

ORIGINAL ARTICLE

Comparison of the diagnostic methods on the canine adenovirus type 2 infection

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ABSTRACT

Background and aims: Canine adenovirus type 2 (CAV-2) infection is typically diagnosed histopathologically since intranuclear inclusion bodies (IN/IBs) are demonstrable in the infected lung. However, it is sometimes difficult to identify IN/IBs, particularly in autolyzed tissues or samples from both early and late stages of infection, and other methods were presently developed. **Methods:** Stray dog samples were evaluated by histopathology, polymerase chain reaction (PCR), and immunohistochemistry (IHC) to investigate the status of the CAV-2 infection on the stray dogs in Korea. Histologic tests were performed, and dogs with pneumonic lungs were further evaluated by IHC and PCR. **Results:** Pathognomonic IN/IBs were identified in 3 of 213 lungs; CAV-2 PCR was positive for 27 of 213 pneumonic lungs. A total of 7 of 27 CAV-2 PCR-positive lungs were IHC-positive. No PCR-negative lung was IHC-positive. Positive results were primarily detected in the IN/IBs of the bronchial epithelial cells, macrophages, and very rarely in the cytoplasm of bronchial epithelial cells. **Conclusions:** IHC was a more reliable diagnostic method than conventional pathologic methods in the present study, and suggests that IHC should be routinely used in the diagnosis of CAV-2 infection. Further, PCR alone may not be adequate for CAV-2 diagnosis.

INTRODUCTION

Infectious canine respiratory diseases primarily occur in dogs housed in groups, including those in rescue shelters, breeding kennels, and veterinary hospitals. Several studies evaluating natural disease outbreaks have demonstrated complex etiologies and infection with various viruses and bacteria. Common infectious causes of respiratory disease in dogs include canine distemper virus (CDV) and canine adenovirus type 2 (CAV-2).¹ A canine coronavirus associated with clinical canine respiratory disease similar to bovine coronavirus has been recently described in the UK.² However, CAV-2 is more commonly associated with infectious laryngotracheitis, or the kennel cough complex. The CAV-2 virus is included in the genus *Mastadenovirus* and the family *Adenoviridae*. *Adenoviridae* are non-enveloped icosahedral viruses with 26- to 44-kb double-stranded DNA genomes,³ and infect a variety of vertebrates including mammals, fish, birds, and reptiles.³ Pulmonary CAV-2 lesions are consistent with those of bronchio-interstitial pneumonia, including the presence of necrosis and large basophilic intranuclear inclusion bodies (IN/IBs) in bronchiolar and alveolar epithelial cells as well as pulmonary macrophages.⁴ Simultaneous CAV-2 and CDV infections have been previously described.⁵ Histopathology is a reliable indicator of CAV-2 infection because large IN/IBs are easily visualized in the infected lung. However, it is frequently difficult to find IN/IBs in lung tissue, (particularly in autolyzed tissue), at the early and late stages of infection, and in cases co-infected with suppurative bacteria.

The companion animal industry has become recently popular in Korea due to rapid national economic growth. Dogs are the most popular companion animal in Korea, and therefore the stray dog population is continuously growing. We investigated the CAV-2 infection status of stray Korean dogs by sampling from a local stray population. Histopathology was not a reliable indicator of CAV-2 infection since IN/IBs were not evident in all cases.^{5,6} Therefore, pneumonic lung tissues were examined for CAV-2 antigen by immunohistochemistry and CAV-2 genes by polymerase chain reaction (PCR) in the present study.

METHODS

Animals

Dogs who died or were killed were included from countrywide rescue shelters in Korea. A total of 565 bodies of stray dogs (comprising 246 female and 318 male dogs) were collected from January–December 2006 and included in the study. Most dogs from shelters were killed unless they were claimed within 30 days of rescue day.

Pathology

Complete necropsies were performed for all dogs. All tissues (including lungs) were fixed in 10% phosphate-buffered neutral formalin. All tissues were processed routinely and stained with haematoxylin and eosin for histopathology. Lung tissues with pneumonic lesions were selected for PCR and immunohistochemistry.

Table 1 Oligonucleotide primer sequences and product sizes for canine adenovirus (CAV) PCR amplification

Primer	Sequence	Position	Expected product size
Forward	CGC GCT GAA CAT TAC TAC CTT GTC	770–791	CAV-1, 508 bp
Reverse	CCT AGA GCA CTT CGT GTC CGC TT	1387–1408	CAV-2, 1,030 bp

(Gene bank No: S38238.1)

Electron microscopy

Lung tissues from dogs with pneumonia and IN/IBs were fixed as a pellet for 20 min by the addition of 2.5% glutaraldehyde (1 mL) in phosphate buffered saline (pH 7.2). Tissues were then pelleted, washed, and post-fixed with 1% osmium tetroxide. Tissues were then embedded in the Eponate 12 kit (Pelco, Redding, CA, USA) using propylene oxide, sectioned with an ultra microtome (Leica LG, Frankfurt, Germany), and double-stained with uranyl acetate and lead citrate. Stained sections were examined under a transmission electron microscope (H-7100AF; Hitachi, Tokyo, Japan).⁷

Polymerase chain reaction

Viral genomic DNA was extracted from the pneumonic lung tissues using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification was performed for the detection of the E3 and flank regions, as previously described.⁸ Primers and product sizes are shown in Table 1. PCR cycling conditions were as follows: denaturation for 15 min at 95°C, 35 cycles amplification for 20 s at 95°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C. Final extension was performed for 5 min at 72°C. PCR products (15 µl) were run on 1% agarose gels at 85 V for 20 min to visualize the amplified DNA. A molecular weight marker was run with the samples to determine the length of the amplified product. Bands were visualized after ethidium bromide staining under UV illumination, and were photographed with the UV gel imaging system.

Immunohistochemistry

Dogs with pneumonic lesions (213 of 565 dogs) were tested to demonstrate the CAV-2 antigens by IHC. Antigen unmasking pro-

cedures were performed by boiling (30 or 60 min), enzyme treatments (protease-1, protease-2, or proteinase K), or both boiling and enzyme treatments to determine the best treatment methodology and time.

Serial sections (3 µm) were mounted on Poly-L-Lysine-coated slides (Sigma Chemical Company, St Louis, MI, USA) and were heated to 60°C, dewaxed in xylene, and rehydrated through graded ethanol. The primary mouse anti-CAV-2 antibody (US Biological, Swampscott, MA, USA) was diluted from 100 to 1 000 times with antibody-diluting buffer. Negative controls were sections of the same tissues without primary antibodies. Antigen was primarily detected with the avidin-biotin kit (Ventana Medical Systems, Oro Valley, AZ, USA) using an automatic immunohistochemical stainer (Ventana). The multimer molecule detection method (Ventana) was used to confirm the cases whose interpretation was ambiguous by avidin-biotin staining.

RESULTS

Pathology

Two hundred and thirteen lungs (37.7%) of 565 dogs had various types of histopathologically evident pneumonic lesions. Pathognomonic IN/IBs were detected in three of 213 pneumonic lungs, mainly in the bronchiolar epithelium and alveolar macrophage. Most bronchial and bronchiolar epithelial cells displayed IN/IBs in the early stages of bronchiolitis (Fig. 1). Necrotizing bronchiolitis and prominent inflammation were evident in the lamina propria and the submucosa of the bronchi and bronchioli (Fig. 2). Macrophages, lymphocytes, and plasma cells were included in thick peribronchiolar lesions.

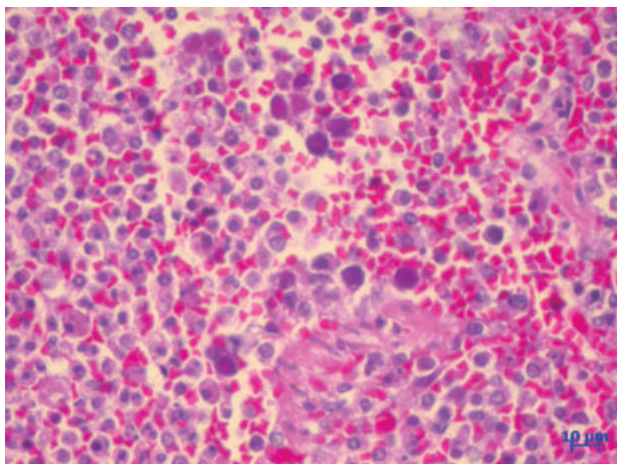


Figure 1 Lung tissue. Numerous prominent basophilic intranuclear inclusion bodies in the nuclei of bronchiolar epithelial cells (hematoxylin and eosin).

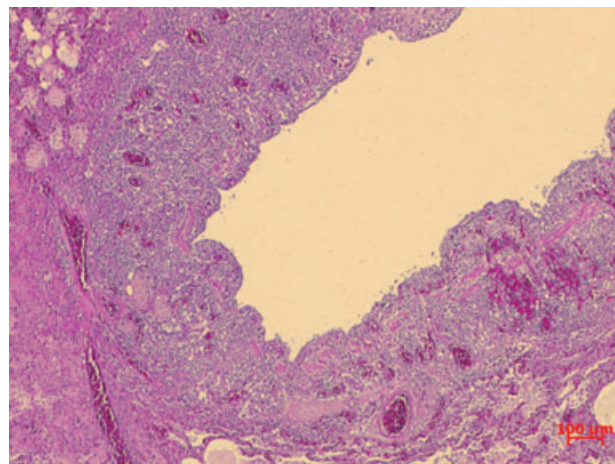


Figure 2 Lung tissue. Detachment and attenuation of bronchiolar epithelial cells and severe diffuse proliferative peribronchiolitis (hematoxylin and eosin).

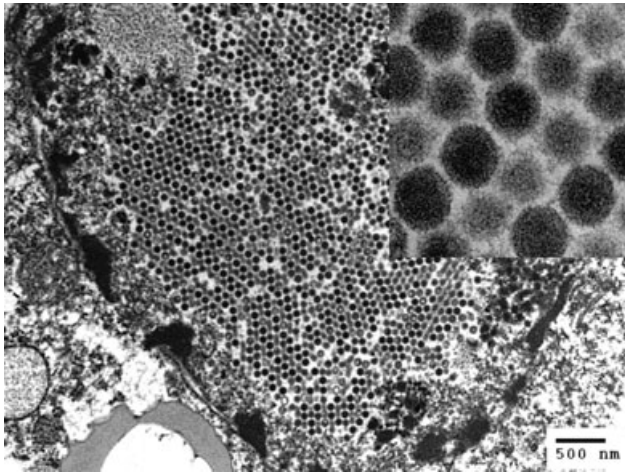


Figure 3 Lung tissue. Numerous viral particles demonstrating a paracrystalline pattern in the bronchiolar epithelial cell nucleus (insert represents the magnified viral particles).

Electron microscopy

Electron microscopy was used to examine the details of IN/IBs in one of the examined specimens. Viral particles were present in the macrophages, bronchiolar epithelial cells, and pneumocytes. Viral particles were identified in greater numbers of cells than expected based on frequency of inclusion in histopathology. Infected nuclei usually contained large numbers of viral particles which measured approximately 70–80 nm in diameter (Fig. 3). Viral particles demonstrated a typical paracrystalline array in the nucleus of the bronchiolar epithelial cell.

Immunohistochemistry and polymerase chain reaction

Positive CAV-2 PCR results (508 bp) were identified in 12.7% (27 of 213) of pneumonic lungs (Fig. 4). The CAV-1 specific gene (1,030 bp) was not amplified in this experiment.

IHC was performed on 37.7% of dogs (213 of 565 dogs). The best result was obtained at 500-times dilution in the iView kit in

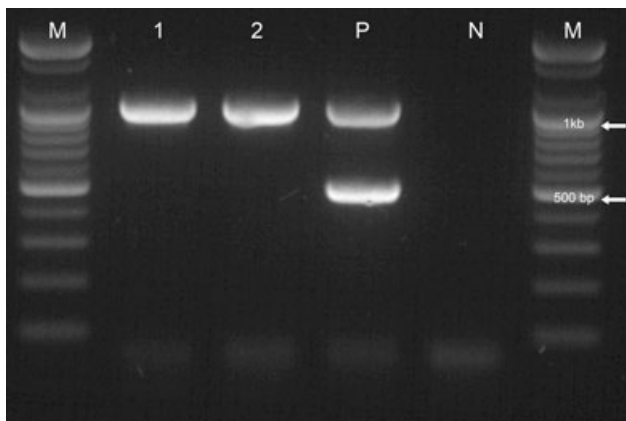


Figure 4 Representative patterns seen with agarose gel electrophoresis of amplified PCR products to canine adenovirus type 2 (CAV-2). M: DNA ladder, 1 and 2: CAV-2 positive field samples, P: CAV-1 and CAV-2 virus, N: DW.

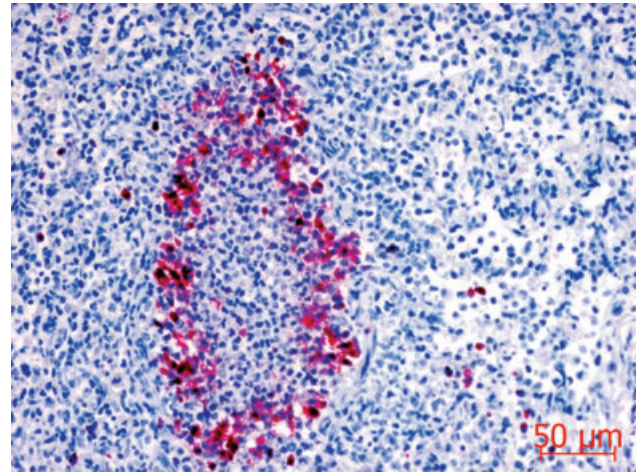


Figure 5 Lung tissue. Red areas represent positive immunohistochemistry reactions using multimer molecules and were stained with alkaline phosphatase/fast red detection kit. Most positive reactions were detected in the bronchial epithelial cells.

the primary antibody titration and 1 000 times in the UltraView kit (Ventana). Further, positive signals were easily detectable by the kit. Protease 2 digestion for 20 min with boiling yielded the best labeling results for antigen retrieval methods. Positive IHC signals were detected in 3.3% of lungs (7/213). All lungs positive by IHC were also positive by CAV-2 PCR, but there were no positive results identified among 188 PCR-negative lungs. Positive results were primarily identified on the nuclei of bronchial epithelial cells. Most epithelial cells in the same bronchus demonstrated similar positive reactions in a single case (Fig. 5). In advanced case, positive signals were identified in the nuclei of the macrophages and detached epithelial cells in the bronchiolar or alveolar lumens in scattered patterns in advanced cases. Positive signals were also detected in the cytoplasm of the epithelial cells of bronchus.

DISCUSSION

Canine infectious respiratory disease (CIRD) is a disease complex characterized by a dry hacking cough. Viruses commonly associated with respiratory disease in domestic dogs include CDV, CAV-2, canine parainfluenza virus type 2 (CPIV-2), canine reovirus type 1 (CRV-1), and canine herpesvirus type 1 (CHV-1).⁵

CAV-2 was first detected from dogs affected by laryngotracheitis in Canada in 1961.⁹ The clinical isolate was designated Toronto A26/61, was characterized as an adenovirus, and was initially categorized as an attenuated canine adenovirus type 1 (CAV-1) strain.¹ CAV-2 is considered one of the most common causes of infectious tracheobronchitis, and results in mild respiratory tract infection. CAV-2 has been implicated in enteritis episodes and has been detected in the brains of dogs with neurological signs.¹

Serological tests for canine viral disease using immunofluorescent methods were performed in Korea in 1995,¹⁰ and 42.5% of dogs from veterinary clinics had anti-CAV-2 antibodies. Anti-CDV and anti-CPIV-2 antibodies were identified at 83.8% and 63.8%, respectively. CAV-2 was less prevalent than CDV and CPIV-2; however, CAV-2 was identified in the present study as one of the important infectious agents of dogs in Korea. A positive ratio to CAV-2 was 12.7% by PCR of lung tissue in the present study. This

Table 2 Comparison of the test results among the three diagnostic methods for canine adenovirus type 2 infection

Immunohistochemistry	Histopathology (PCR)*		
	Positive	Negative	Total
Positive	3 (7)	4 (0)	7 (7)
Negative	0 (20)	206 (186)	206 (206)
Total	3 (27)	210 (186)	213 (213)

*Histopathology: sensitivity = 3/7 (42.9%), specificity = 206/206 (100%), PCR: sensitivity = 7/7 (100%), specificity = 186/206 (90.3%)

result suggested that CAV-2 was widely distributed and may be a common cause of respiratory disease in Korea.

Most cases of CAV-2 infection are histologically characterized by necrotizing tracheobronchitis and bronchiolitis with eosinophilic IN/IBs in epithelial cells.⁶ CAV-2 infection clinically causes severe necrotizing bronchitis and interstitial pneumonia, particularly in the presence of secondary bacterial infections.⁶ Nuclei with IN/IBs are usually round and very large, and inclusions are usually present in alveolar macrophages, pneumocytes, and bronchiolar and bronchial epithelial cells. The size and shape of identified viral particles were similar to those seen in previous studies, and the pattern was also identical with previous reports.^{11,12}

Intracellular CAV-2 antigens were identified in the bronchiolar epithelia, macrophages, neutrophils, and type 2 alveolar epithelial cells.¹³ However, CAV-2 antigens were also detected in a tonsillar epithelial cells in a previous report.¹⁴

Positive signals were primarily detected in the nuclei by immunohistochemical staining in the present study. However, mild positive signals were detected in the cytoplasm of infected macrophages and epithelial cells, suggesting that virus was present in both the cytoplasm and the nuclei, similar to previous reports in cell culture systems.¹⁵ IN/IBs are generally located in the nucleus; however, spherical dark inclusions were observed in both the cytoplasm and the nuclei in MDCK (Madin-Darby Canine Kidney) cells infected with CAV-2.¹⁵ The distribution of these cellular inclusions suggested that they were simultaneously formed in the cytoplasm and the nucleus and did not appear to be secondary to movement of these inclusions between the cellular compartments in the early stages of infection.¹⁵ IHC was reported as a reliable and rapid method for the diagnosis of CAV-1 infection, as previously reported.¹⁶

CAV-1 and CAV-2 can be differentiated by haemagglutination and neutralization test in the laboratory. They are difficult to differentiate in practical field specimens, particularly with infection in the gastrointestinal tract. Therefore, simple PCR methods were developed to detect and differentiate the viruses using a single pair of common primers based on E3 and flanking region sequences.⁸ These primers were designed to detect PCR products for CAV-1 (508 bp) and CAV-2 (1,030 bp), respectively, under the same amplification conditions;⁸ these primers were used in the present study. There were no CAV-1 cases, but 27 of 213 cases were CAV-2 positive. The PCR method used in the present study was effective and could be used for rapid diagnosis and differentiation of the different adenoviruses in canine field samples.

IHC of lung tissue has been previously used to detect several respiratory viruses in dogs.^{5,12} Further, CAV-2 IHC was a more sensitive diagnostic method than histopathological demonstration of inclusion bodies in a previous study.⁵ CAV-2 specific IC/IBs were identified in only three cases in the present study; however, seven cases were IHC positive for CAV-2 antigen detection. The

detection ratio for CAV-2 infection in the present study was higher in IHC than in routine histopathology (Table 2). These results suggested that CAV-2 particles may fail to create detectable inclusions in some cases although viral antigens were present in the lung tissues.

CAV-2 antigens were identified in the macrophage in the early stages of infection and in the epithelial cells in the late stages in a previous study, suggesting that CAV-2 particles are initially phagocytized and concentrated in the macrophages and later replicated in the bronchial epithelium, resulting in the production of the necrotizing bronchitis.⁵ Most bronchiolar epithelial cells in the present study were IHC positive in one case.

CAV-2 antigens were detected by IHC in 7 of 27 CAV-2 PCR positive cases; 20 cases were positive by PCR and negative by IHC, with no evidence of inclusion bodies. Sensitivity and specificity of PCR (100% and 90.3%, respectively) were comparable with IHC results (Table 2). Such cases will have a different final diagnosis than other protocols drawn from individual diagnostic laboratories. Clinical signs, pulmonary histopathological findings, and the presence of other infectious agents may all contribute to the ease of appropriate diagnosis. In conclusion, the IHC test is considered to be a necessary procedure for the accurate diagnosis of CAV-2 infection, with PCR being used as a supplementary method.

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