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RESEARCH ARTICLE

Detection of Canine Distemper Virus from Ocular Swab and Blood Samples in Dog by Real-Time RT-PCR Method

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ABSTRACT

CDV is a highly contagious, systemic and viral infection encountered all around the world. This study aimed to investigation of the the prevalence of CDV seen in dogs in Burdur. For this purpose, ocular swab and blood samples were taken from 65 CDV pre-diagnosed dogs that were brought into Mehmet Akif Ersoy University Veterinary Faculty clinics. Real-time RT-PCR method was applied on the subject samples in order to get a laboratory diagnosis of CDV. In total, 42 of these, 16 from blood and 26 from ocular swab, were detected as positive by real-time RT-PCR. Nine of them were detected as positive both in ocular swab and blood samples. In addition, 11 of 42 samples (33 animals) detected as positive by real-time RT-PCR were already known to have been taken from dogs vaccinated against CDV previously. In the study, test results of the samples taken from diseased dogs and their relations with vaccine history were evaluated statistically as well. In the light of these results, it is recommended to use ocular swab samples as materials in diagnosing CDV and examining all animals (including those vaccinated) with clinical findings in terms of this infection so as to confine the disease.
Keywords: Canine distemper virus, epidemiology, Real-time RT-PCR

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Real Time RT-PCR Metodu ile Köpeklerin Oküler Swap ve Kan Örneklerinden Canine Distemper Virusunun Tespiti

ÖZ

Canine distemper virus (CDV- köpek gençlik hastalığı) çok bulaşıcı, sistemik ve dünya genelinde yaygın olarak görülen viral bir enfeksiyondur. Bu çalışma ile Burdur'da köpeklerde görülen CDV'nun yaygınlığının araştırılması hedeflenmiştir. Bu amaçla Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi kliniklerine gelen hasta hayvanlardan CDV ön tanısı konulmuş 65 köpekten oküler swap ve kan örnekleri alındı. Söz konusu örneklere CDV laboratuvar tanısı koymak amacıyla real-time RT-PCR metodu uygulandı. 16 adeti kan, 26 adeti oküler swaptan olmak üzere toplamda 42 tanesi real time-RT-PCR ile pozitif olarak belirlendi. Dokuz adeti hem oküler swapta hemde kan örneklerinde pozitif olarak tespit edildi. Ayrıca real-time RT PCR ile pozitif tespit edilen 42 örneğin (33 hayvan) içerisinden 11 tanesinin daha önceden CDV'ye karşı aşılanmış köpeklerden olduğu bilinmekteydi. Araştırmada hasta köpeklerden alınan örneklerin test sonuçları ve aşı geçmişleri ile ilişkileri istatistiki olarak da değerlendirildi. Bu sonuçlar ışığında, CDV'nun teşhisinde materyal olarak oküler swap örneklerinin kullanılması ve hastalığı sınırlandırmak için klinik bulgu gösteren tüm hayvanların (aşılılar dahil) bu enfeksiyon yönünden incelenmesi tavsiye edilmektedir.
Anahtar Kelimeler: Canine distemper virus, epidemiyoloji, Real-time RT-PCR

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INTRODUCTION

CDV belongs to Morbillivirus type taking part in Paramyxovirinae subfamily of Paramyxoviridae family (ICTV, 2019). This virus is a single-stranded RNA virus frequently causing fatal infections in domestic dogs. Its genome is approximately 15.690 nucleotide (nt) long and its nucleoprotein (NP) consists of six gene areas coding phosphoprotein, matrix, fusion, haemagglutinin (H) and large proteins (Yi et al. 2012). CDV is considered the most important reason for various deaths of wild racoons (Procyonlotor), grey foxes (Urocyoncinereoargenteus) and striped skunks (Mephitismephitis), as well as affecting the species in all terrestrial carnivore families, including Canidae, Felidae, Hyaenidae, Mustelidae, Procyonidae, Ursidae and Viverridae (Roscoe, 1993). The mentioned terrestrial carnivores play an important role in the epidemiology of the disease (Deem et al. 2000).

CDV, a multisystemic disease, has a complex pathogenesis that might cause various pathological findings such as pneumonia, enteritis and encephalitis. While mortality rates differ between species, they also differ within species (Appel and Summers 1995, Weckworth et al. 2020). Virulence of the agent depends on the strain of the virus, individual immune state, immune response and age of the animal. The death rate among young ones is higher than that of adult dogs (Lednicky et al. 2004, Martella et al. 2008).

The incubation period of CDV might vary between 1-4 weeks (Beineke et al. 2009). Initially, no clinical findings are observed in 25%-75% of the infected animals, yet as the disease progresses, neurological symptoms become evident and death may occur (Pope et al. 2016). CDV primarily infects respiratory lymphoid tissues. During the first viremic phase, where high viral replication is seen, a systemic transmission appears thanks to lymphoid tissues, and this causes immunosuppression and fever (Martella et al. 2008, Beineke et al. 2009). The second viremic phase appears 6-9 days following the infection and contains the generalized infection of parenchymal and epithelial cells (Martella et al. 2008, Beineke et al. 2009, Kapil and Yeary 2011). 1-3 weeks after clinical findings appear, neurological findings typically occur (Kapil and Yeary 2011).

Acute infected animals spread the virus in all their body fluids regardless of whether they show clinical symptoms or not (Appel and Summers 1995). The virus remains persistent in neurons, urothelium and footpads and continues to spread during 15-90 days after recovering from acute infection (Martella et al. 2008, Kapil and Yaery 2011, Pope et al. 2016).

The clinical diagnosis of distemper disease seen in dogs is difficult, for it has a wide range of symptoms that might be confused with other respiratory and intestinal diseases. Laboratory confirmation is needed for suspicious cases (Elia et al. 2016). Urine (Saito et al. 2006), stool (Tupler et al. 2012), nasal discharge (Kim et al. 2001), blood (Cho and Park 2005) and cerebrospinal fluid (Frisk et al. 1999) can be used as diagnosis materials. In the diagnosis of the CDV disease, various methods such as electron microscopy (Sun et al. 2010), immunoperoxidase (Soma et al. 2001), ELISA (Suzuki et al. 2015) and PCR (Shin et al. 2004) are used.

In this study, we aimed to detect CDV genome presence by real-time RT-PCR test in ocular swab and blood samples of 65 dogs which were brought into Mehmet Akif Ersoy University Veterinary Faculty clinics and most of which had neurological symptoms such as unilateral paralysis, diplegia in legs, seizures, myoclonus, rhythmic tonic and clonic convulsions.

MATERIAL and METHODS

Ocular swab and blood samples were collected from a total of 65 dogs which were brought into Mehmet Akif Ersoy University Veterinary Faculty animal hospital between 2015-2020 with symptoms such as respiratory disorders, cough, neurological disorders, ocular/nasal flow, hardening of nose and plantar and sent to Virology Department for diagnosis.

Trizol®Reagent (Invitrogen/Life Technologies) kit was used for viral RNA extraction of all samples. The obtained viral nucleic acid was subjected to the following thermal cycle program by Applied Biosystems One Step (ABD) device. A Real-time PCR test was performed by targeting the gene area that codes N protein in terms of CDV (Table 2 and 3). An 83 bp fragment of N gene area could be replicated by primer-probe sequences given in Table 1.

RESULTS

Ocular swab and blood samples were sent for diagnosis, and CDV genome presence from 65 samples of dogs clinically suggestive of CDV was searched by real-time PCR method. At the end of the study, a total of 42 samples, 16 blood and 26 ocular swab samples, were detected as positive by real-time RT-PCR (Figure 1). 9 of these had positive genome presence following RT-PCR, both in blood and ocular swab samples. Furthermore, 11 of 42 samples (33 animals) detected as positive by real-time RT-PCR were already known to have been taken from dogs vaccinated against CDV previously.

As a result of the applied chi-square test, a significant difference was found statistically between the vaccinated and unvaccinated animals ($p<0.05$). Accordingly, it was believed that unvaccinated animals had a higher possibility of catching the disease than vaccinated ones (66.7%) and not a significant difference but vaccinated animals also had the possibility of getting sick (33.3%). (Table 4) According to chi-square test performed among blood and ocular samples, no significant difference could be

detected ($p>0.05$), and when evaluated by percentage, ocular samples showed a higher positivity (40%) than blood samples did (24%).

Table 1. Primer/probe sequences (Elia et al. 2006)

Oligonucleotides Sequences (5'-3')	
CDV forward	AGCTAGTTTCATCTTAACTATCAAATT
CDV reverse	TTAACTCTCCAGAAAACTCATGC
CDV probe	FAM-ACCCAAGAGCCGGATACATAGTTTCAATGC-TAMRA

Table 2. Thermal cycle program of real-time PCR

	Temperature	Duration	
Reverse Transcription	50 °C	10min.	1
RT-Inactivation	95 °C	2min.	1
PCR	95 °C	15sec.	50x
	55 °C	30sec.	

Table 3. Concentrations of reaction mixture

Each tube	Master Mix
12.5 µl	4X qRT-PCR Master Mix (Verso-1 step-Thermo)
1.25 µl	Enzyme Mix
1.25 µl	RT-enhancer
1.5 ul (9 mM)	$MgCl_2$ (25mM)
0.50µl (0.4µM)	Forward Primer (20 µM)
0.75µl (0.6µM)	Reverse Primer (20 µM)
1.25 µl (0.25µM)	Probe (5 µM)
0.5 µl µl (0.25µM) (50 nM)	ROX
3.5 µl	H_2O
3 µl	Template RNA
25 µl	**Total reaction mix**

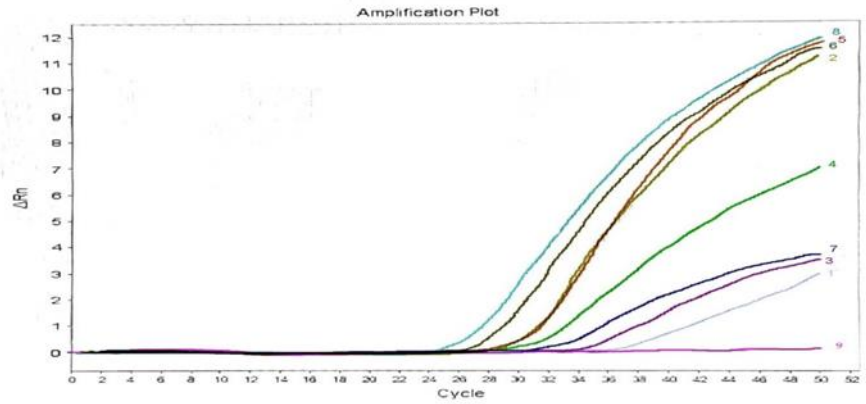

Figure 1: 1,3: Blood sample 2, 4, 5, 6, 7: Ocular swab sample 8: Positive control (MLV vaccine) 9: Negative control Unreal-time PCR results

Table 4. Results of CDV presence by real-time PCR from blood and ocular swab samples of vaccinated and unvaccinated animals.

Sample		**State of vaccination**			
		Vaccinated		**Unvaccinated**	
		n	**%**	**n**	**%**
Blood	**Positive**	3	9,1	13	39,4
	Negative	8	24,2	9	27,3
	Total	11	33,3	22	66,7
Ocular	**Positive**	10	30,3	16	48,5
	Negative	1	3,0	6	18,2
	Total	11	33,3	22	66,7
Statistical values: x^2=9,440 p=0,02					

DISCUSSION

Blood and ocular swab samples collected from 65 dogs suggestive of CDV between 2015-2020 in Burdur province were sent to the virology laboratory for diagnosis. Real-time RT-PCR examined these samples in terms of CDV genome presence, which was detected in 42 samples. 11 of CDV positive samples were reported to belong to vaccinated dogs (according to clinical records). In the studies previously carried out worldwide on this topic, CDV epidemics were reported many times (Laurenson et al. 1998, Nouvellet et al. 2013, Fischer et al. 2016, Ricci et al. 2021). The obtained results were such as to support previous studies. In one of these studies (Budaszewski et al. 2014), CDV positivity was found as 12.2% (19/155) in vaccinated dogs while it was 26.19% (19/155) in our study. Various factors such as suppression of immunity, application of wrong protocols, unsuitable storage conditions, the occurrence of poor immune response and appearance of new antigenic variants capable of escaping from antibodies created by vaccines are among the reasons why vaccinated dogs developed CDV epidemics (Lan et al. 2006). In their study in Brazil, Budaszewski et al. (2014) detected CDV genome presence by PCR test in dogs vaccinated with live vaccine and showing clinical symptoms. The researchers sequenced the obtained isolates and compared them with known CDV vaccine strains. As a result, they revealed that the circulated field strains were different from vaccine strains.

CDV is one of the most important diseases progressing with high morbidity and mortality rates, especially in 3-6-month-old dogs (Feijoo et al. 2019). Although owned dogs are commonly vaccinated, there is no special vaccination program for stray dogs, which constitutes a crucial risk for the disease (Adam et al. 2011). In addition, this study and the previous ones show that CDV cases continuously increase despite vaccination. Therefore, to prevent the spread of the virus and control the number of affected dogs, a timely diagnosis is vital. RT-PCR procedures have recently been developed for fast and reliable diagnosis of CDV (Shin et al. 2004, Yi et al. 2012, Piewbang et al. 2016). In Turkey, its epidemiological presence has been revealed by serological tests such as ELISA (Saltık and Kale 2020) and neutralization tests (Gencay et al. 2004). When we look at the recent studies, even though there are many studies in our country for diagnosing respiratory and digestion system infections (Avci et al. 2016, Aydin et al. 2018, Dinçer 2017, Polat et al. 2019, Timurkan et al. 2018, Timurkan et al. 2021), those about CDV are quite limited (Oğuzoğlu et al. 2018). During these limited amounts of studies, CDV genome presence has been revealed by RT-PCR method. This study is highly significant because that it is one of the first ones in which CDV genome presence has been revealed by real-time PCR.

Although real-time PCR test is an expensive one, it has begun to take the place of conventional PCR since it has a high specificity and sensitivity and requires no process following the reaction; thus the risk of contamination is low (Elia et al. 2006, Pawar et al. 2011). In diagnosing CDV, quite many samples can be taken from animals such as blood (Shin et al. 2004, An et al. 2008), ocular and nasal swab (Saito et al. 2006, An et al. 2008), urine (Shin et al. 2004), vaginal swab (Fischer et al. 2013), lung (Headley et al. 2000, Shin et al. 2004), stomach (Headley et al. 2000), kidney (Namroodi et al. 2013) and bladder (Richter and Motze 1970) tissues. Kim et al. (2006) detected positivity at an important high rate in ocular swab samples of seven dogs infected experimentally. In our study, a higher positivity was also detected similarly in samples collected with an ocular swab (Table 4). They stated that CDV was detected in blood at late stages. Thus blood was not the correct material for early diagnosis, and conjunctiva remained infected for a longer period by being infected earlier than viremia. Furthermore, they associated the reason for this with the exclusion of conjunctiva out of the immune system. Similarly, Shabbir et al. (2011) considered conjunctival swab more reliable than nasal swab and plasma in early diagnosis of CDV. Using a conjunctival swab, Van Drunen et al. (2008) found that the sensitivity and specificity of the immunochromatographic test (IC) was 100% compared to RT-nested-PCR. Lymphocyte and nasal swabs showed low sensitivity and specificity rates. Researchers probably stressed that it was the most suitable sample for early diagnosis of CDV since CDV continuously spread in the eye, which was different from the other samples.

Among the reasons for vaccination failure were various factors such as the quality of the vaccine, the appearing poor immune response and CDV genetic diversity, and the fact that serologically and genetically different variants appeared was considered the basic factor for vaccines not to be able to protect CDV (Martella et al. 2006). Simon- Martinez et al. (2008) stated that H and F proteins, the target proteins in the host immune response, underwent the highest genetic/antigenic changes in CDV. In this study, the fact that vaccinated dogs could also be infected with the disease suggests that gene areas coding H and F proteins of the obtained CDV isolates should be examined in terms of mutations. These isolates should be compared with the existing ones MLV vaccine strains. In this sense, important contributions will be given for developing significant control studies in fighting the disease.

As a result, stray dogs being in the first place, all unvaccinated animals should be considered a potential source of CDV. The disease is mainly characterized by the nervous system and respiratory system symptoms. The infection was also encountered in vaccinated dogs might be ineffective vaccinations and the probability of infection with the

mutant virus. In order to put this forward certainly, advanced phylogenic studies with CDV are needed so that newly circulated variants could be detected and vaccination failures and spreading of the disease could be prevented.

Conflict of Interest: The authors declare that there is no conflict of interest.

Ethical Permission: This study was found in accordance with the principles of the Ethics Committee of Burdur Mehmet Akif Ersoy University with the decision of "Animal Experiments Local Ethics Committee, dated 20.05.2021 and Decision No: 778.

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