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Short communication

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

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Summary

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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, CAV-VP1/CAV-VP2/, and CAV-F1/CAV-R1). The results showed 28 positive samples, including 16 CAV-1 and 12 CAV-2. According to the study, the molecular diagnosis of CAV in faeces could be performed with better results using the primers CAV-VP1/ CAV-VP2 and CAV-F1/ CAV-R1.

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

et al., 2008). 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 ^{o}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 $MgCl_2$, 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 ^{o}C and 35 cycles at 94 ^{o}C for 45 s, 59 ^{o}C in use of CAV-HA1 and HA2 primers, 60.5 ^{o}C in use of CAV-VP1 and VP2 primers and 53 ^{o}C in use of CAV-F1 and R1 primers for 45 s and a final amplification at 72 ^{o}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 CAV-HA1 and HA2 primers had multiple bands in agarose gel (Fig. 1) and the results were unclear. The amplicons for CAV-1 and CAV-2 were 508 bp and 1030 bp, respectively. Then, all samples were tested using the CAV-VP1 and CAV-VP2 (Fig. 2) and CAV-F1 and CAV-R1 (Fig. 3) primers

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

Primers	Sequence	Expected product size
CAV-HA1	5′ CGC GCT GAA CAT TAC TAC CTT GTC 3′	1030 bp for CAV-2
CAV-HA2	5′ CCT AGA GCA CTT CGT GTC CGC TT 3′	508 bp for CAV-1
CAV-VP1	5′ CTG GGC GGG ATT TAG AGG GTG G 3′	704 bp
CAV-VP2	5′ CAA GGG CGT GGG CGG AGT TAG A 3′	
CAV-F1	5′ TGT CAA CAA GGT TTT GTC TTT T 3′	254 bp
CAV-R1	5′ TTT TCA AGG GAC GTG CGT 3′	

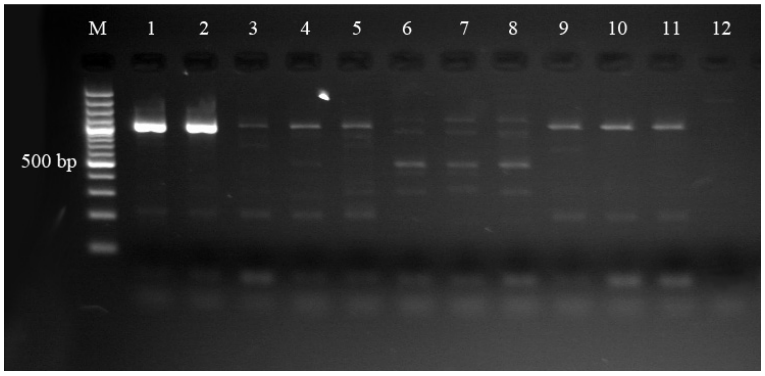

Fig. 1. Agarose gel electrophoresis of PCR amplified CAV-1 and CAV-2 DNA using the primers CAV-HA1 and CAV-HA2. Lane M: 100 bp DNA marker, lanes 1 and 2: positive controls (CAV-2 vaccine); lanes 3, 4, 5, 9, 10 and 11: CAV-2 positive samples (1030 bp); lanes 6, 7 and 8: CAV-1 positive samples (508 bp),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

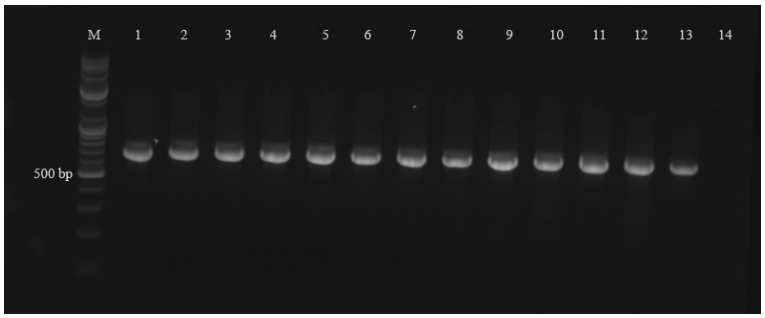

Fig. 2. Agarose gel electrophoresis of PCR amplified (704 bp) CAVs DNA using the primers CAV-VP1 and CAV-VP2. Lane M: 100 bp DNA marker, lane 1: positive control (CAV-2 vaccine DNA); lanes 2 to 13: CAV-positive samples, lane 14: blank.

which PCR product sizes were 704 bp and 254 bp, respectively. Twenty eight (37.33%) positive samples were detected including 16 (21.33%) for CAV-1 and 12 (16%) for CAV-2 (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 CAV-1 and CAV-2 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 intereference 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

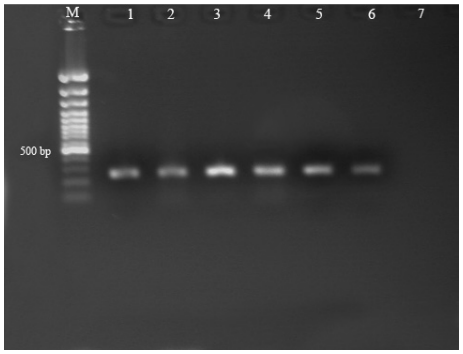

Fig. 3. Agarose gel electrophoresis of PCR amplified (254 bp) CAV-2 DNA using the primers CAV-F1 and CAV-R1. Lane M: 100 bp DNA marker; lanes 1 to 6: CAV-positive samples, lane 7: blank.

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

	Without diarrhoea (n=25)	With diarrhoea (n=50)
CAV-1 positive	–	16 (21.33%)
CAV-2 positive	–	12 (16.00%)
CAV-1 & CAV-2 positive	–	–
Total CAV-positive	–	28 (37.33%)

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.

Yildirim *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%).

Apparently, CAV infection viruses are widespread in dogs that are not immune to the viruses. It has been proved that modified live CAV-2 vaccines are very effective in reducing the circulation of the virus in canine populations. Dogs vaccinated with CAV-2 or CAV-1 develop the immunity to both CAV types (Posuwan, 2010). None of positive cases in our investigation was vaccinated against CAV types. So the vaccination against the disease seems to be necessary to eliminate or control the disease.

According to the results of this study, the primer sets CAV-VP1/ CAV-VP2 and CAV-F1/CAV-R1 are more efficient than

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.

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