Diagnosing respiratory syncytial virus using immunofluorescence and immunohistochemistry methods in caprine lungs with bronchopneumonia

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SUMMARY
The aim of this study was to evaluate the diagnostic interest of direct immunofluorescence and immunohistochemistry for RSV infection in goats with bronchopneumonia from the Elazig region. For this purpose, lungs of 889 slaughtered male hair goats were macroscopically analysed in order to looking for chronic bronchopneumonia lesions. Among the 146 cases of bronchopneumonia found in which mild lesions (according to the extent of consolidation) were prominent (65.8%), there were 105 interstitial bronchopneumonia, 41 catarrhal-purulent bronchopneumonia and 2 verminous bronchopneumonia according to histological criteria. The RSV antigens were detected in 7 cases with interstitial chronic bronchopneumonia using direct immunofluorescence on frozen sections whereas direct immunohistochemistry on paraffine sections failed to detect the viral particles. These results show that immunofluorescence on frozen section was undoubtfully more adequat than immunohistochemistry for RSV detection in situ and they suggest a possible role of RSV in the induction of interstitial bronchopneumonia in goats.

Keywords: Respiratory syncytial virus, goat, lung, chronic bronchopneumonia, pathology, diagnosis, immunofluorescence, immunohistochemistry.

RESUME
Diagnostic du VRS chez la chèvre atteinte de bronchopneumonie.

L’objectif de cette étude a été d’évaluer l’intérêt diagnostique d’une détection directe du virus respiratoire syncitial (VRS) par immunofluorescence et par immunohistochimie chez les chèvres atteintes de bronchopneumonie provenant de la région d’Elazig. Pour cela, les poumons de 889 boucs abattus ont été examinés macroscopiquement. Parmi les 146 cas de bronchopneumonie ainsi identifiés, les lésions les plus intenses (en fonction de l’étendue de la consolidation), 105 présentaient à l’histologie des lésions de bronchopneumonie interstitielle, 41 des lésions de bronchopneumonie catarrhale et purulente et 2 des lésions de bronchopneumonie vermineuse. Les antigènes du VRS ont été mis en évidence dans 7 cas de bronchopneumonie interstitielle par immunofluorescence directe sur coupes congelées alors que les particules virales n’ont été détecté dans aucun cas par immunohistochimie sur coupes incluses en paraffine. Ces résultats montrent que l’immunofluorescence sur coupes congelées est bien plus adéquate que la technique d’immunohistochimie pour détecter in situ le VRS et suggèrent un possible rôle de ce virus dans l’induction d’une bronchopneumonie interstitielle chez la chèvre.

Mots-clés : Virus respiratoire syncitial, chèvre, poumon, bronchopneumonie chronique, anatomopathologie, diagnostic, immunofluorescence, immunohistochimie.

Introduction
Respiratory Syncytial Virus (RSV) which is from the Pneumovirus genus of the Paramyxoviridae family, leads to significant economic losses by causing respiratory system infections in cattle and sheep [2]. RSVs in ruminants have been defined as bovine RSV, ovine RSV and caprine RSV [8]. An antigenic and structural connection has been reported between all RSV, but an especially close connection between bovine RSV and caprine RSV [1, 5, 17, 22]. Previous studies have indicated that all RSV could cause inter-species infections [4, 7, 14, 25].

The virus could also be clinically isolated from goats that did not exhibit respiratory system infection [19]. RSV infection in goats has been evidenced in many areas of the world throughout serological and pathological studies [15, 24, 27]. Contamination occurs through droplet infection, and contaminated animal food and water [23]. Most of the severe epidemics occur in autumn and winter [26]. Clinical symptoms such as fever, sluggishness, nasal discharge, constant and deep breathing, respiratory distress, cough, anorexia are observed in affected animals [6]. Moderate clinical symptoms have been emphasized to occur in experimental RSV infections, whereas intense clinical signs were encountered during mixed infections with bacterial agents such as Mannheimia haemolytica [3]. Necropsy findings are similar in all ruminant RSV infections. Macroscopically, a mucopurulent exudate in the lumen of the bronchus and bronchioles are observed together
RSV Diagnosis in Goats with Bronchopneumonia

with irregular lobular or diffuse mildly collapsed gray-red foci in varying sizes in the cranioventral lobes of the lung. Emphysema may be formed in different regions of the lungs. Histopathologically, bronchitis, bronchiolitis, thickening in the alveolar septum with mononuclear cell infiltrations, lymphoid hyperplasia, hyperplasia in the epithelial cells of the bronchi and bronchioles, acidophilic inclusion bodies in the epithelium of the bronchi and bronchioles and syncytial cells have been reported [11, 13, 19, 24].

Direct and indirect laboratory methods are beneficial in the diagnosis of RSV. Viral antigens may be directly detected using methods like indirect immunohistochemistry, direct immunofluorescence and ELISA [26]. Serological methods such as complement fixation, neutralization, ELISA, indirect immunofluorescence, and indirect haemagglutination tests are used for indirect diagnosis [23].

In this study, it was aimed to determine the prevalence of RSV antigens in goats in the Elazığ province, Turkey using direct immunofluorescence and immunohistochemistry methods in frozen and paraffin sections obtained from caprine lungs with bronchopneumonia.

Material and Method

SAMPLE COLLECTIONS

In the study, the lungs of 889 male hair goats (> 1 year old), that had been slaughtered in a slaughterhouse in Elazığ between January and June 2011 were investigated. Macroscopic bronchopneumonia lesions in the cranial and cardiac lobes were observed in 146 lungs (16.42%). The severity of bronchopneumonia in cranial and cardiac lobes was scored according to the extent of consolidation. In cranial and cardiac lobes, lesions in which 10% or less of lung volume was affected were evaluated as 'mild', those with 10%-20% consolidation were evaluated as 'moderate', and lesions with more than 20% of lung volume affected were evaluated as 'severe'. Some parts of the injured pulmonary tissues were fixed in 10% buffered formalin solution and others were stored at -80°C for frozen sections.

After fixation in 10% buffered formalin, paraffin blocks were prepared throughout routine procedures. Sections 5 µm in thickness were obtained and stained with Haematoxylin-Eosin (HE) and examined under light microscopy. Additionally, sections were stained with Shorr's staining solution according to Page-Green method for the detection viral inclusion bodies [20].

IMMUNOHISTOCHEMISTRY

Immunohistochemical staining was performed with the avidin-biotin-peroxidase complex procedure [13], using commercially available immunoperoxidase kits (Ultravision Detection System, Antipolyvalent, HRP/DAB, Thermo Scientific, Cat No:TP-015-HD). For immunohistochemical staining, deparaffinised tissue sections were placed in citrate buffer solution (10 mM citric acid, pH 6.0) and were kept in a microwave for 20 minutes for antigen retrieval stage. The sections were incubated in 70% methanol with 3% H₂O₂ for 10 minutes to inhibit endogenous peroxidase activity, and were then washed three times in phosphate-buffered saline (PBS, pH 7.4). The sections were treated with blocking solution for 10 minutes. After draining the blocking serum, the sections were incubated with primary antibody [monoclonal mouse anti-RSV (cat no: bio 031, bixo jemelle, Belgium)], diluting to 1:20, 1:50, 1:100 and 1:200 in PBS at 4°C overnight in a humidified chamber. After washing in PBS 3 times, sections were incubated with biotinylated anti-goat polyvalent secondary antibody for 10 minutes. Then the sections were washed three times in PBS and treated with the peroxidase-conjugated streptavidin. After another PBS washing step, the sections were incubated with 3,3’-diaminobenzidine (DAB). After colour change, sections were washed with tap water and then counterstained with Mayer’s haematoxylin. Lung section from a healthy goat was used as negative control. Furthermore, non-immune mouse serum was used instead of primary serum in lung sections with pneumonia.

DIRECT IMMUNOFLUORESCENCE

Using a frozen microtome, sections of 6 mm in thickness were obtained. They were fixed with in 90% acetone solution and washed with PBS (phosphate Buffer Saline, pH 7.4) and the surfaces of the samples were coated with monoclonal mouse anti-RSV fluorescein isothiocyanate (FITC) conjugate (biox jemelle, Belgium, cat no: bio 032), diluting 20 times with PBS-Evans Blue. After incubation for 1 hour at room temperature (25°C) and washing in PBS, sections were examined under a fluorescent microscope with 9% glycerol solution. The negative controls prepared for immunohistochemistry were also used for the immunofluorescence test.

Results

The severity, type and rates of lesions in bronchopneumonia and their distribution according to months in which samples have been collected were summarized in Table I. Although the severity and distribution varied, all bronchopneumonia lesions were seen to be macroscopically characterized by patchy or diffuse, purple-red or gray, focal or irregular lobular atelectatic foci. Among the 146 cases, 65.75% of them exhibited mild lesions, 24.66% moderate lesions and 9.59% severe lesions according to the extent of consolidation. Subpleural emphysema was observed together with thickening in the interlobular areas in bronchopneumonia with severe consolidation. Interstitial bronchopneumonia was the prominent type of pneumonia found and occurred in 71.92% cases of bronchopneumonia whereas verminous and catarrhal/purulent types remained minor (1.37% and 26.71%, respectively). The presence of RSV antigens was investigated using immunofluorescence and immunohistochemical methods in the caprine lungs with interstitial bronchopneumonia (n = 105).
Using the direct immunofluorescence (DIF) test, RSV antigens were detected in 7 out of 105 (6.67%) sampled lungs with interstitial bronchopneumonia. Fluorescence was observed in the bronchus and the alveolar epithelium (figure 1); there was spilling of the cellular content into the lumen, and inflammatory cells invaded the interstitial area (figure 2). In the afore-mentioned cells, the fluorescence reactions were seen to be limited to the cytoplasm, were of varying sizes, and granular in appearance. In addition to positive staining, autofluorescence reactions were present, particularly in the capillary vessels. Cytoplasmic granular fluorescence reactions were not observed in negative control sections. Using immunohistochemistry, RSV antigens could not be determined in any of the 105 cases with interstitial bronchopneumonia.

Only DIF positive interstitial bronchopneumonia cases were examined microscopically in order to evaluate the RSV-related changes in the lung sections. Varying degrees of inter-alveolar septal thickening and fibrosis were the most prominent microscopic changes. The vast majority of the alveolar septa were seen to have thickened irregularly due to an increase in connective tissue together with lymphocyte and macrophage (at a lesser extent) infiltration. Moreover, the other prominent lesions included peribronchial and peribronchiolar mononuclear cell infiltrations together with hyperplasia in the bronchus and the bronchiolar epithelium and lymphoid hyperplasia in the bronchus, bronchioles and areas adjacent to some alveolar septa. The lumens of some bronchioles and alveoli were found to have been filled with neutrophil-rich cellular exudate, and a small numbers of lymphocytes and macrophages were found to have contributed to the inflammation, in addition to neutrophils in some areas. Alveoli adjacent to the bronchioles were seen to have been destroyed and subjected to atelectasis. No viral inclusion bodies could be encountered using the Page-Green method.

**Discussion**

Pneumonia that are microscopically characterized with bronchitis and bronchiolitis, hyperplasia in the bronchus and bronchiolar epithelium, thickening in the alveolar septum, hyperplasia in the epithelial cells of the bronchi and bronchioles, lymphoid hyperplasia, syncytial cell formations and cytoplasmic inclusion bodies, are reported to be formed in RSV infections [6, 13, 24]. Although the microscopic findings in the presented study are similar to those in previously described RSV pneumonias, no virus-related syncytial cells or inclusion bodies could be encountered in the present study. Occurrence of specific viral lesions like inclusion body and syncytial cell formations in the lungs in natural and experimental RSV infections of ruminants would be inconstant according to many factors such as

<table>
<thead>
<tr>
<th>Number of examined lungs</th>
<th>January</th>
<th>February</th>
<th>March</th>
<th>April</th>
<th>May</th>
<th>June</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchopneumonia severity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>9</td>
<td>13</td>
<td>10</td>
<td>41</td>
<td>16</td>
<td>7</td>
<td>96 (65.8%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>5</td>
<td>11</td>
<td>4</td>
<td>11</td>
<td>4</td>
<td>1</td>
<td>36 (24.7%)</td>
</tr>
<tr>
<td>Severe</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>14 (9.6%)</td>
</tr>
<tr>
<td>Bronchopneumonia type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verminous</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (1.4%)</td>
</tr>
<tr>
<td>Catarhal and purulent</td>
<td>6</td>
<td>4</td>
<td>8</td>
<td>11</td>
<td>7</td>
<td>3</td>
<td>39 (26.7%)</td>
</tr>
<tr>
<td>Interstitial</td>
<td>10</td>
<td>21</td>
<td>8</td>
<td>45</td>
<td>15</td>
<td>6</td>
<td>105 (71.9%)</td>
</tr>
</tbody>
</table>

Table I: Severity, type and distribution of bronchopneumonia lesions in goats (889) according to the month of sample collection.
the agent virulence, the virus amount, the duration of the infection period, age and species of the animal [9, 11, 19]. Furthermore, these lesions have been reported to disappear when secondary bacterial infections were developing [6].

Diagnosis of RSV infections is based on macroscopic findings, histopathological findings and detection of viral antigens using immunohistochemical methods [6, 11]. Furthermore, it has been reported that PCR, cell culturing and serological methods could also be beneficial [10, 12, 16, 21]. Previous investigations aiming to determine the incidence of RSV infection have been usually based on serological methods, and RSV seropositivity has been reported to reach 73% in goat populations worldwide [15, 18, 27]. However, only few studies have used immunohistochemistry for evidencing RSV antigens [9, 24]. In the present study, the RSV antigen could not be detected by immunohistochemistry in any of the 105 cases with interstitial bronchopneumonia and only 7 (6.67%) cases were determined positive by the direct immunofluorescence test. In previous studies, it was reported that positive cases could be evaluated as negative because of the inactivation of immunogenic viral epitopes in tissues fixed with formalin [6, 9, 13]. In this study, it was considered that the inability to detect RSV positivity in caprine lungs by immunohistochemistry may result from fixation of samples with formalin. According to the results of the present study, when difficulties in detection of specific histological lesions in natural RSV pneumonias of goats were taken into consideration, the DIF method was found to be a more sensitive method compared to immunohistochemistry for a definite diagnosis, despite difficulties in detailed examinations of cellular structures in frozen sections. Thus, it was concluded that the DIF method could be used as a more appropriate method for a direct diagnosis of RSV infections in goats.

As a conclusion, RSV antigens were found in the present study at a rate of 6.67% using the DIF method in interstitial bronchopneumonia cases in goats in the Elazığ region and the role of this virus in the development of interstitial bronchopneumonia in goats would be out of importance.

References