UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

CARACTERIZACIÓN DE LA PATOLOGÍA PULMONAR EN CORDEROS DE CEBO DEL SUR-OESTE DE EXTREMADURA: FACTORES DE RIESGO Y PARÁMETROS DE ESTRÉS ASOCIADOS

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A mis padres, por ellos, esto fue posible.

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Resumen

Uno de los principales problemas que afectan a la rentabilidad de las explotaciones de ovinos y caprinos es el originado por la patología respiratoria. La infección pulmonar y la inflamación como principal manifestación constituyen una de las causas de morbilidad y mortalidad más significativas en el periodo de cría y cebo de ovino.

Son numerosos los factores que pueden influir en el desarrollo de enfermedad o lesión. Entre otros, contribuyen a su presentación factores inherentes al hospedador, principalmente modelados por la inmunidad del individuo, así como factores inherentes al medio ambiente, tanto aquellos procedentes de la climatología de la zona como otros frutos del manejo en este periodo de vida de los animales.

Por ello, se ha llevado a cabo un estudio para analizar la patología pulmonar ovina encontrada en matadero, así como los posibles factores que pueden concurrir en su presentación durante la estancia de los animales en los centros de clasificación. Se incluirán parámetros fisiológicos (hematológicos, hormonales y de respuesta al estrés), medioambientales y patológicos, así como su posible relación y, en consecuencia, su implicación en la presencia de lesión o enfermedad. Con ello, se pretende apuntar posibles mejoras para paliar las pérdidas directas e indirectas en las explotaciones afectadas.

Por último, se ha realizado un estudio inmunohistoquímico con el fin de analizar la posible evolución de los procesos patológicos encontrados durante la fase de cebo en aras de buscar medidas basadas en la aplicación de productos farmacológicos y biológicos para evitar el desarrollo de lesiones.

Abstract

One of the main problems affecting the profitability of sheep and goat farms constitutes respiratory disease. Lung infections and its inflammatory problems, is a cause of significant morbidity and mortality in the period of breeding and fattening in ovine.

Many factors can influence the development of this disease. Among others, influence in their presentation, inherent host factors mainly associated to the immunity of the animals, and environmental conditions, both those from climatology and other of the area, as well as the result of management in this period of life of the animals.

Therefore, a study was conducted to analyze the ovine pulmonary pathology found in slaughterhouse and the possible factors that can come into your presentation during your stay in the feedlot. For this fact, physiological parameters (hematologic, hormonal and of stress response), environmental and pathologic were evaluated and their possible relationship in order to point out possible improvements to mitigate the direct and indirect losses in the affected farms.

Finally, an immunohistochemical study have been performed in order to analyze the possible evolution of the found pathological processes during the fattening period in order to establish measures based on the use of pharmacological and biological products to avoid the development of severe lesions.

Abreviaturas

- **CC:** Centros de Clasificación.
- **SRO:** Síndrome Respiratorio Ovino.
- **GMD:** Ganancias Medias Diarias.
- **IL-1R:** Receptores de Interleuquina 1.
- **IL-6:** Interleuquina 6.
- **IL-10:** Interleuquina 10.
- **TNF-α:** Factor de Necrosis Tumoral Alpha.
- **CD3:** Cluster of Differentiation 3.
- **CD79α:** Cluster of differentiation 79 alpha.
- **CD68:** Cluster of differentiation 68.
- **DAD:** Daño Alveolar Difuso.
- **IP:** Neumonía Intersticial.
- **PB:** Bronconeumonía purulenta.
- **BI:** Neumonía broncointersticial.
- **INF-γ:** Interferón gamma.
- **IL-4:** Interleuquina 4.
- **IL-5:** Interleuquina 5.
- **ppm:** partes por millón.
- **PMN:** Células Polimorfonucleares.

- **Células NK:** Células Natural Killers.
- **IL-1β:** Interleuquina 1 beta.
- **IL-8:** Interleuquina 8.
- **CPA:** Células Presentadoras de Antígenos.
- **MHC-II:** Complejo Mayor de Histocompatibilidad II.
- **MA:** Medio-Ambientales.
- **RBC:** Red Blood Cells.
- **WBC:** White Blood Cells.
- **N/L ratio:** ratio neutrófilo/linfocito.
- **SD:** Standard Deviation.
- **µl:** Microlitros.
- **µg/dl:** Microgramos/Decilitros.
- **PBS:** Phosphate-buffered saline.
- **TBS:** Tris-buffered saline.
- **O/N:** overnight o Durante toda la noche
- **BALT:** Bronchus-associated lymphoid tissue.
- **H-E:** Hematoxilina-eosina.
- **PRRS:** Síndrome Respiratorio y Reproductivo Porcino.

Índice general

BLOQUE I. Introducción General

Capítulo 1. Introducción

En este capítulo se presenta la motivación de esta tesis doctoral. También se incluyen los objetivos y un esquema de la memoria.

1.1. Motivación

Extremadura es la segunda comunidad autónoma española en censo ovino, con un 19.57% de los animales, sólo precedida de Castilla y León, que alcanza un 19.95%. El sistema de producción ovina ha pasado de mostrar una estructura individual a un sistema más competitivo y más estructurado con la formación de cooperativas y la introducción de centros de clasificación (CC) (López et al., 2002). La producción en Extremadura se caracteriza, principalmente, por la obtención de corderos ligeros, inferiores a los 3 meses de edad y con un peso aproximado a la canal 8.5-13 kg. Este mercado muestra una estacionalidad productiva, con el mayor número de animales sacrificados en los meses de marzo a abril, alcanzando valores máximos en diciembre (MAGRAMA, 2013). La creación de estos CC ha permitido la estandarización de los procesos de producción de corderos. De esta forma, se establece una normalización de los animales, facilitando así la venta del producto, lo que concede ventajas para el ganadero (Miranda et al., 2010a).

Las principales causas de mortalidad en el ovino muestran variación en función de diferentes variables y una de las principales es la edad. Al síndrome respiratorio ovino (SRO) se le atribuye el mayor porcentaje de bajas a partir del tercer periodo de vida, siendo considerado este la época de cebo y recría (González y Ruiz, 2008). La prevalencia de esta enfermedad en cebaderos ha sido estudiada por diversos autores, estableciéndose que entre un 57% y un 85% de muertes en este periodo están asociadas a procesos de tipo respiratorio (Moreno, 1994). Bello et al. (2007) establecieron un 67% de bajas asociadas a procesos respiratorios en los animales

muertos en esta tercera fase de vida, lo que sitúa a este síndrome en un punto estratégico de control de la producción intensiva ovina.

Este síndrome ha sido caracterizado como un problema multifactorial, donde se ven implicados una serie de factores predisponentes y desencadenantes. El papel de los microorganismos, como bacterias y virus, es primordial, ya que actúan como factor desencadenante de la enfermedad. No obstante, hay otros factores a tener cuenta, como, por ejemplo, el manejo (transportes, clasificación, etc.), que pueden predisponer al desarrollo de la enfermedad (Miranda, 2010a). Las condiciones medioambientales también han sido señaladas por algunos autores como causas predisponentes de enfermedad (Lacasta et al., 2008).

El impacto de este complejo en las explotaciones ovinas es muy importante ya que, además de las pérdidas directas ocasionadas por la muerte de los animales afectados, hay otras pérdidas indirectas como las penalizaciones por decomisos en mataderos, los costes asociados a medicamentos y las mermas en la producción (la disminución de las ganancias medias diarias (GMD) y el aumento en el índice de conversión (Goodwin et al., 2004). La realización de una estimación económica fiable es necesaria, a pesar de su dificultad. Además, también es necesario evaluar la implicación desde el punto de vista de la salud animal. Así, al inicio del proyecto que ha dado lugar a la elaboración de esta tesis doctoral, en el grupo OVISO S.C.L., durante el año 2009 se produjeron pérdidas aproximadas de 100.000 € por cebadero medio (cebo de 90.000 corderos al año), debido a la depreciación de la canal por los decomisos de pulmón, asociada principalmente a los meses comprendidos entre marzo y junio (datos facilitados por la empresa). Además, las medidas de lucha contra esta enfermedad han ido encaminadas al uso de vacunas comerciales y al tratamiento de animales enfermos dentro de los CC. Esto también ha supuesto un coste añadido para la producción de corderos.

Por todo lo anterior, se considera necesario estudiar y analizar en profundidad este síndrome, ya que sólo existen estudios someros y desde un punto de vista cualitativo. Sin embargo, no existe una cuantificación en el número de lesiones encontradas ni tampoco se ha descrito una posible relación estadística entre las variables predisponentes que culminan en el desarrollo del mismo en este síndrome. Para ello es necesario también el estudio de las dominantes patológicas asociadas con los diferentes tipos de respuesta inmune a nivel pulmonar. Con todo esto lo que perseguimos es desarrollar un trabajo que pueda contemplar el desarrollo de lesiones con el estudio de las diferentes variables predisponentes, así como, las distintas respuestas inflamatorias que puedan mediar en este síndrome.

1.2. Objetivos

Para el desarrollo de esta tesis, en primer y último lugar se ha estudiado la patología asociada al síndrome respiratorio ovino y los factores asociados, incluyendo la respuesta inmune celular y las interleucinas implicadas en el desarrollo de lesiones.

Los objetivos concretos que se buscaban alcanzar son:

- Estudio de lesiones en aras de establecer una clasificación histopatológica de las lesiones encontradas, además de, buscar la relación de estas lesiones con los diferentes factores predisponentes durante el desarrollo de las mismas (Capítulos 3 y 4).
- Análisis de la influencia del periodo de cebo mediante parámetros hematológicos e inmunológicos que sirvan para determinar la influencia del estrés en la aparición de posibles cuadros patológicos (Capítulo 5).
- Evaluación de la respuesta inmune celular en diferentes cuadros patológicos mediante el uso de marcadores específicos de linfocitos T, B y macrófagos,

para un mayor conocimiento de la enfermedad en condiciones naturales (Capítulo 6).

- Evaluación de la respuesta inmune mediante el uso de interleuquinas específicas para valorar su efecto, así como las implicaciones que puedan tener en los patrones histopatológicos de los animales analizados dependiendo de su condición de citoquina pro o anti-inflamatorio (Capítulo 7).

1.3. Esquema de la memoria

Esta memoria consta de cuatro bloques, en los que se integran ocho capítulos. El primer bloque consta de dos capítulos. En el primero se ha incluido la motivación para la realización de esta tesis doctoral, así como los objetivos planteados para su desarrollo y un esquema de la memoria de tesis. En el segundo capítulo se realiza una revisión bibliográfica exhaustiva de las bases y conocimientos científicos del síndrome respiratorio ovino en la actualidad.

El segundo bloque contiene tres capítulos. El primero de ellos muestra un estudio que describe los principales hallazgos histopatológicos en pulmones de corderos de cebo. Para ello, realizamos un experimento en el que se llevó a cabo una monitorización de los animales durante su estancia en los centros de clasificación. Se consideraron parámetros medioambientales mediante su medición para ver su posible implicación en la aparición del síndrome respiratorio ovino en el Suroeste de Extremadura. En este bloque se aplicaron modelos matemáticos como las redes bayesianas, para analizar de una forma probabilística la relación entre los diferentes factores predisponentes y la aparición de patología. De este bloque surgen dos artículos que conforman los capítulos 3 y 4:

1. Galapero, J., Fernández, S., Pérez, CJ., Ramos, A., Salguero, FJ., Cuesta, JM., Gómez, L. Study of possible influence of seasonal conditions on pathological respiratoy processes in fattening ovine from Extremadura 'South-western Spain' Enviado a la revista Small Ruminant Research para su revisión. Impact Factor 1.275.
2. Galapero, J., Fernández, S., Pérez, CJ., Calle-Alonso, F., Rey, J., Gómez, L. 2016. Identifying risk factors for ovine respiratory processes by using Bayesian networks. Small Ruminant Research. 136, 113–120. Impact Factor 1.275.

En este diseño experimental también se incluyó la toma de muestras para valorar la respuesta inmune innata y diferentes parámetros hematológicos. Los parámetros hematológicos han sido usados para estudiar el efecto de las condiciones de producción en el estrés y su influencia sobre el sistema inmune. De esta valoración se desarrolló un artículo que conforma el capítulo 5.

3. Galapero, J., Fernández, S., Pérez, CJ., García-Sánchez, A., García-Sánchez, L., Gómez L. 2016. Valuation of immune response by using phagocytosis index and parameters associated as markers of animal stress in fattening lambs. Small Ruminant Research. 133, 58–61. Impact Factor 1.275.

En el tercer bloque se realiza un estudio en profundidad de los hallazgos patológicos mediante técnicas inmunohistoquímicas. Este bloque se estructura en dos capítulos. En primer lugar, se estudia la respuesta inmune celular mediante el inmuno-marcaje de linfocitos T, B y macrófagos en los grupos previamente establecidos. De este estudio se obtiene el artículo que se presenta en el capítulo 6.

4. Galapero, J., Fernández, S., Pérez, CJ., Ramos, A., Cuesta, JM., Salguero, FJ., Gómez L. Characterization of cell populations involved in

immune response based on CD3, CD79 and CD68 markers: A study in lung of fattening lambs with different pathological patterns. Enviado a la revista Veterinary Immunology and Immunopathology para su revisión. Impact Factor. 1.681.

Posteriormente se realiza un estudio de diferentes citoquinas pro-inflamatorias y anti-inflamatorias. Con esto, intentamos ver la implicación de estos mediadores en los diferentes procesos patológicos implicados en el síndrome. Para ello, se utilizan los marcadores IL-1R, IL-6 y TNF-α como citoquinas pro-inflamatorias y el marcador IL-10 como interleuquina con función anti-inflamatoria. En el capítulo 7 se recoge el trabajo obtenido de este estudio.

5. Galapero, J., Fernández, S., Pérez CJ., Ramos, A., Salguero, FJ., Gómez, L. Cytokine expression in lung of fattening lambs with different lesional patterns based on IL-1, IL-6, TNF-alpha and IL-10 markers. Enviado a la revista Journal of Comparative Pathology. Impact Factor 1.325.

En el cuarto y último bloque se desarrolla una discusión general donde se contrastan los resultados obtenidos en esta tesis con los establecidos por otros autores. También se presentan conclusiones y líneas futuras de trabajo en base a los objetivos planteados.

Capítulo 2. Base científica

En este capítulo se presenta una revisión bibliográfica sobre las bases y conocimientos científicos del síndrome respiratorio ovino. El capítulo se estructura en las siguientes cuatro secciones.

2.1 Anatomía e histología del pulmón

El aparato respiratorio comprende los pulmones y un sistema de conductos que los conecta con el medio ambiente. Se pueden distinguir dos partes: la porción conductora, que va desde las fosas nasales hasta los bronquiolos, y la porción respiratoria, compuesta por las porciones terminales del árbol bronquial y los alveolos, donde se produce el intercambio gaseoso. Desde un punto de vista anatómico, el aparato respiratorio se inicia con la porción conductora, que está conformada por la tráquea como tubo conector desde el medio ambiente hacía el pulmón. El pulmón es un órgano hueco estructurado en dos partes: pulmón izquierdo, que se compone de dos lóbulos (el apical y el diafragmático o caudal) y pulmón derecho, conformado a su vez por los lóbulos craneal, medio, caudal y accesorio.

Desde un punto de vista histológico, la tráquea está formada por un epitelio pseudoestratificado ciliado que descansa en una lámina propia de tejido conjuntivo laxo. Esta estructura incluye glándulas de tipo mucoso que excretan su contenido a la luz traqueal (Welsch, 2008). Una vez la tráquea llega al pulmón, se bifurca en el árbol bronquial, formando así los bronquios primarios o extrapulmonares (cuya estructura histológica es similar a la de la tráquea) y los bronquios intrapulmonares (secundarios y terciarios).

A continuación, se disponen los bronquiolos, encargados de llevar el aire hasta los lóbulos pulmonares. Se componen de un recubrimiento que evoluciona desde un

epitelio simple cilíndrico ciliado a células cúbicas dispuestas en una sola capa en los bronquiolos de mayor tamaño (Gartner & Hiatt, 2007). En el bronquiolo terminal destaca el epitelio compuesto por células de clara y cúbicas comunicando directamente con el bronquiolo respiratorio, que constituye la primera región de intercambio del sistema respiratorio. Este último presenta un epitelio simple, con capacidad respiratoria, que varía de columnar bajo a cuboides, pudiendo presentar cilios en la porción inicial (Mescher, 2014). El BALT (Bronchus-associated lymphoid tissue; tejido linfoide asociado a bronquios) aparece como acumulación local de tejido linfático que puede formar un solo folículo o una acumulación de varios. Además de estas estructuras, como elementos de defensa en la zona del epitelio bronquial podemos encontrar una infiltración linfocitaria que participa en el control de posibles patógenos.

Los conductos alveolares conforman el inicio de la porción de intercambio respiratorio. Carecen de pared propia, ya que son sólo disposiciones lineales de los alveolos. Un conducto alveolar surge de un bronquiolo respiratorio ramificado y da lugar a una evaginación que se conoce como saco alveolar.

El conducto alveolar está reforzado y estabilizado por un tejido conjuntivo entre los alveolos que se denomina tabique inter-alveolar. Este tabique está compuesto por dos capas de epitelio simple plano separadas por capilares sanguíneos, fibras reticulares y elásticas, fibroblastos y sustancias fundamentales del tejido conjuntivo. En él destacan tres tipos de células. Por un lado, las células endoteliales, que son las más numerosas y que presentan un núcleo pequeño y alargado; por otro, los neumocitos tipo I, que presentan un núcleo alargado con una ligera protuberancia hacía el interior del alveolo, observándose una separación entre ellos. Estos conforman la principal barrera contra el paso de moléculas de la circulación al alveolo. Por último, los neumocitos tipo II, también conocidos como células septales,

son menos frecuentes y normalmente aparecen en grupos de dos o tres células (Mescher, 2014). Además de producir y fagocitar el agente tensoactivo, los neumocitos tipo II se dividen por mitosis para generar neumocitos tipo I (Gartner & Hiatt, 2007).

Además de estos tipos celulares, también se observan macrófagos alveolares, células localizadas en el interior de los alveolos y cuya función es la participación en la respuesta inmune innata mediante la ingestión de partículas y sustancias extrañas, tanto de agentes microbianos como de partículas provenientes de la contaminación ambiental.

Rodeando toda la estructura pulmonar encontramos la pleura, que es una membrana serosa que envuelve al pulmón, dispuesta en dos capas: la capa parietal y la capa visceral. Histológicamente está formada por un mesotelio, compuesto a su vez por un epitelio simple plano asociado a un tejido conjuntivo con capilares sanguíneos y vasos linfáticos.

2.2 Histopatología asociada al aparato respiratorio

Para la óptima comprensión de la histopatología del aparato respiratorio, su análisis comprende dos aspectos fundamentales, el estudio macroscópico y el microscópico, que nos permiten describir diferentes manifestaciones patológicas.

El enfisema es, en sentido estricto, la infiltración de aire y otros gases en el órgano. Pueden definirse dos tipos: alveolar o intersticial. En el primero se observa un aumento de tamaño de la zona alveolar sin fibrosis extra (Caswell y Williams, 2007). Por el contrario, el enfisema intersticial es de más rara manifestación y se define como la presencia de aire en las zonas de tejido conectivo y linfático, incluyendo el septo interlobular, debajo de la pleura y alrededor de vasos y vías aéreas. Los

pulmones que presentan enfisema alveolar se caracterizan macroscópicamente por un aumento de tamaño y palidez asociada.

La presencia de edema también puede ser evidenciada desde un punto de vista macroscópico. Su origen radica en los desequilibrios osmóticos y de presión. Desde un punto de vista macroscópico, el pulmón aparece húmedo, edematoso y no se observa colapso al abrir el tórax. Histológicamente, el edema se detecta por primera vez dentro del intersticio peribronquial/bronquiolar, septo interlobular y pleura, donde es eliminado por el sistema linfático.

La presencia de líquido tiene poco impacto en la función pulmonar. Si la acumulación de líquido supera la depuración por el sistema linfatico, éste se situará dentro de los alvéolos. Cuando esto ocurre se reduce la función del surfactante, lo que implica una disminución en la tensión superficial llegando al colapso alveolar. La consecuencia directa de esto es un descenso de la actividad pulmonar.

Microscópicamente, el líquido del edema es a menudo incoloro y se manifiesta simplemente como expansión y separación de constituyentes de la matriz extracelular intersticial, especialmente alrededor de los vasos sanguíneos y el haz broncovascular. Con un mayor contenido proteico, el edema parece acidófilo, especialmente en los alvéolos, donde es homogéneo, excepto por discretos espacios ocasionales que pueden aparecer atrapados en burbujas de aire. En casos de cronicidad, se acompaña de un aumento difuso del número de macrófagos alveolares.

Las bronconeumonías se caracterizan por la presencia de zonas de consolidación en las áreas más cráneoventrales del pulmón. Oruç et al. (2005) definen esta lesión como áreas de consolidación con una apariencia roja-gris, siendo esta consolidación uno de los criterios más importantes para determinar la presencia de neumonías. Dependiendo de la extensión de la lesión, se cataloga como bronconeumonía lobular

o neumonía lobular. La bronconeumonía lobular diferencia la porción afectada de la porción sana, siendo la localización de la primera principalmente los lóbulos craneales. Sin embargo, la neumonía lobular ocupa la totalidad del lóbulo afectado.

Si bien los procesos exudativos han sido catalogados como responsables de fenómenos de consolidación, Mawhinney et al. (2010) describieron esta característica macroscópica en procesos de neumonía intersticial.

El término neumonía intersticial se aplica para describir un proceso inflamatorio descrito en el septo alveolar e interlobular, que definen la zona septal del pulmón. Suele ser un hallazgo frecuente en lesiones pulmonares. Caswell & Williams, (2007) describen el daño alveolar difuso como una forma menos grave de neumonía intersticial, que se caracteriza por un daño estructural de los neumocitos tipo I o células endoteliales del septo alveolar, la formación de membranas hialinas, la proliferación de los neumocitos tipo II y una posible fibrosis intersticial. En animales con esta lesión, Oruç (2006) encontró un incremento del número de células mononucleares en el septo interalveolar, además de la presencia de macrófagos dentro de las luces alveolares. En algunos casos se ha observado una ligera hiperplasia peribronquial y peribronquiolar.

La presencia de procesos exudativos junto con otros intersticiales suele ser habitual, definiéndose como neumonías atípicas o neumonías broncointersticiales (Caswell y Williams, 2007). En ellas se puede visualizar un aumento del grosor del septo intersticial, acompañado de fenómenos exudativos en la zona alveolar y bronquial.

Los fenómenos de bronconeumonía, normalmente asociados a infecciones de tipo bacterianas, se describen como procesos de tipo exudativo grave con presencia de neutrófilos y macrófagos en las zonas de unión broncoalveolar. Estas lesiones pueden extenderse a otros bronquios y bronquiolos, asociándose también a una hiperplasia peribronquial.

Si tenemos en cuenta los hallazgos macroscópicos y microscópicos, la presencia de consolidación en las zonas craneales ha sido asociada de forma general a formas exudativas, bronconeumonías de tipo catarral, fibrinoso, y purulento (Gázquez et al., 2001; Leite et al., 2002; Oruç, 2006). Pero también se encuentran artículos en los que se citan procesos intersticiales asociados a consolidación pulmonar (Sheehan et al., 2007; Azizi et al., 2013). En ellos se describe la formación de membranas hialinas, junto a la presencia de hiperplasia e hipertrofia de los neumocitos tipo II.

En la pleura, las inflamaciones que se pueden observar son, generalmente, las de carácter fibrino-purulento. En ellas se observan tramas de fibrina distribuidas por el espacio pleural y suelen mostrarse en formas agudas. Cuando esta lesión tiende a la cronicidad, las redes de fibrina se organizan, originándose adherencias pleurales y posteriormente fibrosis. También cabe destacar en estas formas crónicas la posible presencia de abscesos en la cavidad pleural.

Si bien la etiología de esta patología de tipo exudativo suele ser bacteriana, muchos autores consideran que posee un origen multifactorial, en el que se ven implicados tanto agentes bacterianos (Gázquez et al., 2001; Oruç, 2006; Sheehan et al., 2007), como estresantes y de manejo e incluso factores medioambientales (Lacasta et al., 2008).

Dentro de los microorganismos implicados, los hallazgos más numerosos en animales con presencia de consolidación son los pertenecientes a la familia *Pastereullaceae* (Oruç, 2006; Lacasta et al., 2008). Se ha observado que estas bacterias pueden actuar de forma sinérgica con otras presentes en las vías respiratorias. A esto hay que añadir el estatus inmunológico del animal y la presencia de factores predisponentes a la infección y el desarrollo de lesiones pulmonares (Mohamed y Abdelsalam, 2008). Esta acción sinérgica puede provenir de otras bacterias también implicadas en la presencia de lesión pulmonar, como son las

pertenecientes al género Mollicutes, principalmente *Mycoplasma ovipneumoniae* y *Mycoplasma arginini*. De hecho, Dassanayake et al., (2010) describieron un papel fundamental de estas dos bacterias en la predisposición de procesos neumónicos ocasionados por *Mannheimia haemolytica*.

Además de estas bacterias también debe considerarse la implicación de virus en la presencia de lesiones, actuando en solitario (Obi & Ibu, 1989), o bien en concomitancia con bacterias de la familia *Pastereullaceae*, como observaron Rosadio et al. (2011) en alpacas (*Lama pacos*).

2.3 Estrés y parámetros asociados a la producción

Cada día más consumidores son conscientes de la importancia en la elección de un producto de calidad (Blokhuis et al., 2003). Numerosas organizaciones, como la Organización Mundial de la Salud (OMS) y la Organización para el Desarrollo Agrario y de la Alimentación (FAO), apuntan al desarrollo de más controles en las cadenas de producción cárnica para mantener una calidad suficiente en el producto, además de conseguir una sostenibilidad en las explotaciones.

El bienestar animal juega un papel muy relevante en la calidad del producto, y para ello se hacen necesarios controles efectivos. Las diversas normativas y las exigencias de los consumidores han culminado en el hecho de que exista cada vez una mayor transparencia en los productos finales, proporcionando información sobre el animal desde origen al punto de venta (Quintili and Grifoni, 2004).

Los CC de corderos han sido diseñados para mejorar la actividad ganadera, además de estandarizar el mercado de la carne ovina. No obstante, esto ha supuesto una serie de inconvenientes, entre los que destaca el mayor manejo que sufren los animales hasta su llegada a matadero (transporte, reagrupamiento, nuevo ambiente, alimentación, etc.) (Knowles et al., 1998; Parrott et al., 1998; Ibáñez et al., 2002;

Cockramet al., 2004). Estas situaciones pueden generar estrés a los animales que no solo afecta a la calidad de la carne (Sañudo et al., 1998) sino que, además, puede poner en riesgo la propia salud del animal (Lacasta et al., 2008). El medio ambiente también se ha convertido en un componente a tener en cuenta, debido a la acción que diferentes componentes (partículas de polvo, gases, etc.) pueden tener sobre el sistema inmune de los animales (Bonnette et al., 1990; Niekamp et al., 2007).

Cuando un estresor persiste en el tiempo, el eje hipotálamo-hipófisis puede llegar a adaptarse, volviendo a una liberación hormonal hasta niveles basales; en el momento que existe la presencia de otro agente estresor de carácter agudo, la respuesta frente a este, tendrá un mayor grado de intensidad (Kiss, 1994; Dallman, 1993). En algunas ocasiones los niveles de hormona adenocorticotropa y cortisol pueden mantenerse elevados mientras permanezca el agente causal, lo que puede ser causante de un estrés crónico (Dallman et al, 1992; Kiss, 1994).

La serie roja puede considerarse un buen indicador de bienestar animal, ya que se encuentra relacionada con el mecanismo de estrés (Kiss, 1994). La formación de glóbulos rojos, también conocidos como eritrocitos o hematíes, se denomina eritropoyesis. Ésta se encuentra regulada por una serie de factores entre los que destaca, como factor de aceleración, la eritropoyetina. Sus principales funciones incluyen, entre otras, la maduración de prorrubrocitos y la inhibición de la apoptosis eritrocitaria (Semenza, 1994). Igualmente, la eritropoyesis se encuentra influenciada de una forma negativa por numerosos factores, como el factor de necrosis tumoral alfa (TNFα), interferón gamma (IFNγ) o el cortisol, pudiendo llegar a provocar estados de anemia (Anderson et al, 1993; Porter, 1994). Pero no solamente el número de eritrocitos puede aportar información sobre el estado de las células rojas circulantes, sino también diferentes características de los mismos, como su tamaño o su contenido en hemoglobina.

Por otro lado, el sistema inmunitario sufre alteraciones ante situaciones de estrés, incluyendo las que derivan del sistema de producción, el estatus social de los animales y el sanitario. Por tanto, el estrés es un indicador importante de las condiciones de bienestar de los animales. Es más, la respuesta del sistema inmunitario es uno de los mecanismos que el organismo desarrolla para defenderse de los cambios ambientales. La teoría más aceptada es que el estrés tiene un efecto de supresión sobre el sistema inmune, como en casos de privación de alimento o aislamiento (Kannan et al., 2002) o manejos como cirugías o descornamientos (Mellor et al., 2002).

Las citoquinas juegan un papel importante en la activación del sistema inmune y en la diferenciación de las células de la serie blanca, aunque no son las únicas sustancias implicadas (Kidd, 2003). Los glucocorticoides liberados durante un estado de estrés, actúan inhibiendo la liberación de varias citoquinas como IL-4, IL-5 y IFNγ (Richards et al, 2001; Sapolsky et al, 2001). En general, inhiben la síntesis de citoquinas proinflamatorias (Wiegers et al, 2005), siendo la ratio neutrófilo/linfocito un indicador de estrés crónico (Lawrence & Rushen, 1993). Esta ratio disminuye cuando existe un menor nivel de estrés. Sin embargo, no en todos los casos sucede lo mismo. Los estados de estrés pueden suprimir, promover o no tener efectos sobre el sistema inmune en función de su duración o intensidad (Johnson & BIGlone, 1996).

Por otra parte, el neutrófilo es el principal tipo celular presente en la sangre implicado en la respuesta inmune innata. El modo de actuación de esta célula dentro de la respuesta inmune, es mediante la fagocitosis, gracias a la destrucción mediante el uso de metabolitos de los antígenos previamente ingeridos. Factores de tipo genético, factores medioambientales y aquellos propios del manejo pueden producir una disminución de respuesta inmune en los animales (Clappertone et al., 2005; Niekamp et al., 2007). Por ejemplo, se ha observado una mayor función en la

actividad fagocítica de neutrófilos en cerdos ibéricos producidos en sistemas intensivos frente a otros en condiciones extensivas (Rudine et al., 2007; García-Torres et al., 2010).

Un aspecto importante en relación con el estrés y el bienestar animal es el ambiente en el que se crían a los animales. Los sistemas intensivos de producción ovina provocan un cambio sustancial desde la crianza en el medio ambiente hasta otra basada en un sistema de naves. Estas naves suponen un importante punto de controversia en la producción ovina, ya que el diseño y la construcción pueden favorecer el desarrollo de enfermedades relacionadas con el aparato respiratorio. Además, y asociado a la idiosincrasia de los puntos de producción, se ha constatado también una disminución de la actividad en el sistema inmune del animal en las nuevas condiciones a las que se someten. Las premisas establecidas por ITOVIC (1991) consideran unas condiciones óptimas para el cebo de corderos cuando se logra una humedad relativa de 70-80%, una velocidad del aire de 0.2 m/s y un rango de temperaturas de 13-15ºC.

Los principales peligros asociados al sistema de producción, además de todo lo implicado en el manejo de los animales, son las construcciones, ya que, generalmente, han sido creadas sin tener en cuenta una buena ventilación y orientación (Lacasta et al., 2008). Esto hace que aumente el número de animales enfermos en días con condiciones climáticas adversas. Por supuesto, no podemos olvidar la presencia de gases irritantes como el amoniaco, consecuencia inherente al modelo de producción. Una concentración superior a las 10 partes por millón (ppm) podría originar un daño en el epitelio, provocando la pérdida de funcionalidad de los cilios (Caswell & Williams, 2007). De esta forma desaparecería la primera barrera inmunitaria del pulmón. De hecho, en porcino se ha constatado una disminución de

la funcionalidad de los macrófagos alveolares ante la presencia de partículas de polvo (Knetter et al., 2014).

2.4 Respuesta inmune en el aparato respiratorio

El organismo puede sufrir la invasión de agentes extraños, denominados patógenos, que son capaces de producir enfermedad en el órgano hospedador. Para paliar este hecho, los organismos vivos tienen la capacidad de evitar la entrada de patógenos, y para ello utilizan diferentes tipos de defensas: las barreras físicas, la inmunidad innata y la inmunidad adquirida. La tos, el moco y los estornudos constituyen la barrera física de entrada de patógenos al tracto respiratorio.

Quizás sea la inmunidad innata el principal mecanismo de defensa implicado que, gracias a una respuesta celular y química, da lugar a una acción rápida contra el microorganismo. Este tipo de inmunidad, que responde al ataque de agentes patógenos de forma inespecífica, tiene una serie de mecanismos implicados: las células fagocíticas (neutrófilos y macrófagos), las proteínas sanguíneas y las citocinas, las cuales, actúan como mediadores en muchas de las acciones de la inmunidad.

El término “macrófago” fue propuesto por Metchnikoff (1968) para describir unas células grandes con capacidad claramente fagocítica que están presentes en la mayoría de los tejidos conectivos y fluidos corporales. Los macrófagos son los responsables de una respuesta rápida en la defensa o supervivencia del huésped, atacando a microorganismos extracelulares, a células infectadas por virus en el lugar inicial de la infección del huésped y a las células tumorales. Así, en un primer momento, estas células pueden acabar con la enfermedad sin necesidad de que se produzca una estimulación inmune específica, proporcionando una solución local. Pero, además, los macrófagos intervienen en la respuesta inmune específica puesto

que, entre sus funciones, se incluye la presentación de antígenos a las células T "helper". Además, tiene importantes funciones reguladores con respecto a los polimorfonucleares (PMNs), células NK y linfocitos. Muchas de estas acciones reguladoras son realizadas mediante la producción de metabolitos específicos y citoquinas (Kay, 1996).

La movilización de los neutrófilos hacia la zona afectada es modulada por la interacción de leucocitos y citoquinas (Hughes et al., 1992; DeBey et al., 1996; Caswell et al., 2001). La invasión de las vías aéreas por los neutrófilos marca una propia reacción de defensa para el órgano (Soethout et al., 2002). Este tipo celular es, de forma directa o indirecta, el causante de la principal patología pulmonar cuando se origina una lesión de tipo exudativo (Malazdrewich et. al., 2001).

La movilización de neutrófilos no es efectiva "per se" para luchar contra la infección, ya que necesita otros elementos para ello; pero estas células, junto a la movilización de interleucinas y otros componentes celulares, contribuyen al desarrollo de la lesión pulmonar (Yoo et al., 1995; Malazdrewich et al., 2001; Leite et al., 2002; Youssef et al., 2004). En la fase aguda de los procesos neumónicos en rumiantes, las citoquinas inflamatorias, como IL-1β, el TNFα, IL-8 y el IFN-γ, son secretadas por una variedad de células del sistema inmune y no inmune, incluyendo monocitos, macrófagos, fibroblastos, células epiteliales y neutrófilos (Caswell et al., 2001; Malazdrewich et al., 2001). Estos últimos pueden estimular la migración de leucocitos y aumentar la respuesta inflamatoria, dando lugar a la infiltración de neutrófilos adicionales (Ackermann et al., 1999, 2000; Leite et al., 2002).

Otros autores destacan la actividad inmunomoduladora de la IL-10 como interleucina con actividad antiinflamatoria. Es decir, no solamente existen citoquinas que potencian la actividad, sino también se describen otras con clara actividad disminuyendo los efectos causados por los mediadores inflamatorios (Fach et al.,

2009; Redondo et al., 2014). Esta actividad se modula por la inhibición en la producción de otras interleucinas debido a la acción de otros mediadores como la IL-1, IL-8 o TNF-α. Su función biológica principal parece ser la limitación y la finalización de las respuestas inflamatorias y la regulación de la diferenciación y la proliferación de varias células inmunes tales como células T, células B, célula natural killer (NK), células presentadoras de antígeno (CPA), mastocitos y granulocitos.

La importancia de las interleucinas como mediadores de la enfermedad en el ganado ovino ante la presencia de determinados agentes patógenos del ovino, como *Mannheimia haemolytica* (Redondo et al., 2011), virus respiratorio sincitial (Redondo et al., 2014) y *Mycoplasma ovipneumoniae* (Xue et al., 2015) ha sido previamente descrita.

Cuando un agente es capaz de evadir a la respuesta inmune innata, las diferentes poblaciones de linfocitos T actúan para acabar con el agente nosógeno. Bajo los efectos de una desregulación de estos, el pulmón puede sufrir daños, lo que puede asociarse a enfermedades de carácter crónico. Los linfocitos se regulan por la actuación de mediadores como las interleucinas, dentro de las cuales tienen diferentes funciones, moléculas co-estimulantes, regulación epigenética y factores de transcripción. En cuanto a las distintas subpoblaciones de linfocitos T, se ha observado en pulmón, que las células CD4+ (Linfocitos T helper) ayudan a las células B a provocar una respuesta de anticuerpos proveyendo de una retroalimentación a las células dendríticas y mejorando y manteniendo la respuesta de los CD8+ (linfocitos T citotóxicos) (Moore et al., 2001).

Los linfocitos T por sí solos no son capaces de identificar a los antígenos extraños, necesitan que estos les sean “presentados”. Para cumplir esta función de gran importancia, el sistema inmunológico dispone de un grupo de células denominadas “Células presentadoras de antígenos” o CPA. Dentro de éstas se incluyen a los

macrófagos, linfocitos B, células dendríticas y células de Langerhans. Los linfocitos T son estimulados por células dendríticas, que llegan hasta los linfonodos para servir como CPA, siendo esta población linfocitaria distinguible de otras CPA, como son los linfocitos B y los macrófagos (Tony et al., 2003). Estas células comparten la particularidad de que todas expresan en sus membranas moléculas del MHC II, a las cuales se les asocia el antígeno, y de esta manera pueden ser "presentados" a las células T.

El reconocimiento de los antígenos por parte de los linfocitos T se hace en los órganos linfoides, en la mayoría de los casos en los linfonodos. En estos sitios anatómicos residen las células T naive, y es aquí donde se transforman en células efectoras que, después de la expansión clonal y diferenciación, migran para entrar en circulación sanguínea y dirigirse hacia el lugar de la infección.

Los linfocitos B poseen receptores de unión al antígeno (BCR). Estos pueden reconocer antígenos sin una estimulación previa, aunque para una respuesta óptima se necesitan linfocitos T helper tras coestimulación a través de una sinapsis inmunitaria en la que intervienen, además, distintas citoquinas. El heterodímero CD79a/CD79b es el componente de transducción de señales del complejo BCR, responsable de iniciar las vías de señalización intracelulares complejas que rigen la respuesta inmune de células B para el reconocimiento del antígeno exógeno y de unión por las inmunoglobulinas de membrana (Reth et al., 1991).

Existen dos mecanismos de activación de las células B, la activación de antígenos T dependientes y los mecanismos de activación T independiente. Los antígenos proteicos no pueden inducir por sí solos la activación de los Linfocitos B, sino que requieren la estimulación de los Linfocitos CD4+. Las células B específicas unen el Ag a su receptor, lo internalizan y lo procesan en vesículas endosómicas. Estas proteínas endocitadas son degradadas por enzimas presentes en los endosomas y

lisosomas para generar pequeños péptidos que podrán unirse a las moléculas del MHCII. Finalmente, en la membrana del Linfocito B se encuentran el péptido unido a la molécula del MHCII y sus coestimuladores, cuyo fin es poder “presentar” el Ag a los LTh. Gracias a esta estructura, las células B son consideradas también “células presentadoras de Ag” (CPA). Los LTh presentan en su membrana receptores para el MHCII y ligandos para los coestimuladores. Cuando interactúan con la estructura de las CPA (Linfocito B + MHCII y los ligandos) se produce la activación de los LTh. Una vez activados, los LTh, secretan citoquinas para estimular la proliferación y diferenciación del Linfocito B.

Las citoquinas no son específicas para cada Ag, aunque hayan sido secretadas tras la activación de un LTh especifico. Las citoquinas desempeñan dos funciones principales: estimulan la proliferación y diferenciación de los Linfocitos B y determinan qué tipo de inmunoglobulina se producirá por la activación de estas células.

Los antígenos T independientes son activados de diferente forma. No son internalizados, sino que ejercen su acción por señalizaciones intracelulares producidas por el receptor del Linfocito B. Generalmente, la respuesta producida por este tipo de Ag origina Ac de escasa afinidad y un bajo número de células de memoria. La importancia práctica de este tipo de reacciones viene marcada porque muchos Ag de las paredes bacterianas son polisacáridos lo cual sirve de reconocimiento por parte de estos y da lugar a la activación de los Linfocitos B, sin necesidad de mediadores y CPA en la inmunidad frente a las infecciones bacterianas.

BLOQUE II. Anatomía patológica en el SRO: factores medio-ambientales y de manejo asociados

Capítulo 3. Estudio de las condiciones MA en los hallazgos histopatológicos en el SRO

Realizamos un estudio para identificar los principales procesos respiratorios patológicos durante el proceso de cebo de corderos, así como su posible relación con las condiciones estacionales en los cuales se mantenían durante su estancia.

Para ello se seleccionaron un total de 410 corderos de forma aleatoria después de su clasificación seleccionados en cinco centros de clasificación ubicados en Extremadura en la zona 'Suroeste de España'. Las condiciones estacionales (Temperatura y humedad relativa) fueron analizadas durante el período estudiado (desde febrero hasta noviembre), con el objetivo de buscar la relación entre las lesiones encontradas y el periodo donde fueron más patentes.

Una vez extraídos los pulmones en el matadero, las nuestros fueron analizadas en las dependencias de la facultad donde se realizó un examen exhaustivo de las patologías encontradas donde establecimos dos grupos diferentes: A) con ausencia o B) con presencia de consolidación pulmonar. El estudio histopatológico mostró cuatro grupos de lesiones: (1) daño alveolar difuso (DAD); (2) neumonía intersticial 'IP'; (3) bronconeumonía purulenta 'PB' y (4) neumonía broncointersticial 'BI'.

En nuestros resultados vimos diferencias estadísticamente significativas entre la presencia de consolidación y el mes en que los animales estuvieron en el centro de clasificación, lo que revela que los procesos patológicos descritos en corderos de cebo se ven influidos por la estación en la que los animales fueron cebados.

En este estudio pudimos determinar la época del año con más riesgos asociados a la aparición de lesiones asociadas al síndrome respiratorio ovino en corderos de cebo en el Suroeste Extremeño.

Los resultados de este trabajo han sido plasmados en el artículo ‘Study of possible influence of seasonal conditions on pathological respiratory processes in fattening

ovine from Extremadura ‘South-western Spain’ enviado a la revista Small Ruminant Research para su revisión.

Study of possible influence of seasonal conditions on pathological respiratoy processes in fattening ovine from Extremadura 'South-western Spain'

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Abstract

A study was carried out to identify possible relationship between the main pathological respiratory processes in fattening lambs and seasonal conditions. 410 randomized fattening lambs from five feedlots located in Extremadura 'South-western Spain' were evaluated. Seasonal conditions (temperature and relative humidity) were recorded during the studied period (from February until November). A thorough gross pathology examination was conducted to establish two different groups: a) with absence or b) with presence of lung consolidation. The histopathological study showed four lesional groups: (1) diffuse alveolar damage 'DAD'; (2) interstitial pneumonia 'IP'; (3) purulent bronchopneumonia 'PB' and (4) intersttial bronchopneumonia 'BI'. There were significant associations between the presence of consolidation and the month in which animals were in feedlot, and also between these months and the histopathological groups. These results revealed that

pathological respiratory processes in fattening lambs are related with season in which the animals are fattened, allowing us to determine the time of the year with more associated risks to develop respiratory pathology.

Keywords

Lamb; Pathology; Pneumonia; Preventive medicine.

Introduction

In farms, pathological respiratory processes cause direct losses (mortality in fattening lambs) and indirect losses (loss of weight, bad conversion rates and increasing number of pulmonary lesions in the slaughter and therapies) (Goodwin et al., 2004; Lacasta et al., 2008). Among others, temperature and relative humidity have been previously registered as related risk factors (Brodgen et al., 1998; Yener et al., 2009). In fact, the climatic factors which produce an increase of relative humidity have a significant influence on the presence of pneumonia (Lacasta et al., 2008). Lundborg et al., (2005) established most of the extrinsic risk factors for respiratory health are related to indoor housing: insufficient space allowance, overcrowded pens, quick temperature changes, high humidity, air dust and high ammonia concentration.

The main gross pathological lesions found in the lungs are consolidation and atelectatic foci confined to the cranioventral regions and the presence of a pleural fibrinopurulent membrane (Sheehan et al., 2007; Yener et al., 2009). This consolidation can be lobar or lobular depending on the degree of affection (Caswell & Williams, 2007). Histopathological findings associated to consolidation are usually represented by suppurative bronchopneumonia, characterized by alveoli and bronchioles filled with polymorphonuclear inflammatory cells ‘PMN’ (Gázquez et al., 2001; Gonçalves et al., 2010), serofibrinous exudation, degenerated leukocytes and

necrotic debris (Gonçalves et al., 2010; Yener et al., 2009). However, interstitial pneumonia has been also associated to pulmonary consolidation in outbreaks of high mortality. In red deer, Mawhinney et al. (2010) noted hypertrophy and proliferation of type II pneumocytes, with hyaline membrane formation, septal oedema, fibrin deposition and emphysema. This pattern has been established in ovine, although it is often observed associated with peribronchial and peribronchiolar lymphocytic proliferation and luminal exudation (Oruç, 2006).

In Spain, lambs produced for meat are light and less than 3 months old (8.5–13 kg carcass weight). Many of them are Corderex®, a protected geographical indication for light lambs which are fed a concentrate based diet and milk until weaning (45–50days old), and thereafter with concentrate and straw (Sañudo et al., 1998). These animals are usually kept indoors (Alvarez-Rodriguez et al., 2008).

The aim of the present study was to describe the possible association between seasonal conditions of temperature and relative humidity and the presence of different respiratory processes in fattening lambs from Extremadura.

Materials and methods

Experimental design

Four hundred and ten either sex, randomly selected fattening lambs from five feedlots of Extremadura 'South-Western Spain' and with a live weight of 21±3 kilograms, were considered in this study. The slaughter dates were distributed homogeneously from February to November. Animals were grouped by live weight with other animals in pens with a density of 0.5m^2 per animal. Feeding (pellet concentrate and straw) and water were administrated ad libitum. The fattening period was of 21±5 days with a final weight of 25± 4 kilograms. Lungs were collected at the abattoir.

Seasonal conditions were studied from February to November in each fattening period. For this purpose, temperature and relative humidity were registered using meteo station (Data Logger 175H1, Testo®). Two measures per hour of each parameter were recorded and the obtained information was processed by spread sheet (Microsoft Excel®). Mean, ranges and standard deviation of humidity and temperature during the fattening period of the lambs were calculated.

Gross pathology

Digital images of ventral and dorsal side of the lungs were taken. They were classified into two groups depending on whether consolidation was found or not, i.e., absence of consolidation, and presence. Lesional areas and total area were delimited using Adobe Photoshop cs5® software. The percentage of affected lung was calculated by the ratio affected area/total area.

Histopathology

2-mm thick lung samples including the lesioned tissues and undamaged adjacent area of were taken. In those lungs without consolidation areas in the cranioventral regions, 2-mm thick randomly selected samples were taken. These samples were fixed in neutral buffered formalin ‘3.5%, 0.1M and pH 7.2’, routinely processed and embedded in paraffin wax. 5 µm sections were stained with haematoxylin and eosin, Masson´s Trichrome and van Gieson for the histopathological study. Microphotographs were taken using a microscope (Eclipse 80i, NIKON®, Tokyo, Japan) with a digital video camera (DXMI200F, NIKON®).

Statistical analysis

The correlation between the presence of lung consolidation and the month in which animals were in feedlot and, between the histopathological group and the month in which animals were in feedlot were calculated by using the Chi-squared test.

When significant results were obtained, homogeneity analyses were performed by using the Chi-squared test in order to determine whether there was any correlation between consolidation in the lung and the histological group in the considered months. Fisher´s exact test was used as an alternative when the expected frequencies were small, i.e., when the Chi-squared applicability conditions were not met (Cochran's rule, see, Cochran (1954)).

The Pearson´s correlation coefficient was used to examine the linear correlation between mean relative humidity and mean temperature.

The results were considered as significant when p-values were less than 0.05. The statistical software R has been used (R development Core Team, 2008).

Results

Gross pathology

Absence of consolidation was found in 48.8% of the lungs (n=200) and presence in 51.2% (n=210). These lungs showed foci of irregular consolidation in the cranioventral regions and occasionally in the middle and diaphragmatic lobes. In cases of consolidation, pleural surface showed a fibrinous membrane adhered to the rib wall. Lungs with absence of consolidation were apparently healthy and without membrane formation in the lung surface.

Histopathology

The histopathological study revealed four groups of microscopic changes: Diffuse Alveolar damage ‘DAD’. The percentage of findings was 48.1% of sampled animals. This group included ciliary necrosis and compaction and alveolar denudation, characterized by loss of type I pneumocytes and epithelial basement membrane (Figure 1). A mild inflammatory reaction was observed in peribronchial adjacent zone without BALT reaction. Fibrotic processes or pleural phenomena weren´t found.

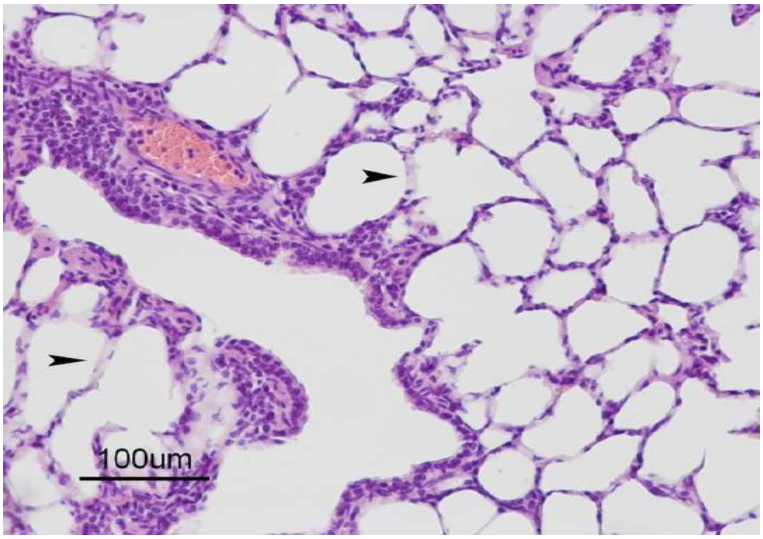

Figure 1. Loss of type I pneumocytes (arrowheads).

Interstitial pneumonia ‘IP’ (34.1% of cases). Lung lesions were characterized by septal damage, loss of type I pneumocytes and proliferation of type II pneumocytes (Figure 2). Septa were thickened (Figure 2), with mononuclear cell inflammatory infiltrates, marked congestion and oedema. Hyaline membranes were observed in junction interstitium and alveoli, composed of the presence of fibrin, eosinophilic proteinaceous material and cell debris. The airways and pleural surface did not show any significant change.

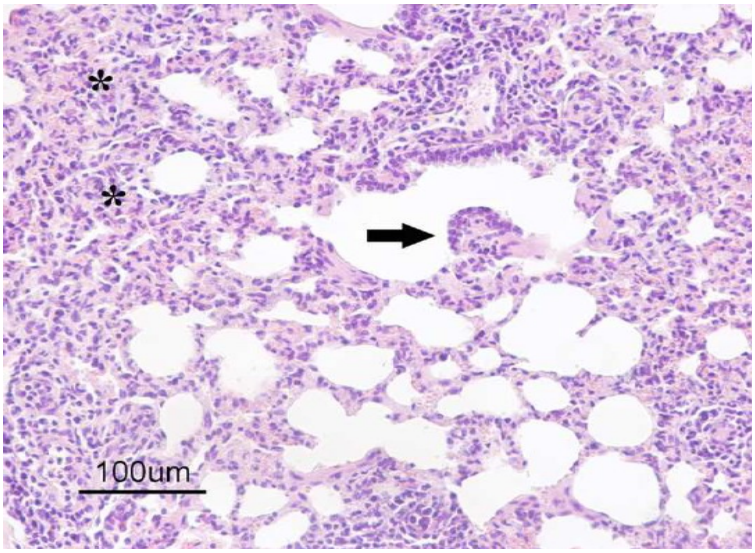

Figure 2. Thickening of alveolar septum (asterik). Hyperplasia and hypertrophy of type II pneumocytes (arrow).

Purulent bronchopneumonia ‘PB’. 12.7% of the cases were included in this group. The predominant lesional pattern was of exudative type. Neutrophils, macrophages and cells debris were observed within bronchial, bronchiolar and alveolar lumina. BALT was enlarged, as a multinodular structure (Figure 3). Perivascular inflammatory processes were mainly formed by mononuclear cells. Alveolar septa did not show any change. Severe fibrinous inflammatory reaction was present on the pleural surface.

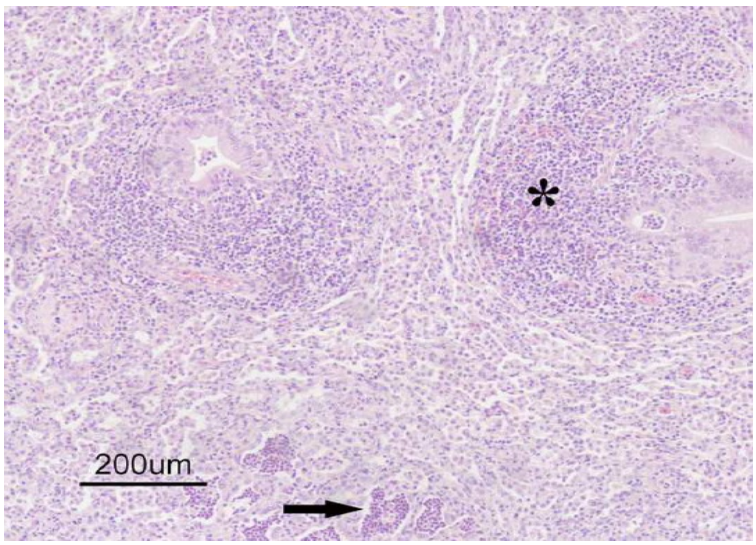

Figure 3. Exudative component in bronchiolar luminal (arrow). BALT hyperplasia (asterik).

Pneumonia together with suppurative bronchopneumonia was also described in 5.1% of the cases. This group was called bronchointerstitial pneumonia 'BI'. Inflammatory reaction within the interalveolar septa and exudative processes were concomitant in all the samples included in this group '1:1 of the studied sample' (Figure 4). BALT was found frequently enlarged, although displaying a diffuse pattern. Alveoli were filled with neutrophils, macrophages and some bronchi and bronchioles showed this inflammatory cell infiltration. However, thickening of interalveolar septum and a marked increase in number of mononuclear cells was also found in areas where exudate wasn´t observed. Pleural surface was affected in these lungs showing similar lesions to the 'PB' group.

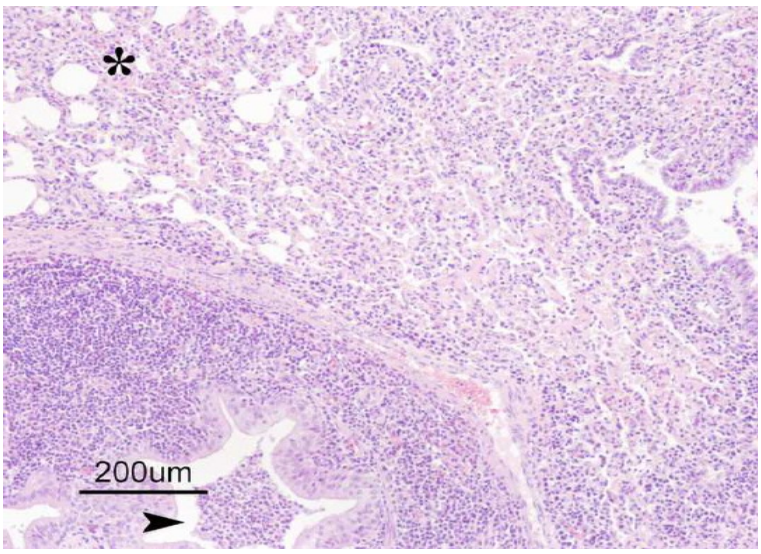

Figure 4. Interstitial infiltrates (asterik) and suppurative processes (arrowhead) concomitantly.

Statistical analysis

A significant negative correlation *(r = -0.704, p < 0.001)* between the mean relative humidity and mean temperature was obtained. Figure 5 shows the mean relative humidity and temperature across the time.

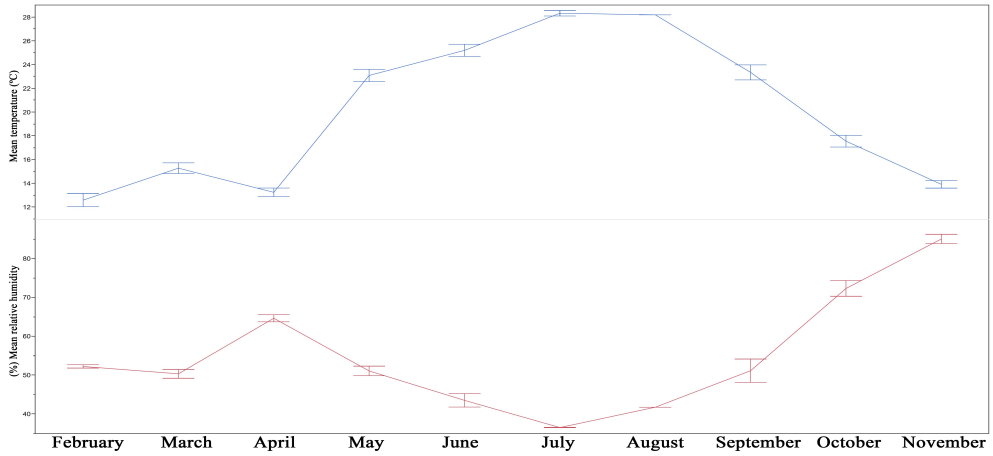

Figure 5. Mean temperature and mean relative humidity across the time.

In lung samples with absence of macroscopic consolidation, diffuse alveolar damage was present. However, samples with lung consolidation were represented by IP, PB and BI. These results were statistically significant by using a Chi-square test *($p < 0.0001$)*. These results are summarized within Table 1.

	DAD	IP	PB	BI	Total	p-value
Absence	69.5%	27.5%	3%	0%	100%	0.0001
Presence	27.6%	40.5%	21.9%	10%	100%	

Table 1. Relationship between macroscopic findings and histological groups.

The Chi-square test showed significant association between months in which animals were in the feedlot and lung consolidation *($p < 0.0001$).* Figure 6 represents the percentages of presence/absence of lung consolidation by months.

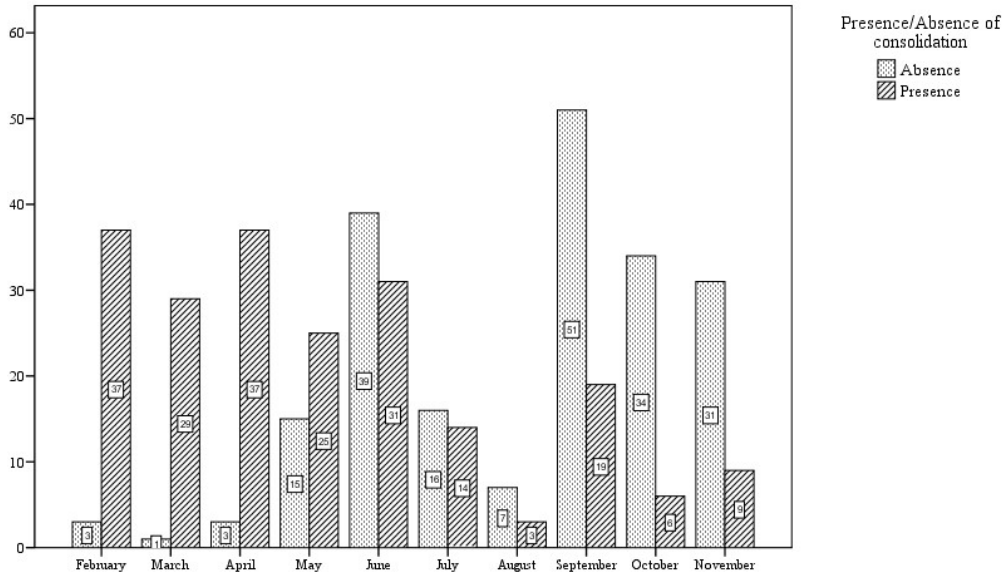

Figure 6. Percentage of absence/presence of consolidation and month in which animal was in feedlot.

The homogeneity analysis led to the following three periods: The first corresponded to February, March and April *(p = 0.728)*; the second was formed by May, June, July and August *(p = 0.169)*, and, finally, the third one from September to November *(p = 0.343)*. The highest percentage of cases with presence of lung consolidation corresponded to February, March and April. However, absence of consolidation was mainly observed in September, October and November. These results are presented in Table 2.

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Month	Absence	Presence	p-value
February	7.5%	92.5%	0.728
March	3.3%	96.7%	
April	7.5%	92.5%	
May	37.5%	62.5%	0.169
June	55.7%	44.3%	
July	53.3%	46.7%	
August	70.0%	30.0%	
September	72.9%	27.1%	0.343
October	85%	15%	
November	77.5%	22.5%	

Table 2. Homogeneity within group for months by considering lung consolidation.

The Fisher's exact test showed significant association between months in which animals were in the feedlot and the histopathological group *($p < 0.0001$)*. Figure 7 represents the percentages of histological groups by months.

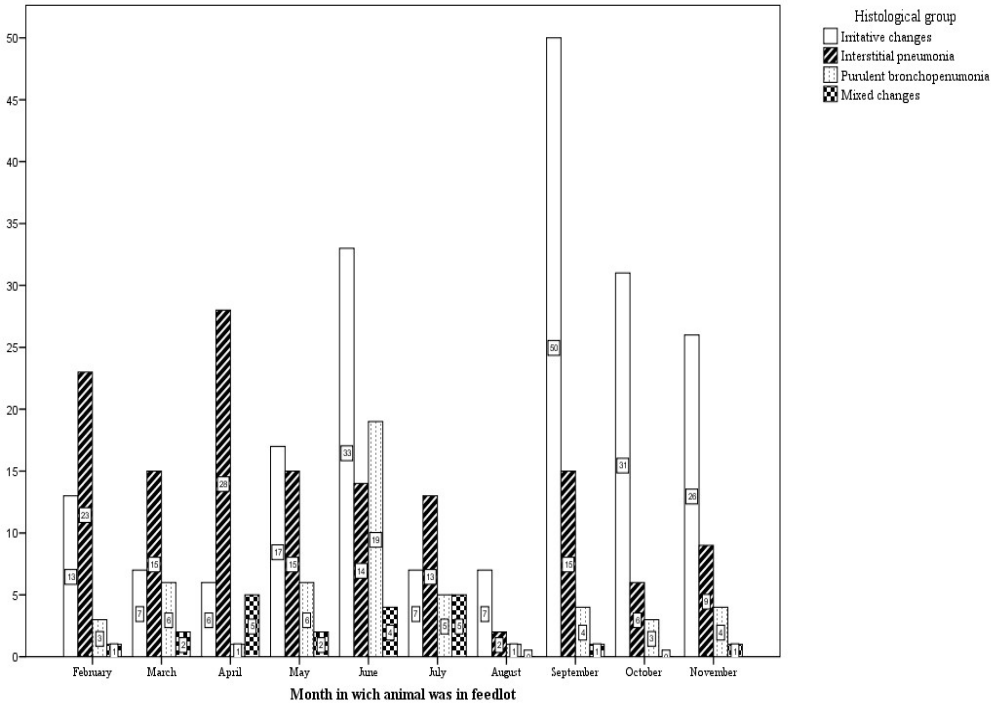

Fig. 7. Percentages of histological groups by months.

The homogeneity analysis led to the following three periods: The first corresponded to February, March, April and July *(p = 0.064)*. The second included May and June *(p = 0.193)* and, finally, the third was composed from August to November *(p = 0.927)*. Diffuse alveolar damages were grouped in August, September, October and November (third period). The most cases of interstitial pneumonia were present in February, March, April and July, in the first period. Suppurative bronchopneumonia was mainly present in March and June, in first and second period. Bronchointerstitial pneumonia showed a similar pattern of appearance to interstitial pneumonia, were grouped in April and July (first period).

These results are presented in Table 3.

Month	DAD	IP	PB	BI	p-value
February	32.5%	57.5%	7.5%	2.5%	0.0641
March	23.3%	50.0%	20.0%	6.7%	
April	15.0%	70.0%	2.5%	12.5%	
July	23.3%	43.3%	16.7%	16.7%	
May	42.5%	37.5%	15.0%	5.0%	0.193
June	47.1%	20.0%	27.1%	5.7%	
August	70.0%	20.0%	10.0%	0%	0.9269
September	71.4%	21.4%	5.7%	1.4%	
October	77.5%	15%	7.5%	0%	
November	65%	22.5%	10%	2.5%	

Table 3. Homogeneity within the group for months by considering histological groups.

Discussion

New intermediate steps have also been adopted, making national meat markets and associated logistics more dynamic and complex (i.e. auction markets, Miranda de la Lama et al., 2010a). Classification centres (CC) have been designated to establish the lamb fattening period. There are several commercial and productive reasons why CC are useful in the lamb meat production chain, including simplification of farm management, scarcity of specialized man power and product standardization. Depending on the live weight at arrival, lambs may have to stay at the CC for several days or even weeks until they reach the appropriate slaughter weight.

Presence of consolidation is an indirect problem in fattening lamb production associated to indirect losses by condemnations in slaughterhouse and a severe economic repercussion in the lamb production systems (Goodwin et al., 2004), giving rise to important direct and indirect economic losses in lamb production (Goodwin et al., 2004; Lacasta et al., 2008). Indirect losses have mainly been associated to lung consolidation processes (Lacasta et al., 2008). For these reasons, our aim was to study the correlation of lung lesions with seasonal factors and to stablish the most important risk periods in these production systems.

Presence of lung consolidation was frequently found, characterized by cranioventral areas of atelectasis with fibrinous membranes in the lung surface. These findings have been pointed by other authors (Sheehan et al., 2007; Yener et al., 2009; Rosadio et al., 2011). Histopathological study revealed four histological groups: diffuse alveolar damage (DAD), interstitial pneumonia (IP), purulent bronchopneumonia (PB) and bronchointerstitial pneumonia (BI). This classification has been performed similarly to those reported by other authors (Oruç, 2006; Caswell & Williams, 2007).

The statistical analyses evidenced a correlation between macroscopic and histological changes, similar to described by Rosadio et al. (2011). Suppurative bronchopneumonia is the most frequently microscopical lesion associated with lung consolidation (Gázquez et al., 2001; Gonçalves et al., 2010). However, Mawhinney et al. (2010) pointed to interstitial pneumonia as the main lesion involved in the so-called “red hepatisation”. In our case, interstitial pneumonia, suppurative bronchopneumonia and bronchointerstitial pneumonia has been described in pulmonary consolidation cases. This was also noted by other authors in ovine (Oruç, 2006) and in caprine (Yener et al., 2008). However, in absence of consolidation, the majority of analyzed samples were grouped in the DAD group.

Previous studies have analysed different possible risk factors in lamb respiratory pathology. Nash et al. (1997) described the age of the animals as an important risk factor. Lacasta et al. (2008) also considered the seasonal conditions as risk factors.

A statistical study was achieved to establish the possible relationship between seasonal conditions and lung consolidation as well as between these factors and histopathological groups. Three periods were determined in relationship to the presence/absence of consolidation and the months in which animals were in feedlot. These differences seem to have relationship with the mean temperature and mean humidity. The months of February, March and April were characterized by a mean temperature and a mean relative humidity different from those considered optimal for fattening lambs (ITOVIC, 1991), fixing 13-16ºC as optimal mean temperature and 70-80% as mean relative humidity. Similar findings have been observed by Lacasta et al (2008). This consolidation was also observed, when the temperature was below 10ºC, possibly associated to a poor ventilation and an increase in relative humidity within the feedlot as already described by Lacasta et al. (2008). In other side, Brscic et al., (2012) observed in calves in winter a lower prevelance than summer. In our study, there was an increase in presence of consolidation observing percentages higher than 90 in this period, this fact would imply management changes in order to prevent these problems. In summer months when was observed a high temperature, there was a high value in presence of consolidation, pointed this to first and second quarter as month with a high risk of pulmonary consolidation processes. This fact, seem to have relationship to pointed by other authors in ovine (Lacasta et al., 2008; Plummer et al., 2012). However, the months of September, October and November, were characterized by a mean temperature and a mean relative humidity closer to the statistical optimum range (ITOVIC, 1991). So, in those months which temperature

and humidity were outside of the optimum range interstitial and suppurative processes have been observed.

Although respiratory processes generally need bacteria to induce illness, the importance of predisposing factors, is well recognized in the development of this pathology in sheep (Brodgen et al. 1998; Ábalos, 2006). Therefore, should be an important factor to take into account not only the presence of consolidation in fattening lamb, as well as pathological processes associated to season. This implies that the first and second quart existed an important value in presence of consolidation however, since February to July was recorded a high value of interstitial and suppurative processes. Suppurative processes were more patents in warmer months and interstitial processes were present in colder months, which imply drugs cost together condemnations in abattoirs (Goodwin et al., 2004) and animal death in these months by severe respiratory processes. These studied months in CC, should increase management measures associated with this type of indoor production. Until now similar reports haven´t found in the cited bibliography, and this, and not only is important the gross lesions and bacteria, but also the months with the most prevalence.

The obtained results confirm the involvement of the environmental conditions in the presence of both consolidation and thereby in the onset and the type of lung inflammatory lesion. The knowledge of these correlations will allow us to establish preventive measures to diminish the associated economic losses in fattening lamb production.

Acknowledgements

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Capítulo 4. Aplicación de redes bayesianas para identificar los riesgos asociados a los procesos respiratorios ovinos

En este capítulo se llevó a cabo un trabajo para identificar los factores de riesgo asociados a la consolidación pulmonar, mediante el uso de redes bayesianas.

Se realizó un experimento con 410 corderos de engorde en cinco CC en Extremadura (Suroeste de España). Durante el período de estudio se registraron las condiciones ambientales (temperatura, humedad relativa y concentración de amoníaco). Además, esto se completó con un estudio microbiológico, buscando posibles patógenos, en nuestro caso bacterias pertenecientes a *Mycoplasma spp.* y la familia *Pasteurellaceae*. Para este fin se obtuvieron por cultivo convencional y se identificaron por reacción en cadena de la polimerasa anidada.

Después del sacrificio, los pulmones fueron recogidos en el matadero y examinados macro y microscópicamente (tipo histológico y consolidación pulmonar). Hasta la actualidad, las redes bayesianas no han sido utilizadas previamente para relacionar la presencia / ausencia de consolidación pulmonar con las condiciones ambientales, *Mycoplasma spp., Pasteurella spp*., y los cambios histológicos.

Los resultados mostraron que los principales factores causantes de los procesos respiratorios inflamatorios ovinos y consolidación pulmonar fueron la temperatura, la humedad relativa y *Mycoplasma spp*.

De este trabajo vimos que el control de estos factores puede ayudar a reducir la incidencia de consolidación pulmonar en los cebaderos ovinos.

Este trabajo ha sido publicado en la revista Small Ruminant Research.

Identifying risk factors for ovine respiratory processes by using Bayesian networks

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Abstract

A proposal is put forward to use Bayesian networks to identify risk factors for pulmonary consolidation. An experiment was conducted with 410 fattening lambs from five feedlots in Extremadura (southwestern Spain). Environmental conditions (temperature, relative humidity, and ammonia concentration) were recorded during the study period. In a microbiological study, *Mycoplasma spp.* and Pasteurellaceae were obtained by conventional culture and identified by nested polymerase chain reaction. After slaughter, lungs were collected and examined macro- and microscopically (histological type and pulmonary consolidation). To the best of the authors' knowledge, Bayesian networks have not before been used to relate the presence/absence of pulmonary consolidation to environmental conditions, *Mycoplasma spp., Pasteurella spp.,* and histological changes. The results showed

that the main factors causing ovine inflammatory respiratory processes and pulmonary consolidation were temperature, relative humidity, and *Mycoplasma spp*. Control of these factors may help reduce the incidence of pulmonary consolidation.

Keywords

Bayesian networks; lung pathology; ovine respiratory processes; preventive medicine; risk factors.

Introduction

Analysing animal health data is a complex task since the relationships between variables are usually not known. In fact, determining information about the way the variables are related is usually an objective of analysis. Lewis et al. (2011) discussed the potential of using Bayesian networks as analytical tools for processing complex animal health data. Specifically, they proposed the use of structure discovery to identify variables that may be associated with health status.

Bayesian networks belong to a family of probabilistic graphical models (see, e.g., Jensen, 1995). These graphical structures are mathematically rigorous and intuitively understandable, and can be used to represent knowledge about an uncertain domain. A Bayesian network is a directed acyclic graph (a graph with directed edges between vertices) with an associated set of probability distributions that enables an effective representation and computation of the joint probability distribution over a set of random variables. The visual representation of the random variables' dependency structure is especially useful. Bayesian networks combine principles from graph theory, probability theory, computer science, and statistics (Jensen and Nielsen, 2007).

These networks can be implemented to search for the structure between variables by finding an optimal directed acyclic graph for the dataset in hand, providing information

about the possible relationships linking the variables involved. The network can then be used to infer conditional probabilities. Bayesian networks can also be built using reliable subjective information provided by an expert instead of searching for the structure. Moreover, a mixed approach can be taken by first searching for the structure based on data, and then adjusting the structure using subjective information.

Bayesian networks have been extensively used in many fields of study, especially in artificial intelligence. They are gradually being introduced for the analysis of data in the veterinary field. BIKendrick et al. (2000) applied Bayesian networks to aid in the differential diagnosis of tropical bovine diseases. Otto and Kristensen (2004) proposed a biological model based on a Bayesian network to determine risk factors for infection with Mycoplasma hyopneumoniae in swine for slaughter. Ettema et al. (2009) used a Bayesian network to estimate the probability of claw and digital skin diseases by combining cow- and herd-level information. Jensen et al. (2009) used a Bayesian network to model the causes of leg disorders in finisher herds. BICormick et al. (2013) applied these networks to the identification of environmental conditions that influence disease in pigs, and Firestone et al. (2013) analysed the associations between risk factors and the infection status of horses in an equine influenza outbreak in Australia.

In the present study, we focus on pathological respiratory processes that lead to direct and indirect losses such as mortality, weight loss, low conversion rates, and greater numbers of pulmonary lesions at slaughter (Goodwin et al., 2004; Lacasta et al., 2008). The agents and risk factors that may influence these processes are numerous. Temperature and relative humidity have been identified as associated risk factors (Brogden et al., 1998; Yener et al., 2009). Indeed, climatic factors that increase relative humidity have a significant influence on the presence of pneumonia

(Lacasta et al., 2008). High mortality and morbidity from these causes during the summer months have been reported (Plummer et al., 2007). In the present work, we take a Bayesian network based approach to analyse conjointly the influence of some possible risk factors on pulmonary consolidation, rather than treating them separately. To the best of our knowledge, Bayesian networks have not previously been used to relate the presence/absence of pulmonary consolidation to environmental conditions, *Mycoplasma spp., Pasteurella spp*., and histological changes. A Bayesian network-based approach is considered to analyse some relevant scenarios for pulmonary consolidation.

Materials and Methods

Experimental design

Four hundred and ten Merina breed lambs and their commercial crossings of both sexes from five feedlots in Extremadura (southwestern Spain) formed the sample for study. The animals' ages ranged from 80 to 100 days, and they were monitored during the feedlot period (15-21 days) from February to November. They were held in pens in the different feedlots at a density of 0.5 m2 per animal. Feed (pellet concentrate and straw) and water were administered ad libitum. The animals had been transported by road from the farms to the feedlots. Twenty-one days later, they were transported to the slaughterhouse at weights of 24-26 kg.

Environmental conditions were registered during each feedlot period. Temperature and relative humidity were recorded using a data logger (175H1, Testo®, Titisee, Germany). Two measurements per hour for each parameter were recorded, and the data were processed in a spreadsheet (Microsoft Excel®). The mean temperature and mean relative humidity during the fattening period were calculated. The ammonia concentration was recorded using an ammonia detector (Gastec GV-100S®,

Kanagawa, Japan). Two weekly measurements were made during each feedlot period, and the mean was calculated. The data were binned into intervals using, in part, the classification proposed by ITOVIC (1991).

Bacteria identification

Nucleic acid purification and amplification of the 16S-23S intergenic spacer region was carried out using primers F2A and R2 to identify *Mycoplasma spp*., as described by Tang et al. (2000). This technique detected the presence of *Mycoplasma ovipneumoniae* (*M. ovipneumoniae*) and *Mycoplasma arginini* (*M. arginini*). Molecular identification of *Pasteurella multocida* (*P. multocida*) was carried out using a polymerase chain reaction. This technique was used to amplify a specific fragment of the gene kmt1 of *P. multocida* with primers KMT1SP6 and KMT1T7, as described by Townsend et al. (1998). *Mannheimia haemolytica* (*M. haemolytica*) was identified by direct hemagglutination, and *Pasteurella pneumotropica* was also isolated.

Macroscopic and microscopic study

Lungs were collected at abattoir. They were photographed on both sides, and classified into two groups depending on the presence or absence of consolidation, i.e., consolidation greater than 0% or equal to 0%, respectively.

For the histological study, samples were fixed in neutral buffered formalin (3.5%, 0.1 M, and pH 7.2), routinely processed, and embedded in paraffin. Sections of 5 µm were stained with haematoxylin and eosin.

Four histological groups were established following the classification of Caswell and Williams (2007). (1) Diffuse alveolar damage: This group included ciliary necrosis and compaction and alveolar denudation, characterized by loss of type I pneumocytes and epithelial basement membrane. A mild inflammatory reaction was observed in

the peribronchial adjacent zone, with no bronchial associated lymphoid tissue (BALT) reaction. No fibrotic processes or pleural phenomena were found. (2) Interstitial pneumonia: This group was characterized by septal damage, loss of type I pneumocytes and proliferation of type II pneumocytes. Septa were thickened, with mononuclear cell inflammatory infiltrates, marked congestion, and oedema. Hyaline membranes were observed in interstitium and alveoli. They consisted of fibrin, eosinophilic proteinaceous material, and cell debris. The airways and pleural surface showed no significant changes. (3) Purulent bronchopneumonia: The predominant lesional pattern in this group was of an exudative type. Neutrophils, macrophages, and cell debris were observed in bronchial, bronchiolar, and alveolar lumina. BALT was enlarged as a multinodular structure. Perivascular inflammatory processes mainly involved mononuclear cells. Alveolar septa did not show any change. A severe fibrinous inflammatory reaction was present on the pleural surface. Pneumonia together with purulent bronchopneumonia was also described. (4) Bronchointerstitial pneumonia (bronchointerstitial pneumonia): Inflammatory reaction within the interalveolar septa and exudative processes were concomitant in all the samples included in this group '1:1 of the studied sample'. BALT was frequently enlarged, although displaying a diffuse pattern. Alveoli were filled with neutrophils and macrophages, and some bronchi and bronchioles showed this inflammatory cell infiltration. However, thickening of the interalveolar septum and a marked increase in the number of mononuclear cells were also found in areas where no exudate was observed. The pleural surface was affected in these lungs, showing lesions similar to the purulent bronchopneumonia group. Microphotographs were taken using a microscope (Eclipse 80i, NIKON®, Tokyo, Japan) with a digital video camera (DXMI200F, NIKON®, Tokyo, Japan).

Data analysis

Six algorithms were tested to build Bayesian networks using the GeNie/SMILE software (Druzdzel, 1999). Specifically, these were Greedy Thick Thinning, Bayesian Search, Essential Graph Search, Tree Augmented Naive Bayes, Augmented Naive Bayes, and Simple Naive Bayes. A cross-validation scheme was used to analyse model performance. The networks were compared using the area under the Receiver Operating Characteristic (ROC) Curve (AUC) and the accuracy rates. The AUC is a measure of how well the model can distinguish between the two groups of pulmonary consolidations (absence/presence). Its value ranges between 0 and 1.

The Greedy Thick Thinning search algorithm (Dash and Cooper, 2004) provided the best results. With this method, initially each node has no parents. The nodes providing the strongest increases in the score in the resulting structure (using the Bayesian Dirichlet with score equivalence and uniform priors, known as BDeu criterion) are added incrementally as parents (Cooper and Herskovits, 1992; Silander et al., 2008). When the addition of parents does not increase the score, they are no longer added to the node. A weakly informative prior distribution is considered. The prior distribution of the parameters is associated with their corresponding nodes and all the possible combinations of the parents, where is the parameter vector and is the combination of states of the parents for node for node. The total number of parameters, i.e., the length of the parameter vector, is the product of the number of states of the node under consideration multiplied by the number of states of every parent node. The maximum number of parent nodes was set to six (one less than the number of variables), allowing all possible relationships among variables.

With this algorithm, an optimized network structure is obtained using the BDeu criterion. The arcs represent the statistical dependence existing between the linked variables, but the relationships illustrated by the Bayesian network may be due to a

causal connection or may be spurious from a practical point of view. Although the final graph is usually represented with directed arcs, the causality or direction of association cannot always be completely confirmed without detailed experimental analysis (Heckerman et al., 1995). A sensitivity analysis was also performed.

After building the network, we analysed the most interesting risk scenarios in terms of probabilities by considering evidence propagation. Evidence propagation is one of the most powerful characteristics of Bayesian networks. It allows the probabilities of each node to be updated via bidirectional propagation of new information through the whole structure. In each scenario, a percentage of 100% is set for one category in one or more nodes in order to show how the environmental and/or bacterial evidence influences pulmonary consolidation. The probability estimates for the different scenarios will be reported in terms of percentages in two tables.

Results

With the specifications presented in the previous section, six Bayesian networks were built using the following variables: mean relative humidity, mean temperature, mean ammonia level, Mycoplasma spp., Pasteurella spp., histological type, and pulmonary consolidation. The environmental variables are continuous, and were binned into intervals according to pre-defined environmental criteria.

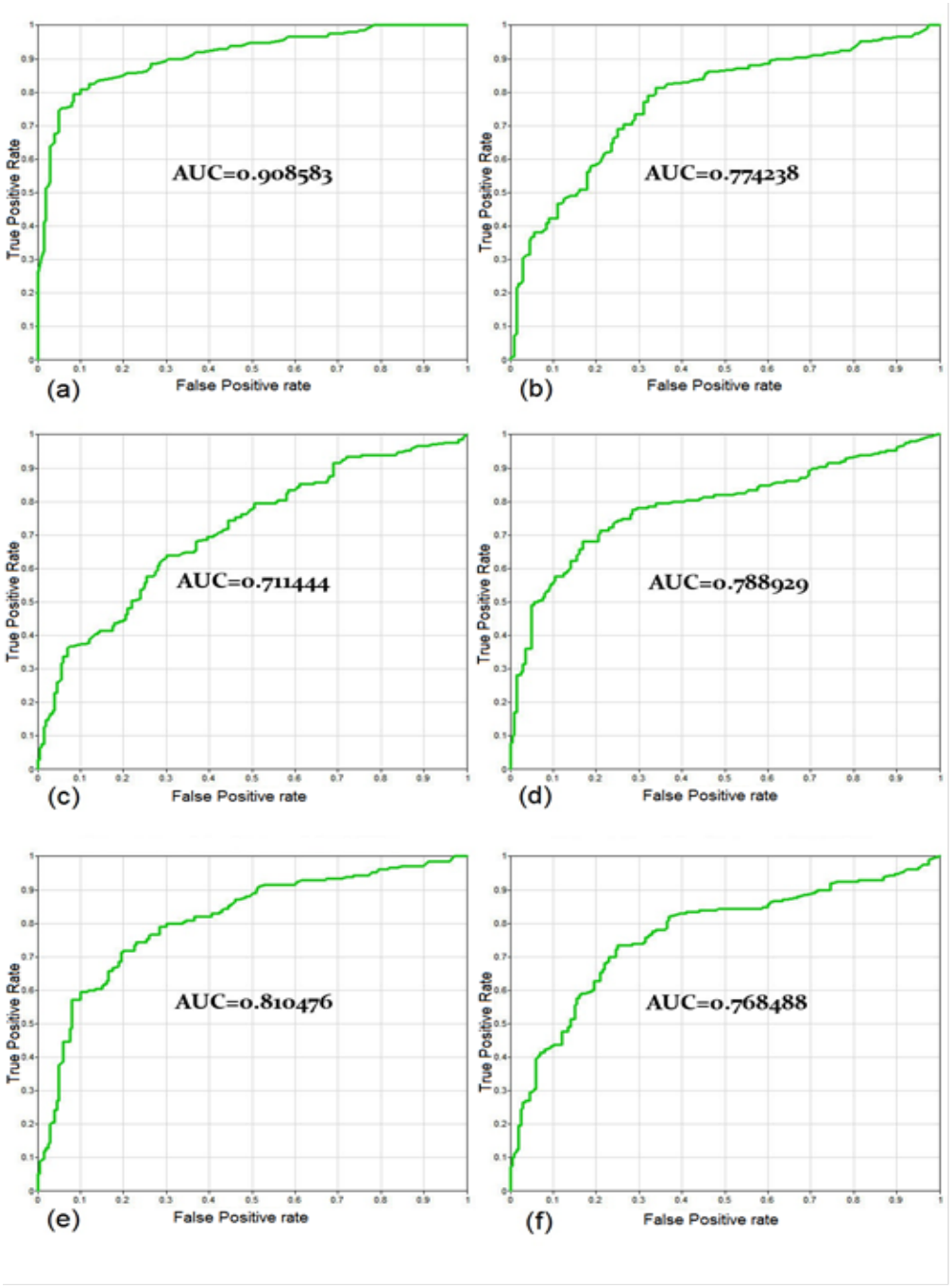

Figure 1. ROC curves and AUC for Bayesian networks built using six algorithms: (a) Greedy Thick Thinning; (b) Bayesian Search; (c) Essential Graph Search; (d) Tree Augmented Naive Bayes; (e) Augmented Naive Bayes; (f) Naive Bayes.

In order to compare the networks, a 4-fold cross-validation scheme was performed with 500 iterations. The AUC and accuracy rates were calculated. Figure 1 shows the ROC curves. The estimated AUC for the Greedy Thick Thinning algorithm was the best, giving a high value (0.9086). This means that the model fits the data quite well. The other algorithms achieved lower AUC values.

In coherence with the AUC criterion, the greatest accuracy rate was also achieved with the Greedy Thick Thinning algorithm (see Table 1). Note that this result is not only obtained for the overall accuracy rate, but also for the presence and absence accuracy rates. Hence, this algorithm provides the best results using both the AUC and the accuracy rates with the cross-validation scheme that was considered.

Algorithms	Accuracy	Absence	Presence
Greedy Thick Thinning	85.6	90.0	79.5
Bayesian Search	71.9	71.0	72.9
Esential Graph Search	66.8	70.0	63.8
Tree Augmented Naive Bayes	75.6	80.5	71.0
Augmented Naive Bayes	75.1	80.0	70.0
Naive Bayes	71.7	78.5	65.2

Table 1. Accuracy rates in percentages for Bayesian networks built using different algorithms.

The network structure and conditional probabilities were calculated based on the collected dataset, using the Greedy Thick Thinning algorithm (see Figure 2). The percentages given on the nodes are estimated conditional probabilities that illustrate how the state of a variable influences the probability distribution for the states of other

variables. Pulmonary consolidation was directly influenced by the mean temperature, mean relative humidity, and histological type, and indirectly by Mycoplasma spp. The joint probability distribution obtained was:

$$P(T,H,A,M,P,HI,C) = P(T)P(H|T)P(A|T,H)P(M|T)P(P|T,M)P(HI|M,H)P(C|T,H,HI)$$

with T being mean temperature, H mean relative humidity, A mean ammonia level, M Mycoplasma, P Pasteurella, HI histological changes, and C pulmonary consolidation.

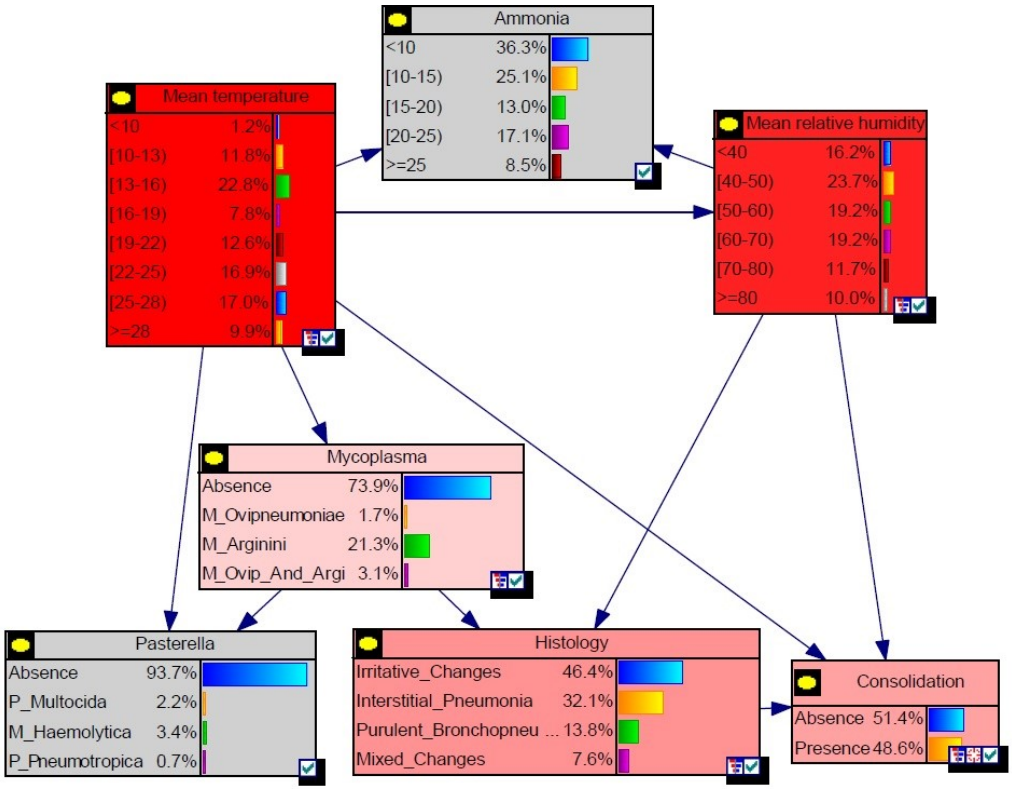

Figure 2. Bayesian network structure and estimated conditional probabilities.

A sensitivity analysis was performed to provide information on how small changes in the preliminary factors impact the terminal events (absence/presence of pulmonary consolidation). In Figure 2, the intensity of the red colouring of the nodes different from that of pulmonary consolidation indicates which variables have more impact in leading to changes in the probability of the target node states when small changes

are made in the probability of their states (the more intense the red, the more impact). Mean temperature and mean relative humidity produce the most important changes in pulmonary consolidation.

In order to make a deeper sensitivity analysis, all the possible cases (single states and combinations) were studied. The impact of the top ten cases on pulmonary consolidation was displayed in a sensitivity tornado chart (Figure 3). The variation displayed in this chart is obtained by adjusting the probability of occurrence for the different states ±20% of their respective values, either several factors at once or one at a time. The interpretation of the tornado chart is simple. Green indicates an increase of 20% in the factor studied, and red a decrease of 20%. The effect of these variations is reflected with a bar, indicating whether the effect is directly proportional (green on the right) or inversely proportional (red on the right). For example, the original probability of consolidation presence was 48.6%, and an increase (see Bar 6 in Figure 3) of 20% in the probability of the mean temperature (state 10-13 degrees), would increase the probability of consolidation presence to 49%. On the other hand, a decrease of 20% in the probability of the mean temperature (state 10-13 degrees) would decrease the probability of pulmonary consolidation presence to 48.2%. Although the model is robust and the appreciated changes are very small, temperature and relative humidity can be pointed to as the two variables causing the greatest variations in pulmonary consolidation when small changes are instituted.

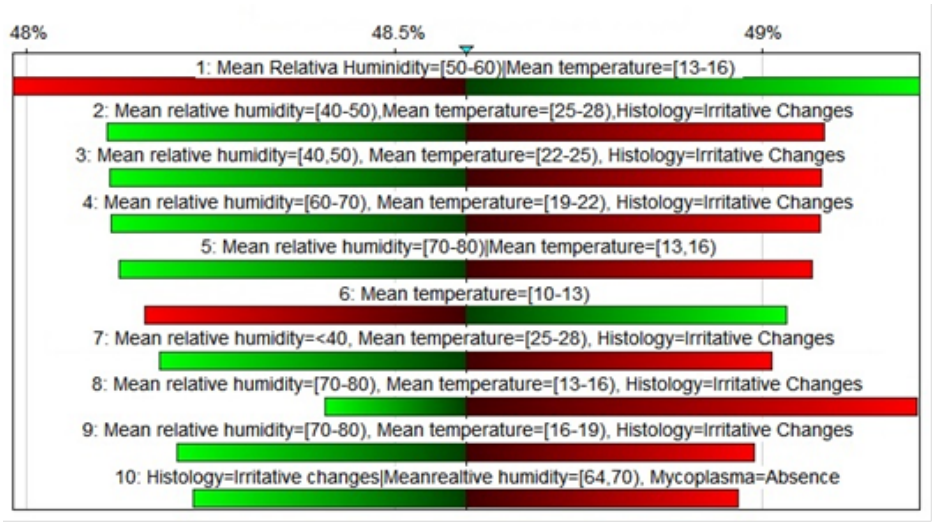

Figure 3. Sensitivity tornado chart for the presence of pulmonary consolidation.

Now we shall focus on analysing various scenarios. Evidence propagation about the state of a given node sheds light on the states of the nodes pointed to by that given node. The probabilities for each state of the nodes can be estimated with the conditional distribution and the joint distribution. For example, if the histology is set to the value diffuse alveolar damage, then the probability of the absence of pulmonary consolidation given that diffuse alveolar damage occurs is:

$$P(C = Absence|HI = IrritativeChanges)$$
$$= \frac{P(C = Absence, HI = IrritativeChanges)}{P(HI = IrritativeChanges)}$$
$$= \frac{\sum_{T,H,A,M,P} P(T,H,A,M,P,HI = IrCh,C = Absence)}{\sum_{T,H,A,M,P,C} P(T,H,A,M,P,HI = IrCh,C)}$$
$$= \frac{\sum_{T,H,A,M,P} P(T)P(H|T)P(A|T,H)P(M|T)P(P|T,M)P(HI = IrCh|M,H)P(C = Abs|T,H,HI = IrCh)}{\sum_{T,H,A,M,P,C} P(T)P(H|T)P(A|T,H)P(M|T)P(P|T,M)P(HI = IrCh|M,H)P(C|T,H,HI = IrCh)}$$

In this case, the probability of the absence of pulmonary consolidation given that diffuse alveolar damage was produced was estimated as 63.3%. The evidence

propagation concept is used to show how one or more variables influence the absence or presence of pulmonary consolidation.

	Scenario					
Variables	**1**	**2**	**3**	**4**	**5**	**6**
Temperature						
<10ºC					100.0	2.7
10-13ºC						2.5
13-16ºC	100.0	100.0	100.0			11.3
16-19ºC						50.1
19-22ºC						11.9
22-25ºC						10.6
25-28ºC						8.4
>28				100.0		2.5
Relative humidity						
<40			4.8	54.1	16.7	
40-50			4.8	23.5	16.7	
50-60		100.0	4.8	5.6	16.7	
60-70			4.8	5.6	16.7	
70-80	100.0		4.8	5.6	16.7	100.0
>80			76.2	5.6	16.7	
Ammonia						
<10	45.4	61.4		24.4	20.0	
10-15	3.2	2.3		24.4	20.0	
15-20	3.6	2.5	100.0	2.2	20.0	100.0
20-25	44.5	31.5		46.7	20.0	
>25	3.2	2.3		2.2	20.0	
Mycoplasma						
Absence	85.4	85.4	85.4	50.6	44.6	81.4
Ovipneumoniae	1.1	1.1	1.1	3.8	11.2	1.8
Arginini	10.8	10.8	10.8	40.8	29.9	13.9
Ovipneumoniae and arginini	2.7	2.7	2.7	4.7	14.3	2.9
Pastereulla						
Absence	91.8	91.8	91.8	95.1	60.1	90.1
Multocida	2.8	2.8	2.8	1.8	14.9	2.4
M. haemolytica	5.1	5.1	5.1	2.3	18.6	6.5
Neumotropica	0.3	0.3	0.3	0.8	6.4	1.00
Histology						
Diffuse alveolar damage	70.7	30.2	52.3	41.9	39.4	68.7
Interstitial pneumonia	15.0	50.3	26.2	28.3	29.8	15.1
Purulent bronchopneumonia	9.7	13.9	13.4	19.4	17.9	11.1
Mixed changes	4.6	5.6	8.1	10.4	12.9	5.1
Lung consolidation						
Absence	85.9	11.4	56.1	56.9	50.0	63.4
Presence	14.1	88.6	43.9	43.1	50.0	36.6

Table 2. presents the evidence propagation for some possible environmental scenarios. The resulting probabilities are expressed in percentages.

Mean temperature and mean relative humidity were negatively correlated (r = 0.704, p<0.001), i.e., when the temperature increased, the relative humidity decreased (see Figure 4).

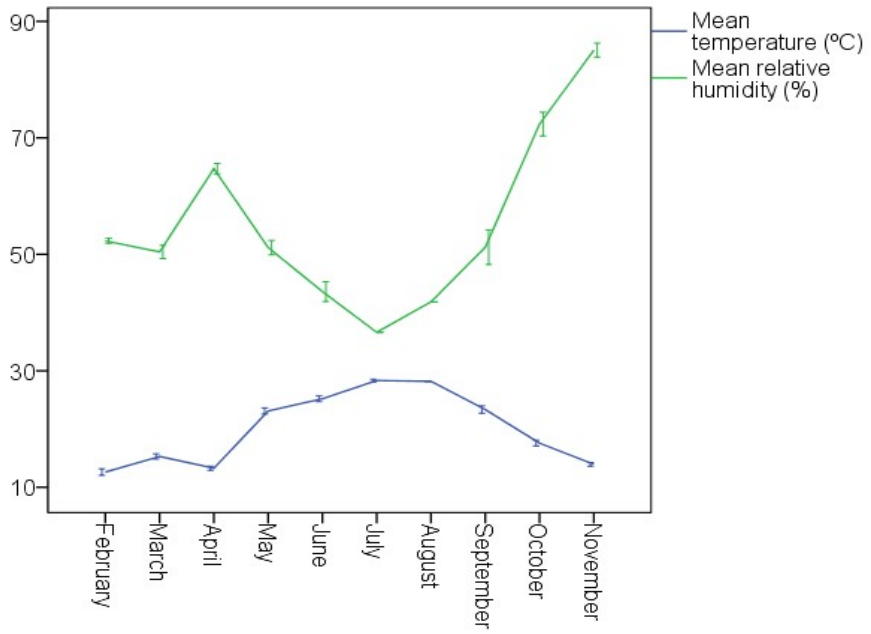

Figure 4. Monthly mean temperature and mean relative humidity.

The Bayesian network shows a relationship between temperature and humidity, and both these variables showed a direct relationship with ammonia levels. According to ITOVIC (1991), ideal conditions of a feedlot correspond to a mean temperature in the range 13-16°C and a mean relative humidity of 70-80%. We define a scenario (Scenario 1) with these two conditions and, as one observes in Table 2, the estimated probabilities of absence in the *Pasteurellaceae* and *Mycoplasma* families are 85.4% and 91.8%, respectively. In addition, the mildest histological type (diffuse alveolar damage) provides the greatest probability (70.7%). In this scenario, the estimated probability of presence of consolidation was low (14.1%). Subsequent scenarios will be compared with this one as reference.

If the optimal temperature (13-16°C) is considered, but with a lower relative humidity, i.e., 50-60% (Scenario 2), the estimated probability of the presence of consolidation increases to 88.6%. In this case, the histology showed an increase in the estimate of interstitial pneumonia with respect to the previous scenario (50.3%). When the temperature is ideal and the ammonia level is in the range 15-20 ppm (Scenario 3), there is mainly associated a high relative humidity (>80). The estimated probability of the presence of pulmonary consolidation is 43.9%, a low percentage with respect to the previous scenario. The estimated probabilities in interstitial pneumonia and purulent bronchopneumonia are greater than the ones obtained in Scenario 1. When the temperature is above 28°C (Scenario 4), the two most probable states of relative humidity are lower than 50 (<40 and 40-50). The probabilities of *M. arginini* and presence of consolidation increase to 40.8% and 43.1%, respectively. Considering the histology, there is a decrease in the estimated probability of diffuse alveolar damage (41.9%), and increases in the estimated probabilities of interstitial pneumonia (28.3%), bronchopneumonia (19.4%), and bronchointerstitial pneumonia (10.4%). When the temperatures considered are below 10°C (Scenario 5), the probabilities of the presence of the *Mycoplasma spp.* and *Pasteurellaceae* families increase with respect to the ideal conditions of Scenario 1. Moreover, there were high estimated probabilities of severe lung inflammation.

Finally, the optimum relative humidity and ammonia levels of 15-20 ppm are considered (Scenario 6). The most likely temperature is 16-19°C, and the estimated probability for the presence of consolidation is 36.6%, which is higher than in the ideal scenario.

Findings related to non-environmental conditions are presented in Table 3. If the absence of the *Mycoplasma spp*. and *Pasteurellaceae* families (Scenario 7) is considered, the estimated probabilities for diffuse alveolar damage and interstitial

pneumonia are 52.6% and 32.9%, respectively. The estimated probability of presence of pulmonary consolidation is 46.9%. When only *M. ovipneumoniae* is considered (Scenario 8), suppurative bronchopneumonia and bronchointerstitial pneumonia reach high probabilities with respect to the previous scenario. However, when *M. arginini* is considered (Scenario 9), there was a mild increase in diffuse alveolar damage (29.2%) with respect to Scenario 8. Both this and the previous scenario indicate the important influence of *M. ovipneumoniae* and *M. arginini* in pathological respiratory processes.

Scenarios							
Variables	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Temperature							
<10ºC	0.3	8.0	1.7	1.0	1.1	1.6	2.0
10-13ºC	11.6	15.4	12.1	10.6	14.0	10.3	12.8
13-16ºC	25.8	15.1	11.5	24.0	23.5	19.2	19.1
16-19ºC	8.8	8.0	2.7	10.2	5.7	6.1	5.5
19-22ºC	15.7	8.0	4.8	12.9	14.34	8.6	10.4
22-25ºC	15.7	8.0	23.1	15.6	17.8	18.8	18.3
25-28ºC	15.3	15.2	25.1	16.8	14.9	21.6	18.3
>28	6.8	22.4	19.0	8.9	8.7	13.9	13.5
Relative humidity							
<40	14.0	22.0	23.8	15.3	14.6	21.1	19.9
40-50	22.8	22.2	28.1	25.6	18.5	30.6	22.0
50-60	19.9	16.7	16.9	12.5	28.8	19.6	18.5
60-70	20.6	17.0	15.0	18.5	25.0	8.4	18.3
70-80	12.5	11.6	7.9	17.3	5.5	9.5	7.9
>80	10.3	10.5	8.5	10.9	7.6	10.8	13.5
Ammonia							
<10	36.03	32.1	37.5	36.7	36.0	36.6	34.2
10-15	25.6	27.1	23.9	25.4	25.0	23.8	26.3
15-20	14.0	10.8	10.0	13.4	12.4	13.3	12.4
20-25	16.2	22.4	19.2	16.6	17.4	18.1	17.6
>25	8.2	7.7	9.4	7.9	9.2	8.3	9.4
Mycoplasma							
Absence	100.0			83.8	75.6	52.5	45.1
Ovipneumoniae		100.0		0.8	1.4	2.9	6.0
Arginini			100.0	13.4	20.5	39.4	39.6
Ovipneumoniae and *arginini*				2.0	2.4	5.2	9.3
Pasteurella							
Absence	100.0	93.7	93.7	93.6	93.78	93.9	93.8
Multocida		2.2	2.2	2.2	2.2	2.1	2.2
M. haemolytica		3.4	3.4	3.5	3.3	3.3	3.3
Pneumotropica		0.7	0.7	0.7	0.7	0.7	0.7
Histology							
Diffuse alveolar damage	52.6	22.0	29.2	100.0			
Interstitial pneumonia	32.9	27.3	31.0		100.0		
Purulent bronchopneumonia	9.8	23.6	25.6			100.0	
Bronchointerstitial pneumonia	4.7	27.0	14.2				100.0
Pulmonary consolidation							
Absence	53.1	45.8	47.1	63.3	45.8	31.7	38.0
Presence	46.9	54.2	52.9	36.7	54.2	68.8	62.0

Table 3. Evidence propagation for some possible non-environmental scenarios (presence of microorganisms and histological groups). Each non-environmental scenario gives a percentage of 100% for one category in one or more variables. The resulting probabilities are expressed in percentages.

In regards to histology, the greatest estimated risk of pulmonary consolidation (68.3%) is obtained when purulent bronchopneumonia is considered (Scenario 12). The estimated probabilities of presence of pulmonary consolidation are 62.0% for bronchointerstitial pneumonia (Scenario 13) and 54.2% for interstitial pneumonia (Scenario 11). However, when only diffuse alveolar damage is considered, the presence of pulmonary consolidation is reduced to 36.7% (Scenario 10). In these four scenarios, the absence of *Pasteurella* is prevalent, with estimated probabilities greater than 90%, whereas the probabilities of absence of *Mycoplasma* range between 45.1% and 83.8%.

Discussion

It is necessary to consider the relationships among variables and their influence on health status in epidemiological studies of animal diseases (Geenen et al., 2011; Lewis et al., 2011). Some authors have used Bayesian networks to determine risk factors by using different parameters as markers of animal health (BIKendrick et al., 2000; Ettema et al., 2009; Jensen et al., 2009; Lewis et al., 2011, among others). Bayesian networks allow the use of information from relationships among all the variables (Pearl, 1988; Neapolitan, 2004). Moreover, evidence propagation is a useful tool to build scenarios providing information for decision-making processes to help to improve animal health. Bayesian networks allow the incorporation of expert knowledge, if required, in subsequent stages of the model at multiple levels. The prior distribution for the first experiment may be weakly informative, but with prior information from experts, the results of the model can change when combining it with the available data. However, especial care must be taken when incorporating expert information since it may not always be reliable.

Several scenarios were especially defined to analyse the effect of the main environmental risks for ovine respiratory syndrome. The evidence was set to represent the best and worst conditions defined in the veterinary literature (ITOVIC, 1991). The relationships between environmental conditions and the occurrence of respiratory problems have been widely studied (Nash et al., 1997; Lacasta et al., 2008). The aim of the present study was to analyse the relationship between potential risk factors such as certain environmental variables and microorganisms implicated in respiratory processes. In our study, the results show that the most important variables influencing the presence of pulmonary consolidation are mainly environmental, specifically, temperature and relative humidity. If these variables are fixed at their optimal values according to ITOVIC (1991) (temperature 13-16 °C and relative humidity 70-80%), the estimated probability of the presence of pulmonary consolidation is very low, and the estimated probability of inflammatory processes (interstitial pneumonia, purulent bronchopneumonia, and bronchointerstitial pneumonia) is small. In this case, the bacterial involvement is lower, in agreement with other authors (Hervás et al., 1996; Niang et al., 1998). However, if these conditions change, then the estimated probabilities of the presence of pulmonary consolidation and of different inflammatory types rise.

The presence of microorganisms appears to be linked to temperature. The Bayesian network shows a direct relationship between temperature and *Mycoplasma spp*., with an increase in the probability of presence of this agent when temperatures higher than the optimal are recorded. These findings agree with those of other authors (Hervás et al., 1996; Niang et al., 1998) who observed an increase in respiratory pathologies associated with the presence of *M. ovipneumoniae* in the warm months (with high temperatures and low relative humidity). Relative humidity also seems to affect pulmonary consolidation. Thus, when the mean relative humidity ranges within

50-60% and the mean temperature is maintained at optimum levels (13-15ºC), an increase in the estimated probabilities of interstitial pneumonia and pulmonary consolidation is obtained. This is possibly associated with irritative phenomena in the respiratory mucosa caused by environmental dryness.

Caswell and Williams (2007) described lung inflammatory processes associated with toxic irritants. In our study, ammonia concentration seems to have an effect on the presence of consolidation as well. Ammonia values are mainly mediated by temperature and humidity. This is in agreement with the results provided by other authors who noted the association between respiratory processes and high concentrations of ammonia in old buildings with poor ventilation (Lacasta et al., 2008). When the ammonia level is fixed at a range between 15 and 20 ppm, an increase in the probability of the presence of pulmonary consolidation, interstitial pneumonia, purulent bronchopneumonia, and bronchointerstitial pneumonia is obtained. This corroborates this parameter's involvement in inflammatory processes.

According to other authors (Alley et al., 1975; Jones et al., 1982; Sheehan et al., 2007), *M. arginini* and *M. ovipneumoniae* seem to be involved in pneumonic processes in sheep. Furthermore, Lin et al. (2008) and Nicholas et al. (2008) found a relationship between *M. arginini* and Pasteurella genus bacteria (*M. haemolytica*). In our study, *Mycoplasma spp*. and histological types are associated with each other, and the same is true for *Mycoplasma spp*. and *Pasteurellaceae* bacteria. However, *Pasteurellaceae* play a less important role for pulmonary consolidation than Mycoplasma spp. Oruç (2006) and Lacasta et al. (2008) identified *M. haemolytica* as one of the main microorganisms in sheep respiratory processes, with this being the main agent isolated in the pulmonary consolidation processes that they studied.

Purulent bronchopneumonia is the most frequent microscopical lesion associated with pulmonary consolidation (Gázquez et al., 2001, Gonçalves et al., 2010). This is

supported by the results obtained in our study. However, Mawhinney et al., (2010), point to interstitial pneumonia as the main lesion of red hepatization. According to our scenarios, the estimated probability of the presence of pulmonary consolidation is 54.2% when interstitial pneumonia is present. This probability is lower than that obtained for suppurative processes. Therefore, pulmonary consolidation can be associated with interstitial and suppurative processes, with interstitial pneumonia being the predominant lesion.

To the best of our knowledge, Bayesian networks have not previously been used to relate the presence/absence of pulmonary consolidation with environmental conditions, *Mycoplasma spp*., *Pasteurella spp*., and histological changes together. Given a model that includes interaction among all the variables, and the fact that evidence propagation allows hypothetical scenarios to be defined, this tool is interesting in the identification and understanding of all the features involved in the study of pulmonary consolidation.

Conclusion

Bayesian networks are generic tools with great potential for use in a wide range of epidemiological disease situations. In the present study, a Bayesian network model has been able to identify risk factors in ovine respiratory processes.

The main factors causing inflammatory processes and pulmonary consolidation in ovine respiratory processes are temperature, relative humidity, and *Mycoplasma spp*. The control of these variables may help to prevent this ovine pathology. The proposed model can be applied to improve conditions on farms, and thus enhance productivity.

Acknowledgements

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Capítulo 5. Factores de estrés asociados a la respuesta inmune innata

Para la realización de este capítulo se llevó a cabo un estudio para evaluar la función de la respuesta inmune mediante el uso del índice de fagocitosis de los neutrófilos. Además de esto diferentes parámetros hematológicos asociados al estrés, fueron también analizados con el fin de obtener el efecto de la fase de cebo en los CC de corderos de engorde.

Para ello treinta y seis corderos de raza Merino fueron elegidos al azar con un peso vivo de aproximadamente 18-20 kg con 70-90 días de vida.

Para llevar a cabo nuestro objetivo se tomaron dos muestras de sangre en dos momentos de su período en el CC, después del proceso de clasificación al inicio del cebo y un día antes del sacrificio.

Nuestros resultados arrojaron un alto contaje de los parámetros analizados comparados con los valores de referencia en el recuento de glóbulos rojos y el hematocrito, mostrando diferencias estadísticamente significativas ($P = 0,003$, $P = 0,004$, respectivamente) entre las dos tomas de muestra.

Sin embargo, se vio un aumento en la relación neutrófilos / linfocitos (relación N / L), una disminución en el índice de fagocitosis y además recogimos un alto valor de cortisol en los dos momentos analizados.

Por ello, obtuvimos que en estas condiciones de cebo los animales pueden ser inmunocompetentes y con ello pueden sufrir una predisposición para el desarrollo de enfermedades.

Todos nuestros resultados fueron plasmados en al artículo publicado en la revista Small Ruminant Research.

Valuation of immune response by using phagocytosis index and parameters associated as markers of animal stress in fattening lambs

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Abstract

The aim of this study was to evaluate immune response function by using neutrophils phagocytic index and parameters associated to stress in order to analyse the effect of feedlot in the fattening lambs classification centre (CC). Thirty-Six Merino lambs were randomly chosen with a live weight about 18-20 kilograms with 70-90 days of life. Two blood samples were taken in two moments of their period in CC, after the classification processes at the beginning of feedlot and one day before slaughter. High values compared to the reference values were found in red blood cell count (RBC) and haematocrit value, with statistically significant differences (P = 0.003, P = 0.004 respectively) between two moments. However, the increase in the neutrophil/lymphocyte ratio (N/L ratio), the decrease in phagocytosis index and high cortisol values point to stress conditions and a predisposition to develop diseases.

Keywords: Phagocytosis; Neutrophils; Welfare; Lambs.

Introduction

In Spain, lamb meat is based on light lambs less than 3-month-old and standardized by its live weight (8.5-13 Kg. carcass weight) (Miranda-de la Lama et al., 2010a). To supply high quality sheep meat products, lamb cooperative producers have developed the denominated classification centres (CC) (Miranda-de la Lama et al., 2010a) where lambs are fattening in feedlots and improve carcass homogeneity and develop a quality mark.

Several steps and processes are involved in this system of fattening lamb (weaning, transport, classification, regrouping) (Miranda-de la Lama et al., 2010a) which have been pointed as stressors (Knowles et al., 1995): between them, previous transport and several loadings stops to the centre and novel environment may be a main stress agent in lamb production systems.

The production system in which animals are raised has a major impact on their immune response (Bonnette et al., 1990; Niekamp et al., 2007). Extrinsic factors (like production system) and intrinsic factors (such as the animals' social status) are both involved in determining physiological status and, ultimately, defensive potential and health of the animals.

The aim of this study was to determine the possible effects of fattening period and its management practices on some indicators of stress and the effect of these on innate immune response.

Material and *methods*

Animal samples

This study was carried out in a CC in Extremadura (South-Western Spain). 36 male and female Merino lambs were randomly chosen after classification within of a pen in order to establish a homogeneity batch with a live weight of 21±3 kg and between

90±20 days. These data were expressed as mean ± standard deviation (SD). Animals were grouped with other animals in pens with a density of 0.5m^2 per animal. Feeding (pellet concentrate and straw) and water were administered ad libitum. Animals are fed by a continuous feeding system from silos by using designated concentrate for fattening lambs. Straw is added ad libitum in mangers. These animals were transported by road from farms to the CC. Classification centres are built with pens of 60 m^2 where approximately 100-120 animals are kept in order to establish a density of 0.5 animals/m^2. Straw beds are replaced every 3 days by using more straw to apply a drying effect in order to avoid the accumulation of ammonia in pens. Ventilation is controlled by closing and opening doors and side windows. Twenty-one days later the animals were transported until slaughterhouse when their live weight was 24±4 Kg.

Animals are transported in a truck. The loading is organized by the proximity of the farms to the classification centre. Used truck had compartments of 0.25 m^2/animal. These compartments have a lateral dividers and non-slip floors.

Blood samples

Two samples per animal were collected in pens one day after the arrival and classification process at the CC and other at the end of the feedlot period before slaughtering. Three 10 ml tubes (without anticoagulant, with EDTA-K_3 and with heparin), within the pen, these animals were restrained by the head and by using a venepuncture system (Vacutainer®) blood samples were recorded. were extracted using venepuncture with Vacutainer® extraction system. These blood samples were kept on ice and taken to the laboratory for routine haematological measurements. Tubes without anticoagulant were centrifuged at 3000 rpm for 10 min and aliquots were frozen at -21ºC until be analyzed.

Haematological parameters

An automatic particle counter (Sysmex F-620®, Norderstedt, Germany) was used to count red blood cells (RBC), white blood cells (WBC) and haematocrit (%). Leukocyte formula was estimated from blood swabs on clean slides, using the rapid panoptic method from Química Clinica Aplicada Inc.® (QCA). 100 leucocytes per sample were counted and identified as neutrophils, lymphocytes, eosinophils, basophils and monocytes in order to calculate the N/L ratio.

Cortisol Measurements

To quantify the level of plasmatic cortisol, Immulite® 1000 (Munich, German) was performed. One tube with a barcode label is needed for the assay and each barcode-labelled unit contain one bead coated with polyclonal rabbit anticortisol antibody. For this purpose, 10 µl of serum sample was added to the unit test that incubated at 37ºC in a persistent agitation. After incubation, the tube with barcode-labelled unit was centrifuged about its vertical axis. After centrifuged, samples were washed to remove supernatant not united to bead. The chemiluminescent substrate was added and light emission was read with a high sensitivity photo counter. The sensitivity of the test was 0.2 µg/dl. Results were expressed in µg/dl and converted to nmol/L by using a correction factor.

Assessment of phagocytosis function

One tube (10 ml with heparin) was used by this test. Briefly, a 3% dextran solution in PBS was added to the blood at a 1:1 ratio, followed by incubation for 30 min at room temperature. The supernatant was collected and centrifuged (650g for 40 min) on a density gradient (Histopaque-1077 together with Histopaque-1119, Sigma®). The neutrophil halo was harvested, washed twice with PBS, and centrifuged at 420g for

10 min. The supernatant was discarded and the pellet was re-suspended in 1.5 ml Hanks' medium. The number of neutrophils was counted with Neubauer chamber, and the suspension was adjusted to a concentration of 1×10^6 neutrophils/ml. Cell viability, determined by trypan blue, was more than 95%. This method was adapted from García-Torres et al (2011) in Iberian pigs.

Phagocytosis was evaluated using the method described by De la Fuente (1985). 200 µl aliquots of the neutrophil suspension (10^6 cells/ml) were incubated at 37 °C in 100% R. H. and 5% CO_2 on MIF plates for 30 min. The adherent cell monolayer was washed with PBS, and 200 µl of Hanks' medium was added to the plates, together with 20 µl of a suspension of latex beads (1.09 µm, diluted to 1% in PBS (Sigma®). After 30 min of incubation, the plates were washed with PBS at 37 °C, fixed and stained, and the number of particles phagocytised per 100 neutrophils (phagocytosis index) was calculated under a microscope (100X). A total of 100 neutrophils were counted and for each cell, phagocytized beads were counted as 0-6 or more. The percentage of cells that phagocytized at least one bead and the average numbers of phagocytized beads were determined (Rudine et al., 2007).

Data analysis

Dependent sample t-tests were applied to find statistically significant difference between the means of each variable at the beginning and the end of the feedlot period. The experimental unit is the animal.

The dependent-sample t-test was applied to compare the difference in the means between pre-and post-scores of the haematological, hormonal and immune response parameters. In order to apply this hypothesis test, the applicability conditions were checked prior to the analysis. This test may be applied to all the pairs of pre-and post-

scores in this study. The results were considered as significant when P-values were less than 0.05. Statistical software SPSS® 19.0 was used.

Results

Several parameters were analyzed at the beginning and end of the feeding period. Elected blood parameters were red blood cells, white blood cells count and haematocrit value and; physiological parameters were neutrophil/lymphocyte ratio, serum cortisol level and phagocytosis function of neutrophils.

Variable	Start of fattening (Mean ± SD)	End of fattening (Mean ± SD)	p-value
Phagocytosis (%)	64.36 ± 30.13	60.17 ± 29.51	0.557
Cortisol (nmol/ml)	61.86 ± 62.28	59.01 ± 62.38	0.833
RBC (10^6/mm^3)	14.06 ± 1.59	12.74 ± 2.13	0.003
WBC (10^3/mm^3)	8.46 ± 5.62	8.51 ± 4.28	0.943
HTO (%)	56.75 ± 5.81	51.62 ± 9.15	0.004
N/L Ratio	0,63 ± 0,37	0,81 ± 0,59	0.034

Table 1. Summary of results of studied parameters.

Red series and WBC were within normal ranges. When red series was analysed, we observed a decrease in red blood cells and haematocrit, with significant difference between the two values obtained in an animal (P = 0.003; P = 0.004). Finally, the white blood cells showed no changes, just seeing a close significance to 1.

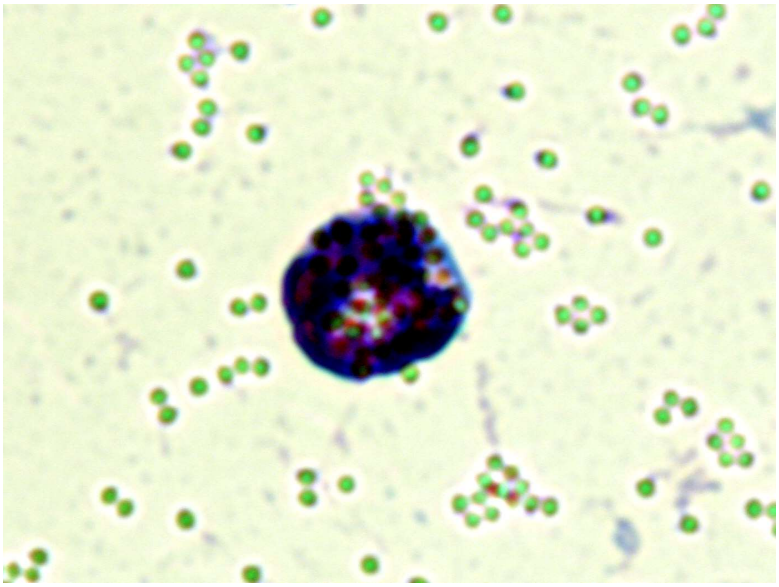

Figure 1. Isolated neutrophils in fattening lamb.

An interesting measure stress indicator is the ratio N/L, in which an increase in the analyzed value in the two sampling in this fattening period was observed and statistically significant differences were observed.

For cortisol, very similar results were obtained between two samples, recording high values compared to normality; a mild decrease although without statistically significant differences (P = 0.833). Isolated neutrophils are shown in figure 1. In the case of phagocytosis index, a low value together a mild decrease without statistical significant differences (P = 0.557) was noted in the studied samples.

Discussion

Classification centres have been established as a commercial strategy to control process prior to slaughter, between them, body weight control and standardize carcass in order to marketing. This production system is associated to management

practices which implies an adaptation to a new situation with the subsequent stress (Ferguson and Warner, 2008).

Numerous studies have suggested the risk of stress factors in fattening lamb production systems (Miranda-de la Lama et al., 2010b). Stress can induce a malfunction of the immune system, with negative repercussions on animal health (Orgeur et al., 1998), which may cause a predisposition to develop diseases (Nash et al., 1997; Brodgen et al., 1998).

Assuming that animal welfare implies numerous complex mechanisms, it is difficult to measure stress objectively using simple techniques, overall interpreting standard physiological, productive and behavioural indicators separately (Hemsworth et al., 1990; Mendl, 1991). In sheep, until now reports about different physiological parameters during the fattened system have not been registered.

In our study, some haematological parameters showed statistically significant differences between the two sampling periods instead of the two samples. RBC count and the haematocrit value were higher at the beginning than at the end of fattening, although showing a higher value than those reported by Lepherd et al. (2009). This higher value in both parameters at the beginning may be explained by the possible negative effects of transport, classification and regrouping on arrival at CC (Cockram et al, 2000; Kannan et al, 2000; Cockram et al., 2004; Ferguson and Warner, 2008). This value shows a decrease that can be due to adaptation of the animal to a new environment, according to Miranda de la Lama et al. (2010b) who noted this detail after twenty eight days in feeding period. Moreover, changes in haematocrit also reflect the mobilization of RBC from the spleen in response to catecholamines (Kent, 1997). This fact is accentuated by long trips associated to dehydration (Cockram et al., 1996; Broom et al., 1996; Chacón et al., 2005; María, 2008), returning to normal range (Bornez et al., 2009; Miranda de la Lama et al., 2010a; Miranda de la Lama.,

2012). For the above exposed, these blood parameters may be used as acute stress markers;.

Cortisol is the primary corticosteroid released during stressful stimuli or stressors (Rhodes et al., 1994), being a good marker of stress grade of the animals. Fernandez et al. (2007) observed an increase of the levels of plasmatic cortisol after classification and mixing at CC. After this process, cortisol values tend to normality. Although the obtained values in cortisol did not show significant differences between two samplings, collected data in this assay show a maintenance of high values during this fattening process, suggesting that lambs do not get fit to new conditions in CC, regrouping or new kennels.

Another useful measure of the effect of stress is the N/L ratio (Blecha, 2000). It is a simple indicator of systemic inflammatory response and has been also associated with chronic stress in animal studies (Puppe et al., 1997; Erminio and Bertoni 2008). Aguayo-Ulloa et al., (2014) indicated a significative decrease of this parameter in enriched kennels. In our case, an increase in N/L ratio was perceived between the classification process and the end of period, similar to the described by Miranda de la Lama et al. (2012a), circumstance that may point to the fattening period as a maintained stressing factor.

There is evidence that stress affects components of the acute inflammatory and innate immune responses (Brown et al., 2008). Neutrophils are the most abundant type of phagocyte, normally representing 50 to 60% of the total circulating leukocytes (Hoffman et al., 2013). They are the innate immune cells that migrate to the site of infectious inflammation. Besides, they also provide signals to other innate immune cells about an invading foreign threat. Today it is known that experimental animals deficient in neutrophils are more prone to develop bacterial or fungal infection as compared to their normal counterparts (Kumar and Sharma, 2010). The phagocytosis

is a method to eliminate agents using enzymatic mechanism and oxidative metabolism. Defects of phagocytosis belong to important factors disposing the animal mainly to infectious diseases (Rotrosen and Gallin, 1987). Previous studies have demonstrated that phagocytic function of neutrophils suffered a significative decrease under effect of stress (Srikumar et al., 2005; Brown et al., 2008).

Until now, there are few studies about phagocytosis index, only some in swine. Rudine et al. (2007) established a worth phagocytosis (74.40%) in intensive pig. In our study, this parameter shows lower values than those above described. Moreover, a decrease in the phagocytic index between both samples of one animal was observed, although without significant differences.

Rhodes et al., (1994) pointed to acute stress might not disturb the immune response; but when stressor agents might be long lasting to limit the immune function. Thus, chronically stressed lambs may show an ineffective response to exposed pathogens on CC.

In conclusion, the study of the previously described parameters together can be a suitable method in order to evaluate stress conditions in fattening period.

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BLOQUE III. Análisis inmunohistoquímico de la respuesta inmune en pulmones de corderos de cebo

Capítulo 6. Estudio de la respuesta inmune celular y respuesta inmune humoral en corderos de cebo

Se realizó un estudio para determinar la respuesta inmune celular en pulmones de corderos de engorde con diferentes patrones patológicos observados, tras su estancia en los centros de clasificación, en el matadero. Un total de 410 animales fueron monitorizados durante su estancia en los CC, una vez en el matadero, sus pulmones fueron recogidos para su evaluación anatomopatológica. Se incluyeron 60 pulmones en el estudio después de su categorización histológica, para ello utilizamos los cuatro parámetros patológicos definidos por nuestro grupo: daño alveolar difuso (DA), neumonía intersticial (PI), bronconeumonía purulenta (PB) y neumonía bronquio-intersticial (BI).

Para el análisis inmunohistoquímico se utilizaron tres anticuerpos, CD3, CD79α y CD68 para analizar la respuesta inmune local analizando en este caso la presencia de linfocitos T, linfocitos B y macrófagos.

El análisis estadístico mostró un aumento en el número de macrófagos y linfocitos B en la luz alveolar en el grupo de PB, lo que indicaría una respuesta celular adaptativa; Una respuesta a nivel septal de linfocitos B y T que podría ser estimulada por la influencia de una variedad de patógenos y la aparición de diversos mediadores inflamatorios; y por último, una alta presencia de linfocitos T y B en BALT que podría ser considerado como responsable de una defensa cronológica más larga en los procesos exudativos. Todos estos detalles apuntan a una evolución de los procesos asociados al síndrome respiratorio ovino, hacia la aparición de forma exudativas durante su proceso de engorde en los centros de clasificación.

Este trabajo ha sido enviado a la revista Veterinary Immunology and Immunopathology para su revisión.

A study in lungs from fattening lambs with different pathological patterns: characterization of cell populations involved in the immune responses based on CD3, CD79 and CD68 markers

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Abstract

A study was conducted to determine the cellular immune response in lungs from fattening lambs with different pathological patterns observed at the abattoir, after their period of stay in the classification center. A total of 410 animals were monitored during their stay in the CC, once at the slaughterhouse, their lungs were collected for their anatomopathological evaluation. Sixty lungs collected at slaughter were included in this study after classification using four different pathological parameters: alveolar damage (AD), interstitial pneumonia (IP), purulent bronchopneumonia (PB) and Bronchointerstitial Pneumonia (BI). Three antibodies to detect CD3, CD79a and CD68 were used to analyses the local immune response by T lymphocytes, B

lymphocytes and macrophages. The statistical analysis showed an increase in the number of macrophages and B lymphocytes in the alveolar lumen of the PB group, which would indicate an adaptive cellular response; a response at septal level of B and T lymphocytes which could be stimulated by the influence of a variety of pathogens and the appearance of various inflammatory mediators; and a high presence of T and B lymphocytes in the BALT which could be considered as responsible for a chronologically longer lung local defense in exudative processes. All of these details point to an evolution of the processes towards exudative forms.

Keywords

Cellular response; Lung pathology; ovine respiratory syndrome;

Introduction

In ovine farms, pathological respiratory processes cause direct losses (mortality in fattening lambs) and indirect losses (loss of weight, bad conversion rates, veterinary interventions, and increasing number of pulmonary lesions in the slaughter associated to partial condemnation) (Goodwin et al., 2004; Lacasta et al., 2008). Lamb pneumonia is regarded as a complex disease, involving interaction among the host (immunological and physiological), multiple agents (bacteria, viruses, mycoplasma) and environmental factors (Brodgen et al., 1998).

The main gross pathological lesions found in the lungs are consolidation and atelectatic foci confined to the cranioventral regions and the presence of a pleural fibrinopurulent membrane (Sheehan et al., 2007; Yener et al., 2009). This consolidation can be lobar or lobular depending on the degree of affection (Caswell & Williams, 2007). Histopathological findings associated to consolidation are usually represented by suppurative bronchopneumonia, characterized by alveoli and

bronchioles filled with polymorphonuclear inflammatory cells 'PMN' (Gázquez et al., 2001; Gonçalves et al., 2010), serofibrinous exudation, degenerated leukocytes and necrotic debris (Gonçalves et al., 2010; Yener et al., 2009). However, interstitial pneumonia has been also associated to pulmonary consolidation in outbreaks of high mortality. In red deer, Mawhinney et al. (2010) noted hypertrophy and proliferation of type II pneumocytes, with hyaline membrane formation, septal oedema, fibrin deposition and emphysema. This pattern has been established in ovine, although it is often observed associated with peribronchial and peribronchiolar lymphocytic proliferation and luminal exudation (Oruç, 2006).

The pulmonary inflammatory response is mediated by several cell types, including the vascular endothelial cells, alveolar macrophages, intravascular macrophages, neutrophils, mast cells, nerve fibres, lymphocytes and airway epithelial cells (Ackerman & Bridgend, 2000). When the lung parenchyma is attacked by a pathogen, an inflammatory response is developed. In this response, different cell types are involved such as macrophages and B and T lymphocytes, among others (Ackerman & Bridgend, 2000).

T cells play a critical role in pulmonary host defense against bacterial, viral and fungal pathogens (Chen & Kolls, 2013). Moreover, insufficient T cell immunity may also increase the likelihood of pathogen dissemination from the lung. Thus, dysregulation of T cell responses during an immune response could also damage host tissues and have detrimental effects for the host itself (Moore et al., 2001; Chen & Kolls, 2013).

To fulfil the function of T lymphocytes, the immune system has a group of cells called antigen presenting cells (APC). T cells are stimulated by dendritic cells that reach the lymph nodes to serve as APC, being this lymphocyte population distinguished from other APCs, as macrophages and B lymphocytes, because T lymphocytes need to be helped by the rest of cells (Vanden-Bush & Rosenbush, 2003).

Macrophages are also involved in the inflammatory response. Two populations have been described in lung, the first one is formed by monocytes that replicate and differentiate into a new population of large, highly active, resident macrophages, known as pulmonary intravascular macrophages (PIMs) (Winkler and Cheville, 1985; Horiguchi et al., 1992; Longworth et al., 1992). The second population is composed of alveolar macrophages, primary phagocytes of the innate immune system, clearing the air spaces of infectious, toxic, or allergic particles that have evaded the mechanical defence of the respiratory tract; being observed a greater number of PIMs than alveolar macrophages in ovine lungs (Warner et al., 1986).

Since PIMs were first reported, research has focused on describing in different species such as swine, sheep and deer (Carrasco et al., 2004), in which PIMs are present and the role that these cells play in hemodynamic control and in the acute inflammatory lung response. Consequently, lung macrophages, including alveolar macrophages, may play a critical role in the induction of lung immunity and in the protection against disease by determining particle translocation from lungs to lymph nodes (Harmsen et al., 1985).

Some authors have carried out analyses of the development of lesions and immunohistochemical findings in different livestock species (Ackerman & Brodgen, 2000; Rodriguez et al., 2000; Gázquez et al., 2001; Mariotti et al., 2007; Redondo et al., 2009; Polledo et al., 2011), although based on specific experimental and natural infectious processes.

To the best of the authors' knowledge, characterization of the local immune response under the influence of different pathological processes observed at the abattoir has not been studied in ovine lungs. The aim of this study was to characterize the distribution of cell populations involved in immune response in fattening ovine lungs with different pathologies in order to know the possible evolution of this disease.

Material and method

Experimental design

A total of four hundred and ten fattening lambs of both sexes were selected from five classification centers in Extremadura (Southwest Spain). Selected animals come from farms with a high prevalence in respiratory processes. Animals were grouped by live weight (21 ±3 kilograms) with other animals in pens with a density of 0.5m^2 per animal. Feeding (pellet concentrate and straw) and water were administrated ad libitum. The fattening period was of 21±5 days with a final weight of 25± 4 kilograms. Animals were identified in pens and the lungs were collected at the slaughterhouse. Once the lungs were analyzed, samples were collected for histopathological analysis; microscopic classification of samples was performed in four histological groups following the established criteria of Caswell & Williams (2007). Fifteen samples from each pathological group were selected to which immunohistochemical techniques were applied in order to evaluate the cell populations in different selected pathological groups.

Histopathology

2-mm thick lung samples including lesioned tissues and undamaged adjacent area were taken. In those lungs without consolidation areas, 2-mm thick randomly selected samples of the cranioventral regions were taken. Samples were fixed in neutral buffered formalin ‘3.5%, 0.1M and pH 7.2’, and routinely processed and embedded in paraffin wax. 5 µm sections were cut with a microtome (Leica RM2255®, Leica microsistemas, Barcelona, Spain), stained with Hematoxylin & Eosin and, after their histopathological study, classified in four pathological groups according Caswell and Williams (2007). Once established the histopathological group to which each sample

belongs, a total of fifteen samples were selected from each group in order to apply immunohistochemical technique.

Immunohistochemistry

Similar sections were cut for immunohistochemical examination. The Avidin-Biotin-Complex (ABC Vector Elite; Vector laboratories®, Burlingame, USA) was used for immunolabelling. All samples were dewaxing in an oven, rehydrated and then treated in hydrogen peroxide 3% in methanol for 15 min to eliminate the peroxidase activity. Samples were washed with TBS 0.01M and pH 7.2. Tissue sections were applied for antigen retrieval following two methods, enzymatic digestion with trypsin/alpha-chymotrypsin (0.5% trypsin and 0.5% alpha-chymotrypsin, Sigma–Aldrich®, Gillingham, Dorset, UK) at 37ºC for 10 min or microwaving by using tris-EDTA pH 9.0; 20 min; 700W according the used primary antibody. Samples were then mounted in Sequenza Immunostaining Centre (Shandon Scientific®, Runcorn, UK). Primary antibody cross-reactivity with tissue constituents was prevented using 1.5% normal serum block which matched the host species in which the link antibody was applied to the sections for 20 minutes. Details of used primary antibodies, specificity, concentration, and incubation time are summarized in Table 1. Sections were washed in TBS and then incubated for 30 minutes with the appropriate biotinylated secondary link antibody (Vector Laboratories®, Burlingame, USA), previously washed twice in TBS. After 30 minutes of incubation at room temperature with Avidin Biotin complex (Vector Elite Kit, Vector Laboratories®, Burlingame, USA), the signal was detected using 3.30-diaminobenzidine tetrahydrochloride (DAB, Sigma–Aldrich®, Gillingham, Dorset, UK), and lightly counterstained with Mayer's hematoxylin (Surgipath®, Peterborough, UK) for 5 min.

Target	Specifity	Comercial origin	Antigen Retrieval	Dilution	Incubation
Human CD3	T cell marker (Polyclonal rabbit anti-human)	DAKO	trypsin/ α-chymotrypsin	1/500	O/N
Human CD79α	B cell marker (clone HM57; Monoclonal mouse anti-human)	DAKO	Microwave-Buffer Tris-EDTA	1/100	1 hour at Room Temperature
Human CD68	Macrophages and monocytes (clone 514H12; Monoclonal mouse anti-human)	ABD Serotec	Microwave-Buffer Tris-EDTA	1/100	1 hour at Room Temperature

Table 1. Details of primary antibodies, antigen retrieval, concentration, and incubation time for the immunohistochemical technique.

The number of immunolabelled cells per mm^2 for each antibody was determined using a method described by Redondo et al. (2003). Briefly, Cells labelled by each antibody were counted in four selected areas (alveolar septa, alveolar lumina, BALT, and bronchial lumina). One hundred fifty measurements were performed in each selected area.

Statistical analysis

Two-way analysis of variance (ANOVA) is the main technique used here for statistical analysis. Each observation was categorized on the basis of two criteria – the pathological group (AD, IP, PB and BI) as well as the antibody marker (CD3, CD79α and CD68). These two factors were considered to address the cell counts in the four previously described lung zones. The possible interaction between the antibody markers and the pathological groups was studied in each lung zone. The Shapiro-Wilks' normality test and the Levene's homokedasticity test were considered to analyze the ANOVA's applicability conditions. The Bonferroni adjustment was considered to determine if there are statistically significant differences between the mean cell counts with the considered antibody markers in a concrete pathological group or with the considered pathological groups in a concrete antibody marker (multiple comparisons).

Two-side *P* values less than 0.05 were considered statistically significant. Statistical analyses were performed by using IBM-SPSS software 19.0®.

Results

Histopathological findings

Absence of consolidation was found in 48.8% of the lungs (n=200) and presence in 51.2% (n=210). These lungs showed foci of irregular consolidation in the cranioventral regions and occasionally in the middle and diaphragmatic lobes. In cases of consolidation, pleural surface showed a fibrinous membrane adhered to the rib wall. Lungs with absence of consolidation were apparently healthy and without membrane formation in the lung surface.

Microscopic study revealed different pathological groups. Lesions in AD group, ciliar necrosis and compaction and alveolar denudation, and loss of type I pneumocytes

and epithelial basement membrane. In interstitial pneumonia group, the found lesions were characterized by septal damage, loss of type I pneumocytes and proliferation of type II pneumocytes. Septa were thickened, with mononuclear cell inflammatory infiltrates, marked congestion and oedema. Hyaline membranes were observed in junction interstitium and alveoli, composed of the presence of fibrin, eosinophilic proteinaceous material and cell debris. In Purulent bronchopneumonia group, the predominant lesional pattern was of exudative type. Neutrophils, macrophages and cells debris were observed within bronchial, bronchiolar and alveolar lumina. BALT was enlarged, as a multinodular structure. Interstitial pneumonia together with suppurative bronchopneumonia was also described. Inflammatory reaction within the interalveolar septa and exudative processes were concomitant in all the samples included in this group. This group was defined as bronchointerstitial pneumonia.

Cell population distribution

CD3 was abundantly distributed around the periphery of BALT and a small number of immunolabelled cells were observed in the medular zone; this fact was described mainly in groups with exudative component like PB group. At the septum level, a large number of immunomarked cells were found in the AD, PI, and BI groups.

CD79alpha showed a high number of immunolabelled cells within exudates in the bronchial lumina in PB group. In BALT, although to a lesser extent that CD3, these cells were also found throughout and surrounding cortical area. Also in the AD and IP groups there were immunolabelled cells diffusely throughout the septal area.

CD68 was observed in greater quantity in the alveolar and bronchial lumina in groups that presented exudates in these zones (PB and BI) within of these exudates. In BALT, only the presence of a low number of immunolabelled cells were observed in cortical area. In AD, IP and BI were observed scattered in septal area.

Statistical analysis

A significant interaction between the pathological group and the antibody marker was obtained in septal zone (P<0.001). Figure 1 (a) shows the effect of markers and pathological groups in the count of cells in this zone. All markers presented statistically significant differences between the mean cell counts in AD and BI (P < 0.001). In both cases CD79α marker (Figure 2.a.; Figure 2.b.) showed a greater mean number of immunolabelled cells followed by CD3 and, subsequently, CD68 markers (Figure 2.c). In IP and PB there were no statistically significant differences between the mean cell counts with the CD79α and CD3 markers; however, the CD68 marker revealed statistically significant differences with the rest of considered markers (P < 0.001) in all pathological groups, being this lower than CD3 and CD79α markers.

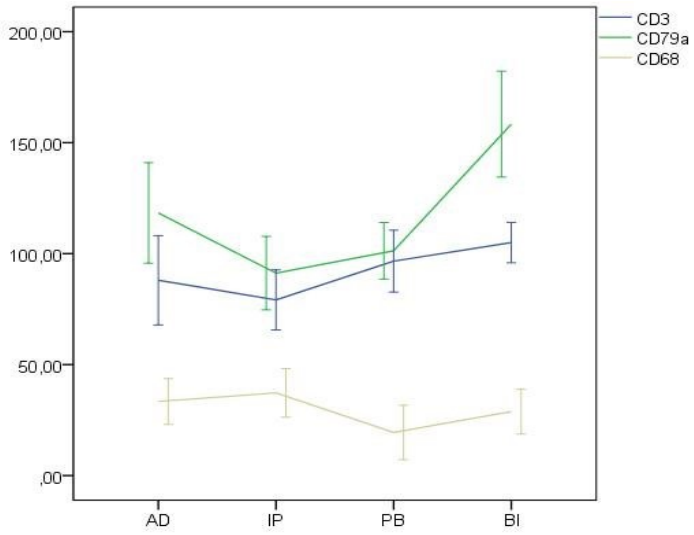

Figure 1a. Means and 95% confidence intervals for the cell count in septal zone.

A significant interaction between pathological group and antibody marker was also obtained in alveolar zone (P<0.001). Figure 1 (b) shows the mean plot. In AD group, no statistically significant differences were observed among the mean cell counts for

the three antibody markers in this zone. In IP, PB and BI groups, statistically significant differences were found between the mean cell counts for the CD68 and CD3 markers (Figure 2.d.; Figure 3.a.) (P<0.001), and between the CD68 and CD79α markers (P<0.001), being the mean number of CD68 immunomarked cells greater than the others in these three groups. The maximum mean number of CD68 immunolabelled cells has been found in PB.

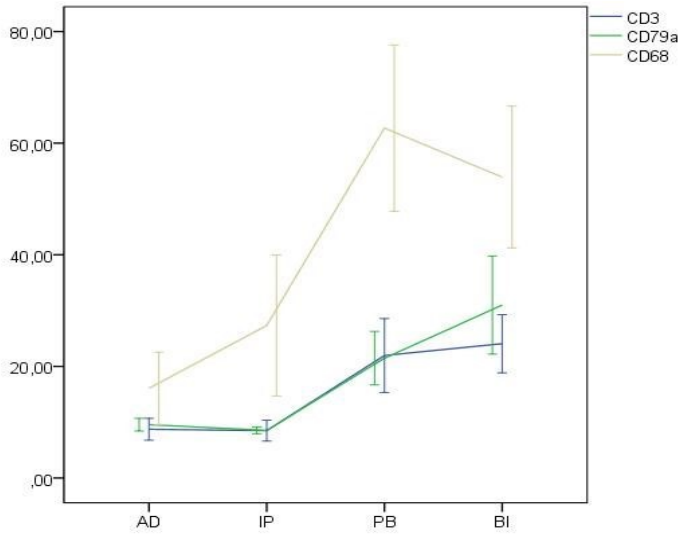

Figure 1b. Means and 95% confidence intervals for the cell count in alveolar zone.

In BALT zone, there is another significant interaction between the pathological group and the antibody marker (P<0.001). The mean plot is presented in Figure 1 (c). In AD group, no statistically significant differences were observed between the mean cell counts for the three markers. However, in IP group, statistically significant differences were observed between the mean cell counts of T lymphocytes (Figure 3.b.) and macrophages markers (P < 0.001) and between B lymphocytes and macrophages markers (P < 0.001), with the CD68 marker (Figure 3.c.) producing the lowest mean number of immunomarked cells. In PB and BI groups, an increasing number of

immunolabelled cells observing significant differences for CD3 and CD79α markers have been observed. In both groups, statistically significant differences were observed among the mean cell counts produced by the three markers (P < 0.001), being the T lymphocytes marker the one obtaining the highest mean number of immunostained cells.

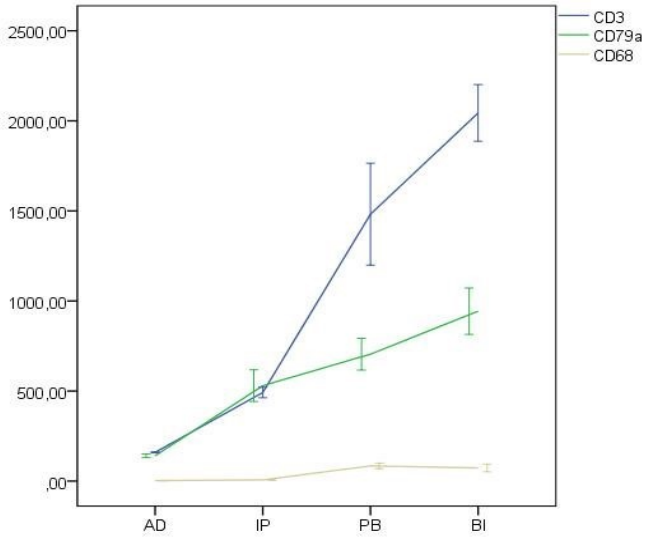

Figure 1c. Means and 95% confidence intervals for the cell count in BALT.

Figure 1 (d) presents the means and their corresponding 95% confidence intervals for the cell count in bronquial lumina zone. As in the previous zones, a significant interaction between pathological group and antibody marker has been found (P<0.001). In AD and IP groups, no statistically significant differences have been found between the mean cell counts for the three markers. In PB group, statistically significant differences were observed between the mean cell counts for CD79α and CD3 markers (P < 0.001) and for the CD79α and CD68 markers (P < 0.001), with B lymphocytes producing the highest mean number of inmunomarked cells. Finally, in BI group, there are statistically significant differences between the mean cell counts

for the three markers (P < 0.001), with the CD79α marker producing the highest mean number of inmunolabelled cells and CD68 marker (Figure 3.d.) the lowest one.

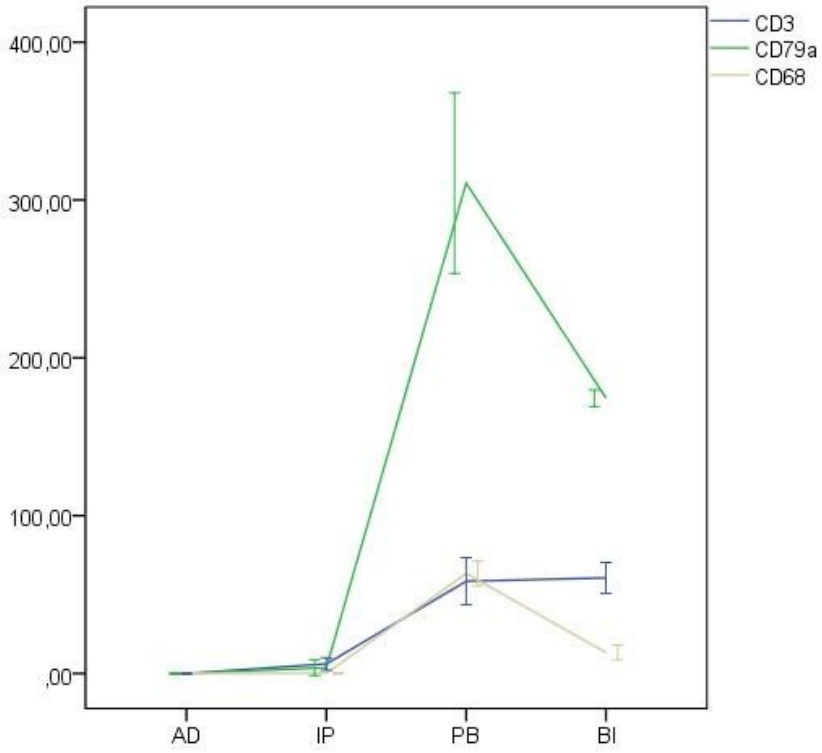

Figure 1d. Means and 95% confidence intervals for the cell count in bronquial lumina.

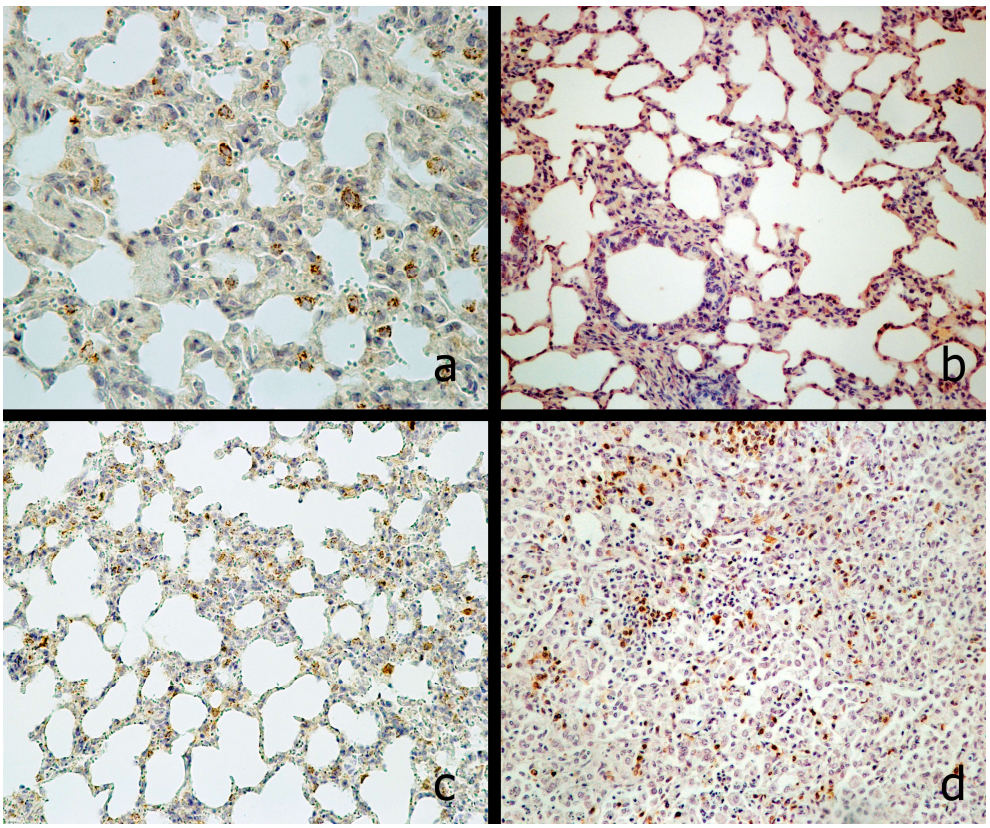

Figure 2. a. CD79α. Interstitial pneumonia group. (40x); b. CD79α. Interstitial pneumonia group. (20x); c. CD68. Diffuse alveolar damaged group. (10x); d. CD3. Purulent bronchopneumonia group. (20x).

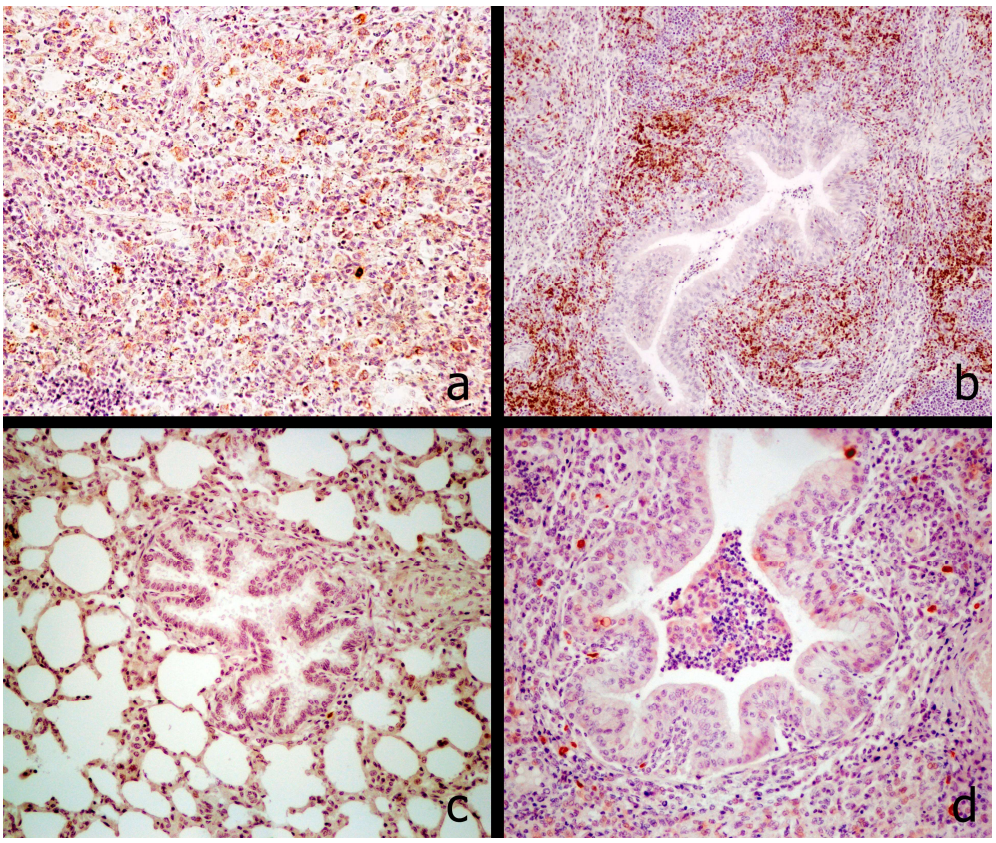

Figure 3. a. CD68. Bronchointerstitial pneumonia group. (20x); b. CD3. Bronchointerstial pneumonia group. (10x); c. CD68. Diffuse alveolar damage group. (10x); d. CD68. Purulent bronchoneumonia group. (20x).

Discussion

The expression of different antibodies associated to cellular immune response in the lungs of fattening lambs with different lesion patterns has been addressed in this paper. The cellular response has been studied in different species such as fallow deer (García-Jiménez et al., 2013), lynx (Peña et al., 2006) or cow (Johnson et al., 2006) in different infections.

An increase in the number of immunostained cells in CD68 marker in the alveolar area has been observed. This increase in the alveolar zone could suggest the importance of these cells in maintaining lung homeostasis, helping thus to keep a proper respiratory function (Ackerman & Brodgen, 2000). Pors et al. (2013)

established macrophages as a first line of defence against pathogens, helping phagocytosis and cytokine production. For this reason, the role of alveolar macrophages is important as a one of the first cells implied in the elimination of the primary agents responsible of the pathological damage at alveolar level. However, septal macrophages also play an important role in more advanced response, as previous studies showed (Carrasco et al., 2004; Gómez-Laguna et al., 2010). In our case, there was an increase at septal level in interstitial pneumonia group, although without reaching alveolar levels. This would also indicate a more advanced stage of disease, matching the results presented by Polledo et al. (2011). This is mainly due to their phagocytic activity and hemodynamic monitoring at pulmonary level.

Lymphoid tissue associated to tissues, in this case bronchus associated lymphoid tissue (BALT), are accumulations of lymphoid cells that act against pathogens. A great number of cells labelled with CD3 in BALT area for groups of broncointerstititial pneumonia and purulent bronchopneumonia has been found. This could support that in infectious stages of the disease, a cellular response could match the possible originating agents (Fragkou et al., 2010). Moreover, Chen & Kolls (2013), discovered that an insufficient T cell immunity may also increase the likelihood of pathogen dissemination from the lung.

A BALT hyperplasia was observed in experimental infections with two strains of *Mycoplasma* agents in goats (Rodríguez et al., 2000). In our case in this area, T lymphocytes were the main involved cells being essential in the inflammation, even in the case of non-specific response. Besides, exudative lesions expressed higher count CD3 cells, in disagreement with Pors et al. (2013) who described a low number of immunostained cells in this kind of lesions; Our hypothesis is that this could be related to the appearance of these cells by the evolution of the disease, which would help eliminate the causative agent

CD79α marker showed a greater number of immunostaining cells in septum and overall in bronchial lumen, mainly in groups of purulent bronchopneumonia and bronchointerstitial pneumonia. Polledo et al. (2011) and García-Jiménez et al. (2013) showed the presence of B lymphocytes as a signal of late infections. However, in our case, the presence of these cells in bronchial areas is attributable to a more specific response to the presence of agents. In presence of pathogens, cellular response associated to exudative lesions is much more effective, being able to neutralize and eliminate more effectively the agent, as indicated by Pors et al. (2013). It is observed an increase in B cells in those groups with exudative component. This fact could be related to a relationship with other cellular components such as macrophages, both acting as antigen presenting cells to lymphocytes T.

By contrast, in septal area, an increase of CD79α marker in diffuse alveolar damage and bronchointerstitial pneumonia group was observed. A possible hypothesis could be related with a possible maintained protective role against these exogenous agents, in agreement with previous authors (Fragkou et al., 2010; Polledo et al., 2011; García-Jimenez et al., 2013). This fact showed the relationship between CD3 and CD79α markers, increasing the efficiency of the immune system in the pulmonary area (García-Jimenez et al., 2013).

In feedlot, animals are exposed to many environmental components. Although B lymphocytes have been related to the fight against bacteria and viruses, Müller et al., (1997) also suggested that an increase of B lymphocytes in pig's lungs was due to high exposure to dust particles. The increase observed in our study cannot only come from acute infection but rather environmental conditions (Galapero et al., 2016); which can produce a similar activation in groups with an interstitial pattern, related with a non-specific response influenced by inflammation mediators. Besides, the shown increase in labelled cells with CD79α marker in the group of bronchointerstitial

pneumonia may be explained due to activation of alveolar macrophages from pathogens and response at septum level by using of different mediators of immune response, as already was pointed in other studies under experimental infections in swines (Redondo et al. 2009; Gómez-Laguna et al. 2010).

An increase in B and T lymphocytes in lymphoid tissue suggests a protective role against different infectious agents (Fragkou et al., 2010). In our study, this increase was recorded, which could indicate a relationship between these two markers to enhance the cellular response, as was mentioned by García-Jiménez et al., (2013), higher in purulent bronchopneumonia and bronchointerstitial pneumonia groups by the possible implication of infectious agents.

Conclusion

According to the obtained results, it can be suggested that macrophages in the alveolar area could be considered as the first line of defence in maintaining respiratory capacity, together with the appearance in the response of B lymphocytes, observing a more specific cellular response to this level. In septal area, there was a further extended process mediated by T and B lymphocytes as a response to different causal agents of pathological processes in fattening lambs as well as the influence of inflammatory mediators. BALT could be considered like a defence center due to the high presence of lymphocytes. To conclude and according to the presence and distribution of the studied cells, respiratory disease described in feedlots evolves from diffuse alveolar damage to exudative forms. This evolution may be influenced by the different predisposing factors which could be influenced by environmental and microbiological components in the response at lung level.

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Capítulo 7. Estudio de la implicación de las citoquinas proinflamatorias y antiinflamatorias en pulmones de corderos de cebo

En este capítulo se presenta un trabajo realizado para analizar la respuesta de diferentes interleucinas en pulmones de corderos de engorde con diferentes patrones patológicos en el síndrome respiratorio ovino (SRO) en condiciones naturales, durante su fase de cebo.

Un total de 410 animales fueron monitorizados durante su estancia en los CC, una vez en el matadero, sus pulmones fueron recogidos para su evaluación anatomopatológica. Un total de sesenta pulmones fueron considerados después de su clasificación en cuatro grupos patológicos.

Un total de cuatro anticuerpos interleuquina 10 (IL-10), factor de necrosis tumoral α (TNFα), interleuquina 1R (IL-1) e interleuquina 6 (IL-6) para evaluar su efecto como mediadores antiinflamatorias y pro-inflamatorias.

Los resultados de este estudio arrojaron un aumento en el número de células inmunomarcadas en el caso de la interleucina 10, en el grupo de daño alveolar en la zona septal y en el grupo de bronconeumonía purulenta en la zona alveolar. En relación al marcador de TNFα, se observó un aumento de las células inmunomarcadas en el grupo de neumonía intersticial, como un control de la actividad de los macrófagos septales. En el caso de la IL-1, presentó un alto valor en el contaje celular en el grupo de neumonía intersticial, así como en BALT; en el área de la luz bronquial, se observó un alto número de células que podrían estar asociados al efecto quimio atrayente de este mediador inflamatorio. En el grupo de bronconeumonía purulenta hubo un elevado número de células inmunomarcadas en la luz alveolar y bronquial. Este hecho podría estar relacionado con una importante respuesta a diferentes estímulos patógenos.

Este aumento en el número de células estuvo patente en la IL-10 como interleucina antiinflamatoria.

Los hallazgos encontrados en el número medio de células inmunomarcadas de IL6 parecen estar relacionados con la inducción de respuesta inmune innata en la zona alveolar, siendo este nivel el centro clave de desarrollo de la respuesta inflamatoria.

Los resultados obtenidos mostraron una respuesta variable en las diferentes áreas estudiadas, posiblemente con origen en los factores predisponentes, siendo una respuesta más inespecífica en la zona septal y BALT, ocurriendo una más específica en el área de la luz alveolar y bronquiolar. Este artículo ha sido enviado para su revisión a la revista Journal of Comparative Pathology.

Cytokine expression in lung of fattening lambs with different lesional patterns included in ovine respiratory syndrome based on IL-1, IL-6, TNF-alpha and IL-10 markers

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Abstract

A study was conducted to determine interleukin response in lungs of fattening lambs with different pathological patterns in ovine respiratory syndrome (SRO) under natural conditions. A total of 410 animals were monitored during their stay in the CC, once at the slaughterhouse, their lungs were collected for their anatomopathological evaluation. Sixty lungs, were considered after classification in four pathological groups. Four antibodies (Interleukin 10 (IL-10), tumor necrosis factor α (TNFα), Interleukin 1R (IL-1), and Interleukin 6 (IL-6) were considered to evaluate anti-

inflammatory and pro-inflammatory cytokines. The obtained results showed an increase in number of immunolabelled cells of interleukin-10 marker in alveolar damage group in septal zone and in purulent bronchopneumonia group in alveolar zone. Regarding TNFα marker, an increase of immunomarker cells was observed in interstitial pneumonia group which could be related to septal macrophages activity. IL-1 presented a high value in counted cells in interstitial pneumonia group in BALT zone; in bronquial lumina, it had a high number of cells which could be associated to chemoattract effect of inflammatory cells. IL-6 showed in purulent bronchopneumonia a high number of immunolabelled cells in alveolar and bronchial lumina. This fact could be related with an important response to different stimuli. An important effect was observed in IL-10 marker like anti-inflammatory interleukin; the found findings in immunolabeling cells of IL6 seem to be related with induction of innate immune response in alveolar zone, being this level the key centre of development of the inflammatory response. IL-1 and TNFα have been recorded like responsible for the development of septal and BALT lesions trough recruitment of lymphocytes and plasma cells at this level.

Obtained results showed a variable response in the different studied areas that could come from the predisposing factors that cause injury, being a more nonspecific response in the septal area and BALT, occurring a more specific response in the alveolar and bronchiolar lumina.

Keywords

Lung; interleukins; pathology; ovine respiratory processes; immune response.

Introduction

In ovine farms, respiratory syndrome cause direct losses (mortality in fattening lambs) and indirect losses (loss of weight, bad conversion rates, veterinary interventions, and increasing number of pulmonary lesions in the slaughter) (Goodwin et al., 2004; Lacasta et al., 2008).

The innate immune response has the ability to control or even eradicate pathogens in short time period by using a wide spectrum of biological responses which include several cell types (e.g., monocytes and natural killer cells) and cell-signalling molecules (e.g., cytokines and chemokines) (Parker et al., 2011).

Several authors have studied the role of cytokines in different respiratory processes in some species, e.g., in ovine (Redondo et al., 2011; 2014), in pigs (Salguero et al., 2005; Gómez-Laguna et al., 2010; 2013) or calves (Ackermann et al., 2004).

Inflammatory cytokines, secreted by a variety of immune and non-immune cell types, including monocytes, macrophages, fibroblasts, epithelial cells, and neutrophils (Caswell et al., 2001; Malazdrewich et al., 2001), can stimulate leukocyte migration, increase the inflammatory response and incite the infiltration of additional neutrophils (Ackermann et al., 1999; 2000; Leite et al., 2002).

Among others, IL-10 is an important immunoregulatory cytokine produced by many cell populations. Its main biological function seems to be the limitation and ending of inflammatory responses and the regulation of differentiation and proliferation of several immune cells such as T cells, B cells, natural killer cells, antigen-presenting cells, mast cells or granulocytes. Besides, Asadullah et al. (2003) suggest IL-10 also has immunostimulatory properties that help to eliminate infectious and non-infectious particles.

However, TNFα, IL-1 and IL-6 have been specified by their pleiotropic effect as pro-inflammatory and upregulate macrophage killing mechanisms. Inflammatory cell

recruitment, acute phase protein induction, protein catabolism and lymphocyte activation and differentiation are also stimulated by these pro-inflammatory cytokines (Biron & Sen, 2001).

The aim of this study was to estimate the expression of different cytokines by using specific antibodies in pulmonary macrophages and their possible effect on the inflammatory response in the fattening lamb lungs to understand their involvement like inflammatory mediators and their possible evolution in these processes in ovine respiratory syndrome.

Material and method

Experimental design

A total of four hundred and ten fattening lambs of both sexes were selected from five classification centres in Extremadura (Southwest Spain) with a high prevalence in respiratory processes. Animals were grouped by live weight (21 ±3 kilograms) in pens with a density of 0.5m2 per animal. Feeding (pellet concentrate and straw) and water were administrated ad libitum. The fattening period was of 21±5 days with a final weight of 25± 4 kilograms. After collection of lungs at the slaughterhouse, these were analyzed and samples were collected for histopathological analysis; microscopic classification was performed in four histological groups following the established criteria of Caswell & Williams (2007). Fifteen samples from each pathological group were selected to immunohistochemical study in order to evaluate the interleukin populations in each group.

Histopathology

2-mm thick lung samples including lesioned tissues and undamaged adjacent area were taken. In those lungs without consolidation areas, 2-mm thick randomly selected

samples of the cranioventral regions were taken. Samples were fixed in Bouin solution and routinely processed and embedded in paraffin wax. 5 µm sections were developed by using microtome (Leica RM2255®, Leica microsistemas, Barcelona, Spain), stained with Hematoxylin-eosin and, after their microscopic study, classified in four pathological groups according Caswell and Williams (2007).

Immunohistochemistry

The avidin-biotin-peroxidase complex (ABC) method was used. All samples were dewaxing oven, rehydrated and then treated in hydrogen peroxide 3% in methanol for 15 min to eliminate the endogenous peroxidase activity. Samples were washed with TBS 0.01M and pH 7.2. Antigen retrieval were subjected by using Tween 20® (Merck, München, Germany) 0.01% in 0.01 M TBS, pH 7.2, for 10 min at room temperature. After pre-treatment, sections were given three 10-min rinses in TBS and mounted in Sequenza Immunostaining Centre® (Shandon Scientific, Runcorn, UK). Primary antibody cross-reactivity with tissue constituents was prevented using 1.5% goat normal serum block for 20 min. before incubation with the primary antibody at 4ºC overnight. Details of used primary antibodies, specificity, concentration, and incubation time are summarized in Table 1.

Specifity	Type	Source	Comercial origen	Fixative	Dilution	Antigen Retrieval
Human IL-1R	PAb	Rabbit	Cultek, SLU	Bouin	1/400	Tween 20®
Human IL-6	PAb	Rabbit	Cultek, SLU	Bouin	1/400	Tween 20®
Human TNFα	PAb	Rabbit	Cultek, SLU	Bouin	1/1000	Tween 20®
Human IL-10	PAb	Rabbit	Cultek, SLU	Bouin	1/500	Tween 20®

Table 1. Details of primary antibodies, antigen retrieval, concentration, and incubation time for the immunohistochemical technique.

Biotinylated goat anti-rabbit antibody® (Vector Laboratories, Burlingame, CA, USA) diluted 1 in 1000 in TBS containing normal goat serum 1.5% was used. The sections were then incubated with the ABC® (Vectastain ABC Kit Elite; Vector Laboratories), before applying the chromogen 3-30 diaminobenzidine tetrahydrochloride (DAB®; Sigma-Aldrich Chemie) in a solution (0.35 g/l) in 0.05 M Tris buffer (pH 7.6) containing hydrogen peroxide 0.1% for 1 min. Slides were counterstained with Mayer's haematoxylin.

Appropriate controls were included in each immunohistochemically run. These included sequential sections with an isotype control for each primary antibody, or the omission of the primary antibody by using TBS instead of the used antibody.

The number of immunolabelled cells/mm^2 for each antibody was determined using a method described by Redondo et al. (2003). Cells labelled by each antibody were counted in four selected areas (alveolar septa, alveolar lumina, BALT and bronchial lumina). One hundred fifty measurements were performed in each selected area.

Pulmonary intravascular macrophages and interstitial macrophages were grouped together and described as 'septal macrophages'.

Statistical data analysis

The main technique for analysing the data was the two-way analysis of variance (ANOVA) since each observation was categorized on the basis of two criteria, the pathological group (Alveolar Damage (AD); Interstitial Pneumonia (IP); Purulent Bronchopneumonia (PB); and Bronchointerstitial Pneumonia (BI)), and the antibody marker (IL-10, TNFα, IL-1, and IL-6) addressed the cell counts in four lung zones (septal, alveolar, BALT and bronquial lumina). The possible interaction between the antibody markers and the pathological groups was studied in each one of the four lung zones. Normality Shapiro-Wilk test and Levene's variance homogeneity test was considered to study the ANOVA's conditions. Bonferroni adjustment was considered to determine if there are statistically significant differences between the mean cell counts with the considered antibody markers in a concrete pathological group (multiple comparisons).

Two-side *P* values lower than 0.05 were considered statistically significant. Statistical analyses were performed using IBM-SPSS 19.0® software.

Results

Histopathological findings

Absence of consolidation was found in 48.8% of the lungs (n=200) and presence in 51.2% (n=210). These lungs showed foci of irregular consolidation in the cranioventral regions and occasionally in the middle and diaphragmatic lobes. In cases of consolidation, pleural surface showed a fibrinous membrane adhered to the rib wall. Lungs with absence of consolidation were apparently healthy and without

membrane formation in the lung surface. These results have already been described by our group (Galapero et al., 2016).

Microscopic study revealed different pathological groups. Lesions in AD group included ciliar necrosis and compaction and alveolar denudation, and loss of type I pneumocytes and epithelial basement membrane. In IP group, the found lesions were characterized by septal damage, loss of type I pneumocytes and proliferation of type II pneumocytes. Septa were thickened, with mononuclear cell inflammatory infiltrates, marked congestion and oedema. Hyaline membranes were observed in junction interstitium and alveoli, composed of the presence of fibrin, eosinophilic proteinaceous material and cell debris. In PB group, the predominant lesional pattern was of exudative type. Neutrophils, macrophages and cells debris were observed within bronchial, bronchiolar and alveolar lumina. BALT was enlarged, as a multinodular structure. Interstitial pneumonia together with suppurative bronchopneumonia was also described. Inflammatory reaction within the interalveolar septa and exudative processes were concomitant in all the samples included in this BI group. These results have already been described by our group (Galapero et al., 2016).

Immunohistochemical findings

At septal level immunolabelled cells were mainly expressed in four markers in septal macrophages, being observed in greater quantity in processes where there was interstitial pneumonia with a thickening of the alveolar septa.

In alveoli, the alveolar macrophages showed a high level of immunolabelling in four studied markers, also highlighting in the cytoplasm of some neutrophils for IL-1 and TNF markers.

In BALT area the immunomarked cells were mainly lymphocytes and macrophages in the four analyzed markers.

The labelling of IL-1 and TNF-α was observed in bronchiolar exudates present within airways, in the cytoplasm of neutrophils and macrophages.

Statistical analysis

The mean and standard deviation of count cells for each antibody in different pathological groups and zones are presented in Table 2.

		AD	IP	PB	BI
Septal Zone	IL-10	35,89±10,09	4,84±1,75	32,39±8,73	27,94±10,90
	TNF-α	21,67±2,75	40,84±4,22	12,88±3,56	23,11±7,78
	IL-1	13,90±1,34	19,72±2,35	11,14±0,32	33,82±8,31
	IL-6	20,39±0,92	28,85±1,40	16,27±4,28	26,50±14,36
Alveolar Zone	IL-10	5,47±0,535	2,18±0,59	92,14±13,48	25,67±12,36
	TNF-α	7,53±0,94	14,71±1,60	52,69±7,22	35,09±19,84
	IL-1	4,35±0,60	5,89±1,73	74,02±4,83	18,11±9,05
	IL-6	4,56±0,82	7,34±1,57	79,38±3,92	28,01±7,93
BALT	IL-10	30,09±6,26	7,95±0,86	49,44±3,46	54,18±8,07
	TNF-α	27,18±0,83	8,63±0,54	37,90±3,95	70,15±10,22
	IL-1	6,90±3,28	27,20±5,81	32,78±1,84	54,13±4,90
	IL-6	0±0	7,72±4,83	9,44±3,53	81,75±48,09
Bronquial lumina	IL-10	0±0	0±0	24,01±5,92	24,24±1,10
	TNF-α	0±0	0±0	16,66±3,07	45,08±37,16
	IL-1	0±0	0±0	9,88±2,83	14,69±6,78
	IL-6	0±0	0±0	68,34±9,57	95,54±76,23

Table 2. Mean and standard deviation of count cells for each used antibody in different pathological groups and four zones.

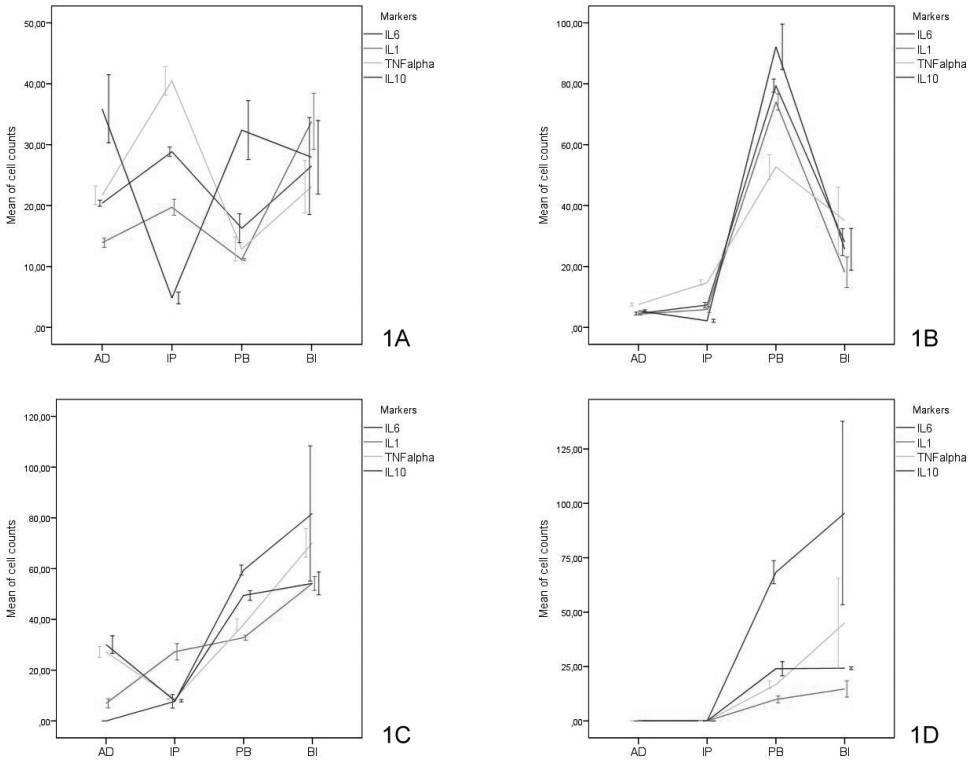

Figure 1. Effect of markers and pathological groups in the count of cells in: (a) septal zone; (b) alveolar zone; (c) BALT zone; (d) bronquial lumina zone.

A significant interaction between the pathological group and the antibody marker was obtained in septal zone (P<0.001). Figure 1 (a) shows the effects of markers and pathological groups on the cell counts in this zone.

In interstitial pneumonia group, all marker pairwise comparisons showed statistically significant differences between the means of immunolabeled cell counts (P < 0.001). In this case, TNFα provided the highest number of immunolabelled cells, whereas IL-10 showed the lowest one. These were the maximum and minimum not only for interstitial pneumonia group, but also for the other pathological groups. On the other hand, in purulent bronchopneumonia and alveolar damage group, IL-10 had statistically significant differences with the rest of markers (P < 0.001). In both cases,

this anti-inflammatory interleukin was expressed with a high mean of cell counts. In addition, in the alveolar damage group, statistically significant differences were found between the mean cell counts of IL-1 and the ones corresponding to the other three markers (P<0.001).

Finally, in bronchointerstitial pneumonia group, statistically significant differences have been reported between IL-1 and TNF-alpha markers (P<0.001), providing IL-1 the greatest mean of immunolabelled cell count.

In alveolar zone, Figure 1 (b), a significant interaction between pathological groups and markers was obtained (P < 0.001). In alveolar damage group, there weren´t statistically significant differences between markers. In interstitial pneumonia group, TNF-alpha had the highest mean number of immunomarked cells, observing statistically significant differences with IL-1 (P=0.013) and with IL-10 (P<0.001). In purulent bronchopneumonia group, the highest mean number of immunolabelled cells was found with IL-10 (Figure 2a), observing statistically significant differences with the rest of markers (P < 0.001). Even more, this mean number is not only the highest one for purulent bronchopneumonia group, but also for the rest of markers in alveolar zone. On the other hand, TNF-alpha had the lowest mean of immunolabelled cell counts, showing statistically significant differences with the others three markers (P < 0.001). Finally, in bronchointerstitial pneumonia group, the highest mean of immunolabelled cells was found with TNF-alpha marker (Figure 2b), reporting statistically significant differences with IL-1 (P < 0.001) and IL-10 (P = 0.007). In addition, statistically significant differences were found between IL-1 and IL-6 (P = 0.004), being IL-1 the marker recording the lowest mean number of immunolabelled cells.

Means and its corresponding 95% confidence intervals for the cell count in BALT zone were shown in Figure 1 (c). In alveolar damage group, two subgroups of

markers with similar mean cell counts were obtained. TNF-alpha and IL-10 (P=1.000) report the highest mean cell counts and IL-1 and IL-6 (P=0.868) report the lowest mean cell counts. Statistically significant differences between the markers in the different subgroups were reported (P < 0.001). In interstitial pneumonia group, IL-1 marker showed statistically significant differences with the rest of markers (P<0.001) (Figure 2c). In purulent bronchopneumonia group, IL-6 provided the highest mean number of immunolabelled cells and showed statistically significant differences with TNF-alpha and IL-1 (P<0.001). Statistically significant differences were also recorded between IL-10 and IL-1 (P = 0.003). In this group, IL-1 obtained the lowest mean count of immunolabelled cells. Finally, in bronchointerstitial pneumonia group, two subgroups of markers with similar mean cell counts were obtained. On the one hand, TNF-alpha and IL-6 (P=0.089) report the highest means of immunolabelled cell counts, being the second marker which showed the highest mean number of immunolabelled cells not only in this group, but also in the rest of pathological groups. By the other hand, IL-1 and IL-10 (P=1.000) report the lowest mean cell counts. Statistically significant differences between the markers in different subgroups were reported (P < 0.005).

A significant interaction between pathological groups and markers was presented in bronquial lumina (Figure 1 (d)). In alveolar damage group and interstitial pneumonia groups, no statistically significant differences were reported between any pair of markers. On the contrary, in purulent bronchopneumonia group, the highest mean number of immunolabelled cells was obtained with IL-6 marker, for which statistically significant differences were obtained between this and the rest of markers (P < 0.001). Similarly, in bronchointerstitial pneumonia group, IL-6 provided the highest mean count of immunolabelled cells, but in this case, the highest value is also for the rest of pathological groups (Figure 2d). Statistically significant differences were

observed between this and the rest of interleukin markers (P<0.001). Besides, there were also statistically significant differences between TNF-alpha and IL-1 (P < 0.001).

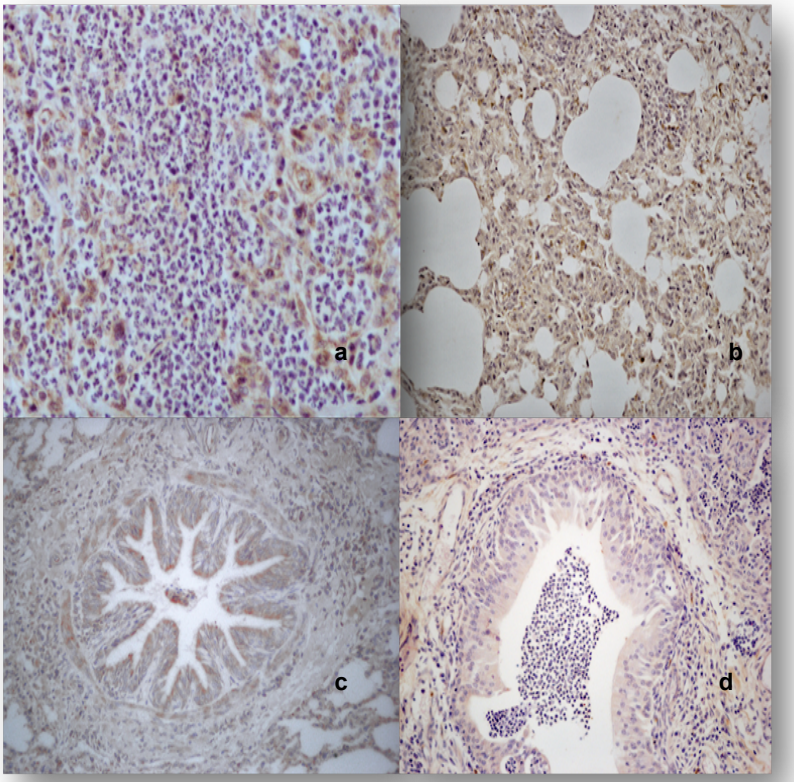

Figure 2. a. IL-10. Purulent Bonchopneumonia group. (40x). **b.** TNFα. Interstitial pneumonia group. (20x). **c.** IL-1R. Interstitial pneumonia group. (20x). **d.** IL-6. Purulent bronchopneumonia group. (40x).

Discussion

Ovine respiratory syndrome with its different pathological expressions generates economical losses and suffering to the animals; so, a good knowledge of the associated immune response is of great interest. Carrasco et al. (2004) proposed the

evaluation of the cytokine response as a valid method in the pathogenesis of respiratory disease in lambs.

Previous studies evaluated the response in experimental infections in ovine (Redondo et al 2011, 2014), bovine (Pedrera et al, 2007) and porcine (Salguero et al., 2001; Redondo et al 2009; Gomez-Laguna et al., 2011).

In this study, the expression of various cytokines has been evaluated to understand the involvement of these in the lung immune response for the different lesional groups pointed by Caswell and Williams (2007) as expressions of this syndrome.

An analysis of different pathological groups and markers was made in which an increase of IL-10 in the septal and BALT areas in alveolar damage group was denoted, being this more steeply in the first one. This fact may indicate a first stage in this syndrome, thus avoiding the development of more serious pathologies in the studied areas. This increase has been previously described, associated with downregulation effect in septal macrophages (Redondo et al., 2009, 2014). However, the highest number of immunostained cells for IL-10 was located in alveolar zone in purulent bronchopneumonia group. Its effect in the alveolar region for this group could be explained as prevention of more serious damage in the respiratory exchange, thus avoiding the recruitment of a greater cellular component by pro-inflammatory cytokines. Similar findings have been described by various authors (Fach et al, 2010; Gómez-Laguna et al, 2010; Redondo et al, 2014). Our results disagreed with the obtained results in other species in this zone, in which it is pointed lowest values (Gómez-Laguna et al., 2013), which seem to be related to the studied agent which causes changes only in septum. This increase in immunolabeling was even more steeply in the cited group than in the bronchointerstitial pneumonia one, possibly pointing to a better control of the pathology in this last group.

Values changes of TNF-alpha were noted. Regard the group of animals with interstitial pneumonia, an elevated activity of TNF-alpha and other pro-inflammatory cytokines was observed. Fach et al. (2010) associated this activity for the recruitment of defence cells in the inflammation sites. This increase in the production of this cytokine may be also associated to a certain activity control by septal macrophages in presence of agents (Gomez-Laguna et al., 2010). In our case, this highest number of immunomarked cells seems to be related with the presence of nonspecific pathogens, which give rise to a greater inflammatory component at this level in the groups with interstitial lesions. This nonspecific response could be associated to environmental conditions as was seen by our work group (Galapero et al., 2016). This activity along with other mediators was observed by Redondo et al., (2014) although in alveolar macrophages, not being noted in our study. Our hypothesis is that the alveolar zone is the area of greater entry of pathogens, which makes that there is a greater regulation of anti-inflammatory interleukins in order to control the pathologic changes, thus avoiding problems in pulmonary homeostasis. In BALT, it remains similar pattern; this could indicate that there was a relationship between anti-inflammatory and pro-inflammatory interleukins to prevent the development of severe pneumonic lesions (Morrison et al., 2000) while maintaining the defence of the lung.

Gomez-Laguna et al. (2010) established IL-1α as one of the determinants of interstitial pneumonia in experimentally PRRS infected pigs. In the present study, high values of this cytokine have been observed in groups with an interstitial component showed a high expression of IL-1R in interstitial pneumonia group comparing with the exudative processes groups. This fact has been recorded in this interleukin in BALT. Besides, this interleukin showed a high number of immunolabelled cells in alveolar zone in purulent bronchopneumonia group,

supporting the hypothesis that IL-1 is considered as neutrophil-chemoattractant and stimulant agent (Van Reeth & Nauwynck, 2000).

Furthermore, IL-1α and TNFα may induce the synthesis of IL-6 in order to stimulate an immune response in the affected area (Van Reeth and Nauwynck, 2000; Mitchell and Kumar, 2004). In our case, their increase was observed in the bronchial lumina and in the alveolar area, which would indicate a greater response against potential pathogens at these levels, along with the presence of IL-10 as a regulator of the activity.

In groups of exudative processes, an important number of immunomarked cells of IL-6 was denoted, which may be due to the stimulating effect of T and B lymphocytes (Parker et al., 2011); highlighting in our study the value in bronchial lumina. This increase in the counting of cells could be related to a major role in innate immune defence against possible microbial pathogens, mainly due to a faster response, according with the obtained results by Parker et al. (2010).

IL-10 showed a high number of immunomarked cells in alveolar zone compared to IL-1 and TNF-α, possibly due to a downregulate in the function of alveolar macrophages and lung inflammation by inhibiting these pro-inflammatory cytokines, in agreement with Redondo et al. (2014). It has been also described its immunosuppressive role in pro-inflammatory cytokines (e.g. IL-6), as was defined by Parker et al. (2010). In our case, this effect between both interleukins has been described in alveolar zone and in BALT zone.

Conclusions

The obtained results showed alveolar zone and bronchial lumina as key centres in the control of the specific response against external pathogens, with the implication of

different pro-inflammatory cytokines and a downregulation effect in the case of IL-10 like anti-inflammatory interleukin.

Septum can be considered as a location with a less specific response, with a greater active cell component in the interstitial pneumonia group, being the effect of anti-inflammatory cytokines less than in other groups. BALT could be considered as a defence centre, showing a rapid response to the attack of agents mainly in the groups with a greater exudative component.

The presence of different predisposing factors develops a non-specific immune response in septum and BALT, and, in absence of an optimal control, originates a specific response with exudative processes in the target sites, alveolar zone and bronchiolar lumen.

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BLOQUE IV. Discusión general y conclusiones

Capítulo 8. Discusión general

Extremadura es una de las regiones de España con mayor censo ovino. Su sistema de producción ha pasado desde el modelo individual a un modelo cooperativo, que aporta una mayor competitividad y una mejor estructuración al sector (López et al., 2002).

El modelo que surge de estas estructuras son los CC, que permiten la homogenización de la producción ovina. Esta razón, principalmente comercial, incluye la unificación en la venta de animales por parte de un grupo de ganaderos cuyo fin es la estandarización del producto. Esto da lugar a la producción en el mercado de animales de igual peso en diferentes explotaciones. Los caracteres de estandarización están basados principalmente en el peso vivo; así pues, la estancia de los animales dependerá en gran medida del tiempo que tarde en alcanzar el peso adecuado para salir hacía el matadero (Imagen 1).

Imagen 1. Vista principal de la clasificadora de corderos.

Durante la realización de esta tesis, desarrollamos un estudio en profundidad de la patología respiratoria. Los procesos patológicos respiratorios originan importantes

pérdidas de tipo directo e indirecto en la producción de corderos (Goodwin et al., 2004; Lacasta et al., 2008).

Dentro de las pérdidas indirectas se encuentran las asociadas al decomiso completo por la presencia de consolidación pulmonar (Lacasta et al., 2008) (imagen 2). Las pérdidas directas son la muerte del animal como podemos ver en la imagen 3.

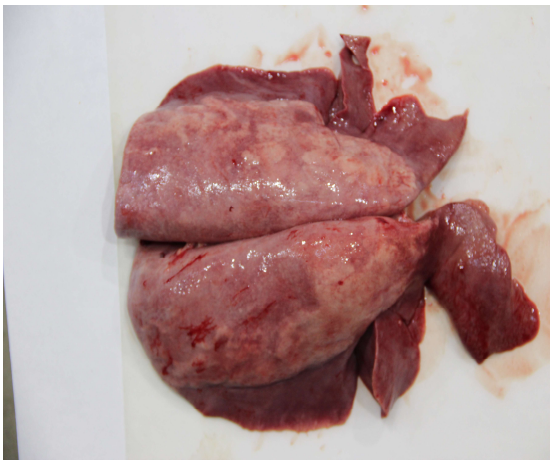

Imagen 2. Presencia de consolidación pulmonar en un decomiso de matadero.

Imagen 3. Cordero muerto en cebadero por SRO.

Si bien es importante el conocimiento de la patología, no es menos el estudio de los factores de riesgo para el desarrollo de la misma. Nash et al. (1997) apuntaron la edad como un importante factor de riesgo; Lacasta et al. (2008) también consideraron las condiciones medioambientales y estacionales.

En nuestro caso, todos los animales fueron agrupados dentro del mismo rango de edad con el objetivo de estudiar la asociación de los parámetros estacionales.

Por este motivo, no hemos pretendido buscar la relación entre la presencia de consolidación y los grupos histológicos hallados en los meses sometidos a estudio, sino que llevamos a cabo un estudio de las relaciones entre las variables (factores medioambientales y microorganismos) y su influencia en el estado de salud del animal. Esto permite un mayor conocimiento de la epidemiología de la enfermedad (Geenen et al., 2011; Lewis et al., 2011).

Algunos autores usan las redes bayesianas como método para determinar los factores de riesgo utilizando diferentes parámetros como marcadores de salud animal (BIKendrick et al., 2000, Ettema et al., 2009, Jensen et al., 2009, and Lewis et al., 2011). En nuestro trabajo las redes bayesianas nos permiten el uso de información sobre la relación existente entre las variables (Pearl, 1988; Neapolitan, 2004). Así, la propagación de evidencias nos sirve como herramienta para construir escenarios, que nos proporcionan información útil para la toma de decisiones. También nos ayuda en la prevención de riesgos en la sanidad animal.

Cuando analizamos la patología macroscópica, la presencia de consolidación se percibió con mucha frecuencia. Ésta se caracterizaba por la presencia de áreas de atelectasia de localización cráneo-ventral, donde se encontraron frecuentemente membranas de tipo fibrinoso en la superficie pulmonar. Estos hallazgos han sido descritos por otros autores (Sheehan et al., 2007; Yener et al., 2009; Rosadio et al., 2011). (Imagen 4).

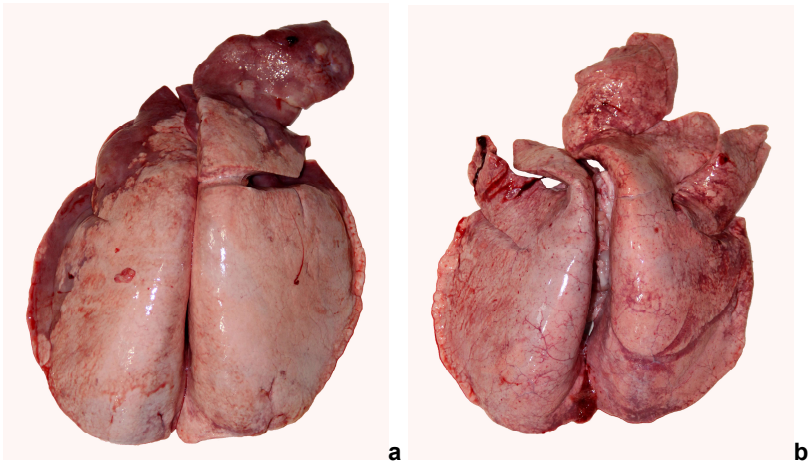

Imagen 4. Fotografías donde se puede distinguir la presencia y ausencia de consolidación en dos pulmones de cordero.

El análisis microscópico de las muestras extraídas reveló cuatro grupos: Daño alveolar difuso (DAD) (imagen 5), neumonía intersticial (NI) (Imagen 6), bronconeumonía purulenta (PB) (Imagen 7) y neumonía broncointersticial (BI) (Imagen 8). Esta clasificación ha sido adaptada a partir de otras similares, para conseguir una mejor adecuación a nuestro método de trabajo, simplificando los procesos con componente exudativo en bronconeumonía purulenta (Oruç, 2006; Caswell & Williams, 2007).

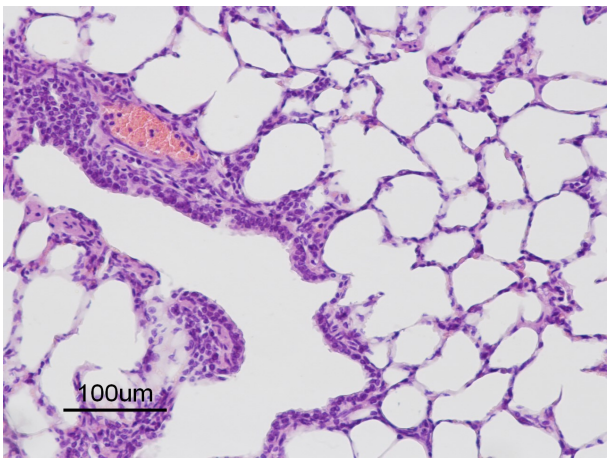

Imagen 5. Grupo de daño alveolar difuso. (H-E)

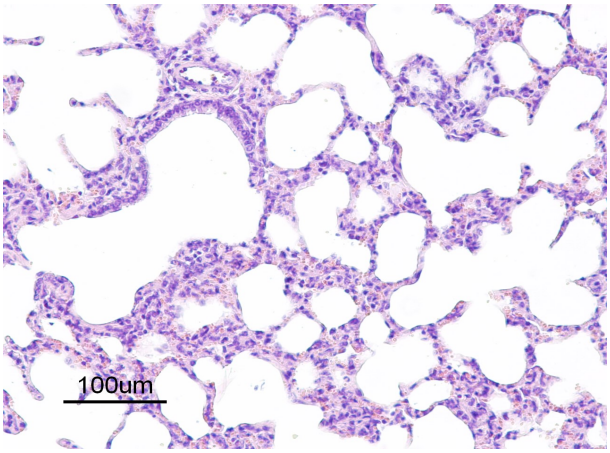

Imagen 6. Grupo de neumonía intersticial. (H-E).

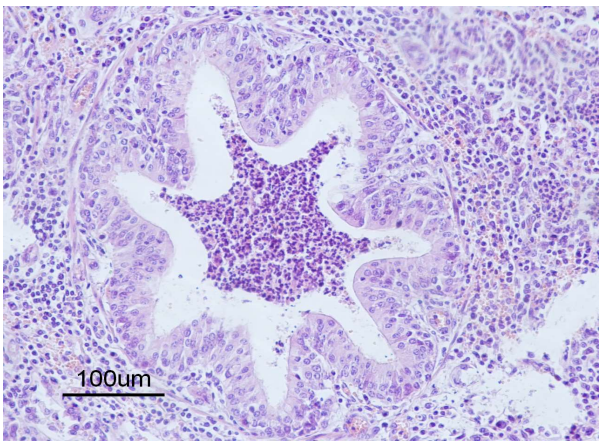

Imagen 7. Grupo de bronconeumonía purulenta. (H-E).

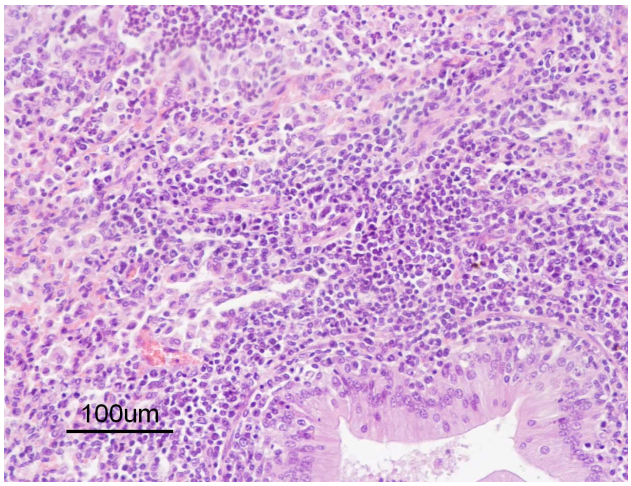

Imagen 8. Grupo de neumonía broncointersticial. (H-E).

Cuando llevamos a cabo el análisis estadístico se evidenció una correlación entre las lesiones macroscópicas y los cambios histológicos, arrojando resultados similares a los obtenidos por Rosadio et al., (2011).

La bronconeumonía supurativa es la lesión que con mayor frecuencia está asociada a procesos de consolidación pulmonar (Gázquez et al., 2001; Gonçalves et al., 2010). Mawhinney et al. (2010), apuntaron hacia la neumonía intersticial como la principal lesión implicada en la presencia de 'hepatización roja'. En nuestro estudio

los grupos de neumonía intersticial, bronconeumonía purulenta y neumonía broncointersticial son los que presentan una asociación con los procesos de consolidación pulmonar. Esto también fue apuntado por otros autores en ovino (Oruç, 2006) y en caprino (Yener et al., 2008). Los resultados obtenidos mediante el uso de modelos con redes bayesianas también apoyan esta afirmación (Galapero et al., 2016). De acuerdo con los escenarios planteados, la probabilidad estimada de presencia de consolidación pulmonar es del 54.16% cuando se observó lesiones de tipo exudativo. En nuestro estudio, la aparición de consolidación tuvo un menor porcentaje de casos asociados a grupos exudativos que la obtenida en los escenarios planteados mediante el estudio con redes bayesianas. Por todo ello, no solo debemos destacar la presencia de procesos exudativos asociados con la presencia de consolidación pulmonar, sino que tenemos que tener en cuenta que la neumonía intersticial puede originar también procesos de consolidación. Sin embargo, en ausencia de consolidación pulmonar la mayoría de los casos analizados se agruparon dentro del daño alveolar difuso.

Aunque es bien conocido que los factores principales para inducir enfermedad proceden de las bacterias (Hervás et al., 1996; Niang et al., 1998), la presencia de factores predisponentes es necesaria para el desarrollo de esta enfermedad. Entre estos factores se encuentran los elementos medioambientales (Brodgen et al. 1998; Ábalos, 2006).

Se realizó un estudio estadístico para observar las relaciones entre los meses en los que el animal estuvo en el cebadero y la consolidación pulmonar y entre aquellos y la presencia en un determinado grupo histológico. Para el primer caso, se determinaron tres periodos en relación con la presencia/ausencia de consolidación según los meses en los que se efectuó la fase de engorde en el centro de clasificación. Esta clasificación de meses donde apareció ausencia o presencia de consolidación tuvo

relación con la temperatura y la humedad relativa media registrada en dichos periodos.

En base a las referencias que se tienen de temperatura y humedad relativa para corderos de engorde (ITOVIC, 1991), de 13-16ºC de temperatura y de 70-80% de humedad relativa, los meses de febrero, marzo y abril, estuvieron caracterizados por una temperatura media y una humedad relativa media consideradas fuera de ese rango óptimo.

Los hallazgos encontrados se asemejan a lo descrito por Lacasta et al. (2008), quienes determinaron que los meses en que las temperaturas eran menores de 10ºC (y posiblemente también con una mala ventilación), concurrían un mayor número de problemas de índole respiratoria. En nuestro estudio los porcentajes de pulmones con consolidación superaban el 90% en los meses con baja temperatura.

Es destacable que los meses donde se registraron los mayores picos de temperatura se visualizara un gran aumento en el número de casos de consolidación. Este hecho también fue descrito por otros autores en el ganado ovino (Lacasta et al., 2008; Plummer et al., 2012). Por tanto, en el periodo desde enero hasta mayo existiría un mayor riesgo de hallazgos patológicos macroscópicos en los mataderos.

Esta presencia de consolidación pulmonar conlleva una pérdida indirecta por decomisos en el matadero, con una importante repercusión económica en los sistemas de producción de corderos de engorde (Goodwin et al. 2004).

Por el contrario, los meses de septiembre, octubre y noviembre se caracterizaron por un registro de temperatura y humedad más cercano al rango óptimo descrito anteriormente (ITOVIC, 1991), viéndose así una disminución considerable en la presencia de consolidación pulmonar.

Sí bien las condiciones medioambientales influyen en la presencia/ausencia de consolidación, también vinculan la aparición de un determinado grupo histológico.

Así, los meses en los que la temperatura y la humedad relativa estuvieron fuera de los rangos óptimos destacaron por un aumento de los casos de neumonía intersticial y bronconeumonía supurativa. Estos hallazgos también fueron observados por Brscic et al. (2012) en terneros, registrándose un aumento de casos de neumonía grave en los meses de verano.

Así pues, los meses donde se registró una menor temperatura, como fue el caso de febrero a abril, se mostraron más patentes los procesos de tipo intersticial. Por el caso contrario, los procesos con un mayor componente exudativo fueron registrados desde abril hasta julio coincidiendo con los mayores registros de temperatura. Esto lleva implícito un mayor coste de producción, no solo por decomisos en matadero, sino además por los costes añadidos en los tratamientos aplicados, unido a la posibilidad de muerte del animal (Goodwin et al. 2004).

Para esclarecer la influencia de estos parámetros en el proceso, se definieron diversos escenarios mediante la aplicación de redes bayesianas para analizar el efecto de los principales riesgos medioambientales en el síndrome respiratorio ovino. La mayor influencia en la presencia/ausencia de consolidación viene determinada por los componentes medioambientales (temperatura y humedad relativa). Cuando se consideran los valores óptimos de acuerdo a lo expuesto por ITOVIC (1991) (temperatura 13-16ºC y humedad relativa 70-80%), la probabilidad estimada de presencia de consolidación pulmonar fue muy baja y la probabilidad de procesos inflamatorios (neumonía intersticial, bronconeumonía supurativa y neumonía broncointersticial) también fue pequeña.

La información temporal obtenida nos llevaría a pensar en la idea de aplicar medidas de manejo en determinados momentos para minimizar el impacto de la patología en los sistemas de producción intensivos.

En este estudio además se consideraron los hallazgos microbiológicos de los pulmones de los animales objeto de estudio. La presencia de bacterias fue más baja que lo presentado por otros autores (Hervás et al., 1996; Niang et al., 1998). Sin embargo, si las condiciones medioambientales cambian, se observa un incremento de la probabilidad estimada en la presencia de consolidación y de diferentes procesos inflamatorios; esto es debido principalmente a que la presencia de microorganismos que puedan originar patología, parece tener relación con la temperatura registrada. La aplicación de redes bayesianas nos muestra como existe una relación directa entre la temperatura y *Mycoplasma spp*., obteniendo un incremento de la probabilidad de la presencia de este agente cuando las temperaturas son superiores a la óptima. Estos hallazgos coinciden con lo expuesto por otros autores (Hervás et al., 1996; Niang et al., 1998), que observaron un incremento de la patología asociada con la presencia de *Mycoplasma ovipneumoniae* en los meses más calurosos, es decir, cuando la temperatura fue alta y la humedad relativa más baja.

Pero la humedad relativa también conlleva cambios por sí misma, cuando se encuentra en el rango del 50-60%, con valores de temperatura dentro de los niveles óptimos (13-15ºC), se produjo un incremento en la probabilidad estimada de presencia de consolidación y neumonía intersticial. Esto puede deberse al efecto irritativo de la sequedad en la mucosa respiratoria.

Caswell and Williams (2007), describieron procesos inflamatorios pulmonares asociados a tóxicos irritantes. En nuestro estudio, la concentración de amoniaco estuvo relacionada con la presencia de consolidación. También cabe reseñar que el registro de altos valores de amoniaco está íntimamente relacionado con los factores medioambientales de temperatura y humedad relativa.

Nuestros resultados coinciden con los obtenidos por otros autores, que describieron una asociación entre altas concentraciones de amoniaco en construcciones antiguas con mala ventilación y la aparición de problemas respiratorios (Lacasta et al., 2008). Cuando los niveles de amoniaco se fijaron en un rango entre 15 a 20 ppm, se observó un incremento en la probabilidad de presencia de consolidación, neumonía intersticial, bronconeumonía supurativa y neumonía broncointersticial. Este aspecto corroboraría su participación en la presencia de cuadros inflamatorios en el pulmón.

Oruç (2006) y Lacasta et al. (2008) identificaron a *Mannheimia haemolytica* como uno de los principales microorganismos involucrados en la aparición de procesos respiratorios ovinos, considerándose como uno de los más importantes agentes aislados en la aparición de consolidación pulmonar.

En concordancia con lo aportado por otros autores (Alley et al., 1975; Jones et al., 1982; Sheehan et al., 2007), *Mycoplasma arginini* y *Mycoplasma ovipneumoniae* parecen estar involucrados en los procesos neumónicos en ovino. Además, Lin et al. (2008) y Nicholas et al. (2008) encontraron una relación entre *Mycoplasma arginini* y las bacterias del genero *Pasteurella* (*Mannheimia haemolytica*). En nuestro estudio se vio una asociación entre *Mycoplasma spp*. y bacterias de la familia *Pasteurellaceae*. Sin embargo, cuando se planteó el escenario de la presencia de agentes de la familia *Pasteurellaceae*, se observó que solo la presencia de ellas registró un papel menos importante que la única presencia de *Mycoplasma spp* (Oruç, 2006).

Cuando abordamos el estudio sobre los factores predisponentes en la aparición de patología respiratoria en corderos de cebo, no hemos de olvidar el lugar de producción de los mismos. En los centros de clasificación se llevan a cabo diversos procesos de manejo que hacen que los animales puedan sufrir procesos estresantes,

como son el transporte, o la clasificación para establecer peso y sexo (Miranda-de la Lama et al., 2010b).

En la actualidad existen numerosos estudios que sugieren la existencia de factores de riesgo en la producción de corderos de engorde (Miranda-de la Lama et al., 2010b). El estrés puede generar un mal funcionamiento del sistema inmune, con repercusiones negativas sobre la salud del animal (Orgeur et al., 1998). Esto puede predisponer a la aparición de enfermedades (Nash et al., 1997; Brodgen et al., 1998). La presencia de estos medios de producción hace que los animales tengan que venir desde su explotación hasta unas instalaciones comunitarias donde se hacen lotes en base a su peso de entrada, siendo puntos críticos en este sistema tanto el transporte como su posterior descarga (Miranda de la Lama et al., 2010a). A partir de aquí, las nuevas infraestructuras implican una adaptación al nuevo sistema y, asociado a esta adaptación, una posible causa de estrés en los animales (Ferguson and Warner, 2008).

En ovino, hasta donde llega nuestro conocimiento, no existen trabajos científicos sobre el comportamiento de diferentes parámetros fisiológicos durante el periodo de engorde. En nuestro estudio algunos parámetros hematológicos mostraron diferencias estadísticamente significativas entre los dos periodos analizados.

El contaje de glóbulos rojos y de hematocrito fueron más elevados al principio del periodo de cebo que al final, y superiores a los descritos por Lepherd et al. (2009). Estos valores altos en ambos parámetros al inicio del periodo pueden ser explicados por los posibles efectos negativos del transporte y el reagrupamiento tras la llegada al centro de clasificación (Cockram et al., 2000, 2004; Kannan et al., 2000; Ferguson and Warner, 2008). Este descenso en los valores observados está en concordancia también con lo descrito por Miranda-de la Lama et al. (2012a), que apuntaban a este descenso como una adaptación a las nuevas condiciones de producción.

Los cambios en los niveles de hematocrito también se asocian a la movilización de los glóbulos rojos desde el bazo en respuesta a las catecolaminas (Kent, 1997), incluso asociado a la deshidratación tras el viaje (Cockram et al.,1996; Broom et al., 1996; Chacón et al., 2005; María, 2008), retornando a valores normales pasado un tiempo (Bórnez et al., 2009; Miranda-de la Lama et al., 2010a, 2012a, b). Por ello, estos parámetros deben ser utilizados como marcadores de estrés agudo.

El cortisol sufre también modificaciones. Su liberación sucede por la existencia de agentes estresores (Rhodes et al., 1994), siendo un buen marcador del grado de estrés de los animales. Fernández et al. (2007) observaron un aumento en los niveles plasmáticos de cortisol después de la clasificación y el reagrupamiento en el centro de clasificación. Después de un tiempo, los valores de cortisol tendieron a una normalidad. Las muestras recogidas a la entrada y la salida del cebadero no mostraron diferencias estadísticamente significativas en el nivel de cortisol medio, aunque en ambos casos los valores fueron elevados, lo que sugiere una falta de adaptación en el periodo de estancia en el centro (Galapero et al., 2016b).

Otra medida usada para analizar el efecto del estrés es la ratio neutrófilo/linfocito (Blecha, 2000). Es un indicador de una respuesta inflamatoria sistémica y ha sido asociada con estrés crónico en los animales analizados (Puppe et al., 1997; Erminio and Bertoni 2008). Aguayo-Ulloa et al., (2014) apuntaban un descenso significativo de los valores de este parámetro cuando se utilizaron corrales con diferentes elementos de enriquecimiento, evitando así problemas de estrés. En nuestro caso percibimos un incremento en la ratio N/L entre el inicio y el fin del periodo de engorde. Esto fue descrito de manera similar por Miranda de la Lama et al. (2012), circunstancia que define el periodo en el centro de clasificación como un factor de estrés sostenido.

Existen resultados previos que muestran cómo el estrés afecta a los componentes de la respuesta inmune innata (Brown et al., 2008). Los neutrófilos constituyen el tipo celular más abundante asociado a la fagocitosis, representando el 50-60% del total de leucocitos circulantes (Hoffmanet al., 2013). Este tipo de polimorfonuclear realiza una migración hacía los puntos de inflamación, íntimamente vinculados a diferentes agentes infecciosos.

Los neutrófilos, mediante diferentes mediadores químicos, ofrecen señales a otras células del sistema inmune cuando existe el ataque de un agente invasor. Los animales con una deficiencia en neutrófilos son más proclives al desarrollo de infecciones de tipo bacteriano y fúngico que otros con valores normales (Kumar and Sharma, 2010). Por tanto, su menor número conllevaría un fallo en la fagocitosis de los agentes invasores, desarrollando el animal enfermedades infecciosas (Rotrosen and Gallin, 1987).

Desde nuestro conocimiento, existen pocos estudios sobre el índice de fagocitosis en corderos de cebo. Rudine et al. (2007), establecieron en su trabajo un valor del 74.40% para el índice de fagocitosis en cerdos en sistemas de producción intensivos. En nuestro estudio obtuvimos unos valores menores, aunque sin diferencias estadísticamente significativas entre los dos muestreos realizados, al inicio y al final del cebo. Rhodes et al. (1994) apuntaron que el estrés no debería provocar daños en la respuesta inmune del animal, pero cuando el agente estresor se mantiene en el tiempo se puede limitar el funcionamiento de este sistema de defensa.

Una vez analizados los principales factores de riesgo inherentes a la aparición de procesos patológicos en el pulmón, otro de nuestros objetivos planteados para el desarrollo de este trabajo, fue la caracterización inmunohistoquímica de las lesiones encontradas. Para ello se llevó a cabo el estudio de las diferentes poblaciones

celulares asociadas a los diferentes patrones histológicos hallados en animales provenientes de centros de clasificación.

La aplicación de técnicas inmunológicas que ayuden a conocer el efecto de determinadas células en los diferentes procesos patológicos observados forma parte del objetivo de esta tesis sobre el estudio en profundidad de la patología pulmonar en corderos de cebo

La aplicación de técnicas inmunohistoquímicas ha sido utilizada por diversos autores para el estudio de diferentes enfermedades en diversas especies como el bovino (Johnson et al., 2006), el ovino (Carrasco et al., 2004), el porcino (Salguero et al., 2001; Gómez-Laguna et al., 2011), así como en especies salvajes (Peña et al., 2006; García-Jiménez et al., 2013). Pero en la especie objeto de este estudio, estas técnicas han sido aplicadas sólo en infecciones experimentales (Redondo et al 2009; 2014).

En nuestro trabajo se registró un aumento en el número de células inmunomarcadas con el anticuerpo CD68 en el área alveolar con respecto al resto de zonas analizadas. Este aumento en la zona alveolar podría sugerir la importancia de estas células en el mantenimiento del homeostasis del pulmón, ayudando así a mantener una función respiratoria adecuada (Ackerman y Brodgen, 2000). Pors et al. (2013), describieron los macrófagos como una primera línea de defensa contra patógenos, ayudando a la fagocitosis y a la producción de citoquinas. Por esta razón, el papel de los macrófagos alveolares es importante y deben ser catalogadas como una de las primeras células implicadas en la eliminación de los agentes primarios responsables del daño patológico a nivel alveolar.

Otro tipo celular implicado en la respuesta inmune es el linfocito B. Polledo et al. (2011) y García-Jiménez et al. (2013), apuntaron que su presencia debe ser considerada como señal de infecciones tardías.

Sin embargo, en nuestro caso, la presencia de estas células en las áreas bronquiales es atribuible a una respuesta rápida frente a diferentes agentes. En presencia de un agente patógeno, la respuesta celular asociada a lesiones exudativas es mucho más efectiva, siendo capaz de neutralizar y eliminar con mayor eficacia al agente, como indicaron Pors et al. (2013). Hemos observado un aumento de las células B en los grupos con componente exudativo, posiblemente como apoyo de otros componentes celulares, principalmente con los macrófagos, actuando ambos como células presentadoras de antígeno.

En los centros de clasificación, los animales están expuestos a diferentes componentes ambientales, como partículas de polvo, amoniaco. Aunque los linfocitos B se relacionan con la lucha contra bacterias y virus, Müller et al., (1997) también sugirieron que su aumento en pulmones de cerdo se debía a una alta exposición a las partículas de polvo. Por ello, el aumento observado en nuestro estudio no sólo puede provenir de una infección aguda, sino de condiciones ambientales que producen una activación similar en los grupos con un patrón intersticial, relacionándose esto con una respuesta no específica que puede verse influida por mediadores de la inflamación. Además, el aumento de las células marcadas con CD79α en el grupo de la neumonía bronco-intersticial puede explicarse debido a la activación de los macrófagos alveolares a partir de patógenos. Esta activación provoca una respuesta a nivel del tabique mediante la implicación de diferentes mediadores de la respuesta inmune, similar a lo descrito en infecciones experimentales en cerdos por Redondo et al. (2009) y Gómez-Laguna et al. (2010).

No debemos olvidar a la hora de realizar un estudio sobre los diferentes componentes celulares que forman parte de un proceso inflamatorio, analizar también los mediadores de la inflamación. Un total de cuatro anticuerpos interleuquina 10 (IL-10), factor de necrosis tumoral α (TNFα), interleuquina 1R (IL-1)

e interleuquina 6 (IL-6) para evaluar su efecto como mediadores antiinflamatorias y pro-inflamatorias.

En el área alveolar se denotaron importantes hallazgos con respecto a los diferentes marcadores utilizados. IL-10 mostró altos contajes de células marcadas en la región alveolar en el grupo de bronconeumonía purulenta. Este hecho podría explicarse por su participación en la prevención de daños más graves que comprometan el intercambio respiratorio, como fue descrito por diversos autores (Fach et al, 2010; Gómez-Laguna et al, 2010; Redondo et al., 2014). Esto, además, debe tenerse en cuenta a la hora de elegir fármacos inmunomoduladores que permitan un tratamiento más efectivo.

Cuando llevamos a cabo el análisis de inmunomarcaje de IL-1, observamos un número elevado de células marcadas en la zona alveolar en el grupo de bronconeumonía purulenta. Este hecho podría apoyar la hipótesis de que esta citocina puede considerarse un mediador para el reclutamiento de polimorfonucleares y agente estimulante de la inflamación (Van Reeth y Nauwynck, 2000).

Además, IL-1α y TNFα pueden inducir la síntesis de IL-6 con el fin de estimular una respuesta inmune en el área afectada (Van Reeth y Nauwynck, 2000; Mitchell y Kumar, 2004). Este hecho ha sido muy destacado en la región alveolar, permitiéndonos considerar esta zona como un centro importante en el control de la enfermedad.

En grupos con procesos exudativos, se registró un número importante de células inmunomarcadas de IL-6, lo que puede deberse a la estimulación que provoca esta interleucina sobre los linfocitos T y B (Parker et al., 2011). Este hecho es especialmente destacado en la luz bronquial para los grupos exudativos. El aumento en el recuento de células podría estar relacionado con un papel importante en la

defensa inmune innata contra patógenos microbianos, principalmente debido a una respuesta más rápida, de acuerdo con los resultados obtenidos por Parker et al. (2010).

El área septal también sufrió variaciones, aunque no de la misma índole, se observó un aumento del marcador CD79α en animales con daño alveolar difuso y en los que mostraban neumonía bronco-intersticial. Una hipótesis podría ser su relación con un posible papel protector mantenido a lo largo del tiempo contra estos agentes exógenos, como proponen otros autores (Fragkou et al., 2010; García-Jiménez et al., 2011; García-Jiménez et al., 2013). En nuestro estudio se vio una relación entre los marcadores de linfocitos B y T, lo cual podría explicarse como una mejora en la eficiencia del sistema inmune en el área pulmonar (García-Jiménez et al., 2013).

También se registró un elevado contaje en los macrófagos septales debido a su importante papel en el desarrollo de patología, como quedó demostrado en estudios previos (Carrasco et al., 2004; Gómez-Laguna et al., 2010). En nuestro caso, hubo un aumento a nivel septal en el grupo de neumonía intersticial, aunque sin alcanzar los niveles alveolares. Esto también indicaría una etapa más avanzada de la enfermedad, coincidiendo con los resultados presentados por Polledo et al. (2011). La acción de estos macrófagos se debe principalmente a su actividad fagocítica y a la monitorización hemodinámica a nivel pulmonar.

Cuando analizamos la presencia de IL-10 en el área septal, se observó un aumento en el grupo de daño alveolar. Este aumento podría estar asociado con un efecto de regulación de macrófagos septales (Redondo et al., 2009, 2014), posiblemente relacionado con procesos patológicos leves.

A nivel septal también se evaluó el efecto de citocinas pro-inflamatorias. Gómez-Laguna et al. (2010) destacaron el papel de IL-1α como determinante de la neumonía intersticial en cerdos infectados experimentalmente con PRRS. En nuestro caso,

valores altos de esta interleucina también se observaron en procesos con un importante componente intersticial. Esto puede indicar una respuesta más inespecífica debido posiblemente a una exposición prolongada a agentes de tipo medioambiental (Galapero et al., 2016). Sin embargo, en los procesos exudativos, cuando ocurre una posible desregulación a nivel pulmonar, su centro de actividad se traslada hacía las luces alveolares y bronquiales.

También en la zona septal se observaron cambios en los valores de TNF-alfa. Este aumento en la producción de esta citocina puede estar asociado a cierto control de actividad por macrófagos septales en presencia de agentes (Gómez-Laguna et al., 2010). Esta actividad, junto con la de otros mediadores, fue observada por Redondo et al., (2014) en macrófagos alveolares, no siendo este hecho manifiesto en los resultados de nuestro trabajo.

Los centros de defensa asociados a tejidos, en este caso a bronquios (BALT), son acumulaciones de células linfoides que actúan contra patógenos. Se ha encontrado un gran número de células marcadas con CD3 en el área de BALT para grupos de neumonía broncointersticial y bronconeumonía purulenta. Esto podría apoyar la hipótesis de que, en las fases en las que actúan agentes de tipo infeccioso en el desarrollo de la enfermedad, la respuesta celular coincide con la presencia de estos agentes patógenos en el foco de inflamación (Fragkou et al., 2010). Por otra parte, Chen & Kolls (2013) descubrió que una insuficiente inmunidad por deficiencia de células T también puede aumentar la probabilidad de propagación de patógenos desde el pulmón.

Rodríguez et al. (2000), observaron hiperplasia de BALT en infecciones experimentales con *Mycoplasma agalactiae* y *Mycoplasma bovis* en cabras. En nuestro caso en esta área, los linfocitos T fueron las principales células involucradas, siendo esenciales en la inflamación, incluso en el caso de respuesta inespecífica.

Además, las lesiones exudativas expresaron un mayor recuento de células CD3+, en desacuerdo con los resultados presentados por Pors et al. (2013), que describieron un bajo número de células inmunomarcadas en este tipo de lesiones. Este aumento en los linfocitos B y T en el tejido linfoide sugiere un papel protector contra diferentes agentes infecciosos (Fragkou et al., 2010).

Se observó una relación entre las interleuquinas antiinflamatorias y proinflamatorias. La función regulatoria de estas citocinas parece tener carácter preventivo, evitando así el desarrollo de lesiones neumónicas graves en el pulmón; además de esto, se encarga de coordinar los diferentes componentes celulares para mantener la defensa del pulmón (Morrison et al., 2000). Con respecto al grupo de animales con neumonía intersticial, se observó una actividad elevada de TNF-alfa e IL-1 en BALT. Fach et al. (2010) asociaron esta actividad al efecto quimiotáctico de células de defensa en los puntos de inflamación. Por todo ello, cabe destacar la importancia del BALT como centro de defensa frente a agentes patógenos en el desarrollo de la actividad inflamatoria, debido a la importancia que tienen las vías aéreas en la fisiología pulmonar, lo cual puede llevar a comprometer la vida del animal.

Capítulo 9. Conclusiones

Primera

El estudio de las condiciones medioambientales nos ha permitido definir los factores predisponentes a tener en cuenta a la hora de evaluar el riesgo de aparición de casos de síndrome respiratorio ovino en corderos de cebo. Además, nos permite determinar los meses del año asociados a un mayor riesgo de aparición de patologías.

Segunda

El uso de modelos gráficos probabilísticos como las redes bayesianas nos permite la evaluación de diferentes situaciones epidemiológicas para la identificación de factores de riesgo asociados a la aparición de síndrome respiratorio ovino en corderos de cebo. Los principales factores predisponentes para el desarrollo de este síndrome tienen su origen en la temperatura, la humedad relativa y la presencia de *Mycoplasma spp*., permitiendo esta información la confección de medidas de manejo para poder aplicar mejoras en los centros de clasificación.

Tercera

La evaluación de diferentes parámetros hematológicos puede ser una herramienta económica y fácil de utilizar para evaluar el estrés de los animales durante su estancia en el centro de clasificación. El control de estos parámetros a la entrada y a la salida a matadero permite una evaluación del efecto de este período sobre los animales. Esto, en su caso, podría permitir el establecimiento de medidas correctoras en su manejo y así, evitar problemas de adaptación durante esta fase de engorde.

Cuarta

Los resultados de nuestro estudio arrojaron una falta de adaptación de los animales a la fase de cebo en los centros de clasificación. Se constata la posible existencia de agentes estresores desde la entrada hasta la salida de los animales con destino a matadero. La evaluación mediante el uso del índice de fagocitosis demuestra una disminución de la funcionalidad de neutrófilos como agentes de respuesta del sistema inmune. Como línea futura debería tenerse en cuenta la introducción de elementos para disminuir ese efecto contrario en los centros de clasificación, siendo la valoración de este índice un método útil para evaluar esas posibles mejoras.

Quinta

La zona alveolar y la luz bronquial deben ser consideradas los puntos diana de una respuesta más específica frente a patógenos externos; esta respuesta está basada en la alta presencia de linfocitos B y macrófagos, así como en una alta expresión de citocinas pro-inflamatorias y de interleucina 10 de origen anti-inflamatorio.

Sexta

El área septal muestra una respuesta inflamatoria más prolongada en el tiempo, observándose un elevado marcaje de linfocitos T y B, además de la presencia de citocinas pro-inflamatorias, las cuales regulan y atraen diferentes poblaciones celulares para el control de los diferentes organismos patógenos en esta zona, pudiendo ser estos de origen medioambiental.

Séptima

El BALT, según los resultados obtenidos, se puede considerar centro de defensa frente a los agentes desencadenantes de procesos inflamatorios en el pulmón,

debido principalmente a la presencia de linfocitos y a los hallazgos obtenidos en la evaluación de las citocinas tanto de origen pro-inflamatorio como anti-inflamatorio.

Octava

Los resultados de este estudio nos desvelan una posible evolución de la enfermedad desde procesos leves a otros de mayor gravedad en el intersticio. Estos últimos, en ausencia de un control eficaz, desencadenan una respuesta de tipo exudativa localizada principalmente hacía las zonas de luz alveolar y bronquial.

Novena

El estudio inmunohistoquímico del síndrome respiratorio ovino nos permite establecer futuras líneas de trabajo sobre la eficacia en la aplicación de diferentes patrones de manejo mediante el uso de diferentes agentes biológicos y químicos en el control de la patología. Estas medidas deberían ir encaminadas a la valoración de la respuesta inmune de los animales en diferentes protocolos vacunales y en caso de instaurarse la enfermedad, valorar el uso de determinados fármacos con carácter inmunomodulador para favorecer la disminución en el número de lesiones, causadas por el síndrome respiratorio ovino.

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Anexo. Publicaciones

- Galapero, J., Fernández, S., Pérez, CJ., Ramos, A., Salguero, FJ., Cuesta, JM., Gómez, L. Study of possible influence of seasonal conditions on pathological respiratoy processes in fattening ovine from Extremadura 'South-western Spain'. Enviado a la revista Small Ruminant Research para su revisión.
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