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Research Paper

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

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ABSTRACT

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

1. Introduction

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

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

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

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especially CD8+ T cells, and a *histiocytic pattern*, with a predominance of histiocytic cells mixed with B cells. The former could be related to a cytotoxic cellular immune response with a low antibody titer and the latter to a stronger humoral response with higher antibody titer. The aim of this study is to evaluate the correlation between individual serologic response against VMV and the histological lesion pattern. The results should be considered when diagnosing and controlling of MV disease, especially in infected but negative animals in serological tests.

2. Material and methods

2.1. Animals

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

2.2. Sampling and histopathology

Tissue samples from the 58 sheep were systematically taken for histopathology from diaphragmatic and apical lung lobes, glandular parenchyma and udder cisterns and CNS from 9 levels of the brain and 9 levels of the spinal cord following a previous description (Polledo et al., 2012a). All the samples were fixed in 10% neutral buffered formalin for 48 h at room temperature. After fixation, samples were embedded in paraffin wax and sections (4 μm) were stained with hematoxylin and eosin (HE) and examined using light microscopy.

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

2.3. Immunohistochemistry

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

2.4. Polymerase chain reaction

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

2.5. Serology analysis

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

2.6. Statistical analysis

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

3. Results

3.1. Histopathology and immunohistochemistry

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

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

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

3.2. Polymerase chain reaction

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

3.3. Correlation between histopahological and serological findings

Fourty-five of the fifty infected animals showed positive optical density (OD) results, three of them presented doubtful results