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Detection and characterisation of porcine circoviruses in wild boars in northeastern Serbia

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Abstract: The objective was to expand and update the knowledge on the presence and genotype diversity of porcine circoviruses 2 and 3 (PCV2 and PCV3) in the wild boar populations from the hunting grounds in northeastern Serbia. The presence of PCV3 was not determined, and PCV2 was confirmed in 40.32% of the organ samples from 124 wild boars hunted from 2018 to 2019, indicating their significance in virus circulation since traditional pig farms with irregular PCV2 vaccination strategies are widespread in this region. The most prevalent genotype was PCV2d, followed by PCV2b and PCV2a in 55.6%, 38.9%, and 5.5% of the examined samples, respectively. Nucleotide sequences of the detected strains were homogenous within the genotype and clustered within the subgroups PCV2d-2, PCV2b-1A/B, and PCV2a-2D with high identity to European, Chinese, and Serbian domestic pig sequences suggesting their origin. Wild boars presented with no clinical or pathological signs of infection, implying that these animals might be less susceptible to disease, particularly since the cofactors present in pig farming systems that support the disease development are absent in the wild. The high PCV2 detection frequency demonstrates the importance of wildlife monitoring to track virus population dynamics, especially in regions with free-range pig farming in order to plan adequate disease control strategies.

Keywords: PCR; PCV2; PCV3; phylogenetic analysis; sequencing; *Sus scrofa*

Porcine circoviruses (PCV) belong to the genus *Circovirus* in the *Circoviridae* family, and represent small, non-enveloped viruses with single-stranded circular DNA containing two open reading frames (ORF): ORF1 encoding proteins associated with viral replication, and ORF2 which

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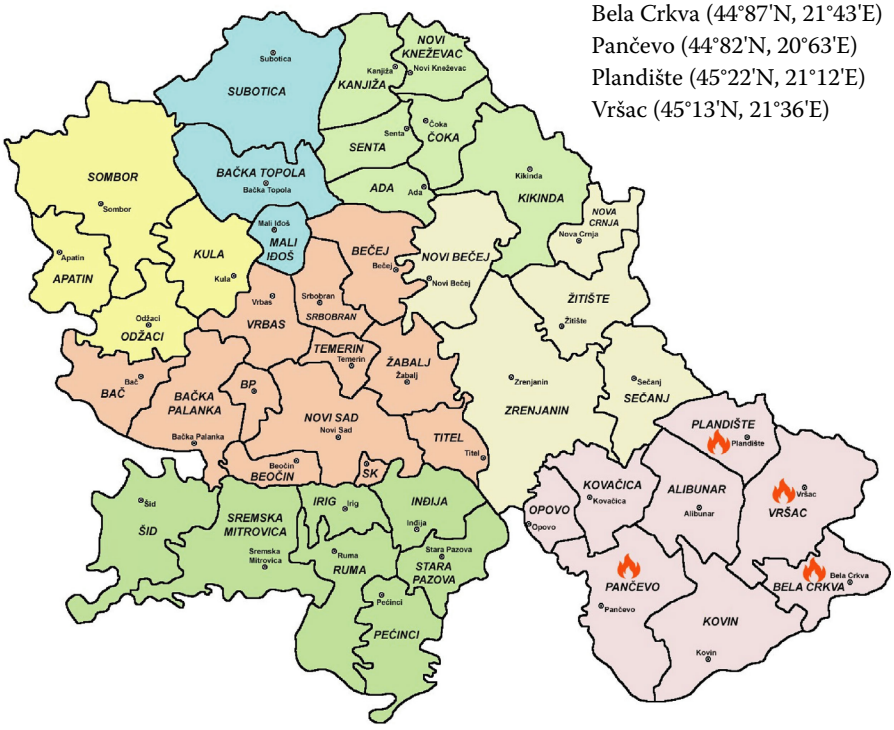
encodes the capsid protein (Segales et al. 2019). Porcine circovirus 2 (PCV2) is connected to the porcine-circovirus-associated disease (PCVAD), including the post-weaning multisystemic wasting syndrome (PMWS) (Savic et al. 2012; Segales et al. 2019). Porcine circovirus 2 is ubiquitous in domestic pigs worldwide and leads to significant economic losses in the pig industry, while contact with wild boars is a contributing factor for spreading the virus (Maioli et al. 2011; Turcitu et al. 2011; Segales et al. 2019). Porcine circovirus 3 (PCV3) has been identified recently in domestic and wild pigs, and its pathogenic potential is unclear (Franzo et al. 2018; Klaumann et al. 2019). The genetic heterogeneity of PCV2 isolates conditioned their separation into eight distinct genotypes, i.e., PCV2a through PCV2h (Franzo and Segales 2018). Two major genotypic shifts took place recently, namely from PCV2a to PCV2b in the beginning of the 2000s, and from PCV2b to PCV2d around 2013 (Xiao et al. 2015; Franzo et al. 2016; Franzo and Segales 2018). Furthermore, PCV2a is divided into four clusters, 2A to 2D, and PCV2b into clusters 1A to 1C, although the strains assigned as PCV2b-1C were reclassified within the PCV2d genotype, further subdivided into PCV2d-1 and PCV2d-2 (Olvera et al. 2007; Xiao et al. 2015). PCVAD outbreaks have been described in Serbia, and PCV2b strains are considered dominant in domestic pigs and wild boars (Savic et al. 2012; Toplak et al. 2012). Due to constant genetic changes occurring as a result of both the nature of the virus and the vaccination influence, PCV2 strain monitoring is considered important (Xiao et al. 2015; Franzo and Segales 2018; Weissenbacher-Lang et al. 2020). However, no recent updates on the PCV2 genetic characteristics are available in our country and wild boar populations in Serbia have never been the main focus of studies. Since traditional pig farming is widespread, the information gathered from wild animal populations may contribute to the tracking of the currently circulating PCV2 strains. This study aimed to update the information on the genetic characteristics of the PCV2 strains in the wild boar population in the hunting grounds of northeastern Serbia and to determine the PCV3 presence as the basis for more extensive studies.

MATERIAL AND METHODS

Samples

Pooled samples of lymph nodes, spleens, and tonsils from 124 shot free-living adult wild boars were collected during 2018/2019 in four hunting ranges in the South Banat district in Vojvodina, northeastern Serbia (Figure 1).



Bela Crkva (44°87'N, 21°43'E)
Pančevo (44°82'N, 20°63'E)
Plandište (45°22'N, 21°12'E)
Vršac (45°13'N, 21°36'E)

Figure 1. Map showing the hunting grounds in the South Banat district in which PCV2-positive wild boars were captured from 2018 to 2019

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The territory covered by this examination is 3 623 km^2. The animals had no clinical or pathological findings of the PCV2-related disease. All the samples were homogenised in phosphate buffered saline (PBS 7.2), centrifuged for 10 min at 1 677 × *g*, and the DNA was extracted using a GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, USA).

PCV2 and PCV3 detection

The detection of the PCV2 *ORF1* and *ORF2* genes, and PCV3 *ORF1* gene were previously reported (Castro et al. 2012; Franzo et al. 2018). The PCV2 strain Stoon 1010 obtained from The Scientific Veterinary Institute "Novi Sad" from Novi Sad, Serbia, and an internal reference PCV3 strain from the Department of Microbiology of the Faculty of Veterinary Medicine, University of Belgrade served as the controls.

DNA sequencing and phylogenetic analysis

The PCV2-positive polymerase chain reaction (PCR) products were purified using a QIAquick Purification Kit (Qiagen, Chatsworth, CA, USA) and prepared with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and PCR primers.

The partial *ORF1* and *ORF2* PCV2 gene segments were sequenced using the Sanger sequencing method with the following protocol: 96 °C for 2 min, 40 cycles of denaturation at 96 °C (10 s), primer annealing at 50 °C (5 s) and elongation at 60 °C (4 minutes). The sequencing was conducted using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The PCV2 nucleotide sequences were compared with PCV2 sequences from the GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence analyses were performed using the MEGA software v7 (Kumar et al. 2016) and the phylogenetic tree was constructed using the neighbour joining algorithm and the Kimura 2-parameter model with 1 000 bootstrap replicates.

The porcine circovirus 1 (PCV1) sequence (FJ-475129) was chosen as the outgroup, and genotype and cluster representative strains were selected (Olvera et al. 2007; Segales et al. 2008; Xiao et al. 2015; Franzo and Segales 2018). The evolutionary distances were determined by the maximum composite likelihood method.

RESULTS

Polymerase chain reaction (PCR)

The PCR analysis showed PCV2 DNA in the samples from 50 animals (40.32%), while no animals were tested positive for PCV3. The highest number of positive animals was detected in Vrsac and Plandiste, followed by Bela Crkva and Pancevo. The hunting grounds of Vrsac, Plandiste, and Bela Crkva represent one geographical entirety (Figure 1). Wild boars roam and cross large distances (from 15 km to 100 km), as depicted by these results.

Sequence analysis and phylogeny

The sequences of the selected eighteen PCV2 strains were submitted to the GenBank under the accession numbers MW550042–MW550059 and their overall similarities ranged from 94% to 100%. The nucleotide similarities of the detected strains and analogous sequences of the representatives of the different PCV2 genotypes varied from 93% to 100%.

The phylogenetic analysis revealed that ten strains (55.6%) belong to PCV2d, seven strains (38.9%) belong to PCV2b, and one strain belongs to the PCV2a genotype (5.5%). The detected PCV2a strain clustered with the PCV2a-2D strains, the PCV2b strains grouped within the cluster 1A/B, and the PCV2d strains clustered with the PCV2d-2 sequences (Figure 2).

The domestic PCV2d strains were 100% similar to each other and were 99–100% when compared to foreign PCV2d-2 strains from Hungary, Italy, and China. The detected PCV2b strains were 99% to 100% homologous between each other and 98–100% in comparison to the PCV2b-1A and 1B representatives from Serbia, Croatia, Slovakia, Italy, Austria, France, the Netherlands, and China. The single PCV2a strain detected in this examination was 100% similar to the PCV2a-2D strain representatives from Germany, Hungary, Austria and France clustering together on the phylogenetic tree.

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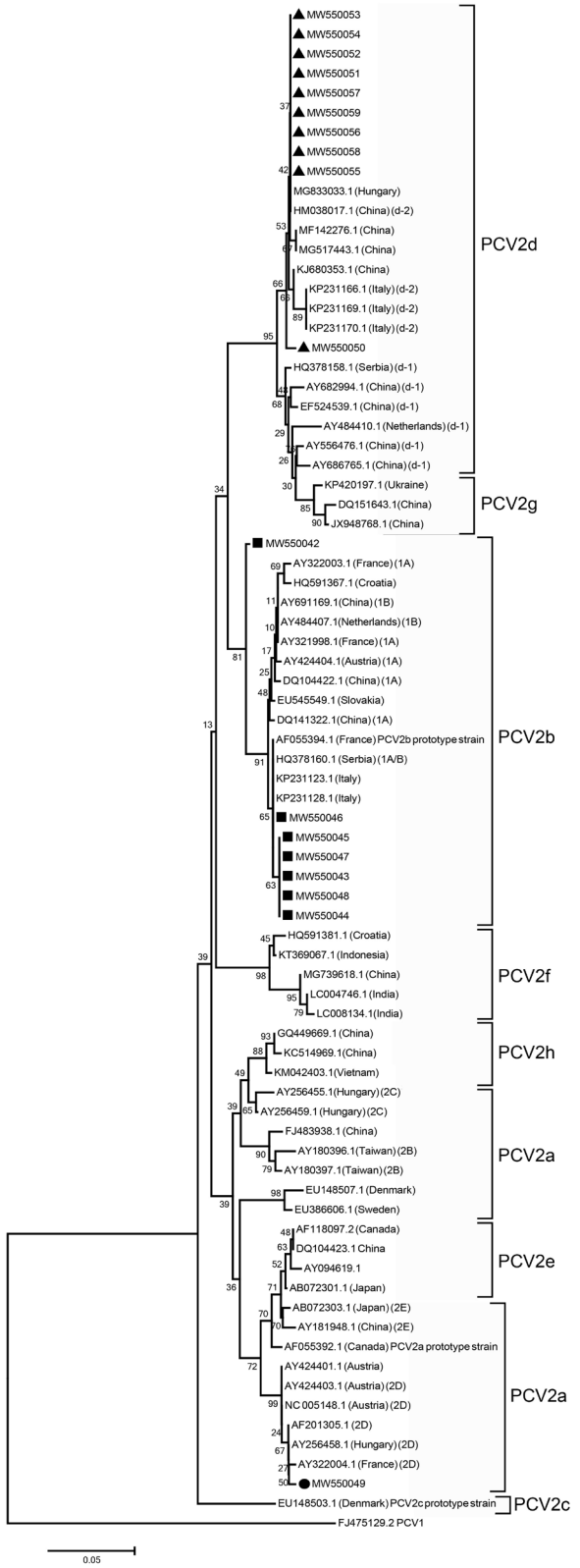

Figure 2. Neighbour-joining tree constructed from the sequences of the present study (MW550042–MW550059) and 60 PCV2 sequences of different genotypes

The sequences from this study are marked by a black triangle (PCV2d), square (PCV2b), and circle (PCV2a)

DISCUSSION

This examination aimed to determine the presence and diversity of PCV2 and PCV3 in the wild boar population in northeastern Serbia. Wild boars are susceptible to PCV3 infections with high prevalence rates (Klaumann et al. 2019), however, we failed to detect its presence. The PCR protocol used in this examination targets the *ORF1* gene as the most conserved region of the viral genome, while the *ORF2* gene, as the most variable and suitable for PCV3 phylogenetic analysis, was excluded for the sample screening (Franzo et al. 2018; Klaumann et al. 2018; Franzo et al. 2020a). Adult wild boars are more often PCV3-positive than younger animals, however, our study shows no specific correlation concerning adults (Klaumann et al. 2019). Different PCV3 circulation patterns, along with the limited sampling area and a relatively low sample number, might all have affected the results of our examination, suggesting a possibility of a false-negative PCV3 prevalence in the examined wild boar population (Tan et al. 2020; Wozniak et al. 2020). Nevertheless, the detected high PCV2 infection frequency suggests, that wild animals might serve as a disease reservoir (Turcitu et al. 2011; Fabisiak et al. 2012). The prevalence of PCV2 infections in the wild boar population varies between countries and among different regions of the same country. Maioli et al. (2011) detected PCV2 DNA in 4.3% of the wild boars hunted in Italy and noted PCV2-positive animals in areas with high domestic pig populations, indicating a possible virus transmission event. Moreover, some studies suggest that the domestic pig PCV2 spills over to wild boar populations (Reiner et al. 2011; Franzo et al. 2020b). The discrepancy in the PCV2 prevalence as a consequence of the different pig farming methods was noted in Romania, showing higher prevalence rates in the wild boar population from areas with traditionally reared pigs (Turcitu et al. 2011; Cadar et al. 2012). Our results support this theory since the pig production in the South Banat district is mostly free-range, contributing to the close contact with wild boars. Porcine circovirus 2 infections of domestic pigs have been previously described in different regions throughout Serbia, however, studies on a larger number of wild boars are not available (Savic et al. 2012; Toplak et al. 2012). Our study revealed the dominance of the PCV2b genotype over the PCV2a one, correspond-

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ing to recent examinations (Dei Giudici et al. 2019; Weissenbacher-Lang et al. 2020). Similar to our results, Song et al. (2020) detected PCV2a in only two out of 91 PCV2 positive wild boar samples. It is regarded that PCV2a was replaced due to vaccination and that PCV2b and PCV2d consequently emerged under selection pressure (Xiao et al. 2015; Weissenbacher-Lang et al. 2020). According to the available data, the dominant genotype in Serbia is PCV2b which is exclusively associated with the occurrence of PMWS in domestic pigs (Savic et al. 2012; Toplak et al. 2012). Nevertheless, our results demonstrate the presence of this genotype in wild boars with no clinical or pathological findings of the PCV2-related disease (Toplak et al. 2012).

Domestic pig PCV2a strains detected in Serbia cluster with 2D representative sequences from Germany and Hungary (Savic et al. 2012). The single PCV2a-2D strain detected in this examination clustered with the PCV2a-2D strains from Germany, Hungary, Austria and France, confirming the low diversity of these strains circulating in Serbia. Similarly, wild boar PCV2a-2D sequences from neighbouring Romanian cluster with analogous European strains (Cadar et al. 2012).

Our results verify the ongoing circulation of PCV2b strains with a high genetic identity among domestic pigs and wild boars (Savic et al. 2012; Toplak et al. 2012). Similarly, the examination of Austrian PCV2b sequences from sixteen consecutive years indicated a low nucleotide diversity within the genotype (Weissenbacher-Lang et al. 2020). Differently, Dei Giudici et al. (2019) demonstrated Sardinian PCV2b strains in different clusters, linking this to different virus introduction events, with a separate Sardinian cluster as a consequence of the local virus evolution. Serbian wild boar PCV2b-1A/B strains grouped with domestic pig PCV2b-1A/B strains previously detected in Serbia and mostly European strains due to the geographical characteristics of Serbia as a common animal trade route between Europe and the East.

Savic et al. (2012) stated that PCV2b-1C is emerging in Serbia, however, these strains were subsequently classified into the PCV2d genotype (Xiao et al. 2015). Our results demonstrate the dominance of PCV2d which corresponds to the global PCV2 genotypic shift, supported by the virus spread in the population through international trade (Song et al. 2020; Weissenbacher-Lang et al. 2020). Moreover, the high PCV2d detection rate in wild boars is possibly linked to an irregular PCV2 vaccination method in small traditional farms (Xiao et al. 2015; Franzo et al. 2020b; Weissenbacher-Lang et al. 2020).

Wild boar PCV2d strains from Serbia clustered with PCV2d-2 strains from Hungary, Italy, and China sharing 99–100% nucleotide sequence similarities. Accordingly, the PCV2d-2 strains that are commonly found in Italy are thought to originate from China (Franzo et al. 2015). Strains identified from 1999 to 2011 mostly belong to the PCV2d-1 cluster, while more recent strains associate within the PCV2d-2 cluster (Xiao et al. 2015). The reclassified PCV2d strain detected in Serbia by Savic et al. (2012) clustered away from the strains in our study along with other PCV2d-1 representatives. Our samples have been collected during the period of 2018 and 2019 as opposed to Savic et al. (2012) who analysed samples from 2009 and 2010. Taking this into account, the above-mentioned dominance of the PCV2d-2 strains over the PCV2d-1 ones in Serbia can only be assumed since there were no recent studies concerning PCV2 diversity in our country. Correspondingly, Dei Giudici et al. (2019) indicate the presence of the PCV2d-2 genotype infecting both domestic and wild pigs in Italy expecting it to become prevalent over time.

Our results confirm the high PCV2 infection frequency in wild boar populations that may serve as disease reservoirs, as opposed to PCV3 which was not detected. Free-range pig production with irregular vaccination procedures is common in Serbia, implying that domestic pigs might be the primary infection source for the wild boar population. However, wild boars might be less susceptible to the disease than domestic pigs, especially since the contributing factors for disease development in domestic pig farming are not present in the wild. Our results correspond to the global PCV2 genotype shift since most of the detected strains belong to the recently acknowledged PCV2d-2 genotype. This paper represents an update on the presence and heterogeneity of PCV2 strains circulating among wild boars in Serbia and serves as a basis for more extensive investigations. Constant monitoring is required to comprehend the PCV2 population dynamics in Serbia in order to develop appropriate strategies necessary for disease control. Further studies are necessary to provide more conclusive evidence on the presence and genetic characteristics of PCV3 in the wild boar population in Serbia.

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Conflict of interest

The authors declare no conflict of interest.

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