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Porcine teschovirus in wild boars in Hungary

Ákos Boros¹, Csaba Nemes², Péter Pankovics¹, Beatrix Kapusinszky³, Eric Delwart^{3,4}, and Gábor Reuter^{1,3,*}

¹Regional Laboratory of Virology, National Reference Laboratory of Gastroenteric Viruses, ÁNTSZ Regional Institute of State Public Health Service, Pécs, Hungary

²Veterinary Diagnostic Directorate of the Central Agricultural Office, Kaposvár, Hungary

³Blood Systems Research Institute, San Francisco, CA, USA

⁴University of California, San Francisco, CA, USA

Abstract

The genus *Teschovirus*, family *Picornaviridae*, currently includes 12 serotypes (PTV 1 to 12) isolated from swine. PTVs have been well studied in domestic pigs, but knowledge about PTVs in wild boars is deficient. Here, we report the first complete PTV genome sequence from 7 (70%) of 10 fecal samples of wild boar piglets (*Sus scrofa*) by RT-PCR and pyrosequencing. Analysis of the wild boar PTV strain WB2C-TV/2011/HUN (JQ429405) showed considerable difference, especially in VP1 (66–74% amino acid identity) compared to the available PTVs. PTV is present in wild boars, and WB2C-TV/2011/HUN represents a novel PTV genotype, provisionally named PTV-13.

Porcine teschoviruses (PTV), family *Picornaviridae*, are non-enveloped viruses with a single-stranded RNA genome of positive polarity that, based on our current knowledge, infect only swine populations. The genus *Teschovirus* currently includes 12 serotypes (PTV-1 to -12) [4, 24]. The majority of PTV serotypes are not pathogenic and circulate worldwide in healthy domestic pigs [4, 11, 13], but PTVs can be associated with a variety of clinical manifestations, e.g., reproductive disorders [6, 11], diarrhea [8, 19], and respiratory disease [16, 25]. In addition to these relatively mild PTV-associated diseases, the syndrome called “teschovirus encephalomyelitis” or Teschen disease [22] caused by virulent strains (e.g., Teschen [AF231769] or Haiti/2008 [GQ914053]) of PTV-1 is currently a rare disease, although severe outbreaks with large economic losses were reported in Europe from 1929 to the 1960s [12]. Outbreaks of “teschovirus encephalomyelitis” were also reported recently in several countries, e.g., Ukraine in 1996–2005, Russia in 2004, Madagascar in 2004–2005, Belarus in 2005 and Haiti in 2008–2009 [7, 18]. The genetic diversity and incidence of currently circulating PTVs is well studied in domestic pig populations [4, 13] but not in wild boars, where only the presence of viruses that are antigenically related to PTV is known based on a report from 1950 [14].

Wild boars could serve as reservoirs for different viruses, including swine vesicular disease virus (SVDV), hepatitis E virus, porcine enterovirus B (PEV-B), foot-and-mouth disease virus (FMDV), and porcine astrovirus, most of which can cause serious diseases [2, 15, 20]. This study reports the first detection and complete genome sequence of PTV from wild boar

*Address for correspondence: Dr. Gábor Reuter Regional Laboratory of Virology ÁNTSZ Regional Institute of State Public Health Service H-7623 Szabadság út 7. Pécs, Hungary Telephone: 0036 06 (72) 514 979 Fax: 0036 06 (72) 514 949 reuter.gabor@ddr.antsz.hu.

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and provides data on the presence, genetic relationship and molecular epidemiology of PTV in wild boars.

Fecal samples from wild boars (*Sus scrofa*) of two age groups (6 and 8 weeks old, n=5–5) were collected from an animal park located in southwestern Hungary in April 2011. None of the sampled boars showed any clinical symptoms at the time of sample collection. The boars were in captive breeding but had no contacts with domestic pigs.

The fecal samples were subjected to viral metagenomics analysis as follows: Specimens were diluted in 0.1 M phosphate-buffered saline (PBS), passed through a 0.45- μ m sterile filter, and centrifuged at $6,000 \times g$ for 5 min. The pellet was treated with a mixture of nucleases to enrich for particle-protected nucleic acids [10]. Nucleic acids were extracted using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Viral RNA and DNA nucleic acid libraries were constructed by sequence-independent random RT-PCR amplification [23]. 454 pyrosequencing using 454 GS FLX technology was then performed as described previously [10, 23].

The pyrosequencing reads from the different samples were assembled *de novo*, and sequence contigs and singletons were compared to the GenBank nucleotide and protein databases using BLASTx. Specific primer pairs were designed based on the sequence contigs and singletons from the pyrosequencing reads to determine the complete nucleotide sequence of a selected PTV using reverse transcription PCR (RT-PCR). The 5' and the 3' ends of the genome were determined using a 5'/3' RACE PCR kit (Roche, Mannheim, Germany) as described previously [3]. Strain specific PTV VP1 primers (WBtv-3131-R: 5'-GGCAGAACAAATCTCACATTGT-3' and WBtv-2306-F: 5'-AGTGCTGGTTGACACACCGTA-3') were designed based upon the sequence contigs of PTV VP1 for screening the fecal samples. PCR products were sequenced directly in both directions using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK) with the specific primers and analyzed on an automated sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Stafford, USA). For the phylogenetic analysis, PTV sequences obtained from the GenBank database and the sequence from this study were aligned using Clustal X, and similarity calculations were performed using the GeneDoc 2.7 software [17]. Phylogenetic trees of the deduced amino acid alignments were created using the maximum-likelihood (VP1) and neighbor-joining methods (P1); the binary tree was constructed across the interval of amino acids 99 to 219 of the VP2 gene, called the EF-loop, using the unweighted pair group mean average (UPGMA) method in the MEGA software (version 5) [21]. Bootstrap values (based on 1000 replicates) for each node are given if >50%. The complete genome sequence of wild boar/WB2C-TV/2011/HUN was submitted to GenBank under accession number JQ429405.

Fecal samples collected from wild boars were subjected to viral metagenomic analysis using the pyrosequencing method. Porcine teschovirus (PTV) sequences were found in seven (70%) of 10 samples. PTV were detected in four (80%) of 5 fecal samples from 6-week-old animals and 3 (60%) of 5 fecal samples collected from 8-week-old animals using the VP1-specific primers, thus confirming the metagenomic results. The VP1 sequences of the Hungarian wild boar strains showed 99% nucleotide (nt) and complete amino acid (aa) sequence identity to each other and 65–72% nt and 66–74% aa identity compared to the known PTV reference sequences.

One of the PTV-positive samples (WB2C) was selected for complete genome characterization. From this sample, 27 PTV sequence contigs and singletons with E-value <10⁻⁵ were found using BLASTx (data not shown). The genome length of wild boar/WB2C-TV/2011/HUN from the polyC stretch at the 5' end was 7108 nt excluding the

poly(A) tail. A long ORF of 6624 nt, encoding a potential 2207-aa-long viral polyprotein precursor, was flanked by a 421-nt-long 5'UTR and a 63-nt-long 3'UTR. The L, P1, P2 and P3 regions were 258 nt (86 aa), 2580 nt (860 aa), 1464 nt (488 aa) and 2322 nt (773 aa) long, respectively.

The percent differences calculated from pairwise comparisons of the complete genome region encoding the structural proteins (VP4–VP1) revealed that wild boar/WB2C-TV/2011/HUN shows only 69–73% nt and 74–76% aa sequence identity to the known PTV serotypes. Of the most closely related available PTV types, those showing highest nt/aa identity to the study sequences in the VP1 protein coding region were PTV-5 strain Vir1806/89 (71%/74%), PTV-7 strain F 43 (72%/72%) and PTV-9 strain Vir2899/84 (71%/73%). This relationship could also be observed in the phylogenetic tree based on the deduced aa sequence of VP1 (Fig. 1), where the wild boar/WB2C-TV/2011/HUN sequence was grouped together with PTVs 5, 7 and 9. Meanwhile, the UPGMA phylogenetic tree constructed from the 120-aa-long section of the VP2 EF-loop of the representative members of the different PTV serotypes shows the separation of the study sequence from PTV-9, with a greater genetic distance (0.11) than the distance (0.08) between the closest two serotypes, PTV-1 and 11 (Fig. 2). In the VP4–VP2 region of the genome, the highest pairwise aa sequence identity was 83% between the study strain and the PTVs. The phylogenetic tree constructed from the deduced aa sequences of the complete P1 genome region of the reference PTV sequences and representative members of the most closely related members of the genera *Cardiovirus*, *Aphthovirus*, and *Erbovirus* illustrated the genetic separation of wild boar/WB2C-TV/2011/HUN from all of the currently available PTV sequences (Fig. 3). The non-structural protein-coding region of the wild boar/WB2C-TV/2011/HUN shows high sequence identity (85–88% nt and 95–97% aa) compared to the available PTVs. The PTV-9 strain Vir2899/84 shows the highest nt/aa similarity with the study sequence in both the structural (73%/76%), and non-structural genome regions (88%/97%).

In this study, we report for the first time the complete genome sequence of PTV, which was present in wild boar piglets less than 8 weeks old in high abundance. The comparison of the overlapping sequence reads gathered from the metagenomic analysis of the different fecal samples shows no signs of other PTV types in addition to the study sequence. Furthermore, the complete amino acid sequence identity in the VP1 region among PTV sequences detected from separate fecal samples suggested that only one dominant PTV type was circulating among these wild boars. This is not surprising, since the study population lives close together. Because of the relatively low sequence similarity of the wild boar PTV VP1 sequences compared to the available PTVs, the complete nucleotide sequence of the wild boar/WB2C-TV/2011/HUN was determined. Several attempts were made to obtain sequence information 5' to the polyC stretch using the 5' RACE method that was successfully applied before [3, 20], but these attempts were unsuccessful, similar to what has been reported by other research groups [5, 24].

Phylogenetic analysis of the VP1 region shows that wild boar/WB2C-TV/2011/HUN belongs to the genus *Teschovirus* and is distantly related to the PTV-9 serotype. According to our current knowledge there are no other reports about the presence of PTV-9, with the exception of the initial study in which PTV-9 was described in 1994 [1]. The distant relationship between the study sequence and the rare PTV-9 isolated from domestic swine could suggest an ancient evolutionary connection between these two strains. This phylogenetic relationship was also observed in the UPGMA phylogenetic tree.

According to our present knowledge, there is no sequence-based PTV “serotyping” method accepted by the ICTV *Picornaviridae* Study Group, and therefore we applied such genome-based methods to investigate the taxonomic position of the study sequence, which was

confirmed by serological experiments [9; 24]. The different PTV field isolates can be typed on the basis of capsid protein sequence comparisons, where heterologous serotypes share less than 90% amino acid identity in the VP4–VP2 genome regions [24]. In the case of wild boar/WB2C-TV/2011/HUN, the highest pairwise amino acid sequence identity was 83% compared to PTV-9. Another strategy for PTV serotyping was based on phylogenetic analysis of the VP2 EF-loop, which has been demonstrated experimentally to be an immunodominant site of PTV-1 strain Talfan and contains serotype-specific information [9]. The UPGMA phylogenetic tree of the EF-loop revealed the separation of the different PTV serotypes identified previously and shows more genetic divergence of the study sequence from PTV-9 than the minimal genetic distance between two different serotypes. The sequence and phylogenetic analysis suggest that wild boar/WB2C-TV/2011/HUN belongs to a previously unknown PTV serotype, tentatively named porcine teschovirus 13 (PTV-13).

The phylogenetic tree based on the complete capsid region shows the separation of the study sequence from all of the previously described PTV serotypes. According to the hypothesis presented by Zell and co-workers [24], the evolution of teschoviruses proceeded in two steps. The first step led to the development of three subgroups, which then subdivided into 11 (now 12) serotypes [24]. The divergence of the three subgroups presented in the P1 phylogenetic tree was preceded by the branching of the study sequence with high bootstrap support, which could indicate the presence of additional, potentially different PTV types circulating among wild boars. Future sequences (especially more PTV-like sequences from different wild boar populations) and studies are necessary to support this assumption.

The fecal samples that were analyzed were collected from clinically healthy wild boar piglets, which could indicate that this PTV does not cause illness with manifested symptoms in the wild boars tested, which is similar to what has been reported recently for other PTV-infected domestic pigs [4, 13, 18]. However, the presence of the same serotypes (e.g., PTV-6 and -11) in field isolates collected from domestic swine without any clinical symptoms and swine with polioencephalomyelitis could indicate that the same PTV serotype can replicate with or without causing manifested symptoms [24].

It is generally accepted that PTVs are present in wild boars. However, to the best of our knowledge, only one historical serological study can be found on this topic, from the year 1950 [14]. Our report provides the first insight into the genetic nature of PTVs present in wild boars based on determination of their complete genome sequences. The occurrence of genetically distant PTV types (and potential further, currently unknown virulent strains) in wild boars differing from the currently circulating PTV strains in domestic pigs could carry a biological hazard to domestic swine populations – not to mention the fact that wild boars could easily cross country borders without any control, could come into direct contact with domestic pigs, and, as a potential reservoir of PTVs and other viruses (e.g., HEV, FMDV, SWDV), could contribute to endemic spread of these agents [15]. Furthermore, pigs of any age are susceptible to infection with different PTVs to which they have not previously been exposed [11]. For these reasons, attention should be drawn to understanding of the diversity of viruses in wildlife reservoirs, which provides epidemiological baseline information about pathogens and may lead to early identification of novel emerging agents.

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Fig. 1. Unrooted maximum-likelihood phylogenetic tree of deduced VP1 amino acid sequences of the representative members of the 12 PTV types (strain names followed by the accession numbers in square brackets) and the study sequence wild boar/WB2C-TV/2011/HUN (JQ429405) in **bold**.

Fig. 2. UPGMA tree showing the genetic relationships and genetic distances between the representative strains of the eleven PTV types and the study sequence wild boar/WB2C-TV/2011/HUN (JQ429405) in **bold** based on a comparison of aa 99 to 219 of the VP2 gene (EF-loop).

Fig. 3.

Neighbor-joining tree based on the deduced amino acid sequences of the P1 region of all of the available PTV strains (serotype name, strain name in brackets and accession number in square brackets), those of members of the genera *Cardiovirus* (encephalomyocarditis virus B variant, EMCV-B; Theiler murine encephalomyelitis virus, TMEV), *Aphthovirus* (equine rhinovirus 1, ERAV-1; foot-and-mouth disease virus A, FMDV), and *Erbovirus* (equine rhinovirus type 2, ERBV), and the study sequence wild boar/WB2C-TV/2011/HUN (JQ429405) in **bold**. Subgroups (sg) 1 to 3 represent the three subgroups within the genus *Teschovirus* identified by Zell and co-workers [23].