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Research Paper

Molecular Characterization of Canine Parvovirus in Iran, 2023

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ABSTRACT

Introduction: Canine parvo virus 2 causes severe and often fatal gastroenteritis and myocarditis in dogs and puppies. Based on the *VP2* gene, this virus is classified into 3 variants: CPV-2a, CPV-2b, and CPV-2c.

Materials & Methods: In present study, 35 rectal swab samples were collected from dogs with clinical signs, including vomiting and diarrhea, and cases with positive results from the rapid test kit. Samples were screened with polymerase chain reaction (PCR) assay to detect the presence of the virus genome. According to the PCR results, all samples were positive.
Results: Out of 35 cases, about 34% received at least one dose of vaccine, and almost 66% were not vaccinated at all. A rapid test was also performed for 34 cases; it was positive for 91% (31 cases) and negative for 9% (3 cases). Phylogenetic analysis of seven samples, which were submitted for sequencing, revealed that 6 of the present isolates (UT-CPV14 to UT-CPV18 and UT-CPV20) were clustered with CPV-2c isolates, and one (UT-CPV19) was clustered with CPV-2b sequences. Homology analysis indicated high similarity (100%) between isolates (UT-CPV14 to UT-CPV18 and UT-CPV20) and isolates K20172c-1, 12B, IZSSI_2021PA43108idAki, BJ001, and CPV-2c/Sull6/2017. UT-CPV19 showed 100% similarity with isolates 19R113-2, YANJI-2, and 15D184. In the present study, we also phylogenetically analyzed a commercial vaccine. Although the homology results indicated almost 98% similarity between the current isolates and the vaccine, the vaccine sequence did not cluster with any groups in the phylogenetic tree.

Conclusion: These results highlight the importance of constantly monitoring antigenic changes of circulating strains and the efficacy of vaccines against them.

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1. Introduction

As in other countries, the number of people willing to keep pets, especially dogs and cats, has increased in recent years in Iran [1]. Like other mammals, their immune system is responsible for ensuring health, well-being, and longevity, as well as protecting them from external invaders such as infectious agents [2]. Among infectious agents infecting canines, CPV-2, and its variants are considered the most common pathogens distributed universally [3]. Despite comprehensive vaccination, canine parvovirus type 2 (CPV-2) remains an ongoing cause of highly contagious and fatal gastroenteritis, particularly in puppies in Iran and the rest of the world [4-7].

The disease is mainly transmitted through fecal-oral route. After infection, it takes 3–7 days incubation period before the onset of clinical signs. Viruses replicate in lymph nodes, subsequently, many viral particles are released into the bloodstream and enter the gastrointestinal tract, where they destroy intestinal cells and form intranuclear inclusion bodies [8]. CPV-2 is a non-enveloped icosahedral virus, approximately 25 nm in diameter, containing a linear single-strand DNA [9]. The genome includes two major open reading frames that encode two non-structural proteins (NS1 and NS2) and two capsid proteins (VP1 and VP2). The VP2 protein is considered responsible for virus's antigenic properties, characterizing the virus host range and tissue tropism [10]. It seems that canine parvovirus is derived from panleukopenia virus due to specific mutations in the capsid protein VP2, which facilitated the host change, permitted the virus to infect canines, and led to lose the ability to infect felines [11]. CPV-2 was identified in 1987 and spread globally between 1978 and 1979. During the 80s, the accumulation of mutations in the original virus (CPV2), which was circulating globally, led to the emergence of two antigenic subtypes named CPV-2a and CPV-2b; in 2000, an additional antigenic subtypes, CPV-2c was identified [4, 12, 13]. According to the previous studies, the genetic difference between the original CPV-2 and the antigenic variants CPV-2a and CPV-2b is determined by five to six amino acid of VP2 protein, including 87, 101, 297, 300, 305, and 426 residues [4]. Furthermore,the differed in residue 426, with types 2a, 2b, and 2c displaying Asn, Asp, and Glu, respectively [14].

Although killed vaccines against CPV-2 are available and can provoke antibody response, prevention of canine parvovirus is mainly achieved through vaccination with modified live vaccines (MLV). These are known to stimulate both antibody- and cell-mediated immune responses, resulting in strong, long-lasting protection against virulent viruses [7, 15, 16]. Despite vaccination, CPV remains one of the major reasons for puppy death [7]. The most common reason for this vaccination failure is the interference with maternally derived antibodies, which are transferred to puppies through colostrum, placenta, and milk, preventing the onset of immunity [16]. In addition to inappropriate vaccination schedules regarding the persistence of maternal immunity,the vaccination of non-responders, and, even more importantly, circulation of different antigenic variants of the virus [7, 16]. Nowadays, the original CPV-2 exists only in commercial vaccines,while other subtypes (CPV-2a, CPV-2b, CPV-2c) are distributed throughout the world canine population [17]. In Iran, Hematzadeh and Jamshidi first isolated CPV using the MDCK cell line and electron microscopy in 2002 for the first time [18]. The first molecular epidemiology study of CPV in Iran was conducted by Askari Firoozjaii et al. in 2011, which showed the presence of CPV-2a and CPV-2b subtypes in collected samples [19]. According to some previous studies in Iran, CPV-2a and CPV-2b are the predominant circulating subtypes in Iran, while CPV-2c been reported with a lower frequency [4, 6, 19-21].

Since all antigenic subtypes are present and circulating among the dog population in Iran, constant monitoring is necessary to determine the predominant antigenic type. The aim of the present study was to perform a phylogenetic analysis of CPV isolated from clinical cases and to update previous phylogenetic data reported about CPV in Iran.

2. Material and Methods

2.1. Sample collection

Thirty-five samples were collected from small animal clinics in Tehran, Iran, between July and October 2023. Fecal swabs were collected from dogs aged 2-12 months that presented with clinical signs, including vomiting and diarrhea, or cases with positive results from a rapid immunochromatography Antigen test kit (AniGen, Seoul, Korea). The samples were transferred to -20 ˚C. Information on each case (including ages, vaccination situation, and rapid test results) is mentioned in Table 1.

2.2. DNA extraction and polymerase chain reaction (PCR)

Total DNA was extracted from rectal samples and a commercial live attenuated vaccine (Himmvac® DHPPL

vaccine, Korea) using the SinaPure Oneviral nucleic acid extraction mini kit (Sinaclon Co., Iran) according to the manufacturer's instructions [22]. A polymerase chain reaction (PCR) method using a specific primer pair (CPVF2: AAAAAGAGACAATCTTGCACCA and CPVR2: TGAACATCATCTGGATCTGTACC) was applied to amplify a part of *VP2* gene to confirm the presence of CPV-2 by amplification of a 747 bp fragment of the CPV viral genome [23-25]. The thermal cycling condition was carried out as follows: initial denaturation step at 94 ˚C for 10 minutes, followed by 35 cycles of 94 ˚C for 30 s, 55 ˚C for 60 s, and 72 ˚C for 60 s, with a final extension step at 72 ˚C for 10 min. The PCR product was analyzed by electrophoresis on agarose gel (1.5%) stained with ethidium bromide.

2.3. Sequencing and phylogenetic analysis

Among all positive samples, 7 were submitted for sequencing by Codon Genetic Company (Tehran, Iran) using the Sanger sequencing method. The sequences were primarily evaluated using BLAST online tool [26], and their quality was subsequently checked with Finch TV software version 1.4.0. Following this, sequences were edited and trimmed using MEGA 7 software. Phylogenetic analysis was performed by MEGA 7 software using the Maximum Likelihood method based on the general time reversible model [27]. For phylogenetic analysis, a dataset comprising 26 nucleotide sequences representing all three genotypes of canine parvovirus (CPV-2a, CPV-2b, CPV-2c) was included. The reliability of the phylogenetic tree was estimated using the bootstrap method with 1000 replicates. The sequences were submitted to the GenBank and are available under the following accession numbers: OQ025284, PP471790, PP471791, PP471792, PP471793, PP471794, and PP471795.

3. Results

3.1. PCR results

All 35 samples tested positive by PCR test. Out of 35 cases in this study, almost 34% (12 cases) received at least one dose of vaccine, while 66% (23 cases) were not vaccinated at all. The CPV rapid detection kit was applied to 34 cases; the rapid test was positive for 91% (31 cases) and negative for 9% (3 cases).

3.2. Phylogenetic analysis

BLAST results revealed that all seven sequences were related to canine parvovirus. Phylogenetic analysis of sequences indicated that only one isolate (UT-CPV19) belonged to CPV-2b genotype (14.3%), while the other sequenced clinical isolates (UT-CPV14 to UT-CPV18 and UT-CPV20) belonged to CPV-2c genotype (85.7%) (Figure 1). Sequences are available in GeneBank under accession numbers OQ025284, PP471790, PP471791, PP471792, PP471793, PP471794, and PP471795. Homology analysis (Table 2) revealed that UT-CPV-14, UT-CPV15, UT-CPV16, UT-CPV17, UT-CPV18, and UT-CPV20 had 100% similarity with isolates K20172c-1 (South Korea, 2017), 12B (Iran, 2021), IZSSI_2021PA43108idAki (Italy, 2021), BJ001 (China, 2019), CPV-2c/Sul6/2017 (Iraq, 2017) and 99.85% similarity with isolate CPV/dog/HCM/20/2013 (Indonesia, 2013). Analysis of the UT-CPV19 showed 100% similarity with isolates 19R113-2 (South Korea, 2019), YANJI-2 (China, 2014), 15D184 (South Korea, 2015), and LONGJING-1 (China, 2015). According to the homology results,the isolates in the present study (UT-CPV14 to UT-CPV20) showed almost 98% similarity with the vaccine strain used for.

4. Discussion

The disease caused by CPV-2, which can cause severe hemorrhagic enteritis in dogs, was primarily recognized in 1978 in the USA. It subsequently spread among throughout the global dog population with high morbidity and frequent mortality [28]. The nucleotide sequence of the gene encoding for VP2 protein, the main determinator of viral host range and tropism, is used to classify of CPV-2 into three genotypes, CPV-2a, CPV-2b, CPV-2c [29-32]. In a study by Faraji et al. (2023), analysis of all positive collected samples based on *VP2* gene showed that they all belong to the CPV-2a genotype. Their phylodynamic results also indicate that this genotype primarily emerged in central Iran, especially in Alborz Province, and the results of mutational analysis indicate a positive selection pressure of CPV-2a genotype [20]. In a study conducted by Nikbakht et al., 50 fecal samples were collected and evaluated for the presence of CPV using different specific primers, which were selected from different regions of the VP2 gene [6].

According to the results of this study, 18 samples were characterized as CPV-2a genotypes and 32 samples were classified as 2b genotypes [6]. In another study by Saei et al., 35 stool samples were collected from healthy and diarrheic dogs. Using specific primers for *VP2* gene, ten samples were positive for CPV. Further analysis showed that only 1 was classified as CPV-2c genotypes, while the others were categorized as CPV-2a and CPV-2b. The results of this study indicate that CPV-2b genotype is

Table 1. Information on cases involved in the present study

Case Number	Age (m)	Vaccination Situation	Rapid Test Results
1	2	No vaccine	Positive
2	2	No vaccine	Positive
3	2	No vaccine	Positive
4	2	No vaccine	Positive
5	2	No vaccine	Positive
6	2	No vaccine	Positive
7	2	No vaccine	Positive
8	2	No vaccine	Not applied
9	3	1 dose	Positive
10	3	1 dose	Positive
11	3	No vaccine	Positive
12	3	No vaccine	Positive
13	3	No vaccine	Positive
14	3	No vaccine	Positive
15	3	1 dose	Positive
16	3	1 dose	Positive
17	3	No vaccine	Positive
18	3	No vaccine	Positive
19	3	No vaccine	Negative
20	3	3 doses	Positive
21	3	No vaccine	Positive
22	3	No vaccine	Positive
23	4	No vaccine	Negative
24	4	No vaccine	Positive
25	4	1 dose	Positive
26	4	1 dose	Positive
27	4	3 doses	Positive
28	5	1 dose	Positive
29	5	No vaccine	Positive
30	6	1 dose	Negative
31	6	No vaccine	Positive
32	6	2 doses	Positive
33	6	3 doses	Positive
34	7	No vaccine	Positive
35	12	No vaccine	Positive

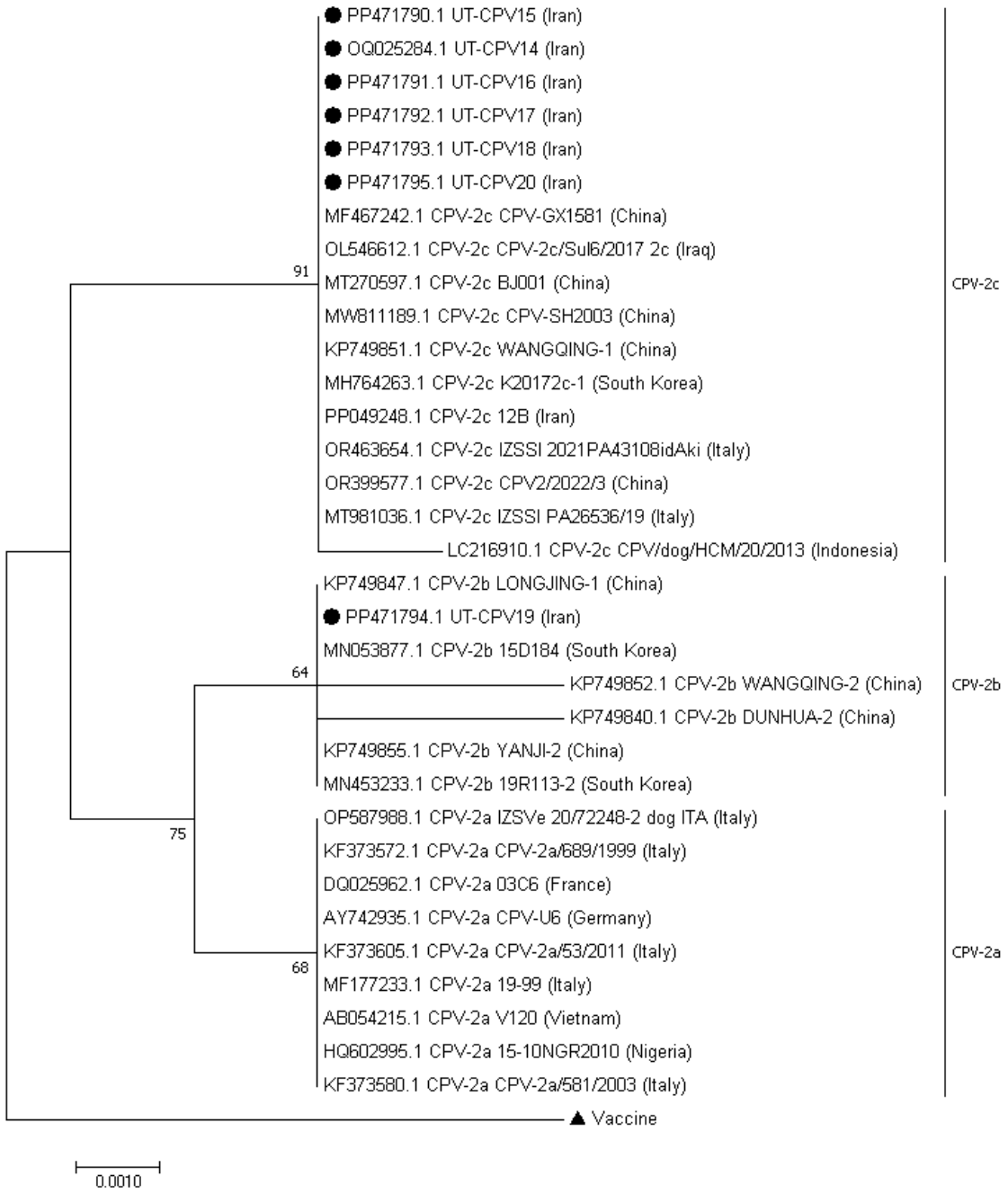

Figure 1. Molecular genetic analysis based on *VP2* gene by using the maximum liklihood method and general time-reversible model

Note: The tree was generated by comparison of the present sequence with 26 other sequences retrieved from NCBI GeneBank. According to the tree out of 7 sequenced samples, 6 were clustered with CPV-2c types, and 1 was clustered with CPV-2b types. Isolates in the present study are marked with black circles, and the vaccine used in the current study is marked with black triangles.

predominant genotype in Northwest Iran, while the two other genotypes also affect dogs [21]. In Another study by Ghajari et al., according to the phylogenetic analysis results based on the *VP2* gene, CPV-2a was predominant among positive samples (50%), followed by CPV-2c (32.1%) and CPV-2b (17.8%) [4]. In another study, Askari Firoozjaii et al., using primers selected from variable regions in VP1/VP2 capsid genes, revealed that out of 44 cases, 39 samples were CPV-2a and 5 were CPV-2b. This study was the first study to confirm the presence of CPV in Iran [19].

Table 2. Nucleotide sequence variation for canine parvovirus virus VP2 segment of clinical samples and vaccine in the present study compared with previous sequences retrieved from NCBI GeneBanck

No.	Isolate Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	UT-CPV14 (OQ025284.1)																			
2	UT-CPV15 (PP471790.1)	100																		
3	UT-CPV16 (PP471791.1)	100	100																	
4	UT-CPV17 (PP471792.1)	100	100	100																
5	UT-CPV18 (PP471793.1)	100	100	100	100															
6	UT-CPV20 (PP471795.1)	100	100	100	100	100														
7	K20172c-1 (MH764263.1)	100	100	100	100	100	100													
8	12B (PP049248.1)	100	100	100	100	100	100	100												
9	IZSSI_2021PA43108idAki (OR463654.1)	100	100	100	100	100	100	100	100											
10	BJ001 (MT270597.1)	100	100	100	100	100	100	100	100	100										
11	CPV-2c/Sul6/2017 (OL546612.1)	100	100	100	100	100	100	100	100	100	100									
12	CPV/dog/HCM/20/2013 (LC216910.1)	99.85	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9								
13	UT-CPV19 (PP471794.1)	99.41	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.3							
14	19R113-2 (MN453233.1)	99.41	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.3	100						
15	YANJI-2 (KP749855.1)	99.41	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.3	100	100					
16	15D184_(MN053877.1)	99.41	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.3	100	100	100				
17	LONGJING-1 (KP749847.1)	99.41	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.3	100	100	100	100			
18	19-99 (MF177233.1)	99.41	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.3	99.7	99.7	99.7	99.7	99.7		
19	CPV-2a/581/2003(KF373580.1)	99.41	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.3	99.7	99.7	99.7	99.7	99.7	100	
20	Vaccine	98.95	98.4	98.4	98.4	98.4	98.4	98.4	98.4	98.4	98.4	98.4	98.2	98.4	98.4	98.4	98.4	98.4	98.4	98.4

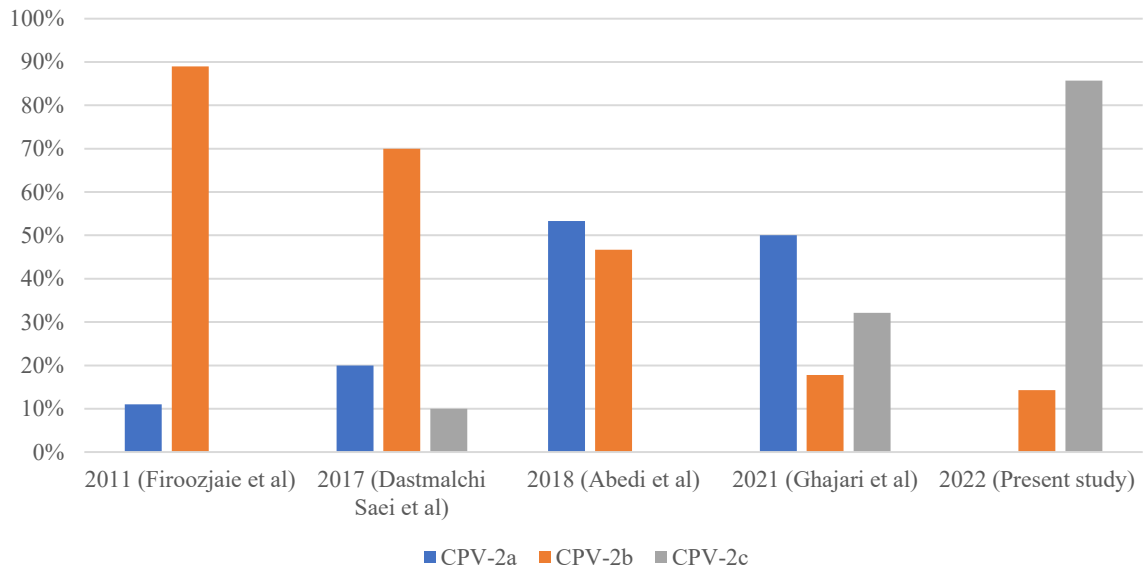

Figure 2. Distribution of CPV-2 genotypes in Iran in different years, based on previous studies

In another study by Abedi et al., out of 60 CPV positive samples, 32(53.3%) bolnged to CPV-2a and 28(46.7%) to CPV-2b [33]. According to previous studies, the prevalence of CPV-2b and 2a subtypes seems to be higher than the other one. However, the present study shows a high prevalence of CPV-2c genotype among collected samples (85.7%) and only one sequenced sample was CPV-2b (14.3%). Altogether, these results indicate that all 3 genotypes of CPV are circulating in Iran. Phylogenetic analysis also revealed that all CPV-2c detected in this study are clustered with isolates from China, Iraq, South Korea, Iran, Indonesia, and Italy. The CPV-2b isolates in the present study are located near other isolates from China and South Korea. No CPV-2a was identified in the present study.

Comparing the distribution of CPV-2 genotypes over different years based on previous reports suggests that the prevalence of CPV-2b has decresed over time, while the prevalence of CPV-2c has increased (Figure 2). This phenomenon may be due to this fact that most commercial vaccines used for immunization against CPV in puppies contain CPV-2b genotypes. Vaccination is considered an effective and the main tool in preventing disease; however, despite its use, different cases of CPV occur, and reports of vaccination failure are documented [10, 34]. One of the major causes of vaccination failure is the interference of maternally derived antibody, and vaccination age is also a significant risk factor for this phenomenon [34]. Common vaccines against CPV are made using the original CPV or CPV-2b variant [16]. Wilson et al., showed that a multivalent vaccine containing the CPV-2b variant could induce a cross-reactive serological response against other field strains like CPV-2a and CPV-2c [35]. Puppies are routinely immunized against CPV in the first months after birth, beginning in the 6-8 weeks, repeating in 3-4 weeks intervals, finishing around 16 weeks, following an annual vaccination [36]. Maternal-derived antibodies against CPV disappear linearly after birth, and their half-life is about 9-10 days. In most puppies, maternal-derived antibodies are reduced by 8-12 weeks of age to a level that allows vaccination, and it has been reported that maternal-derived antibodies will completely diminish by 10-14 weeks of age [37].

Administering the final vaccine dose to puppies younger than 16 weeks, when maternally derived antibodies interference may still impede the development of active immunity, might be one of the major causes of vaccination failure [15]. In a study by Yip et al., the antigen test was positive for 41.2% of vaccinated and 73.2% of unvaccinated diseases dogs [15].

Molecular assays also were positive for 82.4% of vaccinated dogs and 92.7% of unvaccinated dogs [15]. In another study by Singh et al., the molecular results revealed that 75.9% of samples belonged to unvaccinated and 24.1% of samples belonged to vaccinated dogs [38]. Based on the results of the present study, 65.7% of positive cases weren't vaccinated, and 34.3% received at least one dose of vaccine. Among the vaccinated dogs in present study, only 3 cases (25%) were fully vaccinated with three doses of vaccine, while the remaining 9(75%)

had only received one or two doses. These results remark that, in addition to other mentioned reasons, incomplete vaccination can also pose the animal at a higher risk for CPV disease.

Age is also considered a risk factor. Although dogs can be infected at any age, puppies younger than 6 months are more susceptible [34]. In a study by Sayed-Ahmed et al., dogs aged 0-3 months showed the highest prevalence of CPV (68%), followed by 4-6 months (53.3%), while the lowest prevalence was observed in dogs over 6 months (20%) [39]. In another study by Tagorti, out of 54 CPV-infected cases, 70.37% were between 1-3 months and 26.63% were above 3 months [40]. In a study by Behera et al., the results of the age-wise prevalence study indicated that the infection is higher in age group 3-6 (41.37%) than 1-3 months (27.59%), 6-12 months (27.59%) and above 12 months (3.45%) [41]. In the current study, among studied cases, 22 cases were 2 and 3 months (62.86%), 7 cases were 4 and 5 months (20%), 5 cases were 6 and 7 months (14.28%) and only one case was 12 months (2.86%). A comparison of the results of the current study with those of the previously mentioned studies can indicate that puppies younger than 6 months are more susceptible to CPV than those above 6 months.

Although clinical presentations are valuable for diagnosing CPV, this kind of diagnosis is not definitive since different pathogens can cause diarrhea in dogs; therefore detection must always be confirmed via a laboratory test. Immunochromatographic (IC)-based rapid test kits are advantageous because of their lower price and ease of use. However, the efficacies of these rapid test kits are often considered insufficient [42]. In a study by Tinky et al., a PCR test identified 44% of the samples as positive, while an IC strip test showed 36% of samples were positive [42]. In another study performed by Mohyedini et al., the ability of IC test to detect CPV infection in 50 PCR-positive samples was evaluated. Out of 50 samples, the IC test detect CPV in 42 samples (84%) [43]. In the present study, the PCR test showed that 100% of cases were positive for CPV, while the result of the rapid test kit presented that 91% of cases were positive for CPV and showed 9% of cases as negative. This result indicates the importance of molecular tests alongside rapid test kits, particularly when clinical cases represent CPV signs but the rapid tests are negative. In Iran, different commercial vaccines are used for immunization of puppies against CPV. These vaccines are live attenuated multivalant vaccines, which can be used for immunization against other disease such as Distemper, Hepatitis, Parainfluenza, Leptospira, Laryngoteracheaitis and Tracheobronchitis. Some of these vaccines include HIPRADOG 7®, produced by the HIPRA company, which contains canine parvovirus 2c genotype strain C-780916, Biocan® Novel DHPPI, produced by Bioveta company, which contains the CPV-2b strain of canine parvovirus, Nobivac® DHPPi produced by MSD company, which contains strain C154 of canine parvovirus, CANVAC® produced by DYNTEC company, which contains T-86 strain of canine parvovirus, and Himmvac®, produced by the KBNP company, which was used in present study.

5. Conclusion

In the present study, we performed a phylogenetic analysis of CPV isolated from clinical cases. Results revealed that 6 samples out of 7 were considered as genotype CPV-2c, while the other isolates were characterized as CPV-2b, and no CPV-2a were isolated. Constant monitoring of canine parvovirus and assessment of the efficacy of available vaccines is strongly recommended to identify probable mutations that may affect vaccine-induced immunity. In addition, whole genome sequencing of circulating parvovirus will be helpful in this propose.

Compliance with ethical guidelines

We declare that all ethical standards related to animal health and welfare have been respected in present study.

Data availability

The data that support the findings of this study are available upon request from the corresponding author.

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Authors' contributions

Conceptualization, study design, and supervision: Arash Ghalyanchilangeroudi; Sample collection: Shabnam Babazadeh, Arian Abbassioun; Experiments, data analysis and interpretation: Zahra Ziafati Kafi and Soroush Sarmadi; Writing: Shabnam Babazadeh, Soroush Sarmadi, Omid Eghbali, Arian Abbassioun, Alireza Bakhshi, and Fahimeh Jamiri.

Conflict of interest

The authors declared no conflict of interest.

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