

# Molecular Epidemiology and Genetic Evolution of Canine Parvovirus Type 2 in Diarrheic Dogs in Serbia From 2008 To 2020

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## Short Report

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# Abstract

Canine Parvovirus 2 (CPV2) is a causal agent of an infectious disease with the highest fatality rate among dogs. However, in Serbia, it has never been investigated thoroughly. This study was conducted on samples originating from dogs with diarrhea in anamnesis, stored in the sample bank, submitted for various reasons to the Institute of Veterinary Medicine of Serbia. In total, 50 rectal swab samples were collected from the period 2008 to 2020, and consequently tested. Out of 50 rectal swab samples, the CPV2 genome was detected in 14 (28%). This retrospective study showed the presence of three different variants of CPV2 in diarrheic dogs during the last 12 years in Serbia. CPV2a was the most prevalent variant (60%) followed by CPV2b (30%), and CPV2c (10%). Interestingly, CPV2a had been the predominantly detected variant up until 2018. Nevertheless in 2019, there was the first detected occurrence of the CPV2b variant, followed by the first detection of the CPV2c in 2020. This study reports the evidence and distribution of CPV2 throughout the time-lapse from 2008 to 2020, providing new information about the presence and the prevalence of virus strains in Serbia.

## Introduction

Canine Parvovirus 2 (CPV2) is a causal agent of an infectious disease with the highest fatality rate among dogs. It causes gastrointestinal illness accompanied by lethargy, high fever, vomiting, and diarrhea which is often bloody, as well as Myocarditis and Myocardial Fibrosis (Ford et al. 2017). Puppies are the most susceptible, but older dogs can also be affected. Though, there are quite effective vaccines, CPV2 infection is still very prevalent, even in vaccinated dogs, which can be explained in plenty reasons. CPV2 is a single-stranded DNA virus but with a high mutation rate, leading to new genetic variants emergence, which is similar rather to RNA viruses than to dsDNA ones (Shackelton et al. 2005). Therefore, since the immune response induced by vaccine strains does not perfectly match with field viruses, the disease can occur in vaccinated animals as well. Vaccine failure can also be linked to maternal antibody interference. Additionally, environmental contamination (Decaro et al. 2005) with CVP2 mustn't be neglected, in particular considering the infection as a current problem worldwide. Since its emergence as a host variant of Feline Parvovirus (FPV) (Decaro and Buonavoglia 2012), different variants of CPV2 have evolved, replacing the original virus. Currently, CPV2a, CPV2b, and CPV2c are the circulating variants, classified according to the VP2 amino acid residues at the 426th position (Buonavoglia et al. 2001). There were many speculations on the pathogenicity of different variants, emphasizing the higher virulence of a particular variant (Spibey et al. 2008; Moon et al. 2008). However, Franzo et al. (2019) have revealed for the first time, the evidence of an association between CPV genetic clusters and disease severity, highlighting the inheritableness of this feature. CPV2 is usually diagnosed by practitioners using rapid tests whereas the typing itself is of limited importance for them unless it is about discrimination between field and vaccine strains. CPV2 typing can be performed by different molecular methods such as genotyping PCR (Pereira et al. 2000), RFLP (Buonavoglia et al. 2001) and MGB real-time PCR (Decaro et al. 2006), whereas each of these has certain limitations. Therefore, for deeper insight into the virus properties, genome sequencing is nowadays the most utilized approach which enables detection of

mutations not only at expected sites, but also at other parts of the genome. Though, in general, vaccines are effective, monitoring for new emerging variants is of utmost importance for the understanding of viral evolution and subsequently the development of new diagnostic tools that will allow their timely detection, together with improvement of vaccines. Considering so far published data from Serbia, there are no reports on CPV2 infection among dogs, neither its prevalence nor epidemiology. There is a big gap in knowing the circulating strains, virus evolution, and its antigenic characteristics. Therefore, this study aims to contribute to scientific knowledge and society on CPV2 molecular epidemiology, analyzing circulating strains in Serbia from 2008 to 2020.

This study was conducted on samples originating from dogs with diarrhea in anamnesis, stored in the sample bank, submitted for various reasons to the Institute of Veterinary Medicine of Serbia. In total, 50 rectal swab samples were collected from the period 2008 to 2020, and consequently tested. Two dogs sampled in 2020 were vaccinated against CPV2, while other dogs were of unknown vaccinal status. Swabs were prepared by immersing into 1 ml PBS. After vigorous vortexing and centrifugation at 4000 rpm for 5 min, the supernatant was subjected to DNA extraction using IndiSpin Pathogen Kit (Indical, Germany) following the Manufacturer`s recommendations. For CPV2 genome detection, amplification of the VP2 gene was employed using the primers published by Buonavoglia et al. (2001). The reaction mixture was composed of 25 µl of DreamTaq HotStart Master Mix (DreamTaq Hot Start PCR Master Mix (2x), Thermo Scientific), 2.5 µl of each 10 mM primers, and 5 µl of DNA template. The reaction mixture was supplemented with PCR pure water up to 50 µl. The VP2 gene amplification was accomplished using Mastercycler, Eppendorf (Germany) and temperature profile as follows: initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s and elongation at 72°C for 45 s, and a final extension of 10 min at 72°C. PCR products were analyzed in a 1.5% agarose gel stained by Ethidium Bromide and visualized under UV light after electrophoresis at 60V for 1 hour. PCR products showing specific amplification of 583 bp were purified using GeneJET PCR Purification Kit (ThermoFisher Scientific) and sequenced at LGC, Germany. The consensus sequences were obtained using the Staden package 2003, while MEGA 6.0 software and algorithm Neighbor-Joining were used for the phylogenetic analysis. Determination of CPV2 biotypes was performed considering the amino acid at the position 426 position, where CPV2a strains contain asparagine, CPV2b aspartic acid, and CPV2c glutamic acid (Buonavoglia et al. 2001). Out of 50 rectal swab samples, the CPV2 genome was detected in 14 (28%). However, good quality sequences were obtained from 10 samples, that were submitted to NCBI gene bank under accession numbers MW382258-MW382267. Analyzing the amino acid sequences at position 426, it was revealed that strains MW382260, MW382262, MW382263, MW382264, MW382265, and MW382266 belonged to the genotype CPV2a, strains MW382258, MW382261, and MW382267 to the genotype CPV2b, while the strain MW382259 was classified as CPV2c. Mutations at position 555 position nor anywhere else within the VP2 sequence were not observed among Serbian isolates. Samples that were inappropriate for typing originated from 2014 and 2016.

Furthermore, CPV2a strains MW382263, MW382264, and MW382265 belong to the specific new CPV2a biotype which is characterized by the substitution of threonine with alanine at position 440 position (Table 1).

The same classification was obtained by constructing the phylogenetic tree (Figure 1) that showed the evident viral evolution of CPV2b through the time frame from 2019 to 2020.

Even though CPV2 appeared decades ago, and effective vaccines are available, it is still one of the most important intestinal pathogens in dogs and puppies.

CPV emerged from feline panleukopenia parvovirus (FPLV) in the 1970s (Shackelton et al. 2005), evolving independently further on. Since its emergence, CPV2 occurrence was reported from 42 countries (Miranda and Thompson 2016).

Canine Parvovirus 2 infection in dogs in Serbia has never been investigated before by molecular methods. The only information about CPV2 infection in Serbia was given by Savić-Jevdenić et al. (2006) who reported the experimental infection with the field virus and the efficacy of particular laboratory assays for the detection of parvovirus infection. Canine Parvovirus infection in dogs in Serbia, as in other countries (Zienius et al. 2016) is mostly diagnosed in veterinary clinics. In general, typing and further investigations are requested only in cases of outbreaks in kennels when vaccine failure is suspected.

After the initial appearance of CPV2, CPV2a and CPV2b variants emerged during the 1980s (Decaro and Buonavoglia 2012), replacing the original virus. The CPV2c variant occurred in Germany in 1996 (Buonavoglia et al. 2001).

This retrospective study showed the presence of three different variants of CPV2 in diarrheic dogs during the last 12 years in Serbia. However, CPV2a was the most prevalent variant (60%) followed by CPV2b (30%), and CPV2c (10%). Interestingly, CPV2a had been the predominantly detected variant up until 2018. Nevertheless in 2019, there was the first detected occurrence of the CPV2b variant, followed by the first detection of the CPV2c in 2020. Reports from the 2010s showed that the CPV2c variant was already predominant in some countries such as Italy, Germany, and Spain. On the contrary, at the same time, CPV2a and CPVb variants were still more prevalent in Portugal, France, Belgium, UK, Greece, and Bulgaria at the same time (Decaro and Buonavoglia 2012) like it was in Serbia according to our results where the CPV2a was also predominant. However, the prevalence of different variants should be interpreted carefully since its strong dependence on the monitoring system of CPV2 (Miranda and Thompson 2016). Similarly, a recent study from Chile revealed that the CPV2c was widely distributed during the period 2016-2017, but due to the limitations in sample size, closer data on its emergence are not available (Castillo et al. 2020). Variant CPV2c was also the most frequent strain of the virus detected in Australian dogs between 2015 and 2019 (Yip et al. 2020). Italian authors, investigating the molecular epidemiology of canine parvovirus type 2 in Italy from 1994 to 2017, were warning on the emergence of new CPV2b which reappeared a long time after its last detection in 1994, having a new and distinctive profile of the VP2 protein (Battilani et al. 2019) which is, as capsid surface protein exposed to immune response, reported to undergo the majority of changes and variability. Serbian CPV2 isolates were typed based on amino acid residue at position 426 position, considering the substitution at this position in all three biotypes. Additional to the position 426, in Serbian isolates, mutations were detected at position 440 position as well. Namely, isolates MW382263, MW382264, and MW382265 at position 440 position have

Alanine, whereas other strains have Threonine. This substitution was found in 50% of all CPV2a detected in this study. Though it is reported that T440A substitution is associated with the viral evolution (Geng et al. 2015) and virus immunity (Li et al. 2017), this biotype has died off and was not detected after 2010. Though there are continuous changes in the nucleotide sequences, no reports are suggesting more or less virulent strains, but rather similar pathogenetic potential which, for the full manifestation, requires particular field conditions (Decaro and Buonavoglia 2012). VP2 sequences of CPV2 strains analyzed in this study showed 100–99.99% nucleotide identities. Serbian CPV2a diverge into 3 clusters – MW382262 groups together with strains from USA and Italy; strains MW382263, MW382264, and MW382265 having substitution at the 440th position, group with the French strain classified as 2a-like. Since there are no available sequences of isolates between 2011 and 2018, we can assume that the isolate MW382266 from 2018 could be related to the isolate MW382260 from 2011. Serbian CPV2b isolates are closely related and clustered together, hypothesizing the evolution of MW382267 from 2020 from CPV2b strains from 2019. Considering the detection of single CPV2c from 2020, there are unknowns about its evolution and relationships with other Serbian strains. According to the nucleotide sequences and phylogenetic study, the two strains obtained from vaccinated dogs (MW382259/Serbia/2020 and MW382261/Serbia/2020), being distant from vaccine strains, imply the infection with a field strain as a result of vaccine failure. Although commercial vaccines are proven as highly protective against all circulating strains, vaccine failure occurs mainly due to the interference with maternal antibodies. However, continuous monitoring of both circulating strains and the emergence of new ones is a critical factor that enables a better understanding of viral evolution and its epidemiology.

This study reports the evidence and distribution of CPV2 throughout the time-lapse from 2008 to 2020, providing new information about the presence and the prevalence of virus strains in Serbia.

## Declarations

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**CONFLICTS OF INTEREST/COMPETING INTERESTS:** The authors declare that they have no conflict of interest.

**DATA AVAILABILITY:** The datasets generated during the current study are available from the corresponding author on request.

**AUTHORS' CONTRIBUTIONS:** *The study was designed by Vesna Milicevic. Material preparation was done by Zorana Zurovac Sapundzic and Milan Ninkovic, data collection and analysis were performed by Bojan Milovanovic, Dimitrije Glisic and Ljubisa Veljovic. The first draft of the manuscript was written by Branislav Kureljusic and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.*

**ETHICS APPROVAL:** Not applicable

**CONSENT TO PARTICIPATE:** Not applicable

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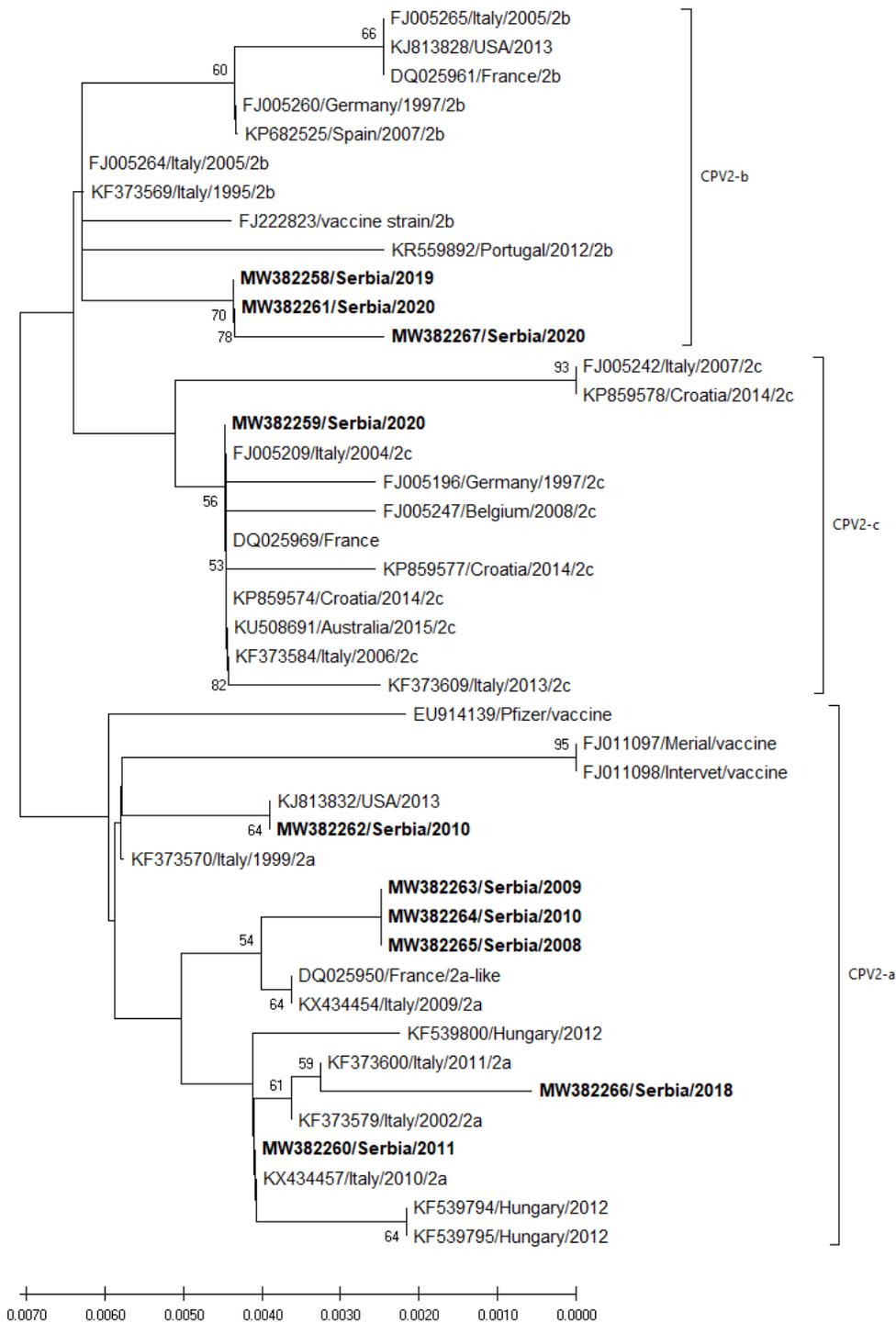
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## Tables

**Table 1.** Amino acid residues and substitutions in the VP2 gene at positions 426 and 440: N – asparagine, D - aspartic acid, E - glutamic acid, T – threonine, A – alanine

Strain	Amino acid residues	
	426	440
CPV2a/KF373600	N	T
CPV2a/KX434454	.	A
CPV2b/KF373569	D	.
CPV2c/KF373584	E	.
MW382267/Serbia/2020	D	.
MW382266/Serbia/2018	.	.
MW382265/Serbia/2008	.	A
MW382264/Serbia/2010	.	A
MW382263/Serbia/2009	.	A
MW382262/Serbia/2010	.	.
MW382261/Serbia/2020	D	.
MW382260/Serbia/2011	.	.
MW382259/Serbia/2020	E	.
MW382258/Serbia/2019	D	.

## Figures



**Figure 1**

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. This analysis involved 43 nucleotide sequences. Evolutionary analyses were conducted in MEGA X.

