample is reported. Legend: (·) homology, (−) deletion, (+++) severe lesions, (++) moderate lesions, (+) minimal lesions, (no) no lesions, (n. c.) not classified, CAN: Canada; CHI: China; ENG: England; ICE: Iceland; ITA: Italy; MEX: Mexico; NOR: Norway; POR: Portugal; SOA: South Africa; SPA: Spain; SWI: Switzerland; USA.

3.2. Analysis of LTR Sequences

The alignment and phylogenetic analysis of LTR nucleotide sequences showed that most samples were closer to the reference Spanish A2/A3 strain 697 (Figures 5 and 6a,b). Comparing samples with the 697 reference strain, Q10 and Q11 showed a 23 nt deletion (9133–9160 nt) in the R region, which appeared longer than the 13 nt deletion present in the reference strains 697, EV1, and in other Spanish strains (C3, 160, 292). Sample Q12 had similar deletions than 697 while other samples showed 2–8 nt deletions in the same tract. No significant differences were found among sequences of samples with a different grade of mastitis ($p > 0.05$). Similarly, as previously described, the TATAbox, the polyadenylation signal, and the AML (vis), which is a site possessing the consensus sequence for the AML/PEBP2/CBF transcriptional factor family [26], were conserved and only a substitution G with A was present in three samples at position 9065 of AML (vis).

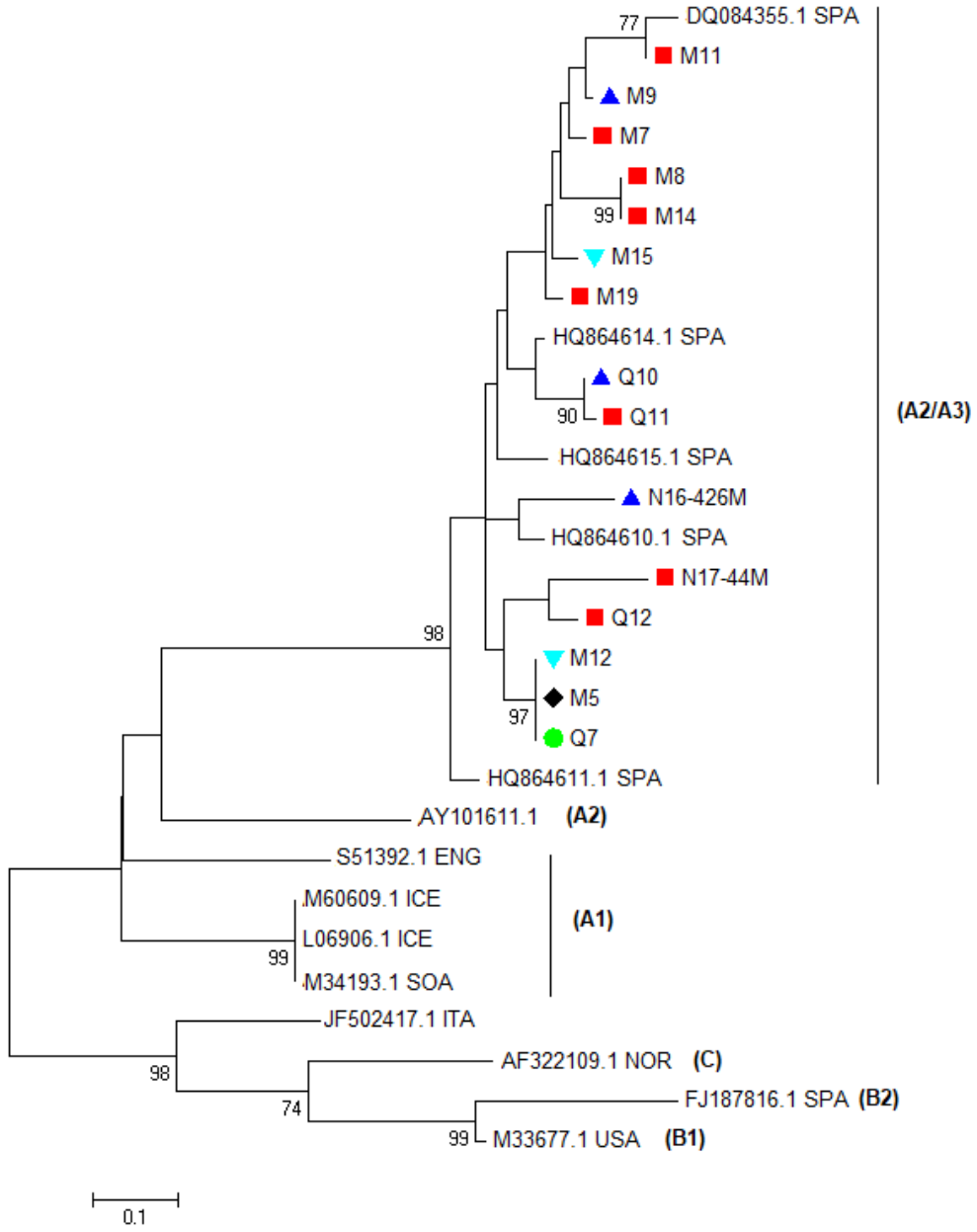
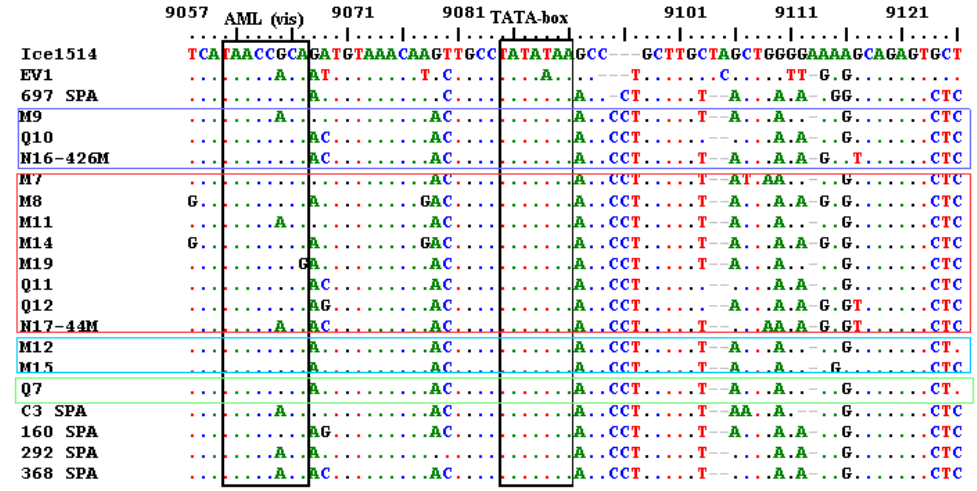
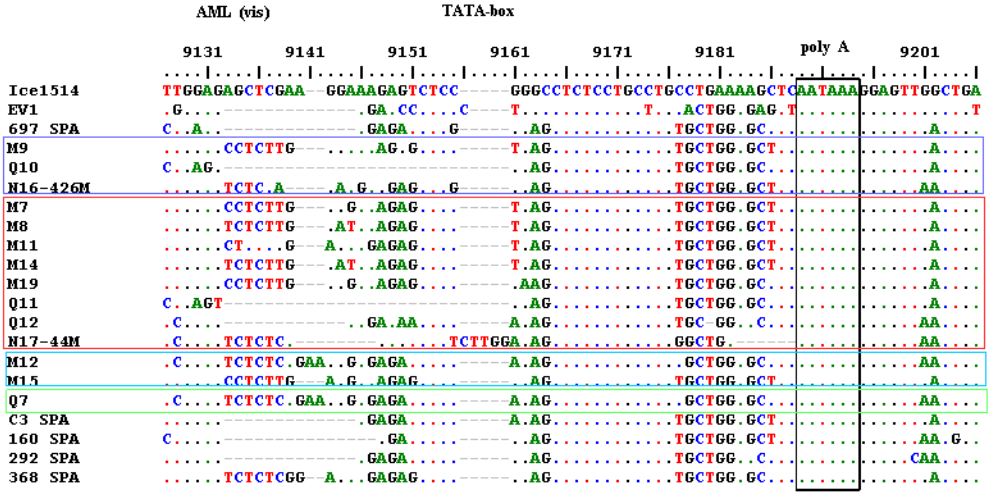


Figure 5. Phylogenetic analysis of the SRLV partial LTR. Sequences of different SRLV genotypes and subtypes available in GenBank were used as reference isolates. Reference sequences are indicated with their accession number and country of origin (CAN: Canada; CHI: China; ENG: England; ICE: Iceland; ITA: Italy; MEX: Mexico; NOR: Norway; POR: Portugal; SOA: South Africa; SPA: Spain; SWI: Switzerland; USA). Samples are indicated with their codes and are labeled on the basis of the score of the mammary lesions observed (▲ severe, ■ moderate, ▼ minimal, ● no lesions, ♦ not available). The phylogenetic analysis was performed with a maximum likelihood (ML) method using the Kimura 2-parameter model with a gamma distribution and with bootstrap values based on 1000 repetitions. Sequences are not deposited in GenBank because they do not reach the minimum length required.



(**a**)



(**b**)

Figure 6. Alignment (MUSCLE) of nucleotide sequences of the LTR region of the SRLV sequences obtained and of some SRLV reference strains. LTR sequence of the isolate Icelandic 1514 (M10609.1) was used as a reference; (**a**) positions from 9057 to 9126, (**b**) posiions from 9127 to 9206. Sequence of reference strains EV1 (S51392.1) was also used. Sequences previously found in Spain in goats (C3—DQ084355.1) and in sheep (697—HQ864615.1, 160—HQ864610.1, 292—HQ864611.1 and 368—HQ864614.1) and showing high homology with our samples were also included. Sequences of the AML (vis), the TATA box element, and the polyadenylation signal are within black squares. Sequences of samples with similar scores of mammary lesions are within colored squares. Legend: (·) homology, (−) deletion, (blue squares) severe lesions, (red squares) moderate lesions, (light blue squares) minimal lesions, and (green squares) no lesions.

4. Discussion

This study describes for the first time *gag* and LTR sequences of SRLVs detected in Spanish Assaf sheep with different grades of histopathological mastitis and their phylogenetic relationships in the context of known SRLV sequences.

Although initially genotypes B viruses were thought to infect only goats, it is not unusual to find reports about infections by type B viruses in sheep and by type A viruses in goats [27–30]. Even in

this study, both genotypes A and B SRLVs have been found in ovine samples. It was not known if the examined sheep had contact with goats, but infection with A genotypes in goats was not reared in contact with sheep, which was reported [31].

The *gag-pol* phylogenetic tree and the pairwise genetic distances comparison revealed that most sequences of the samples were closer to the Spanish A2/A3 isolate 697 while four sequences belonged to subtype B2. Isolate 697 has been previously detected in sheep with neurological diseases from the Spanish region of Castilla y León in Spain and has been classified as A2/A3 because differences between A2 and A3 are often not large enough to separate the two groups [3,25]. Partial sequences of viruses classified as A2/A3 have been detected further seven sheep with neurological signs in Spain [25]. These findings suggest that, in Northern Spain, subtype A2/A3 SRLVs is genetically related to SRLVs, which caused nervous diseases. However, in our cases, neurological signs were not reported. Samples M1, M3, M17, and Q1 were closer to Italian than to Spanish B2 isolates and mammary lesions were found from moderate to severe in three out of four samples while, in sample Q1, lesions were not found. B2 SRLV has been detected in Spain for the first time in SRLV-seropositive adult sheep of the Rasa Aragonesa breed, which shows clinical signs of arthritis [17,32]. Mammary histological lesions were present in 10 out of 13 animals with arthritis, which suggests that udders can be involved even if clinical signs might remain unrecognized until the losses of milk production are significant. B2 viruses have been detected in Italian small ruminants during epidemiological surveys, but data about clinical signs or histological lesions are not reported [4,33].

In addition, good-quality sequences about 800 bp long were obtained from FFPE samples. Fixation in formalin and embedding in paraffin at high temperatures is thought to degrade DNA. Fragmentation of DNA molecules can interfere with their amplification by PCR and with consequent sequencing. In our case, good-quality DNA has been extracted and amplified by PCR from archival FFPE samples, which suggests that this method could be attempted for studying FFPE samples as well as for retrospective investigations.

Analysis of the genetic sequences is important not only for evaluating the spread of SRLV types and subtypes but also for monitoring antigenic variability. Actually, remarkable antigenic variation might be responsible for the misdiagnosis of highly divergent genotypes [34]. The *gag* gene encodes nucleocapsid, capsid, and matrix proteins. Indirect diagnostic assays usually use the capsid protein as the antigen, which helps monitor immuno-dominant epitopes of *gag*-encoded structural proteins. This is useful for detecting antigenic variability in the field and forevaluating and improving the sensitivity of indirect diagnostic tools. Alterations in the amino acid sequences of immuno-dominant epitope regions suggest altered antigenicity, which may affect the sensitivity of serological tests such as ELISA and AGID. The *gag-pol* set of primers used in this study allowed sequencing only of epitopes 2 and 3. Amino acid sequences of epitopes 2 and 3 of type A2/A3 viruses were quite conserved and limited alterations only in three and one samples, respectively. Epitope 2 of B2 isolates had more alterations, which shows single amino acid alterations in three out of four sequences. In addition, more variability was found in the MHR of A2/A3 viruses, which show all but two samples and at least one alteration in comparison with the reference A2/A3 strain 697. The MHR is usually conserved in many retroviruses and is essential for viral assembly [35]. Mutations in the MHR sequence of HIV-1 cause capsid assembly that reduces infectivity [36]. While some studies have been carried out on MHR of human retroviruses, the consequences of MHR mutations on infectivity of SRLVs should be better investigated. MHR of B2 viruses and GG motif of both A2/A3 and B2 viruses in the gag amino acid sequences, AML (vis) motif, TATA-box, and poly-A of both A2/A3 and B2 viruses in the LTR nucleotide sequences were highly conserved, which was previously reported in strains from different geographic areas [31,37,38].

Most LTR sequences showed higher homology with A2/A3 Spanish SRLV isolates. Samples Q10 and Q11 showed a 23 nt deletion in the R region, which appeared longer than the 13 nt deletion observed in type A2/3 reference isolate 697. A 13 nt deletion in this region has been found in sequences of clinically affected sheep and a correlation among this deletion and the appearance

of clinical signs has been suggested [32]. On the contrary, a similar deletion has been found in SRLVs infecting asymptomatic sheep and the lungs of animals infected with viruses carrying the deletion were significantly less affected than sheep infected with viruses without deletion [18]. In the present study, significant correlations among deletions in the R region of the LTR and severity of mammary lesions were not found ($p > 0.05$). Samples with deletions were from sheep with more severe mammary lesions and sheep with moderate to severe mastitis did not show this deletion (see Figure 6b).

Although the severity of mammary lesions was not significantly related to the viral genotype, SRLV sequences from samples with similar grades of lesions (no/minimal and moderate/severe) were most closely related to each other (see Figures 3 and 5 and Table 2). Considering the high economic impact of SRLV diseases, some countries aim to eradicate the diseases by identifying and prematurely culling infected animals. Selecting animals on the basis of serological results could determine the selection of SRLV variants with significant alterations in the antigen sequences. Permanent and extensive surveys should be encouraged in different countries to evaluate the antigenic variability of SRLV and to monitor the sensitivity and specificity of diagnostic tests in detecting these variants. In particular, seronegative animals should be investigated for infections by new viral genotypes not detected by traditional serological tests. Histological screening of different target organs at the slaughterhouse could be a useful tool for selecting samples with lesions, which suggests an SRLV disease in seronegative animals.

5. Conclusions

In conclusion, this is the first study investigating the association between the SRLV sequence analysis and histopathological grading of mammary lesions in sheep. Circulation of SRLVs of types A2/A3 and B2 in Spanish Assaf sheep was confirmed and new viral variants have not been found, but moderate alterations were present in some immuno-dominant epitopes and in the MHR tract.

No significant correlation was found among histological features and alterations in the sequences. Although some sequences obtained from samples with similar grades of mammary lesions appeared closer to each other, more extensive and interdisciplinary studies are required for establishing the existence of viral clusters with a higher or lower pathogenicity for specific target organs.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/10/6/315/s1, Figure S1: Nucleotide alignment of MHR sequences in the *gag* gene of the samples and of some reference strains.

Author Contributions: S.P. and E.G. conceived and designed the experiments. E.G. performed many experiments. S.P., G.R., L.P., J.F.G.M., and V.C. performed some experiments. E.G. and S.P. analyzed the data and wrote the paper. All authors read and approved the paper.

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References

1. Peterhans, E.; Greenland, T.; Badiola, J.; Harkiss, G.; Bertoni, G.; Amorena, B.; Eliaszewicz, M.; Juste, R.A.; Krassnig, R.; Lafont, J.P.; et al. Routes of transmission and consequences of small ruminant lentiviruses (SRLVs) infection and eradication schemes. *Vet. Res.* **2004**, *35*, 257–274. [CrossRef] [PubMed]
2. Preziuso, S.; Renzoni, G.; Allen, T.E.; Taccini, E.; Rossi, G.; DeMartini, J.C.; Braca, G. Colostral transmission of maedi visna virus: Sites of viral entry in lambs born from experimentally infected ewes. *Vet. Microbiol.* **2004**, *104*, 157–164. [CrossRef] [PubMed]
3. Shah, C.; Böni, J.; Huder, J.B.; Vogt, H.R.; Mühlherr, J.; Zanoni, R.; Miserez, R.; Lutz, H.; Schüpbach, J. Phylogenetic analysis and reclassification of caprine and ovine lentiviruses based on 104 new isolates: Evidence for regular sheep-to-goat transmission and worldwide propagation through livestock trade. *Virology* **2004**, *319*, 12–26. [CrossRef] [PubMed]

4. Grego, E.; Bertolotti, L.; Quasso, A.; Profiti, M.; Lacerenza, D.; Muz, D.; Rosati, S. Genetic characterization of small ruminant lentivirus in Italian mixed flocks: Evidence for a novel genotype circulating in a local goat population. *J. Gen. Virol.* **2007**, *88*, 3423–3427. [CrossRef] [PubMed]
5. Ramírez, H.; Reina, R.; Amorena, B.; de Andrés, D.; Martínez, H.A. Small ruminant lentiviruses: Genetic variability; tropism and diagnosis. *Viruses* **2013**, *5*, 1175–1207. [CrossRef] [PubMed]
6. Leroux, C.; Chastang, J.; Greenland, T.; Mornex, J.F. Genomic heterogeneity of small ruminant lentiviruses: Existence of heterogeneous populations in sheep and of the same lentiviral genotypes in sheep and goats. *Arch. Virol.* **1997**, *142*, 1125–1137. [CrossRef] [PubMed]
7. Minguijón, E.; Reina, R.; Pérez, M.; Polledo, L.; Villoria, M.; Ramírez, H.; Leginagoikoa, I.; Badiola, J.J.; García-Marín, J.F.; de Andrés, D.; et al. Small ruminant lentivirus infections and diseases. *Vet. Microbiol.* **2015**, *181*, 75–89. [CrossRef] [PubMed]
8. Lujan, L.; García Marin, J.F.; Fernandez de Luco, D.; Vargas, A.; Badiola, J.J. Pathological changes in the lungs and mammary glands of sheep and their relationship with maedi-visna infection. *Vet. Rec.* **1991**, *129*, 51–54. [CrossRef] [PubMed]
9. Benavides, J.; Fuertes, M.; García-Pariente, C.; Otaola, J.; Delgado, L.; Giraldez, J.; García Marín, J.F.; Carmen Ferreras, M.; Pérez, V. Impact of maedi-visna in intensively managed dairy sheep. *Vet. J.* **2013**, *197*, 607–612. [CrossRef] [PubMed]
10. Gayo, E.; Polledo, L.; Balseiro, A.; Martínez, C.P.; García Iglesias, M.J.; Preziuso, S.; Rossi, G.; García Marín, J.F. Inflammatory Lesion Patterns in Target Organs of Visna/Maedi in Sheep and their Significance in the Pathogenesis and Diagnosis of the Infection. *J. Comp. Pathol.* **2018**, *159*, 49–56. [CrossRef] [PubMed]
11. Anderson, B.C.; Bulgin, M.S.; Adams, S.; Duelke, B. Firm udder in periparturient ewes with lymphocytic accumulations; retrovirus infection; and milk unavailable at the teat. *J. Am. Vet. Med. Assoc.* **1985**, *186*, 391–393. [PubMed]
12. Ryan, D.P.; Greenwood, P.L.; Nicholls, P.J. Effect of caprine arthritis-encephalitis virus infection on milk cell count and *N*-acetyl-beta-glucosaminidase activity in dairy goats. *J. Dairy Res.* **1993**, *60*, 299–306. [CrossRef] [PubMed]
13. Martinez-Navalon, B.; Peris, C.; Gomez, E.A.; Peris, B.; Roche, M.L.; Caballero, C.; Goyena, E.; Berriatua, E. Quantitative estimation of the impact of caprine arthritis encephalitis virus infection on milk production by dairy goats. *Vet. J.* **2013**, *197*, 311–317. [CrossRef] [PubMed]
14. Sihvonen, L.; Nuotio, L.; Rikula, U.; Hirvelä-Koski, V.; Kokkonen, U. Preventing the spread of maedi-visna in sheep through a voluntary control programme in Finland. *Prev. Vet. Med.* **2000**, *47*, 213–220. [CrossRef]
15. Tavella, A.; Bettini, A.; Ceol, M.; Zambotto, P.; Stifter, E.; Kusstatscher, N.; Lombardi, R.; Nardeli, S.; Beato, M.S.; Capello, K.; et al. Achievements of an eradication programme against caprine arthritis encephalitis virus in South Tyrol; Italy. *Vet. Rec.* **2018**, *182*, 51. [CrossRef] [PubMed]
16. Deubelbeiss, M.; Blatti-Cardinaux, L.; Zahno, M.L.; Zanoni, R.; Vogt, H.R.; Posthaus, H.; Bertoni, G. Characterization of small ruminant lentivirus A4 subtype isolates and assessment of their pathogenic potential in naturally infected goats. *Virol. J.* **2014**, *11*, 65. [CrossRef] [PubMed]
17. Pérez, M.; Biescas, E.; Reina, R.; Glaria, I.; Marín, B.; Marquina, A.; Salazar, E.; Álvarez, N.; de Andrés, D.; Fantova, E.; et al. Small ruminant lentivirus-induced arthritis: Clinicopathologic findings in sheep infected by a highly replicative SRLV B2 genotype. *Vet. Pathol.* **2015**, *52*, 132–139. [CrossRef] [PubMed]
18. Angelopoulou, K.; Poutahidis, T.; Brellou, G.D.; Greenland, T.; Vlemmas, I. A deletion in the R region of long terminal repeats in small ruminant lentiviruses is associated with decreased pathology in the lung. *Vet. J.* **2008**, *175*, 346–355. [CrossRef] [PubMed]
19. Preziuso, S.; Taccini, E.; Rossi, G.; Renzoni, G.; Braca, G. Experimental Maedi Visna Virus Infection in sheep: A morphological; immunohistochemical and PCR study after three years of infection. *Eur. J. Histochem.* **2003**, *47*, 373–378. [CrossRef] [PubMed]
20. Ryan, S.; Tiley, L.; McConnell, I.; Blacklaws, B. Infection of dendritic cells by the Maedi-Visna lentivirus. *J. Virol.* **2000**, *74*, 10096–10103. [CrossRef] [PubMed]
21. Edgar, R.C. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **2004**, *32*, 1792–1797. [CrossRef] [PubMed]
22. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [CrossRef] [PubMed]
23. Nei, M.; Kumar, S. *Molecular Evolution and Phylogenetics*; Oxford University Press: New York, NY, USA, 2000.

24. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [CrossRef] [PubMed]
25. Glaria, I.; Reina, R.; Ramírez, H.; de Andrés, X.; Crespo, H.; Jauregui, P.; Salazar, E.; Luján, L.; Pérez, M.M.; Benavides, J.; et al. Visna/Maedi virus genetic characterization and serological diagnosis of infection in sheep from a neurological outbreak. *Vet. Microbiol.* **2012**, *155*, 137–146. [CrossRef] [PubMed]
26. Sutton, K.A.; Lin, C.T.; Harkiss, G.D.; McConnell, I.; Sargan, D.R. Regulation of the long terminal repeat in visna virus by a transcription factor related to the AML/PEBP2/CBF superfamily. *Virology* **1997**, *229*, 240–250. [CrossRef] [PubMed]
27. Kuhar, U.; Barlic-Maganja, D.; Grom, J. Phylogenetic analysis of small ruminant lentiviruses detected in Slovenia. *Vet. Microbiol.* **2012**, *162*, 201–206. [CrossRef] [PubMed]
28. Olech, M.; Rachid, A.; Croise, B.; Kuźmak, J.; Valas, S. Genetic and antigenic characterization of small ruminant lentiviruses circulating in Poland. *Virus Res.* **2012**, *163*, 528–536. [CrossRef] [PubMed]
29. Fras, M.; Leboeuf, A.; Labrie, F.M.; Laurin, M.A.; Singh Sohal, J.; L'Homme, Y. Phylogenetic analysis of small ruminant lentiviruses in mixed flocks: Multiple evidence of dual infection and natural transmission of types A2 and B1 between sheep and goats. *Infect. Genet. Evol.* **2013**, *19*, 97–104. [CrossRef] [PubMed]
30. Santry, L.A.; de Jong, J.; Gold, A.C.; Walsh, S.R.; Menzies, P.I.; Wootton, S.K. Genetic characterization of small ruminant lentiviruses circulating in naturally infected sheep and goats in Ontario, Canada. *Virus Res.* **2013**, *175*, 30–44. [CrossRef] [PubMed]
31. Reina, R.; Mora, M.I.; Glaria, I.; García, I.; Solano, C.; Luján, L.; Badiola, J.J.; Contreras, A.; Berriatua, E.; Juste, R.; et al. Molecular characterization and phylogenetic study of Maedi Visna and Caprine Arthritis Encephalitis viral sequences in sheep and goats from Spain. *Virus Res.* **2006**, *121*, 189–198. [CrossRef] [PubMed]
32. Glaria, I.; Reina, R.; Crespo, H.; de Andrés, X.; Ramírez, H.; Biescas, E.; Pérez, M.M.; Badiola, J.; Luján, L.; Amorena, B.; et al. Phylogenetic analysis of SRLV sequences from an arthritic sheep outbreak demonstrates the introduction of CAEV-like viruses among Spanish sheep. *Vet. Microbiol.* **2009**, *138*, 156–162. [CrossRef] [PubMed]
33. Giammarioli, M.; Bazzucchi, M.; Puggioni, G.; Brajon, G.; Dei Giudici, S.; Taccori, F.; Feliziani, F.; De Mia, G.M. Phylogenetic analysis of small ruminant lentivirus (SRLV) in Italian flocks reveals the existence of novel genetic subtypes. *Virus Genes* **2011**, *43*, 380–384. [CrossRef] [PubMed]
34. Cardinaux, L.; Zahno, M.L.; Deubelbeiss, M.; Zanoni, R.; Vogt, H.R.; Bertoni, G. Virological and phylogenetic characterization of attenuated small ruminant lentivirus isolates eluding efficient serological detection. *Vet. Microbiol.* **2013**, *162*, 572–581. [CrossRef] [PubMed]
35. Purdy, J.G.; Flanagan, J.M.; Ropson, I.J.; Rennoll-Bankert, K.E.; Craven, R.C. Critical role of conserved hydrophobic residues within the major homology region in mature retroviral capsid assembly. *J. Virol.* **2008**, *82*, 5951–5961. [CrossRef] [PubMed]
36. Chu, H.H.; Chang, Y.F.; Wang, C.T. Mutations in the alpha-helix directly C-terminal to the major homology region of human immunodeficiency virus type 1 capsid protein disrupt Gag multimerization and markedly impair virus particle production. *J. Biomed. Sci.* **2006**, *13*, 645–656. [CrossRef] [PubMed]
37. Angelopoulou, K.; Brellou, G.D.; Greenland, T.; Vlemmas, I. A novel deletion in the LTR region of a Greek small ruminant lentivirus may be associated with low pathogenicity. *Virus Res.* **2006**, *118*, 178–184. [CrossRef] [PubMed]
38. Kokawa, S.; Oba, M.; Hirata, T.; Tamaki, S.; Omura, M.; Tsuchiaka, S.; Nagai, M.; Omatsu, T.; Mizutani, T. Molecular characteristics and prevalence of small ruminant lentiviruses in goats in Japan. *Arch. Virol.* **2017**, *162*, 3007–3015. [CrossRef] [PubMed]