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Genetic diversity of porcine circovirus type 2 (PCV2) in the Romanian wild boar population

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ABSTRACT

In this study, we have analyzed 23 PCV2 ORF2 sequences recovered from wild boar population in Romania. The PCV2 sequences were originated from different geographical regions in Romania, and collected between 2008 and 2009 during the classical swine fever virus (CSFV) surveillance campaign. Complete open reading frame 2 (ORF2) nucleotide sequences were obtained and compared with sequences mainly from European and Asian isolates. The Romanian sequences were identified as belonging to previously described clusters 2a and 2b, with high degree of heterogeneity (PCV2 ORF2 nucleotide homology ranged between 90.1% and 100%). Interestingly, for cluster 2a, the majority of the sequences (8 from a total number of 9) clustered mainly with the Asian isolates (especially China, but also India and South Korea), with three exceptions from Europe previously reported in Germany, Belgium and The Netherlands.

Porcine circovirus (PCV) is a small non enveloped circular virus that belongs to the *Circoviridae* family, genus *Circovirus*. The single stranded covalently linked DNA molecule of PCV1 and PCV2 contains two major open reading frames (ORFs): ORF1 (*rep* gene) is involved in virus replication, while ORF2 (*cap* gene) encodes the capsid protein, with major immunogenic proprieties (Hamel et al., 1998). For PCV2, a third ORF has been described (namely ORF3; Liu et al., 2006) that it is not essential for PCV2 replication in cultured cells. Genetically, PCV2 can be further divided into three groups: PCV2a, PCV2b and PCV2c (Dupont et al., 2008).

To date, extensive studies have been made regarding PCV2 circulation and genetic diversity in both domestic and wild boar populations (Knell et al., 2005; Grierson et al., 2004; Wen et al., 2005; Sofia et al., 2008; Csagola et al., 2006), showing a high degree of heterogeneity among isolates within a specific geographic region. However, limited data are available regarding the PCV2 incidence in the Romanian wild boar population (Cadar et al., 2008) and to our knowledge no genetic characterization has been performed yet. Therefore, the present study aimed to draw up a comprehensive genetic characterization of PCV2 isolates from the wild boar population originated from different Romanian regions.

Samples subjected to the study were comprised of 212 sections of spleen originating from five of nine geographical regions of Romania, namely Muntenia (94 samples), Dobrogea (10 samples) Moldova (12 samples), Transilvania (52 samples) and Maramures (44 samples), initially collected for classical swine fever virus (CSFV) surveillance. The age interval of the wild boars ranged from less than 1 year to 10 years and the gender distribution was approximately 73% males and 27% females.

All samples were initially homogenized using the MagNa Lyzer instrument (Roche Applied Science, Mannheim, Germany), following the manufacturer recommendations regarding speed and time, resulting in 10% organ suspensions that were further centrifuged, and the supernatant was collected. The DNA extraction was performed automatically using the MagNa Pure LC Instrument (Roche Applied Science, Mannheim, Germany), with 200 µl of the supernatants of the 10% organ suspensions. The DNA was eluted in a final volume of 100 µl.

The PCV2 screening PCR was performed using previously described primers (Larochelle et al., 2000; Knell et al., 2005; De Boisseson et al., 2004), generating a PCV2 ORF2-based PCR product of 263 nucleotides. Amplification was performed using the FastStart Taq DNA polymerase kit (Roche Applied Science, Mannheim, Germany), following the manufacturer's instructions, in a final concentration of 0.6 µM primers, 2 mM $MgCl_2$, 200 µM of each dNTP and 2 units of enzyme. A volume of 10 µl of extracted

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DNA from each test sample was used for a final volume of 50 μl per reaction.

Samples were loaded on an ICycler instrument (BioRad Laboratories, USA) with a thermal profile that consisted of one cycle at 95 °C for 5 min for initial denaturation and enzyme activation, followed by 35 cycles of 95 °C for 35 s (denaturation), 60 °C for 35 s (annealing) and 72 °C for 45 s (extension). Finally, the DNA strands were extended for 7 min at 72 °C and stored at 4 °C until gel loading.

Gel electrophoresis was performed using a 2% agarose gel stained with ethidium bromide and 1X TBE buffer (Tris Borate EDTA). Negative and positive extraction and amplification controls were incorporated in each test run.

For sample selection for direct sequencing two criteria were used. At least one positive sample from each region was included and if there were more than one positive sample in a certain region, samples from different geographic parts were used. Based on the criteria, 23 PCV2 PCR positive samples were selected for sequencing.

For sequencing PCR, four primers (two primer set) were selected (Knell et al., 2005), amplifying the highly variable ORF2 (capsid protein) coding region of the PCV2 genome. Reaction conditions and reagents were the same as described above for the PCV2 screening PCR, except an adaptation of the annealing temperature to 58 °C and increase of the extension time to 1 min.

Specific amplicons bands were excised from the agarose gels and purified using the MinElute Gel Extraction Kit (Qiagen, Hilden, Germany), following manufacturer recommendation and amplified using the BigDye V 1.1 Cycle Sequencing Kit (Applied Bioscience, Warrington, United Kingdom). Products were purified using CentriSep Spin Columns (Princeton, Applied Bioscience, Foster City, USA) and loaded into a 3130, 4 capillary genetic analyzer (Applied Bioscience, Hitachi High-Technologies Corporation, Tokyo, Japan).

All samples were processed twice for accuracy (two times with each forward and two times with each reverse primer), followed by nucleotide sequences alignment using the Clustal W software included in Bio Edit package (Hall, 1999). The phylogenetic tree was constructed using best homologous sequences retrieved from the NCBI database, together with other published sequences mainly from European countries. One single sequence, previously identified from a clinical case of PMWS in Romanian domestic pigs, was also included (Accession No. DQ233257). Also, a PCV1 sequence isolated from France was used to evaluate the difference between the two PCV types (Accession No. AF012107). A dendrogram was obtained using Mega 4 software, NJ (Neighbor Joining) algorithm, Kimura 2 parameter and bootstrap support of 3000 replicates.

From a total number of 212 wild boar spleen samples, 93 (43.86%) were found to be positive for PCV2 DNA by the screening PCR, which indicates a rather high incidence of PCV2 infected Romanian wild boars. Taking into consideration the traditional situation of backyard farming of domestic pigs in Romania, with real possibilities of close contact between wild and domestic animals, it might be suggested that Romanian wild boar population can act as an active PCV2 reservoir for domestic pigs and vice versa.

The overall PCV2 ORF2 nucleotide homology ranged between 86.7% and 100% for the analyzed sequences ($n = 61$). With the exception of sample ROM 105, nucleotide alignment of ROM 2, 3, 6, 11, 16, 30, 48, 53, 65, 74, 82, 86, 88, 89, 112, 115, 120, 152, 154, 159, 160 and 193 ($n = 22$) ORF2 sequences showed one deletion at position 10/11 of the ORF2 nucleotide coding sequence (position 1042/1043 of the PCV2 genome, Accession No. AY424403), leading to genome shortening. Consequently, by translation, the STOP codon of the samples ROM 3, 53, 89, 112, 120 and 154 was displaced by adding one lysine amino acid at position 234 of the capsid protein (Knell et al., 2005). However, for the remaining sixteen sequences, besides this deletion, a single nucleotide mutation from thymine to adenine at position 3 of the ORF2 nucleotide coding sequence (position 1035 of the PCV2 genome) generated a STOP codon instead of lysine by translation. Therefore, the sequences of ROM 2, 6, 11, 16, 30, 48, 65, 74, 82, 86, 88, 115, 152, 159, 160 and 193 ($n = 16$) maintained the original number of amino acids (a total number of 233 amino acids) (Fig. 1).

Regarding the phylogenetic analysis of the Romanian PCV2 samples, the examination revealed 9/23 samples contained ORF-2 sequences that clustered within PCV2a and 14/23 samples contained ORF-2 sequences that clustered within PCV2b (Fig. 2).

Within the PCV2a cluster, at least two clearly distinct subgroups were identified. The first one comprises of sequences closely related mainly to Asian PCV2 strains, with three exceptions being represented by isolates from Germany (Accession No. AY713470), Belgium (Accession No. EF990645) and The Netherlands (Accession

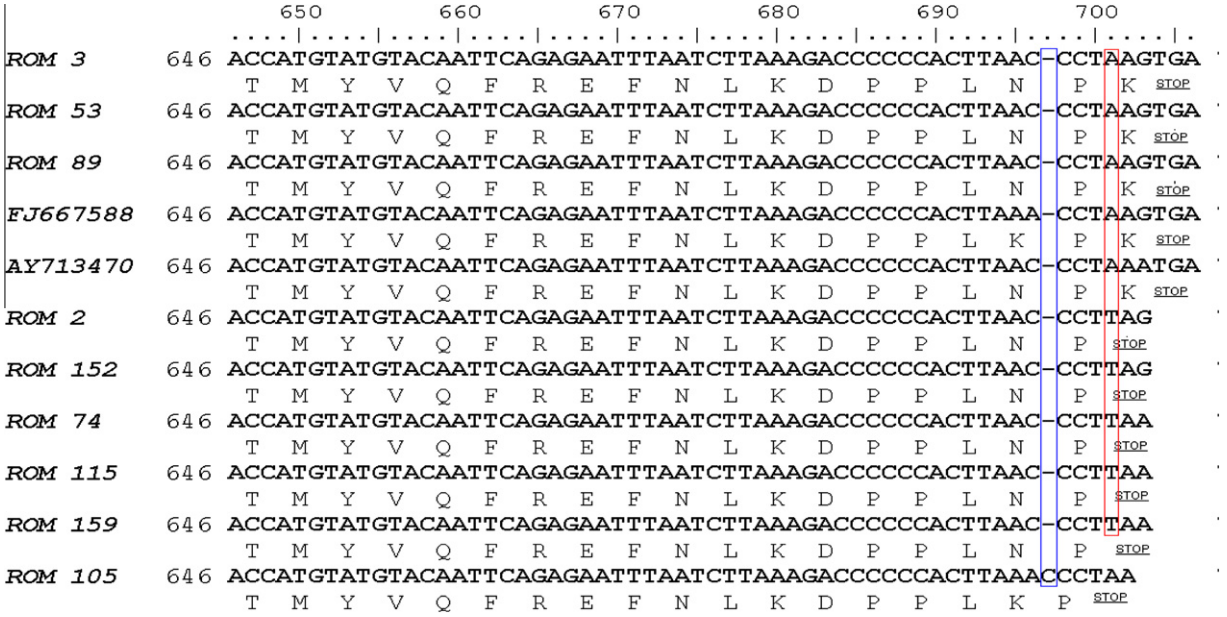

Fig. 1. Nucleotide deletion (blue marked) and substitution (red marked) on the ORF2 reverse complement nucleotide coding sequence and the deduced amino acids. Representatives of the different Romanian sequences (ROM) are presented, and also included are one Chinese strain (Accession No. FJ667588) and one German strain (Accession No. AY713470). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)