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Pneumonia of lambs in the Abruzzo region of Italy: anatomopathological and histopathological studies and localisation of *Mycoplasma ovipneumoniae*

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Summary

The most common forms of inflammation of the lower respiratory tract in lambs are acute enzootic pneumonia, caused mainly by *Mannheimia haemolytica*, chronic enzootic pneumonia (defined as 'atypical' in lambs), the aetiological of which is *Mycoplasma ovipneumoniae* and viral inflammation principally caused by parainfluenza virus type 3. The authors conducted anatomo-pathological and histopathological studies of the most commonly encountered spontaneous lung inflammations in lambs slaughtered in the Abruzzo region of Italy, with special attention to 'atypical pneumonia'. Microbiological isolations and an histo-pathological and immunohistochemical analysis were performed to reveal any possible correlations between causal agents and lesion patterns. Positive results for *M. ovipneumoniae* were compared to those for *Mycoplasma* isolation to evaluate the sensitivity of the two techniques. Of a total of 156 samples, 31 (19.8%) demonstrated involvement of *M. ovipneumoniae*, 15 (9.6%) were positive on microbiological isolation confirmed by typing with biomolecular methods and, finally, histological lesions (atypical pneumonia) were observed in the remaining 16 cases (10.2%). Of these 31 samples, 23 (14.7% of the total) demonstrated postive antigen in alveolar macrophages and giant cells on immuno-histochemical testing. These data revealed the presence of chronic enzootic pneumonia in the Abruzzo area and the importance of immunohistochemistry (in combination with isolation and anatomopathological and histopathological examination) for the diagnosis of pneumonia caused by *M. ovipneumoniae*, as well as the high sensitivity shown by antigen marker expression, even in samples where bacterial load was limited.

Keywords

Histopathology, Immunohistochemistry, Lambs, *Mannheimia haemolytica*, *Mycoplasma ovipneumoniae*, Parainfluenza virus type 3, Pneumonia.

Introduction

Respiratory infections in domestic animals can incur heavy economic losses, due to either mortality or the reduced value of the animal (10). In sheep, the pathogenesis of respiratory diseases is often difficult to establish due to the interaction of various causal agents (1, 14, 15) which can exhibit similar anatomopathological patterns. Therefore, localisation and investigation of the features of the pneumonic and aetiological process are both necessary to identify the causal agent of the inflammation of lungs.

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Mannheimia haemolytica is the most commonly isolated microorganism in cases of ovine pneumonia in both adults and lambs. This microorganism is considered responsible for acute fibrinous pleuropneumonia, sometimes in combination with viral agents (para-influenza virus type 3 virus). In subacute or chronic forms of ovine pneumonia, *Mycoplasma ovipneumoniae* can also be involved (10).

In lambs, a form of chronic enzootic pneumonia is often observed, referred to as 'atypical' or 'non-progressive, proliferative/ interstitial pneumonia'. Caused by *M. ovipneumoniae*, probably in association with *M. haemolytica* (4, 8), this form of pneumonia mainly affects animals in their first year of life and is characterised by the scarcity or absence of clinical symptoms and by its chronic progression. It is predominant in intensively managed farms (7).

The aim of this study was to conduct an anatomopathological and histopathological study of the most commonly encountered spontaneous lung inflammations in lambs in the Abruzzo region, with particular attention to atypical pneumonia. Results were analysed to establish a correlation between pathogenic agent and lesion pattern, thereby improving the definition and understanding of anatomopathological and histopathological patterns of the different forms of pneumonia encountered. In addition, immuno-histochemistry was used for the immune localisation of *M. ovipneumoniae*. Finally, various diagnostic techniques were compared to assess their sensitivity.

Materials and methods

The study was conducted from December 2002 to April 2003 at the Teramo, Chieti and L'Aquila abbatoirs. Lung samples were collected from lambs aged less than 12 months that showed morphological lesions indicative of non-parasitic infectious diseases. A total of 156 lungs were tested. After anatomo-pathological examination, lung samples were divided into two aliquots. One was sent rapidly to the *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale'* (ISZ A&M) for isolation of *Mycoplasma* spp. and other microbiological assays; the second aliquot was fixed in 10% buffered formalin and was processed for histological and immunohistochemical analysis by the Department of General Veterinary Pathology and Anatomo-Pathology of the University of Teramo. Collected lungs were affected by inflammatory processes, in particular apical, medium or cardiac lobes showed areas of atelectasia, hepatisation (red in the acute phase and grey in more chronic stages), emphysema or oedema.

Isolation

A standard bacteriological test was used for *M. haemolytica* isolation according to the IZS A&M standard operating procedures (SOP) (6). Mycoplasma-selective medium (*Mycoplasma* experience®) was used for mycoplasma isolation. Lung parenchymal samples were collected by sterile removal of a 1 cm^3 tissue portion from the border between the healthy and pathological parenchyma. The material was diluted in 10 ml tryptose broth and mechanically homegenised with a Stomacher®. The liquid fraction obtained from the homegenised sample was harvested, centrifuged for 15 min at 399 g and filtered with 0.45 µm millipore filters. A volume of 300 µl primary broth and/or filtered homegenised material was inoculated in three successive dilutions of 3 ml of *Mycoplasma* experience® broth. An additional quantity of 100 µl of primary broth and/or filtered homogenised material was inoculated directly onto agar in 5 cm Petri dishes. Incubation and culture examination were performed in accordance with the protocols described by Nicholas and Baker (12). Isolated strains were then typed by polymerase chain reaction (5).

Histopathology

Samples fixed in 10% neutral buffered formalin (40-50% aqueous formaldehyde solution) were processed under vacuum and imbedded in paraffin. Sections 5 µm thick were cut and stained with haematoxylin and eosin (H&E). Some specimens were also prepared with Alcian blue-periodic acid Schiff (PAS) stains (pH 2.5) to reveal alveolar

mucoprotein material (11), and Congo red (Bio-Optica 04-210822) and acridine orange (Bertalanffy's 1984 technique modified by Beebe in 1999) (2) to identify the composition of the typical concentric laminated acellular bodies.

Immunohistochemistry

Immunohistochemical analysis was performed on sample sections with histological lesions of *M. ovipneumoniae* infections and on samples that tested positive to *Mycoplasma* spp. Lung sections from other species (dogs and pigs) were used as controls to check the specificity of the hyperimmune serum used in the immunohistochemistry test.

Sections were mounted on polylysine slides and incubated at 37°C to encourage sample adhesion. The streptavidin-biotin-peroxidase immunohistochemistry method was performed using the commercial kit (Strept-HRP 6100, Biospa, Milan), including the following reagents: normal non-immune goat serum (NGS), a bivalent biotinylated secondary antibody (goat anti-mouse and rabbit IgG) and a horseradish peroxidase (HRP) detector system.

Sample sections were rinsed in water and then incubated in a solution of 3% hydrogen peroxide in absolute methanol for 15 min at room temperature to inhibit the endogenous peroxidases.

Antigen sites were unmasked using enzyme treatment with a protease (Pronase) for 10 min at 37°C. To reduce background staining, sample sections were incubated in a moist chamber with 5% skimmed milk powder for 30 min at room temperature (13). To eliminate aspecific positivity, after the removal of excess skimmed milk solution, the slides were incubated with NGS in a moist chamber for 10 min at room temperature. Rabbit anti-*M. ovipneumoniae* hyperimmune serum was then added to the slides at a dilution of 1:500 (in a solution of 3% skimmed milk powder) and left at 4°C overnight. Rabbit anti-*M. ovipneumoniae* hyperimmune serum was supplied by the Central Veterinary Laboratory in Weybridge. The specificity of the hyperimmune serum was confirmed by examining strains of *M. ovipneumoniae*, *M. agalactiae*, *M. putrefaciens*, *M. arginini* and *M. mycoides* clusters, using the growth inhibition method. After elimination of primary antibody, sections were first incubated with goat anti-mouse and rabbit IgG and then with a HRP detector system for 10 min, both in a moist chamber at room temperature. Diaminobenzidine (DAB) (Sigma) dissolved in 100 ml Tris buffer, filtered and added to 1% hydrogen peroxide was used as chromogen. Papanicolau's haematoxylin was used as a counter-stain and the sections were then mounted in DPX (Fluka).

Results

Anatomopathological lesions

The most commonly encountered lesions were interstitial pneumonia and reddish areas of atelectasis; atelectasis was accompanied by pleuritis, sometimes fibrinous, with fine fibrous adhesions, especially in the apical lung lobes. The parenchyma presented oedemas, cyanosis with purple-red areas and occasional haemorrhagic areas. Areas of red-brown or red-pink consolidation were observed, especially in the cranial and ventral lung sections which also contained necrotic areas with haemorrhagic extremities. In some cases, the lung tissue was characterised by necrotic foci and widespread whitish nodular masses.

Isolation

Microbiological isolation revealed the presence of *Mycoplasma* spp. in 9.6% of samples (15 cases). Of these, 5 were also positive for *M. haemolytica*. Most of the remaining samples presented aspecific microbial flora or were sterile. Samples subjected to typing (15 cases) were positive for *M. ovipneumoniae.*

Histopathology

The most commonly observed lesion (149 cases or 95.5%) was atelectasis, mainly detected in subpleural areas. In 50 cases (32%) catarrhal bronchitis, alveolar epithelisation, congestion and a marked increase in macrophages, neutrophils, plasma cells and eosinophils in the alveolar and bronchial areas were

observed. A considerable increase in bronchus-associated lymphatic tissue (BALT) was seen in 26 samples (16.6%), which sometimes coincided with the presence of lymphoid, peribronchial and perivascular nodules, found in 10 cases (6.4%). A total of 23 samples (14.7%) (Fig. 1) showed these lesions in association with a desquamative alveolitis (Fig. 2), alveolar epithelisation and the presence of giant cells (Fig. 3). These lesions led to the suspected diagnosis of atypical pneumonia in lambs caused by *M. ovipneumoniae*. In 9 cases (5.7%), fibrinous pneumonia, oedema, boutonneuse hyperplasia of the bronchial smooth muscle and, in some cases, presence of alveolar fibrin, led to a diagnosis of acute enzootic pneumonia caused by *M. haemolytica* supported by the isolation of this pathogen from the samples examined (Fig. 1). In 5 cases (3.2%), lesions attributed to parainfluenza virus type 3 were observed; these were characterised by interstitial pneumonia and necrotising bronchiolitis, associated with a considerable increase in the number of macrophages, presence of alveolar syncytial giant cells, mitotic figures in the respiratory epithelial cells and widespread necrotic areas (Fig. 1).

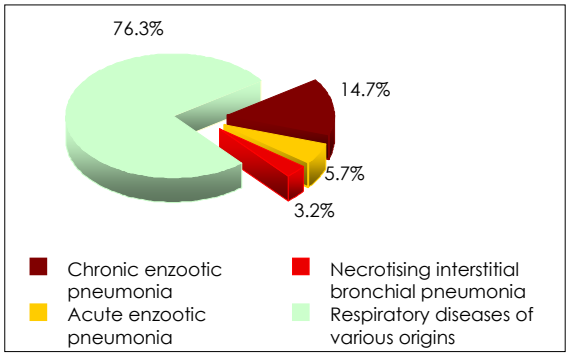

Figure 1
Respiratory diseases detected in 156 lambs in the Abruzzo region of Italy

Some samples clearly showed the presence of verminous pneumonia, with increased numbers of eosinophils, haemorrhages, hyperaemia, nodular concretions of calcified material and, in some cases, parasitic granulomas and strongyle larvae in the bronchi (19 cases or 12%). In 2 cases (1.3%), the presence of *Corynebacterium pseudotuberculosis* was suspected as lesions typical of this organism were observed, namely: inflammatory reaction characterised by epithelioid cells, lymphocytes and plasma cells

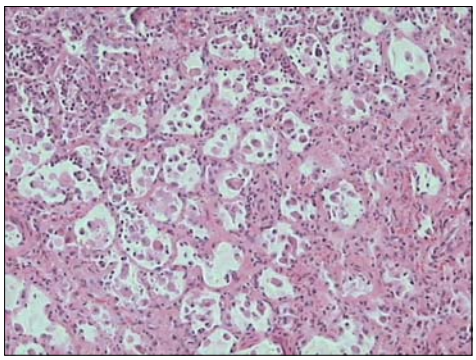

Figure 2
Lamb lung: desquamative alveolitis (haematoxylin and eosin ×40)

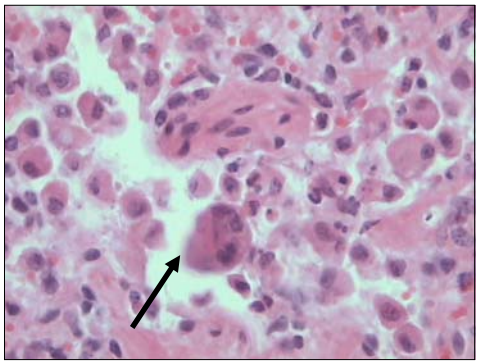

Figure 3
Lamb lung: desquamative alveolitis, macrophages and giant cells (haematoxylin and eosin ×40)

around a central area of purulent necrotic detritus, congestion, atelectasis, catarrhal bronchitis, connective proliferation and BALT hyperplasia. In one sample (0.65%), alveolar mucoprotein accumulation (enhanced with Alcian blue-PAS stain) (Fig. 4), purulent catarrhal bronchitis, lymphoplasma cell infiltrate, neutrophils, foamy macrophages, alveolar oedema, alveolar epithelisation and congestion were observed. A small percentage of samples (1.9% or 3 cases) demonstrated lamellar concretions of protein material, known as Corpora amylacea (Fig. 5), revealed by staining with Congo red and acridine orange (Fig. 6). This indicated that they contained deoxyribonucleoproteins, glyco-proteins and ribonucleoproteins. These proteins are indirectly associated with atypical

pneumonia as they are formed following bronchiolar stenosis and stagnation of exudate, as observed in other studies (9).

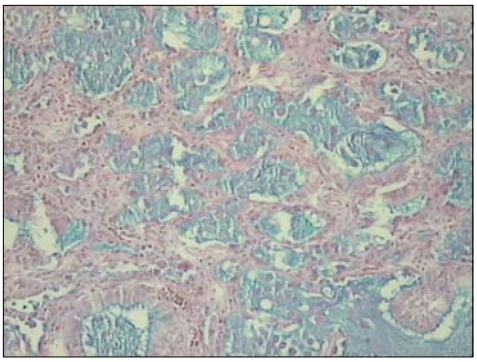

Figure 4
Lamb lung: alveolar mucoprotein accumulation
(Alcian blue-periodic acid Schiff, ×10)

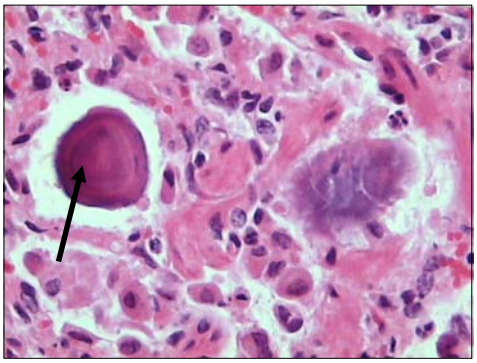

Figure 5
Lamb lung: Corpora amylacea, thickening of alveolar septa
(haematoxylin and eosin ×40)

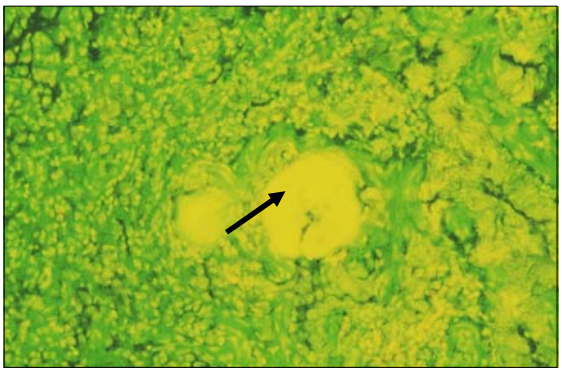

Figure 6
Lamb lung: Corpora amylacea stained with acridine orange
(polarised light ×20)

Immunohistochemistry

Lung sections positive upon microbiological isolation of *Mycoplasma* spp. (15 samples, of which 13 also exhibited histological lesions) and 16 lung section samples that were negative on isolation but contained histological lesions attributable to mycoplasma were used for immunohistochemical tests. A total of 31 lung samples were analysed.

Samples positive on isolation

An immunohistochemical marker was found in 12 cases (7.6%), localised mainly in the alveolar macrophages (Figs 7 and 8), giant cells and, in one case, the bronchiolar epithelium (Fig. 9). In the macrophages, the reaction was expressed as granular brown material, while in the epithelium and giant cells it was more uniform.

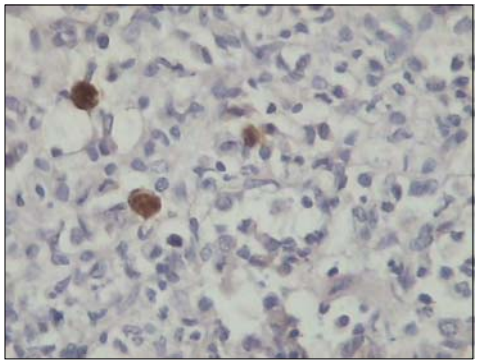

Figure 7
Lamb lung: alveolar macrophages immunohistochemical positivity for *Mycoplasma ovipneumoniae*
(×40)

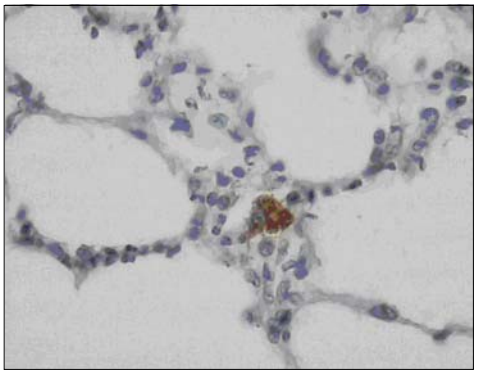

Figure 8
Lamb lung: alveolar macrophages: immunohistochemical positivity for *Mycoplasma ovipneumoniae*
(×40)

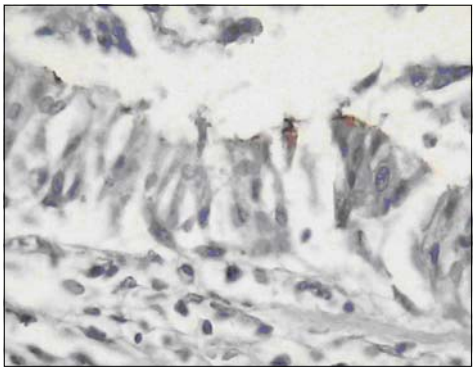

Figure 9
Lamb lung: bronchiolar epithelium: immunohistochemical positivity for *Mycoplasma ovipneumoniae* (×40)

Samples with histological lesions but negative on isolation

A total of 11 samples (7.1%) were positive on immunohistochemical testing. Positive staining was mainly observed in alveolar macrophages. In one case, a positive reaction was demonstrated in desquamating bronchiolar epithelial cells. In another, neutrophils cells showed positive staining in areas with an accumulation of granulocytes, while another gave a positive response to immuhistochemistry staining only in macrophages in the areas of atelectasis. Five cases (3.2%) expressed the marker very strongly and contained numerous positive cells. Lung samples from pigs with enzootic pneumonia that were used as positive controls showed positive antigen response, demonstrating the cross-reactivity of *M. ovipneumoniae* with *M. hyopneumoniae* (16). As expected, lung tissue samples from dogs with canine distemper showed no immunohistochemical marker. In conclusion, 23 samples (74.2%) of the 31 cases tested gave a positve response to immunohistochemical tests; 12 of these (38.7%) also showed histological lesions characteristic of *M. ovipneumoniae* and were also positive upon microbiological isolation of *Mycoplasma* spp. The 11 remaining samples that gave a positive response to immunohistochemistry (35.5%) gave negative results upon microbiological isolation of *Mycoplasma* spp. but exhibited histological lesions (Fig. 10). One immuno-histochemically positive case revealed the simultaneous presence of *M. ovipneumoniae* and *M. haemolytica* when tested both histologically and microbiologically, as described by Brogden *et al.* (3).

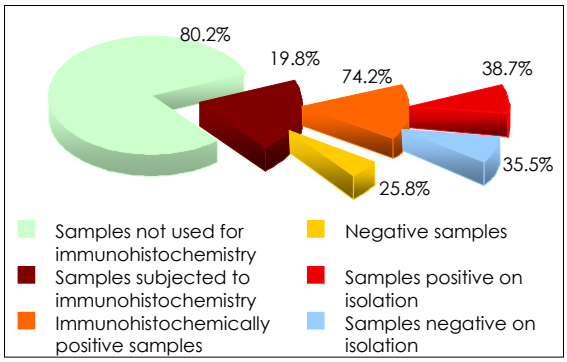

Figure 10
Mycoplasma ovipneumoniae immunohistochemistry results

Conclusions

Our analysis was based on the detection of atypical pneumonia, diagnosed on the basis of anatomopathological and histopathological lesions, microbiological isolation of *M. ovipneumoniae* and especially the expression of immunohistochemical markers. Results showed that the number of samples with microscopic lesions attributable to *M. ovipneumoniae* was greater than the number of samples found positive upon isolation of the micrioorganism. In contrast, nine samples found positive on isolation did not contain any lesions characteristic of this pathogen. However, *M. ovipneumoniae* can be also isolated from lungs in which no pathological changes are observed (4). Of 15 cases in which *Mycoplasma* spp. was isolated, only 7 showed atelectasis, increased BALT, desquamative alveolitis, nodular lymphoid hyperplasia around bronchioles and vessels and a mild macrophage exudate. Moreover, of all the samples tested, 16 contained such lesions without revealing positive results upon microbiological isolation of *Mycoplasma* spp. Among the samples that gave a positive immunohistochemical result, some presented intense marker expression while others had only a few positive cells. This latter may explain the negative results on isolation given the hypothesis of an insufficient bacterial load.

Immunohistochemical testing revealed some important information on the target cells involved in the pathogenesis of *M. ovipneumoniae* infection. Cells that showed positive antigen response follow the pathogenetic course of infection, namely: epithelial cells in the bronchial mucosa, desquamated cells in the bronchial lumen, alveolar cells and inflammatory cells, principally macrophages. Finally, it can be asserted that anatomopathological and histopathological lesions are particularly useful for the diagnosis of atypical pneumonia caused by *M. ovipneumoniae*. Such lesions lead to a suspected diagnosis which needs to be confirmed by microbiological isolation and immunohistochemical testing. In conclusion, this study revealed the not infrequent presence of chronic enzootic pneumonia in the Abruzzo region and demonstrated that the immunohistochemical technique is highly sensitive and specific for the detection of *M. ovipneumoniae* in lung tissues.

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