EVALUATION OF PCR TECHNIQUES FOR DETECTION AND DIFFERENTIATION OF CANINE ADENOVIRUSES IN FAECAL SAMPLES IN SHIRAZ, IRAN

A. MOHAMMADI1, M. MASOUDIAN2 & Y. NEMATI3

1Department of Pathobiology; 2Department of Biotechnology; 3DVM student;
School of Veterinary Medicine, Shiraz University, Shiraz, Iran

Summary


Canine adenovirus type 1 and 2 (CAV-1 & CAV-2) are causative agents of infectious canine hepatitis and infectious canine laryngotracheitis, respectively. Both viruses are shed in faeces and urine of the infected or recovered dogs. A total of 75 faecal samples were collected from diarrheic and nondiarrheic dogs referred to the small animal clinics of the Veterinary School, Shiraz University, Iran. Polymerase chain reaction was performed using separately three sets of primers (HA1/HA2, CAVcVP1/CAVcVP2/ and CAVcF1/CAVcR1). The results showed 28 positive samples, including 16 CAVc1 and 12 CAVc2. According to the study, the molecular diagnosis of CAV in faeces could be performed with better results using the primers CAVcVP1/CAVcVP2 and CAVcF1/CAVcR1.

Key words: canine adenovirus, faeces, molecular diagnosis

Canine adenoviruses are typical members of the family Adenoviridae (Spibey & Cavanagh, 1989). Two types of adenoviruses, canine adenovirus type 1 (CAV-1) and canine adenovirus type 2 (CAV-2) are causative agents of infectious canine hepatitis and infectious canine laryngotracheitis, respectively (Hu et al., 2001; Chaturvedi et al., 2008; Parthiban et al., 2009). Most CAV-1 infections in dogs are subclinical and clinical signs are unapparent, but sometimes the virus causes systemic disease by viremia, and clinical signs. Both viruses are shed in faeces and urine of the infected or recovered dogs, therefore, the main transmission routes of the disease is oronasal exposure to contaminated fluids of infected dogs (Yildirim et al., 2009).

Although there are antigenic relationships and cross-protective immunity between these two viruses, restriction endonuclease analysis has shown that they are genetically different types (Buonavoglia & Martella, 2007).

There are several methods for CAV detection such as enzyme linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), virus isolation, electron microscopy, haemagglutination inhibition (HI), serum neutralization (SN) and complement fixation test (Mochizuki...
Some methods are time-consuming and laborious. Likewise, CAV-1 and CAV-2 can be difficult to differentiate in the laboratory by HI and SN tests, especially when the infection occurs in the digestive tract. Hence, PCR appears to be most suitable for rapid diagnosis and differentiation (Chouinard, 1998; Erles et al., 2004).

The aim of the present study was to evaluate two PCR methods for detection of CAV types in faeces from diarrhoeic and non-diarrhoeic dogs in Shiraz, Iran using the primers HA1/HA2 (Chaturvedi et al., 2008) and the primer sets CAV-VP1/CAV-VP2 and CAV-F1/CAV-R1 (Erles et al., 2004).

A total of 75 faecal samples were collected from diarrhoeic dogs (50 samples) with signs of diarrhoea and vomiting and non-diarrhoeic dogs (25 samples) only with general symptoms such as depression and anorexia referred to the clinics of the Veterinary School of Shiraz University, Iran. Forty five dogs were male and the rest were female. None of diarrhoeic cases in our investigation was vaccinated against CAV. All samples were obtained with sterile swabs and stored at –70 °C until used.

A CAV-2 vaccinal strain was used as a positive control in the PCR assay. Total DNA was extracted from the samples and positive control using the AccuPrep® stool DNA extraction kit (Bioneer co., Korea) in accordance with supplier’s instruction. PCR assay was performed in a 25 µl reaction using three pairs of primers (Table 1) separately for all samples. The reaction mixture consisted of PCR buffer 1×, 1.5 mM MgCl₂, 0.2 mM of each deoxynucleotide, 10 pmol of each primer, 0.5 U of Taq DNA polymerase and 5 µL of template DNA (50 ng). PCR was conducted in an Eppendorf thermal cycler for 5 min at 94 °C and 35 cycles at 94 °C for 45 s, 59 °C in use of CAVc1 and CAVc2 primers, 60.5 °C in use of CAV-VP1 and VP2 primers and 53 °C in use of CAV-F1 and R1 primers for 45 s and a final amplification at 72 °C for 1 min. PCR products were visualized under ultraviolet light after electrophoresis on 2% agarose gel, stained with ethidium bromide.

The samples tested by the CAVcHA1 and HA2 primers had multiple bands in agarose gel (Fig. 1) and the results were unclear. The amplicons for CAVc1 and CAVc2 were 508 bp and 1030 bp, respectively. Then, all samples were tested using the CAVcVP1 and CAVcVP2 (Fig. 2) and CAVcF1 and CAVcR1 (Fig. 3) primers.

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

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Expected product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAV-HA1</td>
<td>5´ CGC GCT GAA CAT TAC TAC CTT GTC 3´</td>
<td>1030 bp for CAV-2</td>
</tr>
<tr>
<td>CAV-HA2</td>
<td>5´ CCT AGA GCA CTT CGT GTC CGC TT 3´</td>
<td>508 bp for CAV-1</td>
</tr>
<tr>
<td>CAV-VP1</td>
<td>5´ CTG GGC GGG ATT TAG AGG GTG G 3´</td>
<td>704 bp</td>
</tr>
<tr>
<td>CAV-VP2</td>
<td>5´ CAA GGG CGT GGG CGG AGT TAG A 3´</td>
<td></td>
</tr>
<tr>
<td>CAV-F1</td>
<td>5´ TGT CAA CAA GGT TTT GTC TTT T 3´</td>
<td>254 bp</td>
</tr>
<tr>
<td>CAV-R1</td>
<td>5´ TTT TCA AGG GAC GTG CGT 3´</td>
<td></td>
</tr>
</tbody>
</table>
which PCR product sizes were 704 bp and 254 bp, respectively. Twenty eight (37.33%) positive samples were detected including 16 (21.33%) for CAVc1 and 12 (16%) for CAVc2 (Table 2).

Diarrhoea is a common syndrome in dogs worldwide but the causative agents are different like canine parvoviruses and adenoviruses. In this study, the samples initially were tested using primers HA1/HA2 that detect CAVc1 and CAVc2 with 508 bp and 1030 bp amplicons respectively, but the results were not satisfactory because multiple bands were produced in the PCR assay. Apparently the primers reacted with the genome of some other organisms in the faeces. It was mentioned that some inhibitory substances in faeces can block the detection of adenoviruses and that chloroform treatment of the faeces will block their activity (Chaturvedi et al., 2008) but the interference from other organism's genome is still a problem. It is possible that filtering of suspended faeces in PBS before DNA extraction being a useful way to eliminate some organisms to obtain a suitable sample for the PCR test.

Erles et al. (2004) applied other primer sets to detect canine adenoviruses

![Fig. 1. Agarose gel electrophoresis of PCR amplified CAV-1 and CAV-2 DNA using the primers CAV-HA1 and CAV-HA2. Lane M: 100 bp DNA marker, lanes 1 and 2: positive controls (CAV-2 vaccine); lanes 3, 4, 5, 9, 10 and 11: CAV-2 positive samples (1030 bp); lanes 6, 7 and 8: CAV-1 positive samples (508 bp), lane 12: blank.](image1)

![Fig. 2. Agarose gel electrophoresis of PCR amplified (704 bp) CAVs DNA using the primers CAV-VP1 and CAV-VP2. Lane M: 100 bp DNA marker, lane 1: positive control (CAV-2 vaccine DNA); lanes 2 to 13: CAV-positive samples, lane 14: blank.](image2)
in bronchoalveolar lavage of dog lungs. We applied successfully these primers in the PCR assay to detect CAV-1 and CAV-2 in faeces. The primers CAV-VP1 and VP2 are able to detect both CAVs in the assay, but the primers CAV-F1 and CAV-R1 are specific for CAV-2. Therefore, these two sets of primers can detect and distinguish CAVs in a two-step PCR assay. In the present study, 16 samples were positive for CAV-1 and 12 samples were positive for CAV-2 that evidenced a relatively high occurrence of the infection in the area. By the way, the study showed that these primers were more efficient than the CAV-HA1/CAV-HA2 primer pair for detection of canine adenoviruses in the faeces.

Yıldırım et al. (2009) investigated the seroprevalence of CAV infection in non-vaccinated Kars dogs in Turkey. They used an ELISA test which could not discriminate the CAV types 1 and 2 but indicated a high prevalence of the infection in their region. In Thailand, canine adenoviruses were prevalent in approximately 9.17 % of dogs with respiratory diseases (Posuwan, 2010), so it was suggested that Thai dogs may be at higher risk for infection with CAV than those in Japan, where the prevalence of CAV was 2.9% (Mochizuki et al., 2008). However, the difference among detection methods is noticeable.

Yoon et al. (2010) reported 12.7% seropositivity to CAV-2 by PCR in lung tissue samples. Also, they previously reported that 42.5% of dogs referred to the veterinary clinics of Korea had anti-CAV-2 antibodies detected by immunofluorescence methods. In our study 37.33% of tested animals were CAV-positive by PCR that shows a high prevalence of the infection in the area with a higher occurrence of CAV-1 infection (21.33%).

According to the results of this study, the primer sets CAV-VP1/ CAV-VP2 and CAV-F1/CAV-R1 are more efficient than the CAV-HA1/CAV-HA2 primer pair for detection of canine adenoviruses in the faeces.

Table 2. Prevalence of CAV-1 and CAV-2 in the faeces of dogs tested by PCR – number (percentage).

<table>
<thead>
<tr>
<th></th>
<th>Without diarrhoea (n=25)</th>
<th>With diarrhoea (n=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAV-1 positive</td>
<td>–</td>
<td>16 (21.33%)</td>
</tr>
<tr>
<td>CAV-2 positive</td>
<td>–</td>
<td>12 (16.00%)</td>
</tr>
<tr>
<td>CAV-1 &amp; CAV-2 positive</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total CAV-positive</td>
<td>–</td>
<td>28 (37.33%)</td>
</tr>
</tbody>
</table>

Fig. 3. Agarose gel electrophoresis of PCR amplified (254 bp) CAV-2 DNA using the primers CAV-F1 and CAV-R1. Lane M: 100 bp DNA marker; lanes 1 to 6: CAV-positive samples, lane 7: blank.
HA1/HA2 primers for molecular diagnostics of CAVs in canine faeces. As 37.33% of tested dogs were CAV-positive, the occurrence of the infection among non-vaccinated dogs in the city of Shiraz could be assessed as significant.

ACKNOWLEDGMENTS

This work was financially supported by the Vice Chancellor for Research, School of Veterinary Medicine of Shiraz University, Iran.

REFERENCES


Paper received 27.04.2011; accepted for publication 16.09.2011

Correspondence:

Dr. A. Mohammadi
Department of Pathobiology,
School of Veterinary Medicine,
Shiraz University, Shiraz, Iran.
phone: 0098 711 613 8677
fax 0098 711 228 6940
e-mail: mohammad@shirazu.ac.ir