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Original

Examination of adenoviruses with molecular and pathological methods in sheep pneumonia cases

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ABSTRACT

Objective. Reveal adenoviruses (AdV) that cause pneumonia in sheep and examine pathologic changes in the pulmonary and mediastinal lymph nodes of naturally infected adenovirus-positive specimens. **Material and methods.** For this purpose, 1459 lungs of sheep slaughtered in a slaughterhouse were macroscopically examined, and pneumonia lesions were detected in 88 (6.03%) of these. The paraffinized tissue sections of these specimens with pneumonia were examined with the immunohistochemical (IHC) and indirect immunofluorescence (IF) methods, whereas their tissue homogenates were examined using the Antigen ELISA and PCR methods for adenovirus positivity. **Results.** Accordingly, the prevalence of adenoviruses was determined as 19.3% for IHC, 22.7% for IF, 20.5% for ELISA and 13.6% for PCR. Hematoxylin-eosin (HE) staining was performed to examine histopathological changes in the specimens that were naturally infected with adenoviruses. The histopathological examinations of the naturally infected lung specimens revealed mainly interstitial pneumonia, as well as catarrhal and verminous pneumonia findings. Consequently, it was determined that the most effective methods in the detection of adenoviruses in sheep pneumonias were found respectively as IF, ELISA, IHC and PCR. The finding that adenoviruses were observed only in the mediastinal lymph nodes of some specimens in the immunopathological methods suggested that the latency. **Conclusions.** The presence of adenoviruses in sheep pneumonia cases was determined with the indirect immunofluorescence, antigen ELISA and PCR methods for the first time. The possibility of the latent nature of adenovirus infection in these species was also discussed for the first time.

Keywords: Adenovirus; ELISA; histopathology; immunopathology; PCR; sheep (*Source*: *MeSH, DeCS*).

RESUMEN

Objetivo. Revelar adenovirus (AdV) que causan pneumonía en ovejas y examinar cambios patológicos en los ganglios linfáticos pulmonares y mediastínicos de muestras positivas para adenovirus infectadas de forma natural. **Material y métodos.** Para este propósito, se examinaron macroscópicamente

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1459 pulmones de ovejas sacrificadas en un matadero, y se detectaron lesiones de pneumonía en 88 (6.03%) de estas. Las secciones de tejido parafinadas de estos especímenes con pneumonía se examinaron con los métodos inmunohistoquímicos (IHC) e inmunofluorescencia indirecta (IF), mientras que tejidos homogeneizados se examinaron usando los métodos ELISA de antígeno y PCR para determinar la positividad de adenovirus. **Resultados.** Por consiguiente, la prevalencia de adenovirus se determinó como 19.3% para IHC, 22.7% para IF, 20.5% para ELISA y 13.6% para PCR. La tinción con hematoxilina-eosina (HE) se realizó para examinar los cambios histopatológicos en las muestras que estaban infectadas naturalmente con adenovirus. Los exámenes histopatológicos de las muestras de pulmón infectadas de forma natural revelaron mayormente pneumonía intersticial, junto con hallazgos de pneumonía catarral y verminosa. En consecuencia, se determinó que los métodos más eficaces en la detección de adenovirus en pneumonías ovinas fueron encontrado respectivamente como IF, ELISA, IHC y PCR. El hallazgo de que los adenovirus solo se vio en los ganglios linfáticos mediastínicos de algunas muestras en los métodos inmunopatológicos sugirió latencia. **Conclusiones.** La presencia de adenovirus en casos de pneumonía ovina se determinó por primera vez con los métodos de inmunofluorescencia indirecta, ELISA de antígenos y PCR. La posibilidad de la naturaleza latente de la infección por adenovirus en estas especies también se discutió por primera vez.

Palabra clave: Adenovirus; ELISA; histopatología; inmunopatología; PCR; ovino (*Fuentes*: *MeSH, DeCS*).

INTRODUCTION

Sheep pneumonias are among the most frequently encountered health problems of this species worldwide, and they result in respiratory distress, loss of yield and sometimes death. In general, infectious agents such as bacteria, viruses and parasites play a role in the emergence of pneumonias (1,2). Viral causes that have a significant place in the etiology of sheep pneumonias lead to damages in the epithelia of the respiratory system and the mucociliary system. Additionally, as they also suppress immunity, they may not only primarily create respiratory system infections but also cause the occurrence of secondary infections (3,4). Adenoviruses, which are among the viral pneumonia causes of sheep, are found abundantly in the world. Adenoviruses that 70-90 nm in diameter, icosahedral capsid structure, non-enveloped linear double-stranded DNA-containing are in the *Adenoviridea* family and are examined in 6 genera. These are *Mastadenovirus, Aviadenovirus, Siadenovirus, Atadenovirus Testadenovirus* and *Ichtadenovirus*. Among these 6 genera, important groups for ruminants are Mastadenovirus and Atadenoviruses. Bovine adenovirus A,B,C and ovine adenovirus A,B,C species are classified in the Mastadenovirus genus, while Bovine adenovirus D,E and ovine adenovirus D are classified in the Atadenovirus genus. (5). Adenoviruses usually create subclinical infections. However, in cases where the clinical symptoms of the disease are observed, there are respiratory distress, coughing and nasal discharge accompanied by deconditioning. Although young lambs are more susceptible to the disease, mortality can also be seen in adults in cases of secondary infections (6,7,8). The virus that is transmitted through direct contact, inhalation, fecal-oral, nasal and tear discharge binds to the coxsackievirus and -adenovirus (CAR) receptors of respiratory epithelial cells and shows a lytic effect on these cells (9,10). In adenovirus infections, the lungs have a pale color, as well as a swollen and elastic consistency. Microscopic examination shows degeneration in the bronchi/bronchioles, proliferation of type II pneumocytes, mononuclear cell infiltration and interstitial pneumonia symptoms such as lymphoid and fibromuscular hyperplasia. Moreover, these viral agents form intranuclear basophilic inclusion bodies in alveolar and bronchial epithelia (6,8,11). The diagnosis of adenovirus infections is made either directly by showing the presence of the antigen or indirectly by detecting the antibodies that are formed against the antigen. Cell culture, IF, IFAT, DFAT, IP, EM, PCR and ELISA tests are utilized for the direct diagnosis of the cause. Serological tests such as SN, AGID, GF and ELISA are used for indirect diagnosis (12,13,14,15).

The vast majority of studies that have been conducted on adenovirus infections that prevalently cause pneumonia in sheep around the world are seroprevalence studies, and

studies what antigen prevalence has been determined using various methods together are almost nonexistent. In this study, adenoviruses causing pneumonia in sheep were identified using molecular and pathological methods, and a comparison was made among these methods. Furthermore, histopathological changes that occurred in adenovirus-positive naturally infected specimens were examined.

MATERIAL AND METHOD

Animal Material. This study was conducted with the approval of the Local Ethics Committee of the Faculty Veterinary Medicine at Atatürk University with the decision numbered 2018/71. Within the scope of the study, 1459 sheep lungs and the mediastinal lymph of these lungs were macroscopically examined, and specimens were collected from 88 piece lungs showing symptoms of pneumonia and lymph nodes of these lungs. While some of these tissue specimens were fixated in a 10% buffered formalin solution for histopathological (HE) and immunopathological (IHC, IF) examinations, some were stored at -20°C for PCR and ELISA analyses.

Histopathological Examination. Routine tissue processing was carried out by subjecting the tissues fixated in the 10% buffered formalin solution to dehydration, clearance and paraffinization steps. After the tissue processing step, 5-µm-thick sections were obtained from all specimens that were embedded in paraffin blocks using a rotary microtome (Leica RM 2235) and taken onto normal and adhesive-coated slides. For the histopathological examinations of the sections taken onto the normal slides, HE staining was performed. The HE-stained preparates were examined under a light microscope (Leica DM 2500).

Immunohistochemical Staining. The tissue sections taken onto the adhesive-coated slides were subjected to deparaffinization and dehydration. The tissues were kept in 3% H_2O_2 for endogenous peroxidase inactivation. To reveal antigen presence, the tissues were boiled in a microwave oven with an antigen retrieval solution. A protein block solution was dripped on the tissues to prevent non-specific antigen binding. After this, the tissues were incubated in the primary antibody (Monoclonal mouse anti-adenovirus type 3 (BAV3) Cat No: BIO 292, BIOX Jemelle, Belgium) diluted with water at a ratio of 1/50 at +4°C for 16 hours. The protein block solution was dripped on the tissues to prevent non-specific antigen binding. The primary antibody diluted with water at a ratio of 1/20 was dripped on the tissues, and the tissues were incubated at 37°C for 1 hour. The incubation of the tissues was facilitated firstly by using the biotinylated secondary antibody, and then, streptavidin-peroxidase conjugate. A 3-3' Diaminobenzidine (DAB) chromogen was dripped on the sections to show the reactions in the tissues, and following counterstaining with Mayer's hematoxylin, the preparates were examined under a light microscope (Leica DM 2500).

Immunofluorescence Staining. As in the immunohistochemical staining process, 1/20 diluted immunofluorescent antibody (Cat No: Goat Anti-Rabbit IgG H&L (FITC)) was dripped on the tissue sections that were subjected to the same procedures including primary antibody incubation, and the sections were left for 45 min. After this, a glycerol/distilled water (1/9) solution was dripped on the sections, the sections were covered with a cover-slide, and they were examined under a microscope with a fluorescent adapter (Zeiss Axio Scope A1).

Antigen ELISA. Homogenization was applied on the tissues that were kept at -20°C, and the Bovine Adenovirus Antigen (ADV-Ag) ELISA Kit (MyBioSource Catalog# MBS2608731, Lot#32305765) protocol was carried out to detect adenovirus presence in these specimens. In our study, the presence of adenovirus was examined by both molecular and pathological methods. Antigen ELISA test was chosen in the study. In this elisa model, the plate base is coated with antibodies. Since the base of the plate is intended to detect Mastadenovirus and Atadenovirus, the test can recognize ovine adenoviruses. Finally, using a microplate reader with a 450-nm filter, the optical densities of the tissue specimens were read by spectrophotometry (Thermo Scientific, Multiskan GO, ABD).

PCR. First, to detect DNA in the homogenized tissues, DNA extraction was performed using the PureLink Genomic DNA mini kit (Catalog no: 2024278, Invitrogen, ABD) protocol. To detect adenovirus DNA in the specimens whose nucleic acids were isolated, the primer pairs used by

Sibley et al. (16) were taken as the reference. The primers in the selected publication (16) are nested PCR-based primers that can recognize both Mastadenovirus and Atadenoviruses. The primers used in the study were checked in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and are capable of detecting all of these species (Mast- and Atadenoviruses). Based on the reports of the same author, the thermocycling of the PCR reaction was carried out. A DNA stain was added to the PCR products obtained with this reaction, the products were subjected to electrophoresis in 0.7% agarose gel, and the bands belonging to the positive specimens were imaged using a UV transilluminator.

RESULTS

Pneumonia lesions were detected in 88 of the 1459 sheep lungs that were macroscopically examined. The rate of adenovirus positivity was found as 19.3% with IHC, 22.7% with IF, 20.4% with ELISA and 13.6% with PCR in pneumonia samples. In our study, the IF method was identified as the most effective method in the diagnosis of sheep adenoviruses, and according to this method, 20 specimens were naturally infected with adenoviruses. The microscopic examinations of the adenovirus-positive specimens revealed that these specimens had interstitial pneumonia (n=12), catarrhal bronchopneumonia (n=6) and verminous pneumonia (n=2).

Macroscopic Results. According to the macroscopic data recorded while collecting the specimens, it was determined that the lungs that were naturally infected with adenoviruses were generally voluminous-edematous, had an elastic consistency, a pale color, were not collapsed, had scarring and occasional atelectatic zones on the dorsal surface (Figure 1A). In addition to these, some specimens had a hard consistency, dark red consolidated zones or parasitic cysts. Mediastinal lymph nodes did not show any significant pathologic finding in the macroscopic examination.

Histopathologic Results. In the cases with interstitial pneumonia with adenovirus infection, it was observed that the interalveolar septa were thickened due to mononuclear cell infiltration and the proliferation of type II pneumocytes, and fibromuscular hyperplasia around the alveolar ducts and bronchioles, as well as bronchial/bronchiolar and perivascular lymphoid hyperplasia, were observed (Figure 1B, C). In the positive specimens with catarrhal bronchopneumonia, the bronchiolar lumina were observed to be filled with an exudate containing leukocytes that were mostly polymorphonuclear (Figure 1D). Parasitic granulomas were detected in the positive specimens with verminous pneumonia. Mild lymphadenitis was detected in some mediastinal lymph nodes (Figure 1E).

Immunohistochemical Results. Adenovirus positivity was detected in 19.3% (n=17) of the 88 specimens with pneumonia that were examined with the immunohistochemical method. Adenoviruses were observed in the bronchial/bronchiolar and alveolar epithelia, macrophages, peri-bronchial/peri-bronchiolar lymphoid tissue and inflammatory cells in the mediastinal lymph nodes. In the specimens with pulmonary positivity, antigen presence was also determined in their mediastinal lymph nodes (Figure 1F, G). On the other hand, while 3 lung specimens did not show antigen presence, positivity was found in their lymph nodes.

Immunofluorescence Results. Adenovirus positivity was detected in 22.7% (n=20) of the 88 specimens with pneumonia that were examined with the immunofluorescence staining method. Adenoviruses were observed in the bronchial/bronchiolar and alveolar epithelia, macrophages, peri-bronchial/peri-bronchiolar lymphoid tissue and inflammatory cells in the mediastinal lymph nodes (Figure 1H, I). On the other hand, while a total of 6 specimens including the ones in the immunohistochemical method and the ones in this method did not show antigen presence in the lungs, immunopositivity was found in their lymph nodes.

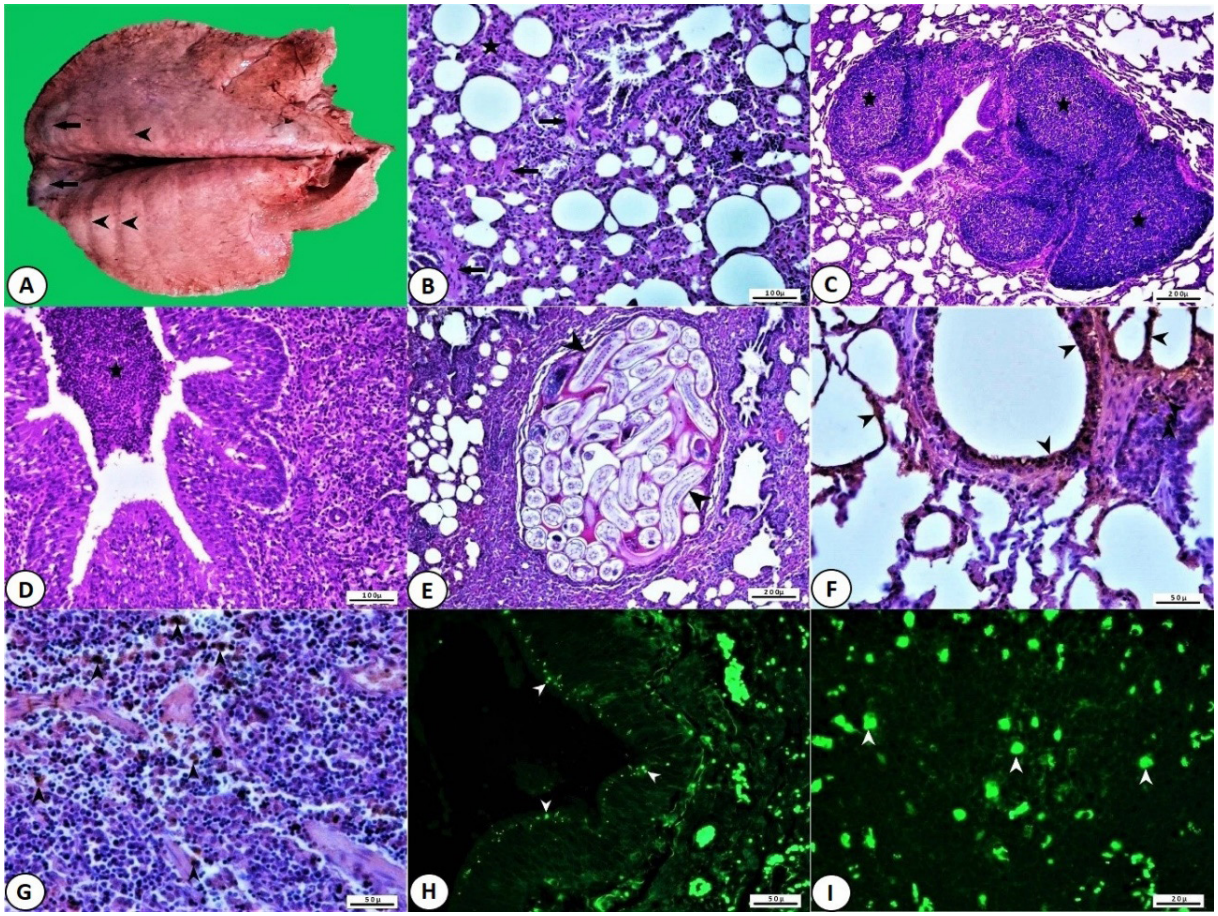

Figure 1. A; Costa traces (arrowheads) and emphysema (arrows) in the lung. B; Interalaveolar thickening (stars) and fibromuscular hypertrophy (arrows), HE, 100µ. C; Peribronchiolar lymphoid hyperplasia (stars), HE, 200µ. D; Catarrhal exudate (star), containing shed epithelium and inflammatory cells in the bronchiole lumen, HE, 100µ. E; Parasite forms in cystic granuloma (arrowheads), HE, 200µ. F; AdV immunoreaction in bronchiole and alveolar epitheliums, IHC, 50µ. G; AdV immunoreaction in mediastinal lymph node, IHC, 50µ. H; AdV immunoreaction in bronchial epithelium, IF, 50µ. I; AdV immunoreaction from mediastinal lymph node, IF, 20µ.

ELISA Results. Adenovirus positivity was detected in 20.4% (n=18) of the specimens that were examined with the antigen ELISA test.

PCR Results. Positive bands (600bp) showing the presence of genetic material belonging to adenoviruses were found in 13.6% (n=12) of the specimens examined with the PCR method. The results on the pneumonia types and examination methods of the adenovirus-positive lung specimens are shown in tables (Table 1).

Table 1. Pneumonia types and examination methods of the AdV (+) specimens**.**

Method	AdV(+) total	Pneumonia types		
		İP	CBP	VP
IHC	17	10	5	2
IF	20	12	6	2
ELISA	18	11	5	2
PCR	13	8	4	1

İP;Intersitisyel pneumonia, CBP; Catarrhal bronchopneumonia, VP;Verminous pneumonia

DISCUSSION

Sheep pneumonia cases caused by adenoviruses are not severe in adults, but they usually result in loss of yield, while those in lambs can result in death. The causes of these cases that cause mucociliary system damage and phagocytic inactivation also lead to the emergence of secondary infections (2,8,17). Studies that have involved experimentally induced adenovirus infections in sheep have reported that the lungs are not collapsed, they have a pale color, and elastic consistency. In microscopic findings, necrotic changes in the respiratory airway epithelium, thickening in the interalveolar septum, mononuclear cell infiltration, fibromuscular and lymphoid hyperplasia cases have been shown (6,11,18). In agreement with experimental studies, this study also demonstrated more findings of interstitial pneumonia in adenovirus infections, but adenovirus positivity was also encountered in the catarrhal and verminous pneumonia cases. Additionally, in this study, inclusion bodies like those in some experimentally induced infections were not observed (18,19).

Most studies on the prevalence of adenoviruses causing pneumonia in sheep are serological, while those that are based on antigen determination are very few. Çeribaşı et al (20) determined adenovirus antigens in 5.1% of sheep lungs with the IP method. Jamshidi et al (21) reported an adenovirus prevalence of 13.6% in goat lungs with the IHC method. In this study, adenovirus positivity (Samples with virus in one or both of the lung and mediastinal lymph nodes were considered infected) was determined in 19.3% of the sheep lungs with the IHC method. Previous studies have not examined mediastinal lymph nodes in terms of adenovirus positivity. In 3 lung specimens in this study, although no antigens were detected in the lungs, antigens were present in the mediastinal lymph nodes of these lung specimens. Although the lytic, latent and oncogenic effects of adenoviruses in other species are known, their latent and oncogenic effects in sheep were not reported (7,11,18). In this study, the results suggested that the viral agent can show latency in the mediastinal lymph nodes of these species. No study that showed adenovirus antigens in paraffinized tissue samples of sheep lungs using the indirect IF method was encountered in the literature. Nevertheless, Çeribaşı et al (20) detected adenovirus presence in 9.4% of sheep lungs using frozen tissue samples with the DFAT (Direct fluorescent antibody test) method. With the immunofluorescence staining method that was used in this study, antigen presence was determined as 22.7%. Additionally, in this method, as in the immunohistochemical staining method, some specimens showed antigen presence only in their lymph nodes. Çeribaşı et al (20) stated that they detected adenovirus presence in more sheep lung specimens with the DFAT method than the IP method. Likewise, in this study, antigen presence was found in more specimens with the IF method than the IHC staining method. Positivity detection was also more selective in the IF method than IHC staining. However, this study also noted some disadvantages of the IF method such as the fact that it does not provide as many morphological details as IHC staining, and it allows a limited working window due to intensity loss.

Among previous studies, no study that determined antigen prevalence with the ELISA method in tissue homogenates of sheep lungs was found. Nonetheless, several studies have been carried out in different parts of the world for antibody detection in sheep serum samples using the ELISA method, and accordingly, the seroprevalence of adenoviruses varies in the range of 69-100% (22,23,24). In this study where antigen presence was investigated in tissue homogenates using the ELISA method, adenovirus positivity was detected in 20.4% of the examined specimens. It was thought that the much lower prevalence value in this study in comparison to the results of previous studies originated from the fact that the presence of antigens was examined in this study, rather than the presence of antibodies. This is because antibody levels are high even after a while following viral infections (12,17). Among the methods they used in their study to investigate herpesvirus prevalence in bovine lungs with pneumonia, Çomaklı et al (25) reported that the highest prevalence was found with the IF method, which was followed respectively by IHC and ELISA. In this study, the IF, ELISA and IHC methods provided antigen prevalence values in descending order. This difference may have been associated with differences in virus and animal species.

The literature review in this study did not reveal any previous study that examined adenovirus presence in sheep pneumonia cases using PCR. Although PCR is a molecular diagnostic method

that is utilized in the diagnosis of many infectious causes with high reliability, its rate of detecting adenoviruses was found to be lower than those in the other methods in this study. While a diagnosis based on antigen-antibody binding was made in the other methods (IHC, IF, ELISA) that were used in this study, the PCR method involves identification with virus-specific hexon and fiber genes (13,26). After the replication of adenoviruses, only 10-15% gain a form of a complete virus particle (27,28). Furthermore, it was reported that 10^4 to 10^5 virus particles are formed in a cell that is infected with adenovirus, and only 1-5% of these have the morphology and capacity to form pathogenicity (29,30). In light of this information, it was considered that not every adenovirus antigen that replicates in the host organism takes a complete virus form, and while these adenovirus particles (procapsid) may provide positive results by forming a complex with antibodies, empty capsids that do not carry the hexon- or fiber-specific genetic materials might not show positivity in PCR. Moreover, results may also differ based on the type of material used in the PCR method (31).

In conclusion, in the investigation of antigen presence, it was seen that the prevalence of adenoviruses in sheep was high. In this study, in which different diagnostic methods were used, the IF method was identified as the most effective method in the diagnosis of sheep adenoviruses, and its effectiveness was followed respectively by ELISA, IHC and PCR. The main finding among those that were considered striking in this study was the possibility of latent adenovirus infections in the mediastinal lymph nodes and that the PCR method that is successfully used in the diagnosis of many infectious agents was found to be weak in the determination of these agents. Furthermore, in contrast to the results of experimental studies, this study revealed infections in catarrhal and verminous pneumonia cases, in addition to interstitial pneumonia cases.

Conflict of interest

The authors declare no conflict of interest.

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