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Molecular detection and characterization of Porcine Kobuvirus in domestic pigs and wild boars in Serbia

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ABSTRACT

Porcine Kobuvirus (PKV) infection is very common in pigs throughout the world. Since it has never been investigated in Serbia, to contribute to the knowledge *of Porcine Kobuvirus*, its role, and distribution, we tested 200 samples from domestic pigs and wild boars. From domestic pigs, 10 fecal, 22 spleen and 68 serum samples, and 100 spleen samples from wild boars were tested. The virus prevalence determined by real-time RT-PCR in domestic pigs was 22% and in wild boars 6%. The phylogenetic analysis of 3D region revealed that Serbian strains are closest related to the Hungarian strain from wild boar from 2011. This is the first report on PKV in Serbia in domestic pigs and wild boars, implying its wide circulation. Although the infection could not be directly related to any clinical manifestation, the frequency of virus found in feces suggests viral affinity to the gastrointestinal tract. However, due to the rather ubiquitous presence of PKV, the clinical and pathological assessment have to be considered when PKV infection is diagnosed.

Kobuviruses, belonging to the family *Picornaviridae*, are one of the smallest viruses, only 27–30 nm in diameter. The genus is comprised of three species: *Aichivirus A* (Yamashita et al., 1991), *Aichivirus B* (Yamashita et al., 2003), and *Aichivirus C* (porcine kobuvirus-PKV) (Reuter et al., 2008). However, there are many kobu-like viruses found, out of these species, found in sheep, dogs, goats, and rodents. Reuter et al. (2008) identified Porcine Kobuvirus in 2008, in Hungary, for the first time. Later on, the viral genome has been detected in many other countries, with the 13–99% prevalence in domestic pigs (Health et al., 2015). Though there are many gaps in knowledge, PKV is being considered as a potential cause of diarrhea in piglets, primarily. However, some authors suggest that PKV can only provoke gastrointestinal disorders in coinfection with other pathogens such as *Rotavirus* and *Coronavirus*. As a support to this observation, the virus prevalence of 65% in the stool samples from healthy pigs in Hungary suggests that the PKV is generally common in the swine populations showing no symptoms (Reuter et al., 2010). On the contrary, Thailand's authors found a very strong positive correlation between PKV infection and acute gastroenteritis in the absence of other pathogens such as the Porcine Rotavirus (Khamrin et al., 2009). *Porcine Kobuviruses*, infecting only pigs, are generally closely related. However, after the discovery of porcine-like *Bovine Kobuvirus* in pigs, the interspecies transmission of Kobuviruses cannot be excluded (Chuchaona et al., 2017). *Porcine Kobuvirus* infection in Serbia has never been investigated, though the very high prevalence in the neighboring countries suggests the high likelihood of the presence in Serbia too. To contribute to the knowledge of *Porcine Kobuvirus*, its role, and distribution, we tested 200 samples from domestic pigs and wild boars, stored in the sample bank. From domestic pigs, we tested 10 fecal, 22 spleen, and 68 serum samples. Domestic pigs, originating from 4 farms, were of different categories: 20 sows, 30 growers, and 50 fatteners. From wild boars, we tested 100 spleen samples originally submitted for classical swine fever (CSF) monitoring. Wild boars originated from central Serbia and were between 6 and 18 months of age. All samples, taken during the period 2017–2019, were from healthy animals, sampled for different purposes such as CSF monitoring. The health status of wild boars was confirmed by hunters, filling the submission forms. The samples were prepared as pools composed of 3 samples. Serum samples were subjected to RNA extraction with no pre-treatment. Fecal and tissue samples were homogenized and prepared as a 10% suspension in PBS. The suspensions were centrifuged at 14000 × **g** for 5 min for fecal samples and at 4000 x g for spleen samples. Supernatants were used for RNA extraction using the commercial kit, Cador Pathogen Mini Kit (Indical Bioscience) following the Manufacturer's Instruction. For the screening purposes, real-time RT-PCR was applied according to Beld et al. (2004) and using Luna® Universal One-Step RT-qPCR Kit, Biolabs. The final

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Table 1
Prevalence of *Porcine Kobuvirus* in domestic pigs.

	Fecal samples		Serum samples		Spleen samples	
	Positive (%)	Tested	Positive (%)	Tested	Positive (%)	Tested
SOWS	2 (100)	2	0 (0)	18	0(0)	0
Growers	0 (0)	0	5 (16.7)	30	0(0)	0
Fatteners	4 (50)	8	1 (5)	20	10	22
Total	**6 (60)**	**10**	**6 (8.8)**	**68**	**10 (45.5)**	**22**

concentration of RT-PCR reaction mixture (25 µl) was 1× Luna Universal One-Step Reaction Mix (20×), 1× Luna WarmStart® RT Enzyme Mix (20×), 0.4 µM of each primer and 0.2 µM of the probe. The template RNA was added in the volume of 5 µl per reaction. Samples from positive pools we tested individually using the described real-time protocol. Positive samples were further on tested by gel-based RT-PCR, to sequence the 3D genome region. The RT-PCR was carried out in 50 µl reaction using QIAGEN One-Step RT-PCR, according to the protocol published by Van Dung et al. (2016). The PCR products of specific lengths were sequenced in Macrogen, The Netherlands. The nucleotide sequences were deposited in the NCBI gene bank under the accession numbers MT268545-MT268549. The sequence analysis and cladogram construction were performed using the Mega X software and UPGMA method (Kumar et al., 2018). The obtained results were statistically analyzed by the chi-square ($\chi 2$) test with 95% confidence, whereas $P < 0.05$ was considered as statistically significant. The chi-square test was performed for the multiple categories of both production category (sows, growers, and fatteners) and sample type (fecal, serum, and spleen samples). Where the result was significant, the chi-square ($\chi 2$) test of independence between each category was applied.

The PKV infection was confirmed in all tested farms. Out of 100 tested samples from domestic pigs, 22 (22%) were positive for PKV genome using real-time RT-PCR: 6 fecal, 10 spleens, 6 serum samples (Table 1), whereas out of 100 wild boar samples, 6 were positive (6%). Related to the age category, 10% of sows, 16.7% of growers, and 30% of fatteners were positive for PKV genome using real-time RT-PCR. However, using gel-based RT-PCR, only 8 samples from both domestic pigs and wild boars were positive, indicating low sensitivity of used gel-based RT-PCR for the samples with the Ct higher than 30. Nucleotide sequences of good quality were obtained from 3 samples from domestic pigs, and 2 from wild boars. The statistical significance was observed comparing results obtained from feces and serum ($p < 0.05$), and from spleen and serum ($p < 0.05$) indicating the highest likelihood of PKV genome detection in feces and spleen. However, results gained from different categories (sows, growers, and fatteners) did not significantly differ from each other ($p > 0.05$). Based on 3D genome region, nucleotide and amino acid diversity between Serbian PKV isolates from wild boars and domestic pigs were 1% being closest related to the isolate from wild boars in Hungary from 2011. The cladogram revealed that PKV sequences were grouped into two clusters: Serbian sequences clustered together with the isolates from a wide geographic range (Hungary, Italy, The Netherlands, China, Brazil, South Korea). The other cluster was composed of Indian, South Korean, and central European sequences from The Czech Republic and Slovakia. (See Fig. 1.)

The results of this study indicate that both domestic pigs and wild boars in Serbia are infected with PKV, confirming that the infection is widely distributed and endemic in the world, as many authors reported so far. The virus prevalence in the Serbian domestic pig population based on this study is lower than in many other European countries (Jackova et al., 2017) but rather similar to the prevalence in the United States (Verma et al., 2013). Certainly, the infection is very common in

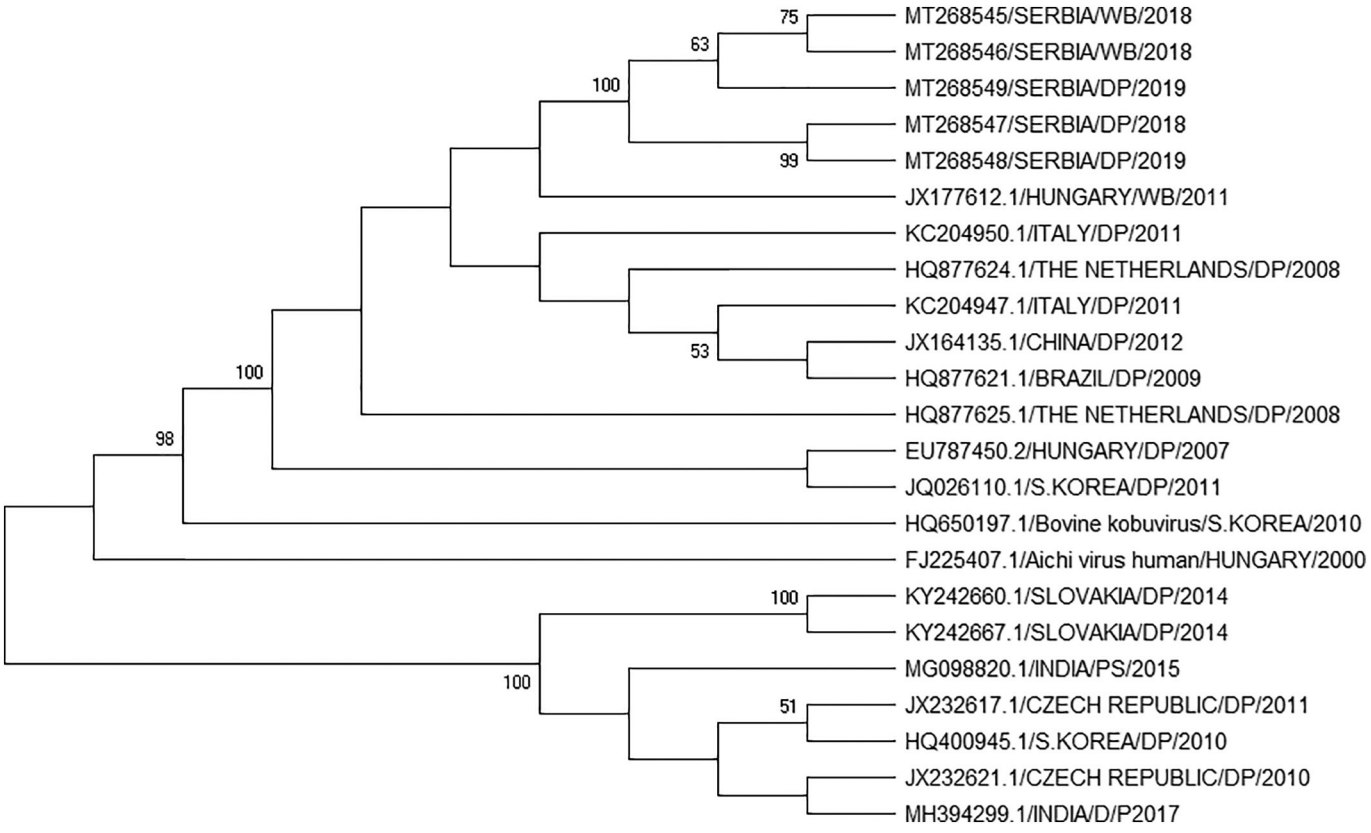

Fig. 1. Cladogram illustrating the relationships among PKV isolates, based on 3D. The analyses were conducted in MEGA X software using UPGMA method. The analysis involved 23 nucleotide sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches; only values > 50% were indicated. Scale bars indicate the numbers of steps for parsimony analysis. DP-domestic pig, WB-wild boar, PS-*PorculaSalvania*.

pigs though the data regarding the prevalence can differ (Di Profio et al., 2013; Di Bartolo et al., 2015). Considering the assumed fecal-oral route of infection, high dense pig populations are more likely expected to have a higher prevalence (Di Bartolo et al., 2015) such as with other similar diseases (Bouwknegt et al., 2011).

The data regarding the infection and its prevalence in wild boars are very limited. Comparing our results to the results of Hungarian scientists (Reuter et al., 2013), the prevalence in Serbia is much lower (6%). However, it should be taken into account that we tested only spleen samples, that are expected to be positive only during viremia. Though there are very limited reports on the pathogenesis, by this finding, it can be concluded that PKV cause viremia. Nevertheless, the question of whether it is due to the escape from the gastrointestinal tract (Reuter et al., 2010) or as a regular part of pathogenesis stays unanswered. Apart from the spleen samples, the viral RNA was found in serum samples, confirming the viremia, which obviously did not lead to the rise of body temperature or occurrence of clinical symptoms. The discovery of PKV infection in wild boars further emphasizes the role of wild boars as the reservoirs of infectious diseases for domestic pigs, as well as zoonotic ones. Certainly, though the insignificant difference in nucleotide levels between a limited number of strains from the domestic and wild boars could indicate strain exchange, the strains from wild boars are on a different cluster. This still could support a certain level of independent circulation of PKV in domestic and wild boar population. The closeness of strains is also related to the short time span strains originated from as well as to the conserved 3D region that was used for phylogenetic analysis. Even though, many authors suggest no geographic clustering, the Serbian strains, based on the 3D region, were branched with the Hungarian strain from 2011. The overall prevalence in Serbia that turned to be lower than in the majority of European countries could also be attributed to the sample type tested since it was shown that the likelihood of PKV detection is significantly higher if feces samples were tested; despite the tested animals had no clinically manifested disorders, the virus clearly shows affinity to the digestive tract. Considering the results by category of animals, interestingly, the sows were at least affected whereby the viremia was not recorded. Though there are no reports on the immune response to the PKV, it could be considered that in older animals there is a protective level of antibodies that prevent the viremia, though the re-infections in older animals were described (Di Bartolo et al., 2015). Khamrin et al., 2014, have shown that in the **human population the** antibody prevalence rises by age. Considering this finding, though the overall difference between tested categories was not significant, the highest proportion of growers in active infection-viremia (16.5%) could be a consequence of maternal antibodies clearance. This could, also, explain that no serum samples from sows were found positive.

In conclusion, this is the first report on PKV in Serbia in domestic pigs and wild boars, implying its wide circulation. Although the infection could not be directly related to any clinical manifestation, the frequency of virus found in feces suggests a viral affinity to the gastrointestinal tract. However, due to the rather ubiquitous presence of PKV, the clinical and pathological assessment have to be considered when PKV infection is diagnosed.

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