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THE GENETIC ANALYSIS OF POTENTIAL NEW HEV-3 SUBTYPE IDENTIFIED ON PIG FARM IN SLOVAKIA

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ABSTRACT

Hepatitis E virus (HEV) represents the causative agent of acute viral hepatitis in the world. The virus belongs to the genus *Paslahepevirus*, species *Paslahepevirus balayani*, which contains 8 different genotypes and at least 14 reference HEV-3 subtypes. Recently we have identified three isolates in a Slovakian pig farm as a new unclassified HEV-3 subtype. The aim of this study was to sequence and analyze additional regions of HEV genome for PER5 isolate, a representative isolate of potential new HEV-3 subtype. The phylogenetic analyses of 749 bp and 828 bp DNA fragments amplified from ORF1 revealed that both sequences fall outside two major clades (3abchij and 3efg) of the HEV-3 genotype as well as the other HEV-3 subtypes. The nucleotide identity with other HEV-3 subtypes indicated low values within the range 79.2–84.1 % as well. The results provide further support for the circulation of a new HEV-3 subtype in the pig farm in Slovakia.

Key words: domestic pig; hepatitis E; HEV-3; sequencing; subtype

INTRODUCTION

Hepatitis E virus (HEV) probably represents the most common causative agent of acute viral hepatitis in the world [1, 10]. In general, the majority of HEV infections are asymptomatic, usually results in an acute self-limiting disease [23], otherwise in immunocompromised individuals HEV infection can become chronic [10]. The primary source of infection is through the faecal-oral route in developing countries. The secondary source, via consumption of raw or undercooked meats or meat products from reservoir animals represents an important risk factor for the emergence of hepatitis E in developed countries [23].

HEV is a single-stranded positive sense RNA with approximately 27–34 nm. The HEV genome (~7.2 kb) expresses three open reading frames (ORFs): ORF1 (~5,082 nucleotides), ORF2 (~1,983 nucleotides), and ORF3 (~300 nucleotides), which is partially overlapped with ORF2 [3, 11]. Virus belongs to the genus *Paslahepevirus*, species *Paslahepevirus balayani*, which contains 8 different genotypes [13, 18, 20, 25, 26, 29, 30]. HEV-3 genotype is found worldwide and represent the predominant genotype in Europe and America [17]. Moreover, HEV-3 genotype has a zoonotic potential and mainly domestic pigs, wild boars as well as deer are the source of infection for people. Humans may be potentially infected

via consumption of raw or undercooked animal products from HEV infected animals [4, 7, 14, 21, 22, 24].

The genetic analysis of HEV-3 revealed that this genotype is quite variable. Based on the phylogenetic analysis of hypervariable region in ORF1, the reference HEV subtypes were described [26]. HEV-3 genotype consists of subtypes divided to 2 main clades; one composed of six HEV-3 subtypes (abchij), the second, composed of three HEV-3 subtypes (efg), as well as ra subtype derived from rabbit genotype [7]. Except of mentioned subtypes, S m i t h et al. [24] provided update of the reference sequences and added three other HEV-3 subtypes namely HEV-3k, HEV-3l, and HEV-3m proposed as novel subtypes [5, 16, 24]. In addition, the novel HEV-3n subtype has been described recently [19].

In Europe, three HEV-3 subtypes (HEV-3c, HEV-3e, and HEV-3f) were the most commonly detected in human and animal samples [6, 17]. While HEV-3 isolates originating from domestic pigs in The Czech Republic, Hungary and Poland belonged mainly into HEV-3efg clade [2, 8, 12, 28], the epizootiological situation in Slovakia was different. In a previous study, we revealed that HEV isolates from Slovakian domestic pigs fall mainly into the HEV-3abchij clade [9]. We also identified the highly divergent HEV-3 isolates with relatively high nucleotide distances (p-distances 0.140–0.178) to other subtypes. This distance was higher than the limit for a new subtype ($P<0.123$) defined by S m i t h et al. [26]. The divergent isolates clustered outside of both major phylogenetic clades and thus remained not classified so far.

The aim of this study was a deeper genetic analysis of a representative PER5 isolate belonging to new unclassified HEV-3 subtype which was identified in Slovakian pig farm. The sequences of additional genomic regions of PER5 isolate were subject for further analysis by bioinformatics tools.

MATERIAL AND METHODS

Sample collection

PER5 isolate, a representative member of the new unclassified genetic subtype, used in this study was collected from randomly selected weaner pig (*Sus scrofa domestica*) originating from a farm in the Eastern Slovakia described by J a c k o v á et al. [9]. The veterinarian who inspected the farm did not recognize any clinical symptoms on a pig of which clinical sample was collected. Rectal swab from a pig was collected using swab applicator (Sarstedt AG & Co, Germany) with transport medium, thereafter delivered to the laboratory and processed.

HEV RNA extraction and reverse transcription

Rectal swabs were resuspended in 1 ml of 0.01 $mol.l^{-1}$ PBS (Merck Millipore Corp., USA) for 30 min., then vortexed at 2000 $rev.min^{-1}$ for 3 min and centrifuged at 14,000 × g for 5 min. RNA was isolated from 400 µl of solution prepared using the MagMAX™ Viral RNA Isolation Kit (Thermo Fisher Scientific, Inc., USA) according to the manufacturer's protocol. The extracted RNA was stored at –80 °C. The cDNA was synthesized in a 20 µl reaction mix comprising 5 µl of extracted RNA, 0.5 mM dNTPs (Thermo Fisher Scientific, Inc., USA), 200 U RevertAid Premium reverse transcriptase with 1xRT buffer (Thermo Fisher Scientific, Inc., USA), 5 µM of gene specific reverse outer PCR primers [5, 15, 31] (Microsynth Austria, GmbH, Austria), 20 U RNase inhibitor (Invitrogen, Inc., USA) and filled with molecular biology grade water (Merck, GmbH, Germany).

Nested RT-PCR and sequencing

A total of twenty pairs of PCR primers available in the literature were used for the amplification of full-length HEV genome [5, 15, 31]. The PCR reaction mix (50 µl) was composed of 1× ThermoPol reaction buffer (New England Biolabs, Inc., USA), 0.2 mM dNTPs (Thermo Fisher Scientific, Inc., USA), 300 nM of outer/inner primers, 1 U Taq DNA polymerase (New England Biolabs, Inc., USA), 4 µl cDNA and molecular biology grade water (Merck, GmbH, Germany). The PCR was carried out under the following thermal profile: 1 cycle at 95 °C for 1 min, and 35 cycles with denaturation at 95 °C for 30 s, annealing (set at various temperature varying between 52–58 °C, which depended on the primers used) for 1 min, extension at 68 °C for 1 min and final extension at 68 °C for 5 min using Thermocycler C1000 (Bio-Rad Laboratories, Inc., USA). Electrophoresis was carried out in 2 % agarose gel, stained with GelRed™ (Biotium, Inc., USA) and visualized by Gel Doc EZ imager (Bio-Rad Laboratories, Inc., USA). The PCR products of expected size were purified and sequenced by the Sanger method using the PCR primers by a commercial company (Microsynth Austria, GmbH, Austria).

Phylogenetic analysis of HEV sequences

For bioinformatics analysis, the partial ORF1 sequences from PER5 sample were edited and aligned using the programs SeqMan, EditSeq and MegAlign (Lasergene, DNASTAR, Inc. USA). The sequences were first checked against the NCBI GenBank database using nucleotide BLASTn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide identity (nt. id.) was calculated using the program MegAlign. Than the phylogenetic trees were separately constructed using the cut off 749 bp and 828 bp nucleotide sequences (GenBank Acc. No. OQ378957 and OQ378956, respectively). The molecular evolution model tests were performed by MEGA11 [27]. The models with the lowest BIC scores (Bayesian Information Criterion) were used for phylogenetic analysis. The model tests indicated that The Maximum Likelihood analyses of 749 bp DNA fragment should be carried out using the Tamura-Nei model with Gamma distribution plus evolutionarily Invariable sites model (TN93+G+I). The Maximum Likelihood phylogenetic analysis of 828 bp fragment was set to the General time reversible model with Gamma distribution plus evolutionarily Invariable sites model (GTR+G+I). The bootstrap support values of branches were calculated from 1000 replicates. The HEV-1 Burma strain (M73218) was used as outgroup.

Ethical considerations

All procedures concerning the animals were performed in compliance with the national guidelines for animal care.

RESULTS

Despite various pair of primers from literature were applied to target parts of HEV genome, neither single PCR was successful to obtain DNA fragment from PER5 isolate. Fortunately, two nested PCRs using the primers referred as 3506-7 and 3154-77 by Meng et al. [15],

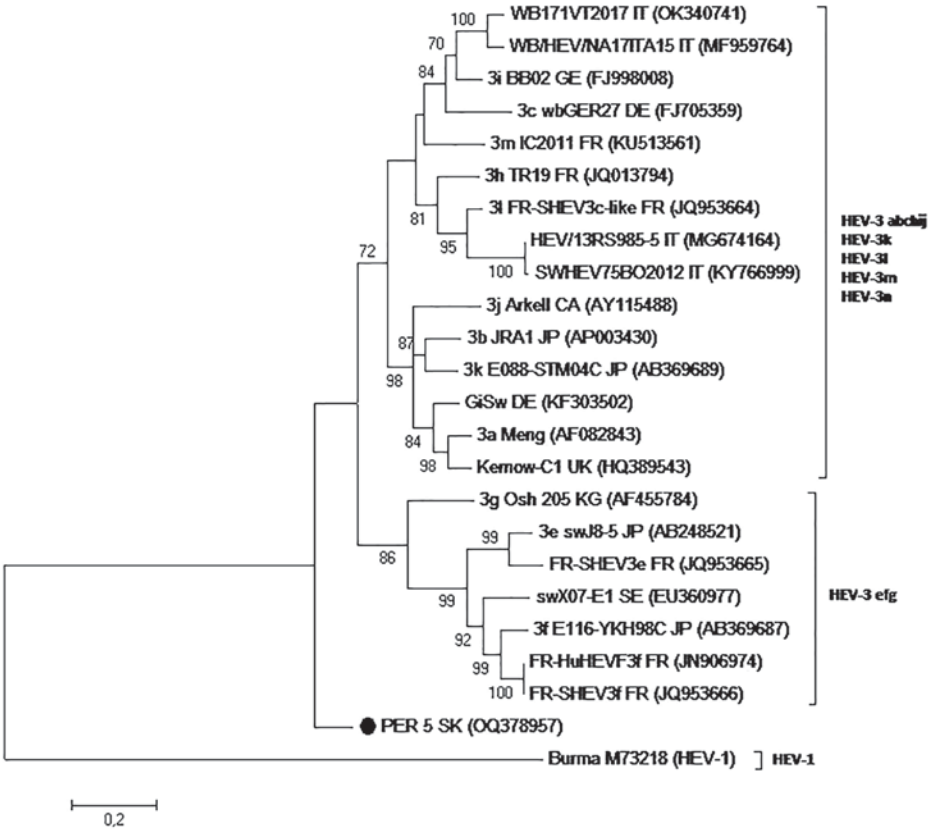


Fig. 1. Phylogenetic tree of HEV nucleotide sequences based on partial ORF1 (749 nt). Bootstrap values > 70 are indicated at their respective nodes. Slovak HEV PER5 sequence are indicated by a black circle. Twelve HEV-3 reference subtypes according to Smith et al. [19] are signed by corresponding letter of subtype. All sequences are denoted by name sequences, ISO code country of origin and NCBI GenBank Acc. No. in brackets. The scale bar indicates nucleotide substitutions *per site*

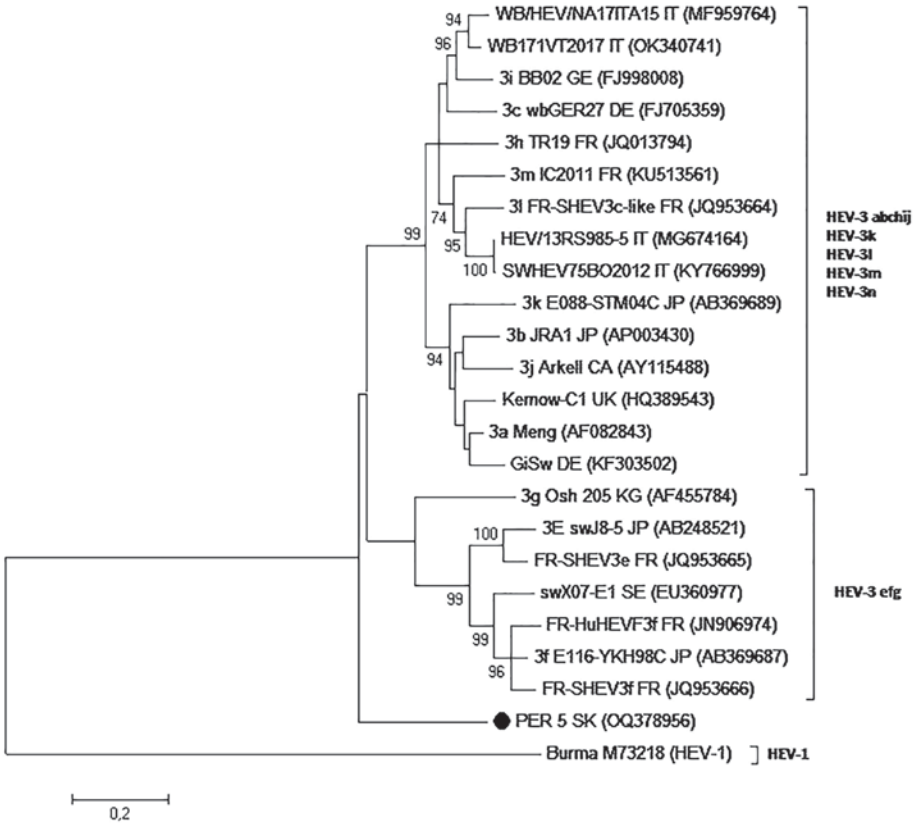


Fig. 2. Phylogenetic tree of HEV nucleotide sequences based on partial ORF1 (828 nt). Bootstrap values > 70 are indicated at their respective nodes. Slovak HEV PER5 sequence are indicated by a black circle. Twelve HEV-3 reference subtypes according to S m i t h et al. [19] are signed by corresponding letter of subtype. All sequences are denoted by name sequences, ISO code country of origin and NCBI GenBank Acc. No. in brackets. The scale bar indicates nucleotide substitutions *per site*

successfully amplified two regions of ORF1. They flanked the positions 3,209–4,324 (for the primer set 3506-7) and 4,189–5,181 (for the primer set 3154-77) in accordance with genome numbering for the representative Meng HEV-3a isolate (GenBank Acc. No. AF082843). The PCR products were sequenced and compared to sequences from the NCBI GenBank database. The blasting in GenBank indicated that DNA fragments were located in the RNA helicase (Hel) and RNA-dependent RNA polymerase (RdRp) regions of HEV-3 genotype, respectively.

For next analysis, the 749 bp and 828 bp DNA fragments long were cut off from two regions amplified. The results of nucleotide identity showed that the Slovakian strain displayed low values: 79.2–84.1 % (749 bp fragment) and 80.4–83.9 % (828 bp) when compared to the established HEV-3 subtypes.

In the phylogenetic analysis, our two sequences were aligned with twenty-two HEV-3 subtype sequences, including reference subtypes proposed by S m i t h et al. [24] as well as other HEV-3 subtypes deposited in GenBank. The phylogenetic trees revealed similar results with both partial OFR1 sequences. The PER5 sequence analysed formed a part of the separate clade clustering outside of two major clades (3abchij and 3efg) of the HEV-3 genotype (Fig. 1, Fig. 2).

DISCUSSION

This study was focused on further genetic characterization of the representative PER5 isolate, which was identified in our study as a potentially new HEV-3 subtype [9]. We were able to amplify new regions in ORF1 which represent together with the sequences obtained previously [9] around one third (2,212 bp) of the HEV genome.

In our study, a total of twenty pairs of primers available in the literature were used for amplification of HEV genome [5, 15, 31]. The amplification of various genomic regions using single PCR primers was not successful most probably due to lower viral content in the tested sample.

However, two nested PCRs with primers proposed by M e n g et al. [15] provided sufficient amount of DNA for sequencing.

The results of nucleotide identity varied in both genomic regions of ORF1 (749 bp and 828 bp) in the range 79.2–84.1 % nt. id. indicating that PER5 isolate was not close related to the HEV-3 subtypes, which have been already classified. This fact is in agreement with low values of the nt. id. between subtypes already detected in other studies. For example, M i u r a et al. [16] obtained 81.0–87.0 % nt. id. of human HEV-3k with other HEV-3 subtypes in Japan. Similar, 81.4–89.9 % nt. id. of swine HEV-3l subtype as well as 80.9–88.6 % nt. id. of wild boar HEV-3n subtype with HEV-3 subtypes have been reported [5, 19].

Additional support for distinct feature of PER5 sequence provided the phylogenetic study. It is striking that in all four regions tested the same results of phylogenetic analysis were obtained [9, this study]. The sequences of PER5 isolate always formed a cluster separated of other identified subtypes, including new proposed HEV-3 (HEV-3k, HEV-3l, HEV-3m, and HEV-3n) subtypes. These data also support the idea that HEV-3 isolates circulating in a Slovakian pig farm belong to new subtype. The existence of unclassified HEV-3 subtypes was also described in other countries (Italy, Japan). They have been just recently proposed as new HEV-3 subtypes (HEV-3k, HEV-3l, HEV-3m, and HEV-3n) based on full-genome sequences [5, 16, 19, 24].

We realize that at present it is generally accepted that data based on nucleotide identity, p-distances between intra- and inter- subtypes or phylogenetic trees constructed with partial sequences do not completely fill in the criteria for the identification of new HEV-3 subtype [8]. So, for definitive conclusion that new HEV-3 subtype circulate in pig farms in Slovakia, the full-length genome sequence analysis is required [17, 26]. Next experiments in the laboratory will be focused to sequence the gaps to obtain complete genome sequence of HEV-3 PER5 isolate using PCR primers designed from our new sequence database created during this project.

CONCLUSIONS

In this study, we genetically characterized another regions of HEV genome in PER5 isolate from domestic pig. The phylogenetic analyses of two DNA fragments confirmed the same results; PER5 isolate clustered out of two major HEV-3 clades (3abchij and 3efg). To conclude, this study provides further evidence that the PER5 isolate from naturally infected domestic pig in Eastern Slovakia probably represents the HEV-3 subtype that has never been identified so far. The subsequent studies will be focused to obtain full genome sequence of unclassified Slovak PER5 isolate.

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