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Original Article

Molecular and Serological Detection of Canine Distemper Virus (CDV) in Rural Dogs, Iran

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Abstract

Background and Aims: Canine distemper (CD) is a deadly infectious disease of Canidae family. CD is a multi-systemic viral disease and is specified by wide range of clinical symptoms. The manifestations are not always indicative of CD, therefore a laboratory confirmation is necessary for suspected cases.

Materials and Methods: Different clinical specimens of 19 CD suspected unvaccinated dogs were examined for canine distemper virus (CDV) infection by reverse transcription polymerase chain reaction (RT-PCR), Nested-PCR, and serum neutralization (SN) test during 2008-2011. RT-nested PCR assay was adjusted for detection of CDV nucleoprotein (NP) in prepared samples.

Results: In samples of 3 out of 19 (15%) dogs, CDV NP gene was confirmed by RT-PCR while RT-PCR and combination with Nested-PCR (RT-nested PCR) presence of CDV NP gene was detected in various samples of 14 (73%) dogs. So efficiency of RT-PCR along with Nested-PCR raised 58%. Among different kinds of obtained samples, conjunctival swabs and kidney tissue biopsies were found to be suitable for analysis of CDV RNA. Additionally CDV antibody was detected in 11 out of 18 serum samples (61%) by SN test, but detection of neutralizing antibodies didn't comply with RT-nested PCR results.

Conclusion: Results of this study indicated that Nested-PCR is a sensitive and applicable method for the diagnosis of CDV.

Keywords: Canine Distemper Virus; RT-nested PCR; Nucleoprotein; Serum Neutralization Test

Introduction

Canine distemper (CD) that is caused by canine distemper virus (CDV; order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Morbillivirus*), is causative agent of important viral disease of wild and domestic canids, with a high morbidity and mortality rate. CDV has a negative sense single-stranded RNA genome almost 15690 nucleotides in length making six structural proteins, namely: Nucleoprotein (NP), Phospho protein (P), Matrix protein (M), Fusion protein (F), Hemagglutinin protein (H), and Large protein (L). Gastrointestinal, respiratory, and neurological signs specify the CD. It is highly

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transmissible acute or sub-acute fevered infection that has been recognized since 1760 (1, 2). Even if vaccination with attenuated virus against CDV has been permitted for the control of infection, this infection remains an important disease of dogs and epidemics of CD have been documented in many countries (3-6).

The high price of the vaccine, vaccine miscarriage, and absence of cautiousness about the significance of vaccination, other than the lack of a national pet registration policy and laboratory-based disease diagnostic services are some of the reasons that cause perpetuation of the CDV in Iran.

Developing of more sensitive and specific diagnostic methods on the basis of reverse transcription polymerase chain reaction (RT-PCR) amplification, compared to outdated assays (that are time consuming and also in some instances not specific) such as indirect fluorescent antibody test, virus isolation, immunocytochemistry, enzyme-linked immunosorbent assay and serum neutralization (SN) test, have been facilitated to identify infected animals faster and more sensitive than ever before (7-10).

Above all, RT-PCR combined with Nested PCR (RT-nested PCR) seems faster and it is a more sensitive method for the identification of CD (8, 9, 11).

Even if diverse viral gene sequences have been classified for recognition of CDV in biological specimens, the conserved nucleoprotein (NP) gene is considered to be a better objective for amplification of particular fragments from all CDV strains (3, 7, 8, 12).

The aim of current investigation was to examine the advantages of Nested-PCR, RT-PCR (based on CDV NP gene detection) and SN test for the laboratory diagnosis of CD in different samples of clinically suspected dogs for the first time in Iran.

Methods

Animals

Samples were collected from 19 mixed breeds dogs (3 dead and 16 living dogs) which were less than one-year old. The population consisted of unvaccinated dogs of both sexes showing distinguished signs of CD, such as fever, tonsillitis, diarrhea, coughing, conjunctivitis, vomiting and purulent nasal discharge. However, the population did not show any signs of neurological disturbance. The samples were obtained from Veterinary medical teaching hospital of University of Tehran (Center of Iran) and local clinics of Golestan province (North East of Iran). Clinical signs and histories, such as age, sex, vaccination and breed were noted (data is not shown).

Biological samples and processing

The following biological samples were analyzed in this study: whole blood (Number of sampled dogs (n) = 18), Ocular swab (n = 19), serum (n = 18), various organs (n = 3, lung, bladder, kidney, intestine, stomach). The owner's consensus was achieved before gathering the clinical samples.

Blood samples were poured into the EDTA tubes. Three ml of blood was diluted 1:1 by phosphated buffered saline (PBS) and were gently mixed. PBMCs were separated through centrifugation at 2000 rpm for 30 min at 4 °C. The supernatant was thrown away and lymphocytes were suspended in 250 µl of PBS (Ph 7.2) and utilized for the analysis. 1 mg of homogenized internal tissue was kept in 1ml PBS (Ph 7.2), centrifuged at 13000 rpm for 15 min, and was filtered twice. Ocular discharges were wiped with sterilized cotton sticks and then eluted with 0.5 ml PBS (Ph 7.2). 2 ml of the serum were diluted 1:1 with PBS (Ph 7.2). The samples were kept in -70 °C till they were used for the investigations.

Total RNA Extraction

Total RNA was removed from all specimens, Vero cells infected with Onderstepoort CDV vaccine strain (Ond-CDV), Alk strain of measles virus, and ultra-pure water as a negative control, using RNA extraction kit (Bioneer Co, Korea) following the manufacturer's introductions.

cDNA synthesis (RT Reaction)

The RNA was reverse-transcribed into cDNA using tow step RT-PCR kit (Vivantis, Malaysia) providing the manufacturer's recommended reaction conditions. The

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amplified cDNA was immediately stored at -20 ^{0}C until it was used for PCR.

Primers

Two sets of primers goaled to a conserved section of the CDV NP gene with sequences regained from GenBank were prepared and used for the studies:

CDV-1:

Forward, 5'-GGGTCGAAAGCTCAAGGAC3'

CDV-2:

Reverse, 5'-CTGACACTAGCTGAGCCTCTTC-3'

CDVa:

Forward, 5'-CCTGCTCGCTAAAGCAGTG-3'

CDVb:

Reverse, 5'-CCCTCCCATGGAGTTTTCA-3'

Primer pair CDV-1&CDV-2 were used for PCR as outer primers and pair CDVa &CDVb were used for Nested-PCR as inner primers.

The predictable sizes of amplicons were 777/778 bp for outer primers at 37-56 and 795-816 position of Ond-CDV strain and also were 520bp at 154-175 and 658-676 position of motioned CDV strains, for the inner primers. All primer sequences were made by Cinagene Company in OD 5 (Cinagene Co, Iran)

PPolymerase Chain Reaction (PCR)

PCR, using outer primer, was carried out in reaction mixture containing: 1µl (0/04 UnitUl^{-1}) Taq polymerase, 5 µl cDNA, 17 µl 1X PCR master mix (Vivantis, Malaysia) (0.4mM Tris–HCl, pH 8.3; 2.5mM $MgCl_2$; 500mM KCl; 0.1% gelatine, 1µl 10mM dNTPs each), 1µl (10mM) each primer up to 25µl.

The reaction mixture was incubated at 95°C for 5min. Amplification was done in 35 cycles with denaturation at 95°C for 1min, annealing at 47°C for 1min and elongation at 72°C for 1min. The final elongation was accomplished at 72°C for 5min.

Nested-PCR

Nested-PCR was performed using internal set of primers and 1µl of the first PCR product. Nested-PCR cycling qualifications were equal to those used for the RT-PCR. The positive and negative CDV controls were excluded in each run of both molecular methods.

Electrophoretical analysis of amplicons

After amplification, 5µl of the reaction mixture were transferred to 1% agarose gel colored by ethidium bromide and evaluated by electrophoresis in TAE buffer (90mmolL^{-1} Tris-HCL, 90 mmolL^{-1} Tris-HCL acetic acid, 2 mmolL^{-1} EDTA) at continuous voltage (100 V) for 60min. Products were examined under ultraviolet (UV) light after electrophoresis with 100bp (Fermentas, St. Leon-Roth, Germany) ladder as a DNA size marker

PCR product sequencing

Positive Nested-PCR results were separated from the agarose gel using Gel Purification kit (Bioneer, Cat No: K-3034, Korea). Purified DNA was sequenced bi-directly using inner amplification primers in ABI 313 DNA sequencing instruments (Seq Lab Co, Germany).

Specificity of the Reaction and Sequence analysis

Alk strain of measles virus was also prepared for controlling the specificity of RT-PCR and Nested-PCR.

Also the sequence characteristics were checked against sequences deposited in the GenBank using BLAST software (http://www.ncbi.nlm.nih.gov/).

Serologic test

To investigate the presence of anti-CDV neutralizing antibody in serum samples, standard SN test was performed (13). 50 µl Heat inactivated and serially two fold diluted sera were mixed with the same volume of CDV suspensions containing 200 $TCID_{50}ml^{-1}$. Serum-virus mixtures were kept at 37 ^{0}C for 1 h. After incubation, 100µl Vero cells were added to each well of plates and titration plates were incubated at 37°C in 5% Co_2 for 3-5 days (14).

The neutralization ability of the sera were controlled by inhibition of the OP-CDV induced cytopathic effect (giant cell formation) and test cut-off point was ≥32 (Carmichael *et al*. 1980).

Results

RT-PCR and Nested-PCR

Different kinds of samples (conjunctival swab= 3, kidney= 1, lung= 1) of three dogs were positive for RT-PCR assays. Besides, Nested-PCR products of the supposed size were

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Table 1. Diagnosis of CD in unvaccinated dogs bases on RT-PCR, Nested-PCR, and SN test.

Dog No.	RT-PCR results				Nested-PCR results				SN test results
	S	Wb	T	Cs	S	Wb	T	Cs	S
1	-	-	Na	-	-	-	Na		
2	-	-	Na	-	-	-	Na	+	+
3	-	-	Na	-	-	-	Na	+	+
4	-	-	Na	-	-	+	Na	+	+
5	-	-	Na	-	+	+	Na	+	-
6	-	-	Na	-	-	-	Na		+
7	-	-	-	+	+	+	-	+	-
8	-	-	Na	-	-	-	Na	+	+
9	-	-	Na	-	-	-	Na	+	+
10	-	-	Na	-	-	-	Na	+	
11	-	-	Na	-	-	-	Na		+
12	-	-	Na	-	-	-	Na	+	
13	-	-	Na	-	-	-	Na	+	
14	-	-	+	+	+	+	+	+	-
15	-	-	-	+	+	+	+	+	+
16	-	-	Na	-	-	-	Na	+	+
17	-	-	Na	-	-	-	Na	+	+
18	-	-	Na	-	-	-	Na		+
19	Na	Na	Na	-	Na	Na	Na		Na

T: Tissue, Cs: Conjunctival swab, S: Serum, Wb: Whole blood, Na: Not available

amplified in additional 11 samples (conjunctival swab= 11, kidney= 2, lung= 1, intestine= 1, whole blood= 5, bladder= 1, serum= 4) (Table. 1 & Fig. 1).

The positive samples were totally identified in 14 out of 19 unvaccinated by RT-Nested PCR (table. 1). CDV Ag had been detected in all conjunctival swabs by rapid Ag test kit (Anigen Co. South Korea).

Specificity of RT-nested PCR

The expected 777-778bp and 520bp fragments of NP gene were obtained from Ond-CDV strain infected cells but not from cells infected with Alk strain of measles virus.

Also CDV-specificity of the obtained 520bp amplicons confirmed by sequencing and identified NP sequences are available in GenBank under accession numbers JN941240, JN941241, JN941242, and JN941243.

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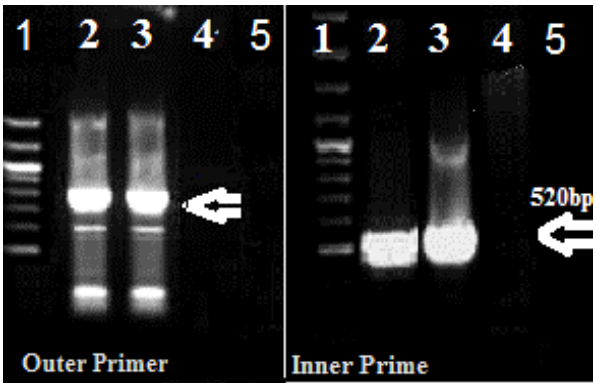


Fig. 1. RT-Nested PCR results on agarose gel (1%) electrophoresis of different samples by outer and inner primer of NP gene: Lanes1: molecular marker (100-bp ladder); lanes 2: CDV Onderstepoort strain; lane 3: Suspected sample; lanes 4: Ultrapure water and 5: Alk strain of measles virus. Numbers on the left are molecular sizes (in base pairs).

Serum neutralization test

11 out of 18 serum samples (61%) of CD-suspected dogs were diagnosed as positive for CDV infection, based on the serum neutralization test. CDV gene was detected by RT-nested PCR in 3 SN negative samples.

Discussion

The expansive signs of CD make it difficult to be easily diagnosed from other respiratory and enteric disease (1). Consequently an early precise detection of CD is of great importance for pertinent and in time treatment and also may lead to quarantine of infected dog to prevent the spread of the virus.

CD is a common disease of unvaccinated dogs in Iran but due to lack of information, little is known about the feature of CD. Avizeh and colleagues reported 17.52% prevalence of CDV antibody in rural dogs, based on IFA, in Iran (15).

In this study RT-PCR, Nested-PCR and SN test were used for comparative diagnosis of CD in samples that were obtained from 19 unvaccinated dogs. This implies the first study on the molecular diagnostic of CDV in Iran.

Due to dissemination of CDV in various organs (Urinary, Gastrointestinal, Respiratory system, and Neurologic system) after entering the body, CDV could be isolated from different kinds of sample tissues and fluids (Whole blood, Serum, conjunctival and Nasal swabs, Urine, and CSF) (7,16).

Among these specimens whole blood and serum samples have been mostly used for CDV detection (5, 7, 13).

In this study maximum obtainable sample of individuals containing whole-blood, conjunctival swab, tissue and serum samples were investigated.

RT-PCR, detected CDV in 3 out of 19 (15%) CD suspected dogs but by Nested-PCR, detection of CDV was (14/19)73%.

Compared with the diagnostic effectiveness of the tow PCR methods, Nested-PCR raised the efficiency of CDV detection up to 58%.

This result was expectable as other authors such as Kim et al. and Jozwik *et al* have been introduced Nested-PCR more sensitive than RT-PCR in CDV detection (8, 9).

In this study presence of NP gene was confirmed in 4 (22%) serum and 5 (27%) whole-blood samples. These range of sensitivity didn't match with previous reports that mentioned by Frisk and colleagues. They detected CDV in 86% serum samples and 88% whole- blood (7). Also Amud and colleagues reported sensitivity of 20% (1/5) and 0% (0/6) when the whole blood and serum, were used respectively as clinical samples for diagnosis of CDV in dogs with neurological disturbances, (17).

They reported that serum and whole blood was not appropriate samples for CDV detection by RT-PCR when the dogs show only neurological disturbance without the characteristic systemic signs of the disease (18).

This disagreement in results of similar studies can be due to the existence of insufficient viral RNA, activity of endogenous RNase, presence of partially degraded RNA, occurrence of neutralizing antibody titer, and utilization of different primers.

Among the 14 distemper-positive dogs, CDV RNA was detected by RT nested-PCR in 66%

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of the kidney tissues, 33% of the lung tissues, 33% of the bladder tissues, and 33% of the intestine tissues. Regarding to small numbers of tissue samples a definite correlation between CDV infection and its distribution in different tissues can't be concluded; however, in this study kidney tissue was the most suitable tissue for CDV detection.

In this study conjunctival swabs (73% positive for CDV detection) was facilitated to earn higher identification rates, than the other specimens (Table 1).

Kim *et al* obtained similar result in their survey which CDV has been identified in conjunctival epithelium before viremia and for a longer period of time (16). Also they have been posed the theory that CDV is not subjected to rapid exclusion by immune system in conjunctiva.

Similarly Shabbir *et al* introduced conjunctival swab the most reliable sample for early detection of CDV by molecular surveys, as compared to nasal swab and plasma samples (18).

Providing adequate specificity and sensitivity is necessary in molecular studies. In this study no amplification was achieved for Alk strain of measles virus, denoting high specificity of the primers that were designed.

Also sequencing of amplifications revealed that the two primer pairs which used in this study were useful and specific for detecting of CDV nucleoprotein gene in samples.

CDV antibody detected in 61% (11/18) serum samples. Dogs number 5, 7, and 14 which were CD positive by Nested-PCR, turned out to be negative by SN test. These findings justify noncontributory role of neutralizing antibody titers for assessing the etiology of the CD diagnosis. On the other hand, neutralizing act against the Ond-CDV strain may not be relevant to neutralizing activity in contradiction of field isolates.

Moreover in sever CDV infected dogs; the antibody titer may become low because of strong immunosuppressive attributes of CDV (19).

Another propensity for impossibility of CDV antibody detection in serum samples, in which the presence of virus was proved by Nested-PCR, can be due to insufficient time for secretion of the antibody against CDV via immune system of individual.

The negative Nested-PCR results were possibly false negative and could be due to the presence of antibody against CDV or the presence of negligible levels of CDV RNA in the samples. Autolytic degradation of CDV RNA due to produced endogenous RNase should be considered as a probable source of false-negative results too.

Our study demonstrated that the use of different body fluids and tissues can increase the sensitivity of RT-PCR and Nested-PCR in CDV detection.

In a similar manner Frisk and colleagues, used whole blood, serum and CSF and reported the same conclusion (7).

Results of the current study have been indicated that RT-PCR plus Nested-PCR is a rapid, sensitive and specific method for diagnosis of CDV, in addition molecular surveys can be a valuable supplement to laboratory methods in case of doubt in false-negative results with SN test.

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