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Comparative Detection of Canine Parvovirus by Differential PCR, Conventional PCR and Nested PCR tests: Detecting Antigenic Variants by Differential PCR

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ABSTRACT

Canine parvovirus (CPV) is a pathogen causing hemorrhagic enteritis in puppies and mainly transmitting via feco-oral route. In this study, stool samples were collected from a total of 35 animals suspected of CPV. The samples were examined by Conventional PCR, Nested PCR and Differential PCR tests. 20 out of 35 dogs (57.1%) were detected positive by conventional PCR, 31 (88.6%) by nested PCR and 30 (85.7%) by differential PCR. CPV 2a was stated as the most common antigenic type, male animals and 0–3-month-olds had a high rate of becoming sick and vaccinated animals might also catch the disease, rarely. Accordingly, it is recommended to focus on studies providing molecular epidemiology surveillance in order to detect the existing subtypes and develop reliable diagnosis and vaccination methods.

Türk Tarım – Gıda Bilim ve Teknoloji Dergisi, 10(2): 254-259, 2022

Diferansiyel PCR, Konvansiyonel PCR ve Nested PCR Testleri ile Canine Parvovirus'un Karşılaştırmalı Tespiti: Diferansiyel PCR ile Antijenik Varyantların Tespiti

MAKALE BİLGİSİ

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ÖZ

Canine parvovirus (CPV), yavru köpeklerde hemorajik enterite neden olan ve esas olarak feko-oral yolla bulaşan bir patojendir. Bu çalışmada, CPV'den şüphelenilen toplam 35 hayvandan dışkı örneği toplandı. Örnekler konvansiyonel PCR, Nested-PCR ve Diferansiyel PCR testi ile incelendi. 35 köpekten 20 tanesi (%57,1) konvansiyonel PCR ile 31 tanesi Nested-PCR (%88,6) ile ve 30 tanesi de (%85,7) diferansiyel PCR ile pozitif olarak belirlendi. CPV-2a'nın en yaygın antijenik tip olduğu, erkek hayvanların ve 0-3 aylıkların hastalığa yakalanma oranının yüksek olduğu ve nadir olarak da aşılı hayvanların da hastalığa yakalanabileceği belirlendi. Bu doğrultuda mevcut subtipleri tespit etmek, güvenilir teşhis ve aşı yöntemleri geliştirmek amacıyla moleküler epidemiyoloji sürveyansı sağlayan çalışmalara ağırlık verilmesi tavsiye edilmektedir.

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Introduction

Canine parvovirus (CPV) belongs to the genus Parvovirus under the family Parvoviridae. It has a linear single stranded (SS) negative sense DNA. Its genetic material with a length of 5.3 kb. contains two open reading frames (ORFs). The first one of these codes two non-structural proteins (NS 1 and NS 2) and the second one codes two structural proteins (VP 1 and VP 2). During CPV-2 infection, digestion of VP2 by the host protease causes VP3 protein to form (Xu et al., 2015). 54 VP2 copies and 6 VP1 copies code the viral capsid (Vihinen-Ranta et al., 2002). The main capsid protein, VP2 also determines viral tissue tropism and host range (Hueffer et al., 2003; Nelson et al., 2007). A pleiotropic nuclear phosphoprotein, NS1 is responsible for viral replication and inducing cell apoptosis (Saxena et al., 2013; Gupta et al., 2016). It is a worldwide crucial disease-causing severe enteritis and systemic diseases in dogs. The virus is genetically related with feline pan leukopenia virus (FPLV), mink enteritis virus (MEV) and blue fox parvovirus (BFPV) (Özkul et al., 2002).

CPV-2 infection was first diagnosed during 1970s. Thanks to the evolution it underwent from those times to our day, it was divided into three types as CPV-2a, CPV-2b and CPV-2c (Decaro ve Buonavoglia 2012, Zhao et al., 2017). CPV-2a is swiftly able to transform into other variants genetically and antigenically. If 426th protein of VP2 is Asp, it becomes CPV-2b and if it is Glu, it becomes CPV-2c (Buanavoglia et al., 2001). In some cases, dogs might be infected together with more than one virus strain (Vieira et al., 2008). The occurring genetic variations cause difficulties for the antigen to be selected in order to be used in both vaccination and diagnosis tests (Shackelton et al., 2005). Moreover, the presence of maternal antibodies might hinder the expected return from the vaccines. For this reason, diagnosis is of vital importance in CPV-2 prophylaxis (Nandi & Kumar, 2010).

Clinical symptoms appear following an incubation period of 3-7 days and are mainly characterized by vomiting, hemorrhagic diarrhea, fever, dehydration and myocarditis in non-vaccinated dogs (Miranda et al., 2015).

In order to diagnose CPV, there are various methods such as cell culture (Parthiban et al., 2011), haemagglutination (HA) (Castro et al., 2010), haemagglutination inhibition (HI) (Khatri et al., 2017), electron microscope (EM) (Li et al., 2019), indirect fluorescent test (IFT) (Li et al., 2019) and ELISA (Proksch et al., 2015). However, these methods are considered not sensitive enough (Decaro & Buonavoglia, 2012). For this reason, molecular methods with high sensitivity and specifity are commonly used in routine diagnosis (Kowalczyk & Jakubczak, 2016). Since genetic material of CPV-2 can be detected in early stages of the infection, modifications such as PCR and Real time PCR (Kaur et al., 2016), multiplex PCR (Parthiban et al., 2010) and nested PCR (Jian et al., 2012) are prominent methods.

The aim of this study is to detect the sensitivity of conventional PCR and differential PCR compared to nested PCR and to reveal the prevalence of CPV strains.

Material and Methods

Collecting Samples

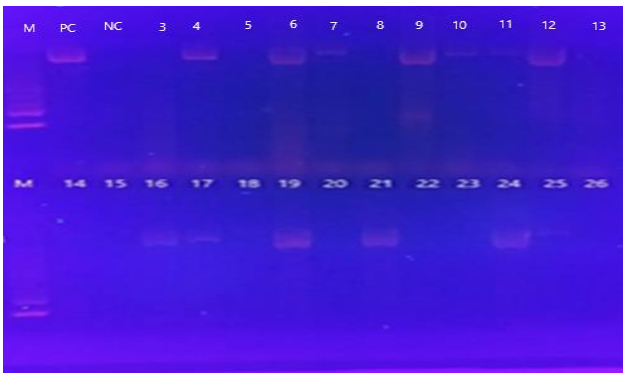
Around Burdur region, stool samples were collected from 35 different race and gender vaccinated and non-vaccinated puppies of 1-20 months old which were brought into Burdur Mehmet Akif Ersoy University Veterinary Faculty Animal Hospital Clinics.

Extraction Process

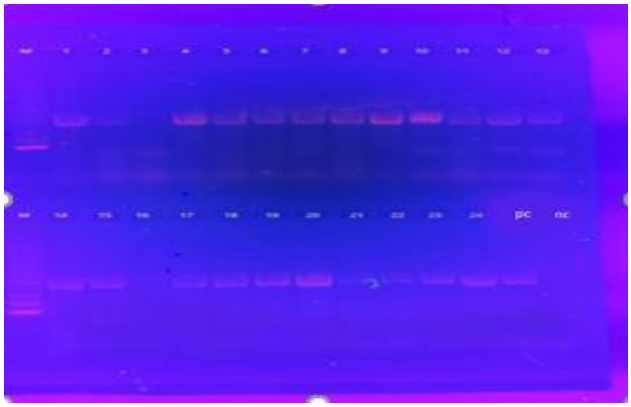
After the stool samples were mixed and crushed at the rate of 1/10 in PSB with 10x antibiotics, they were centrifuged at 3000 rpm for 20 minutes. The extraction of the obtained supernatant was carried out according to the protocol determined by the producing company (Virus Nucleic Acid Isolation Kit, GeneDireX, Taiwan). The DNA extraction products obtained at the end were kept at -20°C until they were used in PCR tests.

PCR

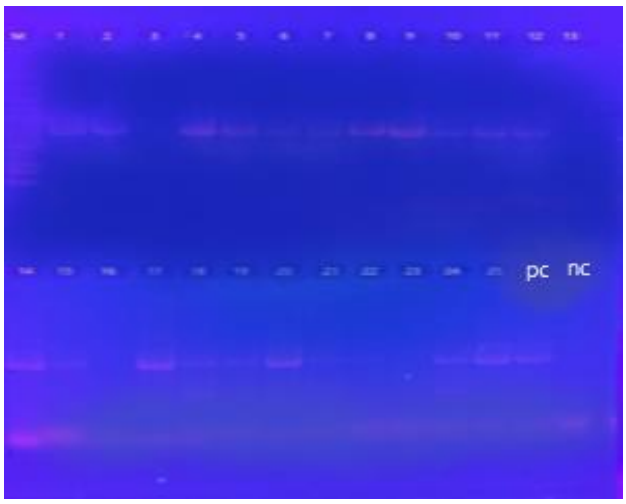
Primers published by Mizak and Rzezutka (1999) were selected for PCR (Table 1). 15µl DNA, 5µ l10x PCR buffer (with 15mM MgCl2), 1µl forward and reverse primer (25 pm/µl)1µl dNTPs mix (10 mM), 0.5µl of MgCl2(50 mM), 1 U *Taq* DNA polymerase reaction mixture was prepared and completed with nuclease free water to 50µl. After pre-denaturation stage at 95°C for 2 minutes, the reaction was subjected to thermal cycler at 35 cycles for various processes such as denaturation for 20 seconds at 95°C, binding for 10 seconds at 55°C, stretching for 1 minute at 72°C and final stretching for 5 minutes at 72°C.



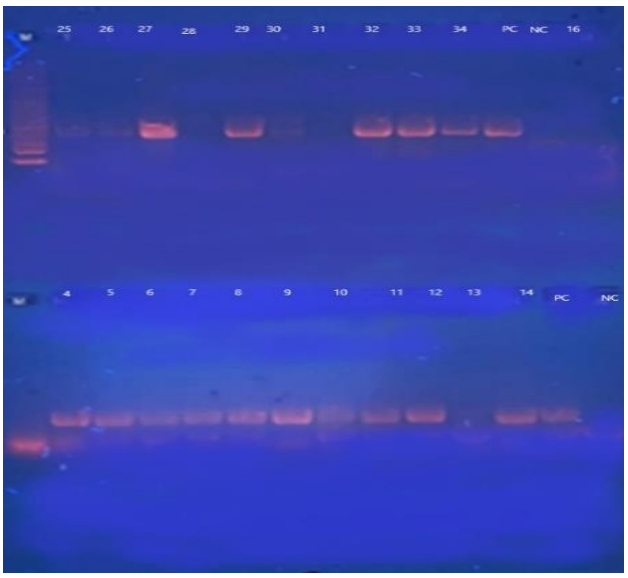
Picture 1. Conventional PCR image. M: 1kb Marker, PC: positive control, NC: negative control and samples



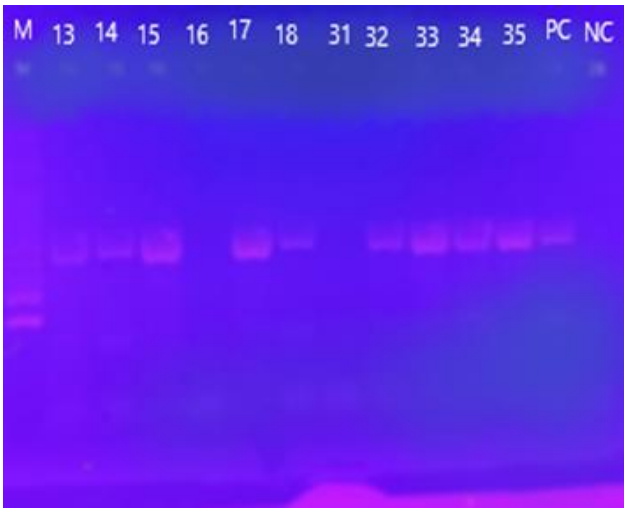
Picture 2. Nested PCR image. M: 100bp Marker, PC: positive control, NC: negative control and samples



Picture 3. CPV-2 PCR image. M: 100bp Marker, PC: positive control, NC: negative control and samples



Picture 4. Top row CPV-2a PCR image. M: 100bp Marker, PC: positive control, NC: negative control and samples. Bottom row CPV-2b PCR image. M: 100bp Marker, PC: positive control, NC: negative control and samples



Picture 5. CPV-2c PCR image. M: 100bp Marker, PC: positive control, NC: negative control and samples

Nested PCR

Primers published by Kumar et al. (2011) were used for nested PCR (Table 1). After pre-denaturation stage, thermal cycle program was carried out as 20 seconds at 95°C, 10 seconds at 60°C, 1 minute final at 72°C and 5 minutes at 72°C (Table 2). A reaction mixture was prepared by adding 12.5µl Mastermix (2X) Taq DNA polymerase 1µl forward and reverse primer (25pmol/µl) from 2µl 1. Reaction PCR product and the final volume was completed by nuclease free water as 25µl. Both in PCR and in nested PCR, a healthy dog was used as negative control and the vaccine virus as positive control.

Differential PCR

In order to detect various antigenic variants, separate PCR reactions for CPV-2, CPV-2a, CPV-2b, CPV-2c were created by using differential primers (Table 1) published by Kumar et al. (2011). For this, the reaction mixture was completed with nuclease free water as 2µl DNA, 1µl forward and reverse primer (10 pmol/µl), Mastermix (2XAmpMaster^{TM} containing 10 µl Taq DNA Polimerase, GeneAll, Korea) and 20µl final volume.

Thermal cycle conditions were the same for each test except for annealing temperature heat (Table 2). 10 µl Ethidium Bromide (EtBr-Sigma, Germany) for 100 ml. was added into 1.5% agarose gel (Sigma, Germany) thawed in 0,5xTAE (Triacetate – Ethylene Diamine Tetra Acetic Acid) buffer. Agarose gel was placed into electrophoresis tank (Serva Electrophoresis Gm BH, Heidelberg, Carl-Benz, Blue Marine 100, Germany). As for 100 basepair (bp) DNA ladder (Solis Bio Dyne 100 bp DNA Ladder, Tartu Estonia), after PCR products including positive and negative controls were placed, power source (Serva, Blue Power Plus, Germany) was set to 100V and 50 mA and samples were ran. The gel was examined under transilluminator (UVP Inc., Upland CA, USA) and bands developed following PCR were evaluated.

Statistical Analysis

Nested PCR was determined as the golden standard and its results were compared with conventional and differential PCR. The effect of age and vaccine states on CPV was also evaluated. In order to detect the effect of gender and vaccine states via SPSS 25.0, Mann Whitney-U test was applied, and to detect the effect of age range, Kruskal Wallis test was performed. Values smaller than $P<0.05$ were accepted as statistically significant. Besides, the frequency values were given in Table 3 in detail.

Results

In the present study, samples were taken from 35 dogs with CPV clinical findings and 20 samples (57.1%) by PCR with 1198bp product size, 31 (88.6%) samples by nested PCR with 442bp product size and 30 (85.7%) samples by differential PCR that detects more than one variant were found positive in terms of CPV (Table 3). When nested PCR test and conventional PCR test were analysed by both Mann Whitney U and chi square tests, no statistically significant difference was observed ($P>0.05$). When nested PCR was compared with differential PCR and analysed by both Mann Whitney U and chi square tests, a statistically significant difference was found ($P<0,05$).

Table 1. Primers for PCR, Nested PCR and Differential PCR

Test	Primer	Sequence	PS. (bp)	AT. (℃)	GR.
PCR	CPV-FP	5'AGCTATGAGATCTGAGACAT-3'	1198	55	VP2
	CPV-RP	5'AGTATGTTAATATAATTTTCTAGGTGC-3'			
Nested-PCR	CPV-FP	'TGAGCTGCATTTAGTTTTGA-3	442	50	VP2
	CPV-RP	5'TGTTTGCCATGTATGTGTTAGTCT-3'			
Differential PCR	CPV-2FP	5'-CTGCTACTCAGCCACCAACT-3'	719	59	VP2
	CPV-2-RP	5'-AGGTGTTTCTCCTGTTGTGGT-3'			
	CPV-2a FP	5'-AGAGCATTGGGCTTACCACC-3'	379	60	VP2
	CPV-2a RP	5'-ATCTTCCTGTATCTTGATGTGCT-3'			
	CPV-2b FP	5'-TGTATTGCTACCAACAGATCCA-3'	178	59	VP2
	CPV-2b FP	5'-TGGTGCATTTACATGAAGTCTTGG-3'			
	CPV-2c FP	5'-GTGGTTCTGGGGGTGTGG-3'	470	60	VP2
	CPV-2c RP	5'-AGCTGCTGGAGTAAATGGCA-3'			

PS: Product Size, AT: Annealing Temperature, GR: Gene Region

Table 2. Thermal cylicling condition

	Temperature	Time	
Conventional PCR	95°C	2 min	1
	95°C	20 sec	
	55°C	10 sec	X 35
	72°C	1 min	
	72°C	5 min	1
	Temperature	Time	
Nested PCR	95°C	2 min	1
	95°C	20 sec	
	60°C	10 sec	X 35
	72°C	1 min	
	72°C	5 min	

When positive data was examined according to Nested PCR, 17 (48.6%) of these were found 0-3 months-old, 8 (22.9%) were found 3-6 months old and 6 (17.1%) were found 9 months old and over. Similarly, when differential PCR was examined, 16 (45.7%) out of 30 positive ones were 0-3 months old, 8 (22.9%) were 3-6 months old, none (0%) was 6-9 months old and 6 (17.1%) were 9 months old and over (Table 3).

When gender state between the ones determined by nested PCR as positive was evaluated, there was no statistically insignificant difference following Mann Whitney U test (P>0,05), however out of 31 animals, 20 (57.1%) were male and 11 (31.4%) were female (Table 2). As for the data observed following differential PCR, there was no statistically significant difference following Mann Whitney U test (P>0,05) and 19 (54.3%) were male and 11 (31.4%) were female (Table 3).

When vaccination state of positive ones by nested PCR was evaluated, there was a statistically significant difference following Mann Whitney U test (P<0,05) and 5 (14.3%) were vaccinated and 26 (74.3%) were non-vaccinated. Out of those determined as positive following differential PCR, a statistically significant difference was observed (P<0,05) and 4 (11.4%) were vaccinated and 26 (74.3%) were non-vaccinated (Table 3).

In the present study, subtypes of CPV-2, CPV-2a, CPV-2b and CPV-2c were detected using differential primers (gel electrophoresis image number).

Out of 30 samples found positive by differential PCR, 30 (100%) were found positive for CPV-2a, 27 (90%) for CPV-2b and 29 (96.6%) for CPV-2c. Therefore, the most commonly observed antigenic types were CPV-2a and CPV-2c, respectively. When we looked at the rate of having more than one antigenic type in the same animal, CPV-2a and CPV-2c were detected in 29 (96.6%), CPV-2a and CPV-2b in 27 (90%) and all variants (CPV-2, CPV-2a, CPV-2b, CPV-2c) in 26 (86.6%) (Table 3).

Discussion

CPV-2 is an enteric pathogen commonly seen in dogs worldwide (Marulappa and Kapil, 2009). Clinical, haematological and histopathological features of CPV-2 strains (CPV-2a, CPV-2b and CPV-2c) were revealed by studies conducted (Decaro et al., 2007; Moon et al., 2008). Between 1979 and 1982, CPV-2 began to leave its place to CPV-2a in the USA, Australia, Belgium, Denmark, France and Japan (Parrish et al., 1988). On the other hand, CPV-2b, appeared in 1984 and later took the place of CPV-2a in many countries (Pereira et al., 2000). CPV-2a and CPV-2b might substitute each other when two single-nucleotide polymorphisms (SNPs) in the viral capsid change places. The fact that Asn or Asp amino acid existed in 426th position of VP2 protein is responsible of this change. Moreover, that Ile or Val amino acid existed in 555th position might cause this (Parrish et al., 1991). There are studies stating that the prevalence of CPV-2b (Ahmed ve ark 2012), CPV-2a (Clark ve ark 2018, Giraldo-Ramirez ve ark 2020) or CPV-2c were denser (Lin et al., 2017; de Oliviera et al., 2020). In Turkey, 2a (Yilmaz et al., 2005, Timurkan ve Oğuzoğlu 2015, Karapinar ve ark 2018) and 2b (Yilmaz et al., 2005, Karapinar et al., 2018, Timurkan et al., 2019) variants were stated commonly.

Table 3. Description of samples and Results of PCR, Nested PCR, Differential PCR

Sample No	PCR	Nested-PCR	Differential PCR				Age(ay)	Sex	Vaccination status
			2	2a	2b	2c			
1	+	+	+	+	+	+	20	M	-
2	-	+	+	+	+	+	3.5	M	-
3	-	-	-	-	-	-	2	F	+
4	+	+	+	+	+	+	2	M	-
5	-	+	+	+	+	+	15	M	-
6	+	+	+	+	+	+	4	M	-
7	+	+	+	+	+	+	2	F	-
8	-	+	+	+	+	+	4	F	-
9	+	+	+	+	+	+	15	M	-
10	+	+	+	+	+	+	2	F	-
11	+	+	+	+	+	+	3.5	M	-
12	+	+	+	+	+	+	20	M	-
13	-	+	-	+	-	+	3	F	+
14	-	+	+	+	+	+	2	F	+
15	-	+	+	+	+	+	2	M	-
16	+	-	-	-	-	-	3	M	+
17	+	+	+	+	+	+	5	F	-
18	-	+	+	+	+	+	2	M	-
19	+	+	+	+	+	+	2	M	-
20	-	+	+	+	+	+	15	F	-
21	+	+	+	+	+	+	18	M	-
22	-	+	-	+	-	+	2	M	+
23	-	+	-	+	-	+	1	M	-
24	+	+	+	+	+	+	4	F	-
25	+	+	+	+	+	+	2	F	-
26	-	+	-	+	+	-	2	M	-
27	-	+	+	+	+	+	4	M	-
28	+	-	-	-	-	-	15	M	+
29	+	+	+	+	+	+	3	M	-
30	+	+	-	-	-	-	2	M	+
31	-	-	-	-	-	-	3.5	M	+
32	-	+	+	+	+	+	3.5	M	+
33	+	+	+	+	+	+	2	M	-
34	+	+	+	+	+	+	2.5	F	-
35	+	+	+	+	+	+	1	M	-
Total	20	31	26	30	27	29			

In this study, parallel to the results of previous studies, CPV-2a and CPV-2b strains were densely detected and in addition to this, presence of CPV-2c strain at the rate of 96.6% was also found.

In the study, while 20 positive (57.1%) cases were detected by conventional PCR, 31 (88.6%) were found by nested PCR (Table 3). This state might explain that nested PCR was 100 times more sensitive. Nested PCR has the ability to detect swiftly and correctly even the virion rarely existing in the sample as 10^6particle/gram. Therefore, it might be helpful for diagnosis of subclinical infections in the early stages, for preventing and controlling the disease (Kumar et al., 2011). Out of 31 animals found positive by nested PCR, 20 (57.1%) were male and 11 (31.4%) were female (Table 3). In their studies, Aktaş et al. (2011) and Karapınar et al. (2018) stated that male dogs had a higher rate of catching the disease compared to female ones. Houston et al. (1996) stated that this condition might be because male dogs were more exposed to infective agents since they had a higher tendency to stroll around.

This study shows that CPV is mostly observed in puppies under 1 year old. The results are similar to the studies carried out previously (Sakulwira et al., 2003; Yılmaz et al., 2007; Pinto et al., 2012). The fact that the infection was detected most in dogs of 0-3 months old was confirmed by many studies carried out on this topic (Mittal et al., 2014; Duijvestijn et al., 2016; De la Torre et al., 2018). As stated by Decaro & Buonavoglia (2012), the reason why adult animals were greatly resistant to CPV might be considered as decreased sensitivity because of age and immunity caused by vaccination or previous infections.

The possible presence of viral co-infections could be important for us to understand the epidemiology and the universal dynamics of CPV-2 subtypes. When an infection occurs with more than one circulated pathogen, faulty results might be observed if PCR and other diagnosis tests are more sensitive to one of the strains (Clark et al., 2016; Barbosa et al., 2017). While co-infections by more than one CPV-2 subtype were not commonly reported, they were stated by Perez et al. (2014). New generation sequencing methods make it possible to correctly and sensitively detect the presence of pathogens circulated together (Parker et al., 2017). These techniques are especially suitable for CPV-2

studies as the recombination between the variants infected together is considered one of the possible mechanisms of the viral diversity and the occurrence of new genotypes (Perez et al., 2014; Miranda & Thompson, 2016). For absolute typing of the samples detected in the present study in line with the points above, sequencing process and molecular characterization is planned to be carried out. As a result, in order to detect the occurrence of new variants and to develop reliable diagnosis and vaccination methods, studies providing molecular epidemiology surveillance should be focused on.

Ethical Approval

The ethics committee report of this study was obtained from Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee (Authorization number: 781)

Conflict of İnterest

The authors declare that there is no conflict of interest for this study.

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