



Short Communication

Detection of Infectious Canine Hepatitis Virus in Vaccines by PCR

Vishal Chander *, Sukdeb Nandi , Rishendra Verma

¹Centre for Animal Disease Research and Diagnosis (CADRAD), Izatnagar, U.P. India 243122.

*Corresponding author: drvishal@gmail.com

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ABSTRACT

Canine adenovirus 1 (CAV- 1) and CAV- 2 cause infectious canine hepatitis (ICH) and infectious canine laryngo- tracheitis (ICLT) in dogs, respectively. The disease is characterized by fever, anorexia, increased thirst and abdominal pain with swollen liver and, in case of CAV-2, respiratory symptoms are also seen. The corneal opacity (blue eye) and interstitial nephritis may occur 1- 3 weeks after the clinical recovery as a consequence of the deposition of circulating immune complexes after CAV- 1 infections in dogs. CAV- 1 is genetically and antigenically distinct from canine adenovirus 2 (CAV- 2). Both viruses are shed in faeces and urine of the infected or recovered dogs, thus urine and faeces are important sources of infection to healthy dogs. A galaxy of immunoprophylactic agents based on CAV- 2 is available in the market for use in dogs to control infectious canine hepatitis in dogs. In spite of vaccination against CAV infections in dogs, the outbreak has been reported occasionally in dogs in India. In this study, PCR has been employed to amplify the genomic DNA of CAV in the vaccines and it has been found that CAV- 2 strains are present in 4 of 7 vaccines tested.

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Infectious canine hepatitis (ICH) is a systemic disease of *Canidae* and *Ursidae* caused by canine adenovirus 1 (CAV- 1). The disease is characterized by fever, apathy, anorexia, increased thirst, abdominal pain with swollen liver, diarrhea and frequently dyspnoea (Appel, 1987). In addition, ocular and nasal discharges are also prominent (Appel and Carmichael, 1979). CAV- 1 is genetically and antigenically distinct from canine adenovirus 2 (CAV- 2) and is mainly associated with respiratory symptoms such as paroxysmal cough with variable expectoration and nasovascular discharge (Benetka et al., 2006; Schaer, 2010). CAV- 1 replicates in vascular endothelial cells and hepatocytes and produces an acute necrohemorrhagic hepatitis with a more severe clinical course in young than adult dogs (Greene, 1990) whereas CAV- 2 replicates in respiratory epithelium (Decaro et al., 2007). Corneal opacity (blue eye) and interstitial nephritis may occur 1-3 week after clinical recovery due to deposition of circulatory immune complexes in CAV- 1 infection (Wright, 1976). Both viruses are shed in faeces and urine of the infected or recovered dogs, thus urine and faeces are important sources of infection to healthy dogs (Macartney et al., 1988; Tham et al., 1998). Current methods of detection of CAV infections are based on hematological findings (lymphopenia and neutropenia) (Mosallanejad et al., 2010; Schaer, 2010), virus isolation (Decaro et al., 2007; Macartney et al., 1988; Schaer, 2010), serological assays (Ditchfield et al., 1962; Jacobs et al., 2007; Pratelli et al., 2001), histopathology (Boomkens et al., 2004; Chvala et al., 2007; Chouinard et al., 1998; Mosallanejad et al., 2010; Park et al., 2007; Schaer, 2010; Yoon et al., 2010), immunohistochemistry (Chvala et al., 2007; Chouinard et al., 1998; Rodriguez-Tovar et al., 2007; Schaer, 2010; Yoon et al., 2010) and indirect haemagglutination assay (Ditchfield et al., 1962). The serological tests are usually laborious and take 2- 3 days to be

performed and show higher titre after infection with virulent virus in contrast with modified live virus vaccines (Chouinard et al., 1998). Again, CAV- 1 and CAV- 2 can be difficult to differentiate in the laboratory by serological tests (Hu et al., 2001; Parthiban et al., 2009). With the advent of polymerase chain reaction (PCR), not only the detection but also differential identification of CAV-1 and CAV- 2 infections has become possible (Hu et al., 2001).

Since the use of modified live CAV- 1 vaccines has been found to cause adverse reactions, either a killed or an attenuated CAV- 2 vaccine has been used to protect dogs from CAV- 1 infection and found to be effective and more safe (Yin and Liu, 1985). Widespread vaccination has reduced very effectively the circulation of CAV- 1 in the canine population (Appel, 1987). However, recent report of some outbreaks of ICH in dogs in India has raised the doubt about the efficacy of vaccine in protecting against ICH. In this study, a rapid PCR based detection based on E3 region of genome of the virus responsible for variability among diverse adenoviruses has been reported. This also aids in differentiation of CAV- 1 and CAV- 2 depending on the degree of virulence and host specificity of the virus. The assay can be used to test the vaccines available in the market for the presence of CAV strain in order to find out the cause of vaccine failure and disease outbreak.

Infectious canine hepatitis vaccines

2 µL each of ICH vaccines namely Vanguard- 5 (Pfizer, USA) containing canine distemper, canine adenovirus 2, canine parvovirus, canine parainfluenza; Megavac- 6 (I) (Indian Immunologicals, India) containing live attenuated canine distemper, canine adenovirus 2, canine parvovirus; Megavac- 6 (2) (Indian Immunologicals, India) containing inactivated *Leptospira canicola* and *L. icterohaemorrhagiae*; Duramune PC

(Ford Dodge USA) containing canine corona virus and canine parvovirus; Canigen DHPPi (Virbac Carros, France) containing canine distemper, canine adenovirus 2, canine parvovirus, canine parainfluenza; Biocan- DHPPi (Bioveta, Czech Republic) containing canine distemper virus, canine adenovirus 2, infectious laryngotracheitis virus, canine parvovirus and parainfluenza; Biocan- L (Bioveta, Czech Republic) containing *L. canicola*, *L. icterohaemorrhagiae* and *L. grippotyphosa* were used directly in the PCR. As a negative control cell culture medium was used in the reaction. The cell culture supernatant of the CAV 1 and CAV 2 adapted in MDCK cell line were respectively used as a positive controls.

The PCR was performed in 200 µL thin layered PCR tubes (Axygen, USA) with a reaction volume of 50 µL. The forward (5' CGCGCTGAACATTACTACCTTGTC 3') and reverse (5' CCTAGAGCACTTCGTGTCCGCTT 3') primer set (Imperial Biomedics, India) (Kiss et al., 1996; Hu et al., 2001) specific for E3 gene of CAV- 1 and CAV- 2 were used in this study. The reaction mixture contained 200 µM of dNTPs, 10 pmol of each primer, 1X PCR reaction mixture containing 15 mM MgCl₂ and 2 µL of each of the 7 vaccines as source of template DNA. Amplification was performed in a thermocycler (Applied Biosystems, USA). 1 µL of DNA polymerase was added to above reaction mixture after initial denaturation at 94 °C for 5 minutes in the thermocycler. The cyclic condition was denaturation at 94 °C for 30 sec, primer annealing at 58°C for 1 min and extension at 72°C for 1 min. The cyclic condition was repeated for 30 times and a final extension at 72°C was given for 10 minutes. After PCR, the amplified products were analyzed on 1 % agarose gel containing ethidium bromide to a final concentration of 0.5 µg/ml. 10 µL of amplified product was mixed with 2 µL of bromophenol (6X) dye and loaded into the well and run along with 100 bp to 1 Kb DNA ladder in 1X TAE electrophoresis buffer at 5 volts/cm². At the end of the electrophoresis, the gel was visualized under the UV transilluminator and results were recorded.

The cell culture adapted positive controls revealed 508 bp and 1030 bp DNA products in case of CAV 1 and CAV 2 respectively. (Figure 1 and 2) The sensitive PCR assay reported here revealed the amplified product of 1030 bp from 4 vaccines and the findings are in accordance with results of Chaturvedi et al., (2008) (Figure 2). There was no amplified product in the agarose gel in the negative control. This PCR assay is based on E3 gene present in the genome of mammalian adenovirus (*Mastadenovirus* and some *Siadenovirus*). E3 gene is present in all genera as compared to E1 and E4 regions present in some and causes variability among diverse adenoviruses and show evidence of different virulence degree and host specificity (Harrach and Benko, 2007). E3 region is responsible for weakening host defense mechanisms and therefore severe damage or death can also occur if normal inflammatory response is not suppressed by E3. Also E3 gene is not essential for growth in cells in culture (Flint et al, 2004).

The polymerase chain reaction has been applied for the diagnosis of canine adenoviral infections in clinical samples and shown to be rapid, sensitive and specific diagnostic method (Boomkens et al., 2004, 2005; Chaturvedi et al., 2008; Decaro et al., 2007; Hu et al., 2001; Park et al., 2007; Parthiban et al., 2009; Schaer, 2010; Yoon et al., 2010). But no report has been available to detect the presence of CAV strain present in the vaccines available in the market for use in dogs. Since the use of modified live CAV- 1 vaccines has been found to cause adverse reactions, CAV- 2 vaccines are usually used as an alternative in the prevention of ICH that are still effective but more safe (Decaro et al., 2007). In pups, maternally derived antibodies (MDA) represent the main protection against CAV infections

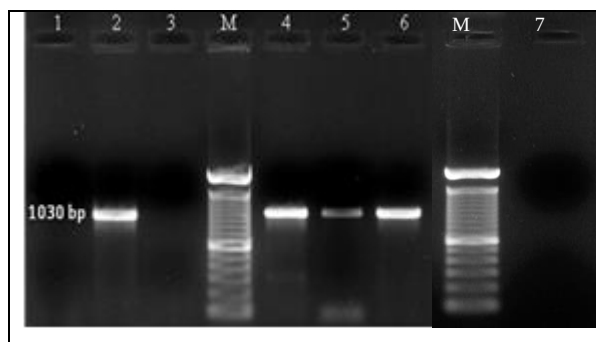


Figure 1: Agarose gel showing the amplification of 1030 bp DNA product of E3 gene of CAV 2; 1- Megavac 6 (2); 2- Megavac 6 (1); 3- Duramune; M-100bp marker; 4- Canigen DHPPi; 5- Biocan DHPPi; 6- Vangaurd 5; 7- Biocan L

but at the same time they may interfere with CAV vaccination (Appel, 1987). Further, a large pool of stray dogs in India is not covered under the vaccination umbrella and urine or faeces of infected or recovered dogs or dogs with sub- clinical form of infection form an important source of CAV- 1 and CAV- 2 (Macartney et al., 1988; Tham et al., 1998). With the reporting of CAV- 1 infections in dogs, it is of obvious suspicion whether the vaccine strain used in the vaccine is effective in protecting against ICH in dogs. Another reason of vaccine failure or ineffectiveness of the vaccination is that as the CAV- 2 is present in the vaccine along with other virus and bacteria and there may be competitive interference /suppression of one antigen by other potent antigen to be processed by the immune system in eliciting the protective immune response. Further, it is to be noted that field veterinarians do not follow either the uniform strategy in vaccinating the pups at the particular age or prior estimating the MDA.



Figure 2: Agarose gel showing the amplification of 1030 bp DNA product of E3 gene of CAV 2 and 508 bp product of CAV 1; 1- Sample1; 2- Sample2; M-100bp marker; 3- Sample3

In the present study, it has been observed that out of 7 commercially available vaccines tested, 4 namely, Megavac- 6 (1), Canigen DHPPi, Biocan DHPPi and Vangaurd- 5 contain the CAV- 2 as vaccine strain to provide protection against both the CAV- 1 and CAV- 2 which is in accordance with the results of Decaro et al. (2007). On the other hand, Megavac- 6 (2), Duramune PC and Biocan L did not contain any CAV as there was no amplification of DNA in PCR.

CAV infections occur occasionally in dogs in India (Parthiban et al., 2009). CAV- 1 and CAV- 2 infections in dogs in most cases are differentiated clinically from each other, but they have the same morphological features under the EM and same CPE on cell cultures. CAV- 2 can also infect the intestinal tract, one of the major target organs for CAV- 1 (Hamelin et al., 1986; Macartney et al., 1988).

The diagnosis of CAV infections is usually based on serological tests (Ditchfield et al., 1962; Jacobs et al., 2007; Pratelli et al., 2001) and virus isolation (Decaro et al., 2007; McCartney et al., 1988; Schaer, 2010).

PCR detection of CAV-1 in livers of infected dogs has also been reported (Kiss et al., 1996; Chouinard et al., 1998). However a number of diseases CDV, CPV, CCoV having similar type of symptoms often make it difficult to detect the specific pathogen accurately. In India, a large proportion of a total of about 25 million dogs mostly stray dogs are not vaccinated against the disease and harbour the pathogen to be transmitted to other susceptible dogs. The CAV-1 is still circulating in the dog populations and occasionally it is responsible for a severe often fatal disease especially in animal shelters and breeding kennels when virus spread is ensured by close contact between the animals. Further, vaccinations are often carried out on pups with high titres of maternally derived antibodies that prevent an active immune response. This PCR based assay was applied to differentiate and cross validate whether vaccine strain is CAV-1 or CAV-2 as mentioned on the vaccine labels and to validate this test for future field investigations as well. From the study, it is clear that out of 7 vaccines tested 4 namely Megavac-6 (1), Canigen DHPPi, Biocan DHPPi, Vanguard-5 contain CAV-2 whereas Megavac-6 (2) and Duramune does not contain any CAV-2 as revealed in the products (Fig. 1). So, it is recommended that owners should check the product/vaccine for the presence of CAV-2 before being used it in dogs against CAV infections.

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Conflict of interest:

The authors have no conflict of interest including any financial, personal or other relationships with other people or organizations.

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