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Detection of canine distemper in conjunctival and nasal swabs of dogs

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Abstract: The present study was undertaken to detect the presence of canine distemper virus (CDV) in suspected dogs by immunochromatographic (IC) strip test and nested RT-PCR. A total of 100 samples (50 each of conjunctival and nasal swabs) were collected from dogs suspected of canine distemper. These samples were tested by IC strip test using Ubio quickVET canine distemper virus Ag detection kit for preliminary diagnosis and then subjected to molecular detection by nested RT-PCR. Twenty eight samples were found positive by the Ubio quickVet kit and out of these positive samples 17(34%) were conjunctival swabs and 11(22%) were nasal swabs. A total of 50 samples were subjected to nested RT-PCR for the detection of CD virus RNA by amplification of N gene and desired amplicon of 419bp was observed in nine samples, out of which 6 (24%) were conjunctival swabs and 3(12%) were nasal swabs. Nested RT-PCR of N gene is the reliable technique for the detection of CDV and conjunctival swab samples would be the most suitable and practical specimen for the early detection of CDV infection.

Key words: Canine distemper, Conjunctival swabs, Dogs, Nested RT-PCR

Introduction

Canine distemper (CD) an important viral disease of dogs is characterized by pneumonia, encephalitis, diarrhea, conjunctivitis, nasal and digital hyperkeratosis and pustular dermatitis (Dungworth, 1993).CD causes high mortality in dogs and has high morbidity rate (Greene and Appel, 2006). The virus is mainly transmitted by aerosol route and targets the mucous membrane and lymphoid tissue of infected dogs (Appel, 1987).CD mostly affects dogs of the age of 3 to 6 months however, non-immune dogs of any age are susceptible to the virus (Greene and Appel, 1998). CD is caused by the virus belonging to family *Paramyxoviridae*, genus *Morbillivirus*. The virus has negative sense single stranded RNA genome which encodes for six structural proteins viz. two membrane glycoproteins (fusion F and hemagglutinin H), envelope-associated matrix (M) protein, phosphoprotein (P), large polymerase (L) , nucleocapsid (N) protein and two non-structural proteins viz. C and V. Among the structural proteins, N protein is the most abundant and main regulator of the virus replication and transcription (Sidhu *et al*. 1993). H protein the major determinant of tropism and cytopathogenicity (Von Messling *et al*. 2001) plays an important role in induction of protective immunity (Diallo, 1990) and is used to assess genetic variations between CD virus isolates (Lednicky *et al*. 2004 and Hashimoto *et al*. 2001).

The diagnosis of CD depends upon various laboratory tests like indirect immunofluorescence assay (IFA), enzyme linked immunosorbent assay (ELISA), immunoperoxidase linked assay (IPA), serum neutralization test (SNT), immunocytochemistry and *in situ* hybridization. Serological diagnostic tests like ELISA, IFA, IPA and SNT have little diagnostic value as vaccine induced anti-CD virus antibodies may interfere with the interpretation of serological testing (Frisk *et al*. 1999 and Kim *et al*. 2001). Although immunohistochemistry is a highly sensitive and specific method for detection of CD virus in tissue obtained from postmortem but is not suitable for diagnosis of distemper in living animals. Moreover these methods are laborious and time consuming and have limited usefulness for clinical specimens. Thus a rapid, sensitive and specific CD virus detection assay is required as the contagious nature and the high mortality rates of CD make it necessary to speed up the diagnostic procedure and to develop techniques which are highly sensitive and specific for the CD virus detection. Molecular diagnostic assays like Reverse-transcriptase-polymerase chain reaction (RT-PCR) combined with nested PCR is a sensitive and specific diagnostic tool for canine distemper (Saito, 2006 and Namroodi *et al*. 2013). Thus in the present study two diagnostic tests have been employed for the detection of canine distemper in clinical specimens.

Materials and Methods

A total of 100 samples (50 each of conjunctival and nasal swabs) were collected from dogs suspected of canine distemper i.e. exhibiting clinical signs of CD which included nasal and ocular discharge, vomition, diarrhea and/or haemorrhagic diarrhea and

neurological signs. The samples were collected in phosphate buffer saline (pH 7.2) from small animal veterinary clinics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab during the period from October 2014 to March 2015 and kept at -80^{0}C till further use.

The conjunctival and nasal swabs collected were subjected to detection of CD virus by using the Ubio quickVET Canine Distemper Virus Antigen Rapid Test Kit according to manufacturer's instructions. The results were interpreted within 5-10 minutes of the test as negative when only control band was visible, positive when both control and test bands were visible and invalid when no control band was visible.

Reverse transcriptase polymerase chain reaction (RT-PCR): The RNA was extracted from conjunctival swab (500 µl), and nasal swab (500 µl) (Sambrook and Russell, 2001) using Trizol (1:2) followed by phase separation with chloroform (200 µl). The RNA in the aqueous phase was precipitated by adding an equal volume of isopropanol (500 µl) and then extracted by centrifugation at 12,000 rpm for 15 min at 4°C.The resultant pellet was washed with 75% ethanol (500 µl) and dried at room temperature. The extracted RNA was eluted in 20-25 µl of nuclease free water and incubated at 60-70°C for 5 min to enhance complete dissolution of RNA. The RNA was reverse-transcribed into cDNA using first strand cDNA synthesis kit (Thermo Scientific) according to manufacturer's instructions. The components were briefly centrifuged and placed in thermocycler (Applied Biosystems, Veriti) for one cycle each of cDNA synthesis at 40 °C for 60 min and inactivation at 70°C for 5 min. The amplified cDNA was stored at -20°C until it was used for PCR.

The complete nucleoprotein coding region of CD virus was amplified using the two set of primers with minor modification (Kapil *et al*. 2008).The outer set of primers included primer 1 5'-ATTTGGGATTGCTTAGGA-3'and primer 2 5'-GGCGCTCATC TTGGACAT-3'and the internal set of primers included primer 3 5'-GTTAGCTAGTTTCATCCT-3'and primer 4 5'-GGTCCTCTGT TGTCTTGG-3'. The 50 µl reaction mixture for the PCR included10 ìl of cDNA, 0.5 µl Taq polymerase (5 U/µl), 5.0 µl PCR Buffer (10X), 1.0 µl dNTPs (10mM) , 1.5 µl MgCl2 (50mM),1µl of each outer set of primers (20 pmol/ µl) and 30.0 µl RNase free water. The reaction mixture was incubated in thermocycler (Applied Biosystems, Veriti) at 94°C for 3min followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 50°C for 45 sec and elongation at 72°C for 1min. The final elongation was done at 72°C for 7min. For nested PCR 10 µl of PCR product was subjected to amplification using internal set of primers. The cycling conditions were same as PCR. The nested PCR products were resolved on 1% agarose gel using 1X Tris Acetate EDTA (TAE) containing 0.5 µg/ml of ethidium bromide, following electrophoresis at 80 volt for 60 min. The bands were observed under UV light and documented photographically.

Results and Discussion

Canine Distemper is one of the severe and infectious viral diseases of dogs causing high mortality in non-vaccinated dogs and also sometimes in vaccinated dogs. The diagnosis of CD on the basis of clinical signs is difficult as the main signs which include nasal discharge, ocular discharge and diarrhoea can be seen in other respiratory and enteric diseases too. Various techniques have been employed for the detection of CD virus like indirect immunofluorescence assay (IFA), enzyme linked immunosorbent assay (ELISA), virus isolation, serum neutralization test (SN), immunocytochemistry *in situ* hybridization and nested RT-PCR (Appel, 1987 and Namroodi *et al*. 2013).

In the present study, a total of 100 samples (50 each of conjunctival and nasal swabs) were collected from dogs suspected for canine distemper. These 100 samples were screened by Ubio quickVET Canine Distemper Virus Antigen Rapid Test Kit as a preliminary screening of CD. Out of these 100 samples, 28 samples were found to be positive. Out of these 28 positive samples, 17(17/50*100, 34%) were conjunctival swabs and 11(11/50*100, 22%) were nasal swabs. In the present study we have found more number of conjunctival swab samples to be positive as compared to nasal discharges. Similarly, many other workers in their study examined various samples by RT-PCR for the diagnosis of CD and revealed that conjunctival swab samples are the most suitable specimen (Namroodi *et al.* 2013; Kim *et al*. 2006; Shabbir *et al*. 2010). The reason could be that the conjunctiva and the eyes probably become infected at the time of generalized viraemia in the early course of the infection. There are also possibilities of aero-exposure of CD virus in conjunctiva while challenging and of contamination through lacrimal duct. Additionally, CD virus replicating in the conjunctival sac or orbital cavity is not subjected to rapid exclusion by immune system. That's why the virus appears earlier and is eliminated later from conjunctival fluid leading to its higher detection rates and moreover, conjunctival swab samples are easy to obtain and non-invasive (Kim *et al*. 2006 and Shabbir *et al*. 2010).

A total of 50 samples (25 each of conjunctival and nasal swabs) were subjected to RT-PCR for the detection of CD virus

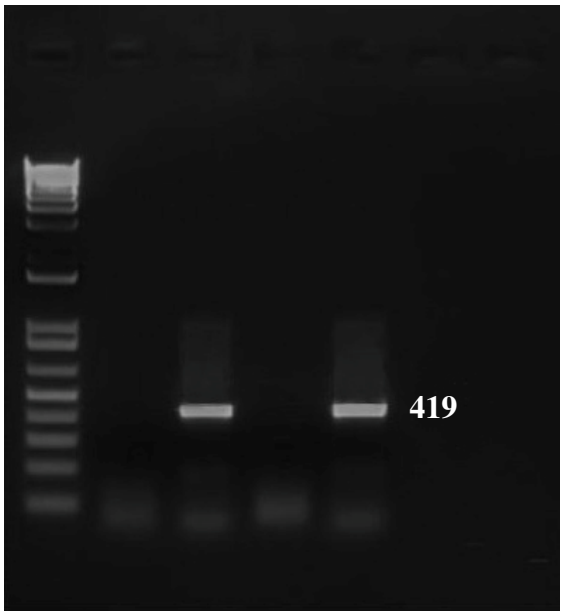

Fig-1: Reverse transcriptase polymerase chain reaction

RNA by amplification of N gene and nested PCR product of 419bp was observed in nine samples (Fig. 1). Out of these 9 positive samples 6(6/25*100, 24%) were conjunctival swabs and 3 (3/25*100, 12%) were nasal swabs. Hence, nested RT-PCR can be used as a specific, sensitive and reliable diagnostic tool for canine distemper detection. Similarly other workers too concluded in their study that nested RT-PCR is a reliable method than RT-PCR for the diagnosis of CD infection (Kim *et al*. 2001 and Namroodi *et al*. 2013). Also, Rzezutka and Mizak (2002), Jozwik and Frymus (2005), when compared various techniques for detection of CD virus in clinically suspected cases concluded that two-step RTPCR is most sensitive, specific and reliable method for diagnosis of CD infection. The possible reason for the variation in the results of the two methods used in the study for the detection of CD virus could be that most of the kits can detect more than one or two analytes (antigen) spontaneously, suffer from low biomolecules affinity towards analytes and tendency of cross-reactivity as sometimes the antibodies to the target in the test line may bind to a different epitope on the test line.

As non-specific reactions were common in conventional antigen detection methods. Hence, more samples were found positive in conventional methods than nucleic acid based method viz., PCR as PCR is based on genome of the virus which gives accurate diagnosis of CDV infections (Muthiah *et al*., 2008). This study concludes that Nested RT-PCR of N gene is the most reliable technique for the detection of CDV and conjunctival swab samples would be the most suitable and practical specimen for the early antemortem diagnosis of CDV infection.

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