

A Molecular Survey and Genetic Diversity of Hepatitis E Virus in Domestic Swine From Slovakia

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Abstract

Background: Hepatitis E virus (HEV) is agent causing hepatitis worldwide. Originally considered to be limited to developing countries, this virus was also detected in developed countries. In recent years an increasing number of reports indicate that farmed domestic pigs are widely infected with HEV in several European countries including neighbouring countries the Czech Republic and Hungary. The HEV status in Slovakia is still missing.

Results: In this study, the circulation of HEV among domestic swine in Slovakia and genetic diversity of the virus was studied. Overall HEV RNA was detected in 53/388 (13.7%) pig rectal swabs in five production stages (age categories) with statistically highly significant differences ($P < 0.01$, $\chi^2=28.444$). No HEV was detected in suckling piglets and sows. The highest HEV incidence (29.6%) was observed in weaners and then declined in growers and fatteners. Twenty-eight partial sequences of ORF1 (242 bp) and seventeen of ORF2 (304 bp) were genetically analysed. Phylogenetic analysis and p-distance comparisons confirmed in both ORFs that all Slovak HEV sequences belong to the genotype HEV-3, major clade 3abchij with higher identity to 3a and 3i subtypes. Three sequences created a new branch, outside of major clades 3abchij and 3efg, showing high nucleotide p-distances (0.140-0.178). They could represent a novel subtype.

Conclusion: This is the first report to fill the epidemiological gap about HEV infection in pigs in Slovakia. The results indicated a lower incidence of HEV in Slovak pig farms than observed in other European countries. While most HEV isolates were typed as HEV-3 clade

3abchij, three sequences were most probably a candidate for a new HEV-3 subtype.

Background

Hepatitis E virus (HEV) is one of the most common agent causing hepatitis worldwide. Originally considered to be limited to developing countries, it has recently also been shown to be widespread in developed countries [1, 2]. A recent surveillance report of hepatitis E virus infection in the European Union/European Economic Area (EU/EEA) countries showed a tenfold increase between 2005 and 2015 [3].

Hepatitis E virus is a small virus with the diameter of 27–32 nm, generally considered to be non-enveloped [2]. The HEV genome consists of a single-stranded positive sense RNA with a size of approximately 7.2 kb containing three open reading frames (ORFs) and short untranslated regions. The ORF1 encodes a non-structural proteins, ORF2 encodes the glycoprotein that forms the viral capsid and ORF3 encodes a small multifunctional protein involved in virus morphogenesis and release [4]. HEV, a highly variable virus, belongs to the family *Hepeviridae*, genus *Orthohepevirus A* which is subdivided into seven genotypes. Genotypes HEV-1 and HEV-2 are restricted to humans. Genotype HEV-3 was detected by Meng et al. [5] in domestic pigs for the first time in the USA and subsequently over the world in different animal species (e.g. wild boar, mongoose and deer) and humans. Similarly, members of genotype HEV-4

have been detected in both animals and humans. On this basis HEV-3 and HEV-4 are considered to be zoonotic [6, 7]. The genotypes HEV-5 and HEV-6 have been found only in Japanese wild boar [8]. The genotype HEV-7 has been isolated in dromedary camels and is also considered to be zoonotic [9].

The HEV genotypes have been subdivided into numerous subtypes. Subdivision into 24 subtypes has been proposed by Lu et al. [10], but classification of HEV subtypes is still under discussion. The inconsistencies concerning the subtype classification were demonstrated using different methodological approaches [11, 12]. Subsequently, Smith et al. [13] proposed reference sequences for HEV subtypes to improve communication between researchers and to help to clarify the epidemiology of this important pathogen. The use of common reference sequences for each subtype today assists with the interpretation of epidemiological and evolutionary studies of HEV. HEV-3 subtypes 3a, 3b, 3c, 3h, 3i, 3j form one major clade-3abchij, while subtypes 3e, 3f and 3g form another clade-3efg [14]. These two clades correspond to the previously named groups 3-I and 3-II [15], groups 3.1. and 3.2. [11] and group 1 and 2 [16]. The values of nucleotide p -distances among HEV genotypes and subtypes show a complex pattern with multiple hierarchies of relatedness [13].

Several studies have confirmed that domestic swine are suspected to be one of the main reservoirs of HEV [7, 17]. In recent years an increasing number of reports indicate that farmed domestic pigs are widely infected with HEV in several European countries [18–22] including neighbouring countries the Czech Republic and Hungary [23–26]. The HEV status in Slovakia, an EU member country geographically lying in Central Europe, is still missing.

The aim of this study was to gain the first insight into the circulation of HEV in Slovak domestic swine at different stages of production. Molecular-genetic analysis of nucleotide sequences was focused on the typing of HEV isolates to extend our knowledge of genetic variability of the virus.

Results

Detection of HEV RNA in different production stages

Overall, 53 out of 388 (13.7%) rectal swabs were tested positive for HEV RNA (Table 1). HEV positive samples were detected in 13 of 25 farms. The farm size did not affect the HEV incidence. No positive sample was detected in the suckling piglets (≤ 4 weeks) or the sows. The circulation of HEV started among weaners (5–10 weeks) in which the highest HEV presence (29.6%) was observed. Lower numbers of positive animals were detected in growers and fatteners, 14.9% and 14.1%, respectively. Highly significant differences were statistically confirmed ($P < 0.01$, $\chi^2 = 28,444$) among the five production categories.

Sequences and phylogenetic analysis

Twenty-eight PCR products from the ORF1 gene (242 bp) and seventeen from the ORF2 gene (304 bp) were sequenced. They were compared to sequences from the NCBI GenBank database representing swine

HEV strains belonging to the reference sequences of genotypes and subtypes proposed by Smith et al. [13].

Sequence analysis of the partial ORF1 gene showed a nucleotide sequence identity ranging from 81.4 to 100%. HEV sequences obtained from the same farm owner but different sites were almost 100% identical. The phylogenetic analysis based on the partial ORF1 gene revealed that all Slovak HEV sequences were clustered within the genotype HEV-3 with separate branches according to the farm of origin (Figure 1). Four HEV sequences (MO17, SEO1, SEO6, SPO6) clustered unambiguously into the 3a subtype with nucleotide p -distances ranging from 0.085 to 0.106 and a high nucleotide identity (88.0–91.3%) to the 3a reference strain. Three sequences RIV1, RIV14, and RIV18 showed higher p -distances = 0.123 to the 3a reference strain and a lower nucleotide identity (87.2–87.6%). Other analysed sequences could be classified into the major clade 3abchij with ambiguous classification into subtypes, except for three remarkable sequences. These three sequences (PER5, PER11, PER14) clustered in the phylogenetic tree outside both major clades (3abchij and 3efg) of the HEV-3 genotype and created a separate new branch showing high p -distance values 0.140–0.178 and low nucleotide identity (81.4–84.7%) to the reference strains of all HEV-3 subtypes.

The analysis of the ORF2 sequences supported results achieved with ORF1. Very similar nucleotide sequence identities (81.2–100%) and the phylogenetic and sequence analysis of the partial ORF2 (Figure 2) confirmed the clustering and subtyping of Slovak HEV sequences with ORF1.

Discussion

In this work we describe for the first time the detection and genetic characterization of HEV in Slovakia. While the incidence of HEV varied from 18.6–22.9% in different European pig herds [18–20, 22–25], the percentage of HEV RNA positive samples in Slovak herds was lower (13.7%) and the virus was not detected in half (12/25) of the farms investigated.

In our survey, the presence of HEV depended on the age of the pigs. No HEV was detected in the youngest category of suckling piglets (≤ 4 weeks) or in the oldest category – sows. The highest percentage of HEV (29.6%) was detected in weaners with the incidence gradually decreasing in growers and fatteners.

The absence of HEV in suckling piglets might be explained by protection with specific maternal antibodies [5] although some authors detected HEV in sucking piglets ranging from 9–11.8% [25, 29, 30]. A higher incidence of HEV in weaners is consistent with the results of several laboratories [18, 19, 29–32]. Weaners are the most critical category of animals, because declining maternal antibodies results in weaker immunity and the highest susceptibility to infections, including HEV [5]. Immunity is stronger in older animals and it corresponds with our data on lower detection of HEV in growers and fatteners. Very similar observation of HEV in adult pigs older than 6 month has been reported by other authors [30, 32]. A significant decline of HEV detection has been described in adult domestic pigs (> 6 months) in Corsica, a French region hyper-endemic for HEV [33]. A strong decline of HEV from 100% in weaners to 40% in growers and 0% in fatteners observed by Seminati et al. [19] in Spain is in agreement with the trend

observed in our work. No doubt, despite the lower incidence of HEV in older pigs, many pigs at slaughter age are infected with HEV. It should be emphasized that slaughtered HEV infected pigs are a significant risk factor for the introduction of virus to the food chain [34] and represent a risk of foodborne transmission of HEV to the human population [35].

HEV is not always detected in sows as we demonstrated in this work and as has been reported for several Spanish pig farms [19]. A very low HEV prevalence in sows was detected by other laboratories [18, 30, 36]. On the other hand, 53.4% HEV prevalence in old sows (1–5 years) and 38.6% in young sows (11–15 months) was reported in Italy [20]. Pig farms with a high HEV prevalence may in future have difficulty sharing their sows with other farms.

The sequencing of partial fragments from the ORF1 and ORF2 regions revealed genetic diversity and enabled typing of viral isolates. The phylogenetic analysis in both genomic regions (Fig. 1 and Fig. 2) indicated that all Slovak HEV sequences were clustered within the genotype HEV-3 and formed separate branches according to their origin. Most European pig HEV isolates are usually found in this genotype along with several human isolates.

Within the HEV-3 genotype it is difficult to divide sequences into subtypes. At present, there are several combined criteria for this typing. The classification into HEV-3 subtypes is based on the comparison with the reference strains and the value of nucleotide p -distances (≈ 0.123) within subtypes as proposed by Smith et al. [13]. In addition, the location of sequences in the phylogenetic tree and the percentage of nucleotide identity also assist with the typing of HEV-3 isolates.

Looking closer at the phylogenetic tree prepared from ORF1 sequences (Fig. 1) it is clear that Slovak HEV-3 nucleotide sequences were grouped within a mixed 3abchij cluster. The comparison based on nucleotide p -distances (0.085–0.106) showed that four HEV sequences from three farms (MO17, SEO1, SEO6, SPO6) unambiguously belong to the 3a subtype together with the reference strain 3a Meng (AF082843). This is also supported by a high nucleotide identity (88.0–91.3%) of those sequences with the reference strain 3a. Three sequences RIV1, RIV14, RIV18 showed p -distances = 0.123, which is close to the limit for a subtype. Despite their lower nucleotide identity (87.2–87.6%) they were located in the branch with reference strain 3a.

Six Slovak HEV-3 sequences (MO4, MO8, PERviet3, PERviet7 and KLAO2, KLAO3) belonged within the 3i subtype (see 3i cluster on Fig. 1). Their typing was based not only on their position in the phylogenetic tree but also on the nucleotide p -distances (0.119) with the reference strain 3i BB02 (FJ998008). This conclusion was supported by comparison with the Italian strain WBHEVNA17ITA15I (MF959764) which has been recently confirmed as subtype 3i by De Sabato et al. [37]. When comparing six Slovak sequences to this strain, the low nucleotide p -distances (0.076–0.106) and high nucleotide identity (88.4–91.3%) also support their typing into 3i subtype. However, to be correct, this conclusion is disturbed by the observation that p -distance of the discussed six Slovak sequences is in the range from 0.110 to 0.114 with the reference strain wbGER27 (FJ705359) for subtype 3c, as well.

Another 12 Slovak sequences (see top of the tree – Fig. 1) compared with the Italian 3i strain mentioned (MF959764) showed similar low p -distances in the range from 0.097 to 0.106 and nucleotide identity from 88 to 88.4%. This data together with clustering in the maximum likelihood phylogenetic tree (Fig. 1) support a conclusion that they belong to the subtype 3i.

Most HEV sequences from Western European countries such as the Netherlands, France, Italy, Spain and Sweden are clustered into subtypes 3e, 3f and 3g (3efg major clade) [15, 17, 21, 22]. Surprisingly, none of Slovak HEV sequences clustered into 3efg clade despite all HEV isolates from the neighbouring country Czech Republic published so far have fallen into subtypes 3f and 3g [23, 24]. When looking at HEV isolates from another neighbouring country Hungary they have been clustered into the subtypes 3e and 3a [25], indicating a possible relationship to 3a isolates from Slovakia, most probably due to the common trade of animals.

The last three Slovak HEV sequences (PER5, PER11, PER14 – Fig. 1, marked with a question mark) originating from a farm in Eastern Slovakia require special attention. They are highly divergent and cluster outside both major 3abchij and 3efg phylogenetic clades. These sequences showed relatively high nucleotide distances (p -distances 0.140–0.178) when compared to $p \leq 0.123$ defined by Smith et al. [13] for the subtype. A new branch in the phylogenetic tree and a low nucleotide sequence identity (81.8–84.7%) compared to the other reference strains of HEV subtypes most probably indicate a new HEV-3 subtype. This conclusion is in agreement with the recently proposed novel subtype 3l with p -distance > 0.129 [38], which is close to the p -distance 0.140–0.178 determined for the three Slovak unclassified HEV sequences.

The analysis of HEV-3 sequences in ORF2 (Fig. 2) confirmed results from ORF1 for the subtype 3a (p -distances 0.070–0.083). The clustering of other sequences into HEV-3 subtypes was not unambiguous (3i and 3c with p -distances 0.120–0.123) but the classification into major clade 3abchij was confirmed as with ORF1. The sequences PER5, PER14 created a separate branch outside clades 3abchij and 3efg with the p -distance values of 0.147–0.187 again confirming them as candidates for a novel HEV-3 subtype.

Conclusion

This is the first report to fill the epidemiological gap about HEV infection in pigs in Slovakia. The results indicated a lower incidence of HEV in Slovak pig farms than observed in other European countries. It is promising that HEV did not circulate on all farms, nor in all production categories. Nevertheless, the occurrence of HEV in fatteners (slaughter age) is a risk to public health, because HEV is considered to be a foodborne pathogen with potential zoonotic transmission. While most HEV isolates were typed as HEV-3 clade 3abchij, three sequences were most probably a candidate for a new HEV-3 subtype. Our work also indicates that systematic genotyping of HEV isolates even from a small geographic region may lead to unexpected results of interest to the international community.

Material

Sample collection

A total of 388 rectal swabs were collected from pigs (*Sus scrofa domestica*) of different production stages (different age categories) from 25 pig farms located in four districts of Slovakia. These farms housed from ≤ 100 to ≥ 1000 animals. The majority of farms focused on two or three production stages of pigs, namely weaners, growers and fatteners (see below). Only two conventional closed farms had over 1000 pigs and all production categories including sows and piglets. Nine farms had less than 100 animals.

Samples were collected using swab applicators (Sarstedt AG & Co, Germany) from five production stages: suckling piglets (≤ 4 weeks, $n=53$), weaners (5–10 weeks, $n=81$), growers (11–16 weeks, $n=67$), fatteners (≥ 17 weeks, $n=135$) and sows (1–3 years, $n=52$). The health status of all pigs was evaluated by qualified veterinarians on each farm. All pigs were asymptomatic with no clinical signs observed.

HEV RNA extraction and reverse transcription

Rectal swabs were eluted in 1 ml of 0.01 mol/l PBS (Merck Millipore Corp., USA) for 30 min, vortexed at 2000 rev. min⁻¹ for 3 min and centrifuged at 14,000 x g for 5 min. Total RNA was extracted from 140 μ l supernatant using the QIAamp® viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) by robotic station (QIAcube GmbH, Hilden, Germany) according to the manufacturer's instruction. Supernatants and extracted RNA were stored at -80°C. The cDNA was synthesized in a 20 μ l reaction mix comprising 5 μ l 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 μ M of gene specific reverse outer primers [17, 27] (Microsynth Austria, GmbH, Austria), 20 U RNase inhibitor (Invitrogen, Inc., USA) and molecular biology grade water (Merck, GmbH, Germany). The mix was incubated at 65°C for 5 min then chilled on ice. Subsequently, the mix was incubated at 50°C for 30 min to synthesise cDNA and finally at 85°C for 5 min to terminate the reaction.

Nested RT-PCR and sequencing

The detection of HEV was based on the amplification of a 287 bp fragment of methyltransferase (MTase) in ORF1 using outer and inner primers [27] and a 348 bp fragment of capsid protein in ORF2 using primers by Meng et al. [5]. The PCR reaction mix (50 μ l) was composed of 1x ThermoPol reaction buffer (New England Biolabs, Inc., USA), 0.2 mM dNTPs (Thermo Fisher Scientific, Inc., USA), 300 nM of outer primers, 1 U Taq DNA polymerase (New England Biolabs, Inc., USA), 4 μ l cDNA and molecular biology grade water (Merck, GmbH, Germany). The first 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 at 55°C 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). For the second PCR with inner primers a similar thermal profile was used. The size of PCR products was checked by electrophoresis in 2% agarose gel after staining with GelRed™

(Biotium, Inc., USA) and visualized by Gel Doc EZ imager (Bio-Rad Laboratories, Inc., USA). PCR products with the expected size of 287 bp and 348 bp were purified and sequenced by the Sanger method with the PCR primers by a commercial company (Microsynth Austria, GmbH, Austria).

Phylogenetic analysis of HEV sequences

Partial ORF1 (242 bp) and ORF2 (304 bp) sequences (primers were omitted) were edited and aligned using the programmes SeqMan, EditSeq and MegAlign (Lasergene, DNASTAR, Inc. USA). Sequences were first checked against the NCBI GenBank database using nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Two representative phylogenetic trees were constructed based on the partial ORF1 and ORF2 nucleotide sequences. The model tests and *p*-distance calculations were performed by MEGA6 [28]. Maximum Likelihood phylogenetic analysis of partial ORF1 gene using the GTR+G+I (General time reversible model with Gamma distribution plus evolutionarily Invariable sites) model was used. For the ORF2 gene the K2+G+I (Kimura-2 parameter model with Gamma distribution plus evolutionarily Invariable sites) model was employed. Models with the lowest BIC scores (Bayesian Information Criterion) were used for phylogenetic analysis. The bootstrap support values of branches were calculated from 1000 replicates. All 45 Slovak HEV nucleotide sequences obtained in this study were submitted to NCBI GenBank database under accession numbers: MT408248-408292.

Statistical analysis

The incidence of HEV was statistically analysed by production categories (suckling piglets, weaners, growers, fatteners, sows) using the chi-square (χ^2) test with confidence limits of 95%, $P < 0.05$ (statistically significant) or 99%, $P < 0.01$ (highly statistically significant) using GraphPad Prism 5 for Windows (GraphPad Software, USA).

Abbreviations

HEV

hepatitis E virus; ORF:open reading frame; RT-PCR:reverse transcription - polymerase chain reaction

Declarations

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Authors'contributions

AJ and SV conceived and designed the study; RM and JN collected defined samples; AJ, KD, SS performed laboratory analysis, molecular genetic studies and statistical analysis; AJ and SV contributed to the writing and revision of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated and/or analysed during the current study are available in the GenBank repository (MT408248 to MT408292). All data and additional files are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

None of the pigs were killed with the purpose to fulfil the objectives of the present study. The sample collection work was approved with informed consent from the pig farm owner under condition that the name of farms will be anonymous. The protocol for collection of clinical samples followed the guidelines stated in the Guide for the Care and Use of Animals (protocol number 3323/16–221/3) which was approved by the State Veterinary and Food Administration of the Slovak Republic and by Ethics Commission of the University of Veterinary Medicine and Pharmacy in Kosice, Slovakia.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interest.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

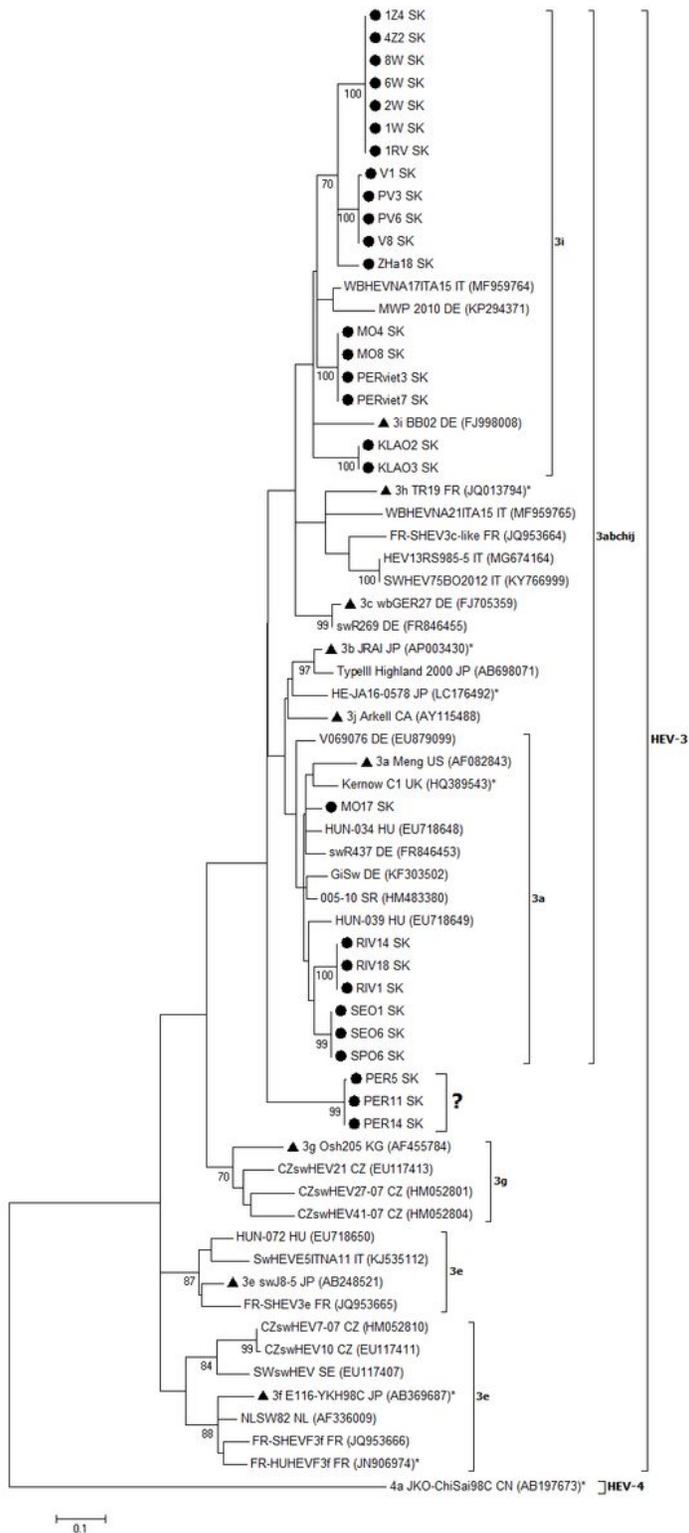


Figure 1

Phylogenetic tree of HEV nucleotide sequences based on partial ORF1 (242 nt). The maximum likelihood phylogenetic tree was built with GTR + G + I substitution model and a bootstrap resampling process (1000 replications) was used to assess node support. Bootstrap values >70 are indicated at their respective nodes. The tree included 66 HEV sequences: i) 28 Slovak HEV sequences are indicated by a black circle ii) 38 HEV sequences were selected from NCBI GenBank database, HEV-3 reference subtypes

according Smith et al. [13] are indicated by a black triangle. Highly diverse sequences outside known subtypes are marked with a question mark. The HEV-4 strain was used as an outgroup. All sequences are denoted by name sequences, ISO code country of origin and NCBI GenBank accession number in brackets. Human HEV sequences are marked with an asterisk. The scale bar indicates nucleotide substitutions per site.

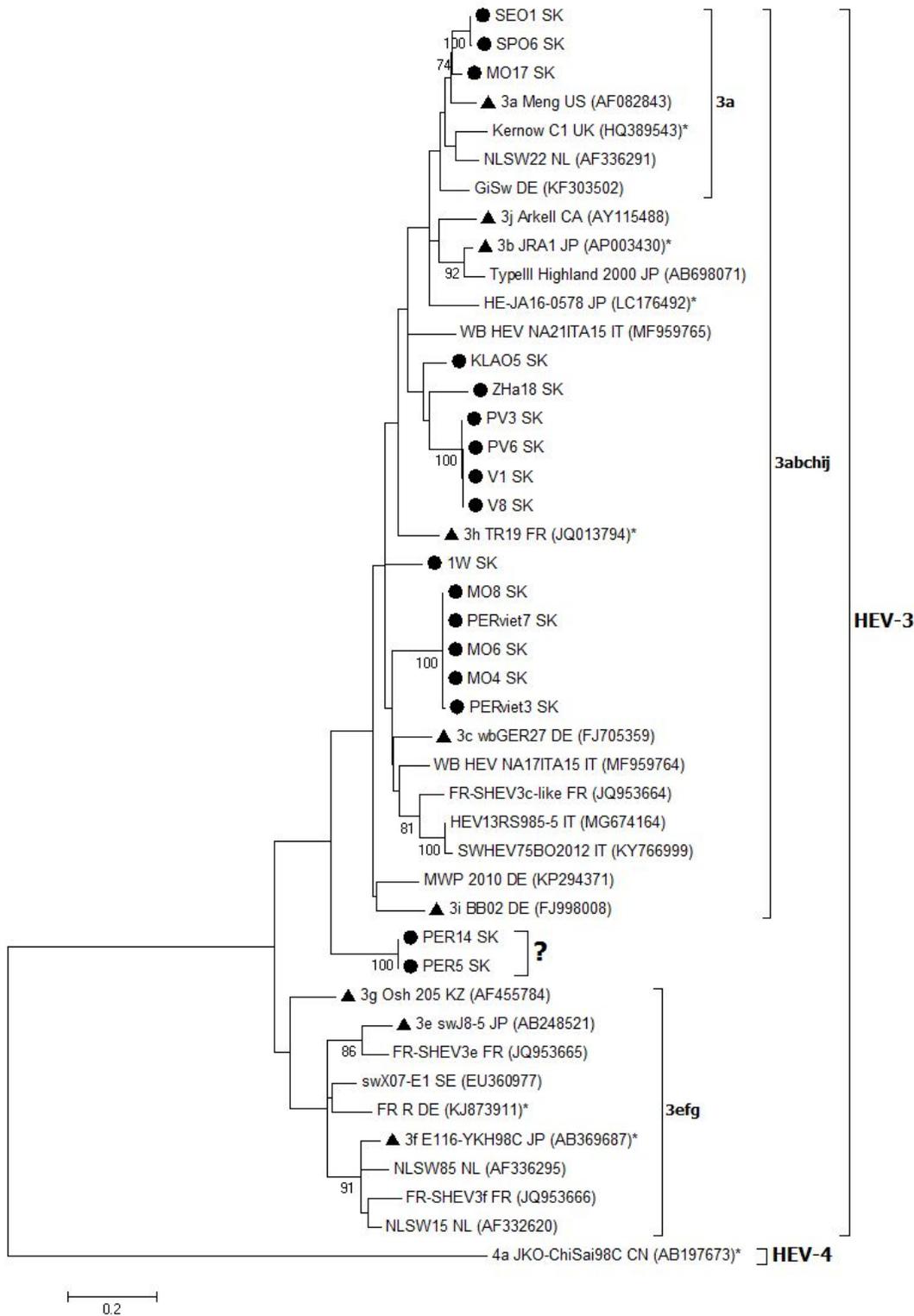


Figure 2

Phylogenetic tree of HEV nucleotide sequences based on partial ORF2 (304 nt). The maximum likelihood phylogenetic tree was built with K2 + G + I substitution model and a bootstrap resampling process (1000 replications) was used to assess node support. Bootstrap values >70 are indicated at their respective nodes. The tree included 44 HEV sequences: i) 17 Slovak HEV sequences are indicated by a black circle ii) 27 HEV sequences were selected from NCBI GenBank database; HEV-3 reference subtypes according Smith et al. [13] are indicated by a black triangle. Highly diverse sequences outside known subtypes are marked with a question mark. The HEV-4 strain was used as an outgroup. All sequences are denoted by name sequences, ISO code country of origin and NCBI GenBank accession number in brackets. Human HEV sequences are marked with an asterisk. The scale bar indicates nucleotide substitutions per site.

Supplementary Files

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- [Table1.JPG](#)