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ORIGINAL PAPER

Screening and Molecular Characterization of Hepatitis E Virus in Slaughter Pigs in Serbia

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Abstract

Hepatitis E virus (HEV) is a zoonotic virus that can cause acute hepatitis in humans. Besides the fecal–oral route, transmission can occur by consumption of undercooked pig liver. Genotype 3 is the most frequent genotype found in Europe. Studies on HEV in slaughter-age pigs have not been conducted in Serbia so far. Pork meat production and consumption in Serbia is on average, higher than in the rest of Europe. With the aim to identify the circulating HEV genotypes, pig livers and swab samples from three pig slaughterhouses located in three different sub-regions of Serbia were collected. A nested RT-PCR was used to amplify the hypervariable HEV ORF-1 region (334 bp). The amplicons yielded in this study were sequenced, and a molecular phylogeny analysis based on the maximum likelihood method, including HEV sequences reported in several other countries, was performed. The average prevalence of HEV genotype 3 in 3-month-old pigs was 34%. Phylogenetic analysis revealed the majority of HEV amplification fragments from Serbia were grouped in four clades within sub-genotype 3a and were also genetically related to German, Italian, Slovenian, and American HEV sequences. Sub-genotypes 3b and 3j were also found in a single pig each. This study provides the first analysis of the genetic diversity and circulation dynamics of HEV in pigs at slaughterhouses in Serbia.

Keywords Hepatitis E virus · Slaughterhouse · Pigs · Serbia

Introduction

Increased urbanization, and conversion of the food market from locally oriented toward globally driven, has pushed policy makers to impose more stringent hygienic standards and various control strategies for pathogenic bacteria, viruses, and parasites. Unsafe raw food, poor storage infrastructure, poor personal hygiene, improper handling methods, inadequate cooking and cross contamination of cooked food with uncooked, raw food are just some of the common causes of foodborne diseases that need to be prevented by these new measures. Foodborne illnesses caused by food viruses are a significant and emerging global problem, and therefore, an important cause of reducing economic growth in many countries of the world (Shirazi et al. 2018). In industrialized countries, the norovirus is causative agent of the majority of viral foodborne diseases (De Aceituno et al. 2013). Nevertheless, other foodborne viruses such as hepatitis E virus (HEV) are deemed an emerging risk in veterinary public health, especially if animal reservoirs are not known, as the virus is zoonotic (Meng et al. 1998).

HEV is a small, non-enveloped icosahedral virus with a single-stranded, positive-sense RNA genome approximately 7.2 kb in size. The RNA consists of short, untranslated regions and three major open reading frames (ORF1, ORF2, and ORF3). HEV is a member of the Ortohepevirus genus in the Hepeviridae family (Smith et al. 2014). Four species, which are named from Orthohepevirus A to Orthohepevirus D, belong to the genus Orthohepevirus. According to Smith et al. (2014), Orthohepevirus B are separated into four proposed subtypes, and they are mainly detected in domestic

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chickens. Orthohepevirus C is divided into two genotypes that are mostly detected in rats (HEV-C1) and carnivores (HEV-C2), while Orthohepevirus D has been detected in bats. Orthohepevirus A is the well-researched type, and furthermore, it comprises seven genotypes, of which genotypes 1–4 can infect humans (Doceul et al. 2016). Genotypes 3 and 4 are common to humans and other animal species (Dalton et al. 2016), while genotypes 1 and 2 commonly infect humans through water (Purcell and Emerson, 2008). There are multiple transmission routes through which people can be infected with HEV genotypes 3 and 4. In underdeveloped countries the most important route of transmission still is the consumption of feces-contaminated water. However, in industrialized countries usual route of transmission is consumption of infected animal tissues and food products (Moor et al. 2018; Park et al. 2016), but the infection can also occur during organ transplantation or blood transfusion. Different types of animals can be infected with genotypes 3 and 4, but domestic pig (*Sus scrofa domesticus*), deer (*Cervidae*) and wild boar (*Sus scrofa*) are at the top-ranking, most important animal reservoirs with these genotypes (Aprea et al. 2018; Caruso et al. 2017). The liver is the target organ for HEV and is where it replicates, but it was also detected in several other tissues. In pigs, the main route of transmission is fecal–oral. Pigs start to shed the virus in feces between 1 and 2 weeks after infection, and the shedding continues for up to 2 months (Widen 2016). Infected pigs are usually without any apparent clinical symptoms, although in some cases, mild to moderate, acute, self-limiting hepatitis can occur (Meng, 2010). As a consequence, HEV-infected pigs enter the slaughterhouse as healthy animals, and hence, their tissues and meat can pose a risk to human consumers. Pork liver and pork liver products are the most obvious sources of foodborne HEV. Pavio et al. (2014) found that 17.3% of foods containing raw pork liver tested positive for HEV. Di Bartolo et al. (2015) also detected HEV in sausages sold in Spain and Italy, while Szabo et al. (2015) discovered HEV in livers and sausages sold in Germany. Furthermore, the virus has also been found in muscle tissue that could also be a source of infection in humans (Rivero-Juarez et al. 2017). Some researchers suggest that general food heating recommendations (70 °C/2 min) may not be efficient for total HEV inactivation (Johne et al. 2016). If the virus is present in tissue or in another food matrix, it exhibits a high degree of resistance to heat treatment (Ricci et al. 2017).

In humans infected with HEV, illness is usually asymptomatic (Sridhar et al. 2015; Weigand et al. 2018). A certain percentage of cases suffer icterus with nausea, fever, abdominal pain, vomiting, and hepatomegaly after an incubation period that lasts between 2 and 8 weeks (Park et al. 2016). Fatal outcomes in humans are rare, occurring in approximately 2% of cases (Park et al. 2016). The disease is usually self-limiting, but sometimes it can become chronic, and among pregnant women, it can be highly lethal, with mortality rising to 25% (Aggarwal and Jameel 2011).

The aim of this longitudinal study was, therefore, to assess the importance of the domestic pig population in Serbia as a potential reservoir of HEV. A prevalence survey in different counties in Serbia was carried out to determine the prevalence of HEV in slaughter pigs, and the phylogenetic relationship between circulating HEV genotypes.

Materials and Methods

Study Details and Sampling Protocol

Sampling was undertaken between January and May, 2018. A total of 345 samples were taken: pig liver samples collected from slaughterlines; environmental swabs from the same slaughterhouses; and unrelated pig livers sampled at retail. Three counties in western, northern, and central Serbia were carefully chosen (Fig. 1). In each county, one high-throughput slaughterhouse was selected where a total of 100 clinically healthy pigs were sampled on the slaughterline. The pigs from each slaughterhouse were divided into two age groups: 3 months old (50 samples) and 6–12 months old (50 samples). From each individual animal, liver was sampled with a sterile scissors and forceps from the lobe under the gall-bladder after its removal. All samples were collected in sterile 50 mL Falcon centrifuge tubes and stored in a deep freezer at − 70 °C until further processing.

During the same visit to each slaughterhouse, five pooled environmental swabs were collected from different sites/surfaces on the slaughterline that had contact with offal during manipulation—hooks hanging liver, inspection tables, knives, sharpener, and offal collection containers. Just prior to swabbing, dry cotton swabs (Copan, Italia) were moistened in 5 mL of Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Germany).

Next, several retail stores were selected within each county and ten samples of pig livers were collected independently from other samples and without any correlation to the pigs sampled at slaughter, mentioned above.

All samples were stored in a deep freezer at − 70 °C until further processing.

Sample Preparation and RNA Extraction

Firstly, 100 µg of each liver sample was homogenized in 1 mL of Trizol (Invitrogen, USA) and with 600 µg of zirconia beads using a BeadBeater homogenizer (Biospec, USA). Next, 200 µL of chloroform was added. The mixture was vortexed for 2 min, incubated for 10 min at room temperature, and centrifuged at 12,000×*g* for 10 min at 4 °C. Phase separation was facilitated using phase lock heavy

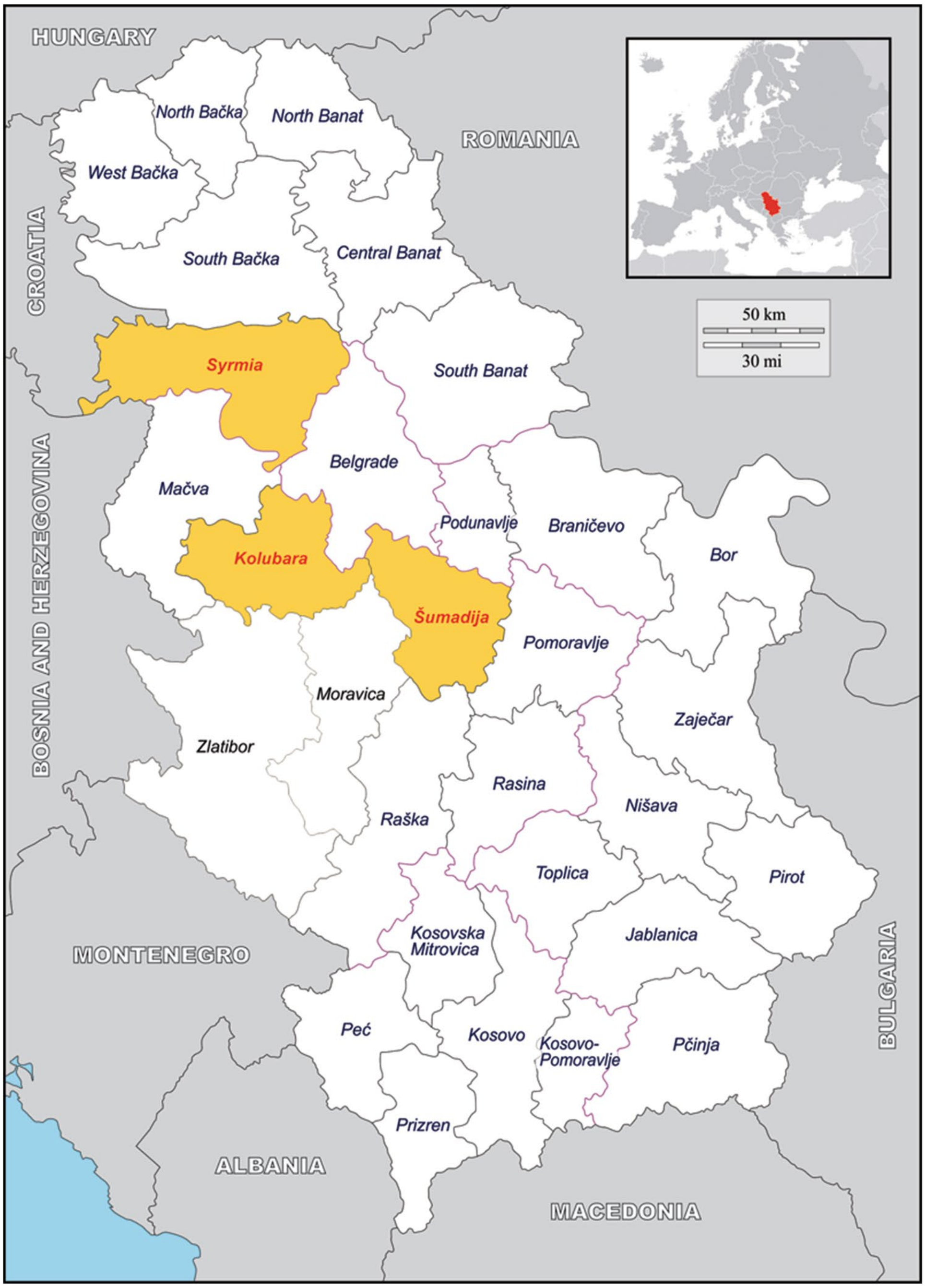

Fig. 1 Map showing counties (in yellow) in Serbia where livers and swabs were sampled (Color figure online)

gel tubes (5 Prime, Germany). The upper aqueous phase was collected and stored at − 70 °C until RNA extraction. Total viral RNA was extracted from samples using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

Environmental swabs were first vortexed for 30 s and the suspensions were then centrifuged for 15 min at 2500×*g*. Total viral RNA was extracted from the supernatants using the aforementioned RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

Detection and Amplification of HEV RNA

A quick and highly sensitive real-time PCR (RT-PCR) assay was implemented to detect HEV RNA, using previously published but partially modified oligonucleotides (Jothikumar et al. 2006). Modification included replacement of adenosine with degenerated nucleotide "R" in JVHEVF forward primer (5′-GGTGGTTTCTGGRGTGAC-3′) in position 5323 (nucleotide positions are in accordance with GenBank accession number AF060669). Reverse primer, JVHEVR

(5′-AGGGGTTGGTTGGATGAA-3′) and probe JVHEVP (5′-TGATTCTCAGCCCTTCGC-3′) were not modified. The primers and probe were designed in the overlap ORF2/3 region of the HEV genome. TaqMan probes were labeled with the fluorophore and quencher molecules FAM/Black-hole Quencher 2 (Microsynth, Switzerland). TaqMan RT-PCR was performed in 20 µL reaction volumes comprised of 5 µL of 5× Reaction Mix (RNA UltraSense One-Step Quantitative RT-PCR System, Invitrogen, USA), 0.5 µL of 50 mM $MgSO_4$, 0.5 µM of forward primer, 0.9 µM of reverse primer, 0.25 µM of probe, and 5 µL of total RNA extracted, for each sample. The reactions were carried out in 96-well optical reaction microplates (Agilent, USA) in an AriaMX Real-Time PCR machine (Agilent, USA). RNAs were reverse transcribed and amplified according to the following program: 1 cycle at 55 °C for 60 min and 95 °C for 5 min, followed by 50 cycles of 95 °C for 15 s, 60 °C for 60 s, and 65 °C for 60 s. Previously identified HEV-positive liver samples (GenBank acc. no. MG051648), confirmed as HEV by Sanger sequencing, and a known negative liver sample were included as positive and negative controls in each run. All samples with a cycle threshold value (Ct) for detecting HEV lower than 40 were interpreted as being HEV-positive, and all other samples were interpreted as being HEV-negative.

Nested RT-PCR

A nested reverse transcription polymerase chain reaction (nRT-PCR) analysis was performed using the external primers HEV-cs and HEV-cas to amplify a 472 bp fragment and the internal primers HEV-csn and HEV-casn to amplify a 334 bp fragment located at the 5′ end of the ORF1 region encoding RNA-dependent RNA polymerase (Johne et al. 2010). The initial RT-PCR was performed with an One-Step RT-PCR Kit (Qiagen, Hilden, Germany) in a 20 µL volume, using 5 µL of total extracted RNA, 0.6 µM of the external primers, and 0.8 µL of the enzyme mix. The second amplification was performed with a Taq PCR Core Kit (Qiagen, Hilden, Germany) in a 50 µL volume, using 2 µL of the RT-PCR product, 0.5 µM of the internal primers, 2.5 mM of $MgCl_2$, and 2 U of enzyme. The RT-PCR conditions were as described by Johne et al. (2010), and all amplifications were performed on an AB2720 thermal cycler (Applied Biosystems, Foster City, USA). Negative controls (extraction and amplification) were incorporated into each step of the detection process. The nRT-PCR products were visualized in 1.5% agarose gel stained with SYBR Safe Dye (Invitrogen, Carlsbad, USA). Bands of the expected size were excised and cDNA was purified by Wizard SV Gel and the PCR Clean-Up System (Promega, Madison, USA), following the manufacturer's instructions.

Sequencing and Molecular Phylogeny Analysis

All samples positive for the presence of 334 bp PCR fragments were subjected to Sanger sequencing (GATC-Eurofins, Cologne, Germany). Phylogenetic analysis was attempted on all samples containing HEV RNA detectable above a lower limit threshold corresponding to a cycle threshold (Ct) value of 40 and on a proportion of samples with lower Cts. Only sequences with high quality electropherograms were considered for the analysis. ORF1 sequences were compared to HEV3 sequences reported in the NCBI database (GenBank) that corresponded to humans, pigs, and other animals from around the world. Editing, multiple sequence alignment, choice of a nucleotide substitution model, and molecular phylogeny analysis by the maximum likelihood method were performed using parameters included in the MEGA X software (Kumar et al. 2018). Statistical confidence of the phylogenetic relationships was determined by bootstrap analysis, with 1000 replications and using Kimura's two-parameter model. Accession numbers for sequences obtained from the NCBI database are listed in the phylogenetic tree. Sequences obtained in this study were compared to sub-genotype 3 reference sequences proposed by Smith et al. (2016). Selected sequences ($n = 26$) from the Serbian HEV-positive samples were deposited in the GenBank database under accession numbers MK474091 to MK474114, and MK570855 to MK570856.

Results

Out of 345 samples tested, originating from three distinct counties (Fig. 1), a total of 59 samples (17.1%) were positive for HEV. Grouped by animal/age categories, HEV was detected in a total of 51 livers (34%) collected from 3-month-old pigs originating from all three counties, while it was not detected at all in 6–12-month-old pigs. No HEV was found in liver sampled at retail in any of the counties. Among the three counties where HEV was found, the highest HEV prevalence was in Syrmia County (82%), while the lowest prevalence (8%) was noted in Kolubara County (Table 1).

On average, positive pig livers from Šumadija, Kolubara, and Syrmia counties had Ct values ± 2SD of 33.50 ± 5.21, 28.79 ± 6.89, and 27.30 ± 5.83, respectively.

Out of 15 environmental swab samples, 8 were positive for the presence of HEV RNA. The highest HEV prevalence in swabs was in Syrmia County (100%). The highest Ct values were detected in swabs from Kolubara county (ranging from 36.43 to 36.79), while the lowest Ct's were detected in swabs from Syrmia county (ranging from 24.29 to 34.02).

Out of all 59 HEV-positive samples (both livers and swabs), only 26 samples in which Ct value was < 30 were

Table 1 Prevalence of HEV in pig-related samples grouped by Serbian county

Sample category	County		
	Šumadija	Kolubara	Syrmia
Liver pigs 0–3 m	12% (6/50)	8% (4/50)	82% (41/50)
Liver pigs > 6 m	n.d. (0/50)	n.d. (0/50)	n.d. (0/50)
Liver at retail	n.d. (0/10)	n.d. (0/10)	n.d. (0/10)
Swabs from slaughterline	n.d. (0/5)	60% (3/5)	100% (5/5)

further sequenced. This group consisted of 25 HEV-positive pig livers and one swab. The sequencing results of the partial RNA-dependent RNA polymerase gene (334 nucleotides in the ORF1 region) and phylogenetic tree constructed using all 26 sequences from this study plus other representative HEV sequences (Smith et al. 2016) revealed that 23 of them were clustered into HEV-3a subtype (Fig. 2). All HEV sequences from Šumadija County had very high mutual homogeneity and they formed a separate cluster (cluster 4), which differed from all other sequences, while sequences from the two other counties formed another three clusters (Fig. 2; clusters 1, 2, and 3). Next, two samples belonged to HEV-3b and HEV-3j subtypes, respectively. Remaining one HEV-positive swab was also clustered in Cluster 2 within HEV-3a subtype. In general, the detected HEV sequences shared 78.3–100% nucleotide identity to each other indicating their high genetic diversity. The sequences detected on different types of HEV-positive samples (liver and swab from Syrmia county) originating from the same slaughterhouse shared 99.2–100% nucleotide identity (Table 2).

Beside HEV 3a subtype, two other sequences were identified as subtypes 3b and 3j, each in one pig. By analysis of the hypervariable region of ORF1, Serbian strain HEV-3b grouped closely with reference Japanese gt3-hHev1 strain while Serbian HEV-3j strain grouped with reference Swine hepatitis E virus strain Arkell (Fig. 3). In the slaughterhouse in Syrmia County, the liver sequence obtained from one particular pig was not identical to the related swab sequence from the same pig.

Discussion

Currently, there are many published data that have been reported by different countries in Europe on the role of pigs as potential HEV reservoirs. Nevertheless, there are still some gaps in our understanding of the role of HEV in the food chain. In Serbia, scientific and research data addressing this topic are still scarce. We examined liver samples from domestic pigs because their tissues are subject to human consumption, and, as already known, these animals play the roles of host and reservoir of HEV. For the purposes of stratification, our pig livers were divided into two age categories. Although the vast majority of pigs are slaughtered at 6-12 months of age, due to Serbs' pork consumption habits and culture, a significant number of pigs under the age of 3 months are also slaughtered. Hence, this was the primary rationale to include these in the study. Next, pigs younger than 2 months of age may be protected by passive immunity, but after that period, piglets easily get infected (Casas et al. 2011). It has also been accepted that breeding sows can play a role as HEV reservoirs and transmit the virus to the suckling piglets after the initial post-natal protection time has passed (Monini et al. 2015).

In this study, all tested livers from pigs aged 6 to 12 months were HEV-negative. Raspor Lainšček et al. (2017) found similar results, showing that the HEV prevalence in Slovenia in pigs older than 6 months was quite low (0.25%). However, reported data from other European countries show different prevalence data in this older age category of pigs. For example, Feurer et al. (2018) found the HEV prevalence in pigs of 6 months or older was 2.8% in France. Similar HEV prevalence rates were reported in pigs in this age category in other countries: 4% in Germany (Wenzel et al. 2011), 5% in the Czech Republic (Di Bartolo et al. 2012), 6% in Spain (Casas et al. 2011), 6.5% in the Netherlands (Bouwknegt et al. 2007), and 3% in the UK (Berto et al. 2012).

Unlike the older animals, in the group of livers from piglets up to 3 months, the overall HEV prevalence was 34%. In neighboring Hungary, a HEV prevalence of 36% for this age category pigs was reported by Forgách et al. (2010). Di Bartolo et al. (2011) and Ruggeri et al. (2013) reported that the prevalence in Italy was 30%, which is also similar to our results. However, it is quite peculiar that in Slovenia, which is adjacent country, the HEV prevalence in this age category of piglets was as half lower than in surrounding countries, at the level of 12.1% (Raspor Lainšček et al. 2017). Similarity of prevalences could be attributed to the geographical adjacency of aforementioned countries to Serbia.

Differences in the HEV prevalence in the two age categories of pigs in Serbia compared to other European countries could be explained as follows: due to intensive swine production (and significantly higher number of animals) in EU versus Serbia, it is possible that some pigs in EU occasionally become re-infected due to co-infection with PRRSV, urine discharge, and developing chronic HEV infection (Widen 2016; Clemente-Casares et al. 2016; Salines et al. 2017). Next, it is also possible that pigs imported in Serbia (usually at the age of 7 weeks) become infected during 4 month fattening and after seroconversion which usually occurs at age of 6 months titer of anti-HEV

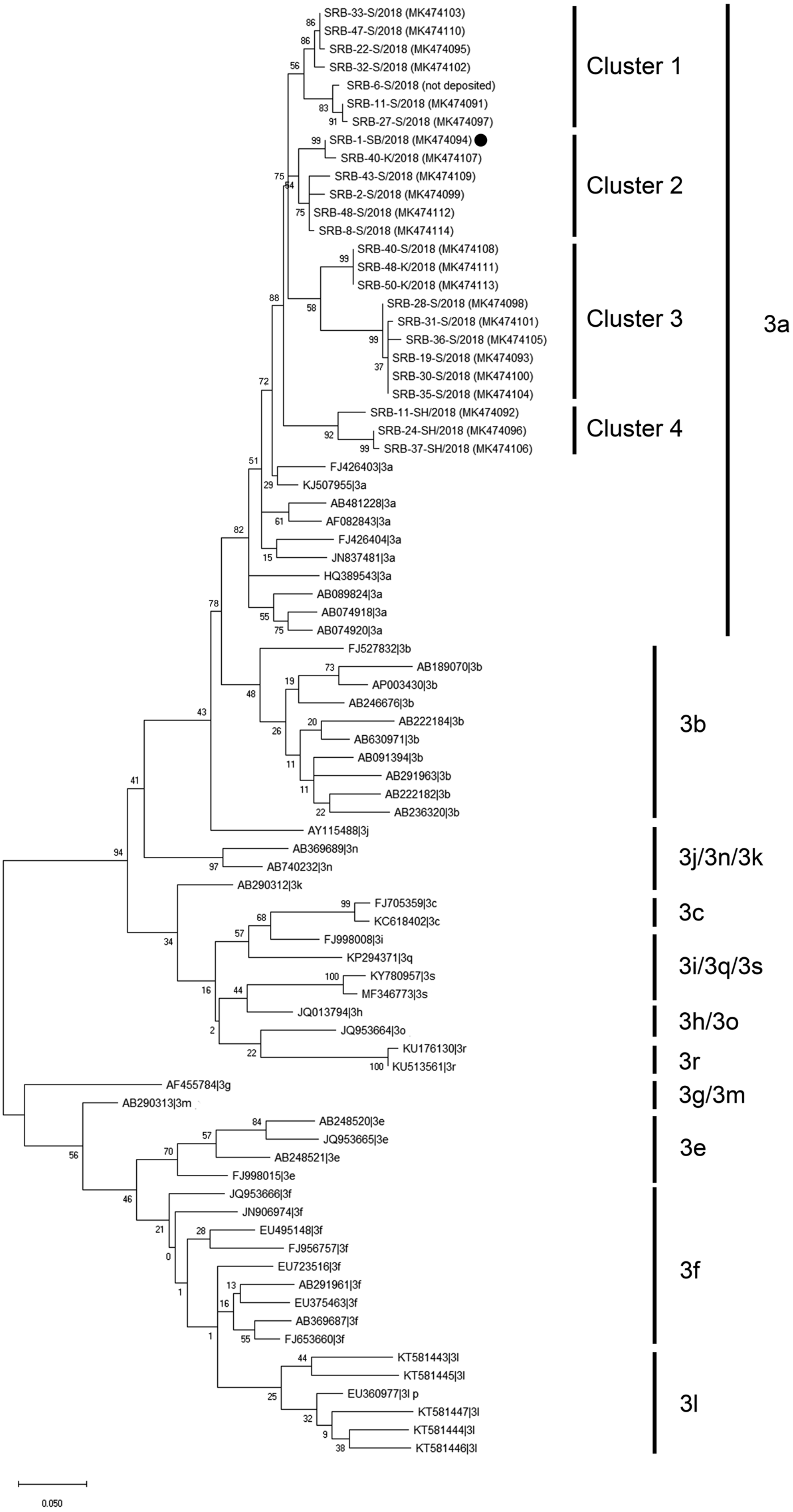

Fig. 2 ORF1-based phylogenetic tree. Unrooted phylogenetic tree, constructed using HEV ORF1 334 bp-length partial sequences, and based on a maximum likelihood analysis employing the General Time Reversible model (Nei and Kumar 2000). The tree with the highest log likelihood (−4474.78) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. This analysis involved 80 nucleotide sequences. There were a total of 265 positions in the final dataset. Bootstrap values acquired after 1000 replications are shown (branch lengths measured in the number of substitutions per site). Serbian sequences were compared only to reference sequences of sub-genotype 3, as proposed by Smith et al. (2016). Black circle denotes HEV-positive swab

Table 2 ORF1 (334 bp) sequenced HEV-positive pig-related samples with data on type of sample, sex and age of pig, county of origin, HEV subtype, and accession number in GenBank

Name of positive sample	Sex of pig	Age	County	HEV subtype	Accession No
SRB-11-SH/2018	Male	0–3	Šumadija	3a	MK474092
SRB-24-SH/2018	Female	0–3	Šumadija	3a	MK474096
SRB-37-SH/2018	Male	0–3	Šumadija	3a	MK474106
SRB-2-S/2018	Female	0–3	Šumadija	3a	MK474099
SRB-8-S/2018	Female	0–3	Syrmia	3a	MK474114
SRB-11-S/2018	Female	0–3	Syrmia	3a	MK474091
SRB-19-S/2018	Female	0–3	Syrmia	3a	MK474093
SRB-22-S/2018	Female	0–3	Syrmia	3a	MK474095
SRB-27-S/2018	Male	0–3	Syrmia	3a	MK474097
SRB-28-S/2018	Male	0–3	Syrmia	3a	MK474098
SRB-30-S/2018	Male	0–3	Syrmia	3a	MK474100
SRB-31-S/2018	Male	0–3	Syrmia	3a	MK474101
SRB-32-S/2018	Male	0–3	Syrmia	3a	MK474102
SRB-33-S/2018	Male	0–3	Syrmia	3a	MK474103
SRB-35-S/2018	Male	0–3	Syrmia	3a	MK474104
SRB-36-S/2018	Male	0–3	Syrmia	3a	MK474105
SRB-40-S/2018	Male	0–3	Syrmia	3a	MK474108
SRB-43-S/2018	Male	0–3	Syrmia	3a	MK474109
SRB-47-S/2018	Male	0–3	Syrmia	3a	MK474110
SRB-48-S/2018	Male	0–3	Syrmia	3a	MK474112
SRB-40-K/2018	Female	0–3	Kolubara	3a	MK474107
SRB-48-K/2018	Female	0–3	Kolubara	3a	MK474111
SRB-50-K/2018	Female	0–3	Kolubara	3a	MK474113
SRB-1-SB/2018	–	–	Syrmia	3a	MK474094
SRB-17-S/2018	Female	0–3	Syrmia	3j	MK570855
SRB-25-S/2018	Female	0–3	Syrmia	3b	MK570856

antibodies is still high enough to protect pigs from re-infection which can easily occur due to low biosecurity level on Serbian small-scale farms and cross contamination between younger and older age categories.

The present study confirmed the overall potential risk for HEV cross contamination on the slaughterline. Swabs were collected from surfaces that contact pigs in all three slaughterlines and eight HEV-positive swabs were found in two slaughterhouses. Since it is not possible to detect an HEV-infected animal in a slaughterhouse, it is highly recommended to implement continuous education and training of workers to prevent further dissemination of the HEV virus through the abattoir. Strict implementation of Good Hygiene Practices and the HACCP procedures at all production stages is further required, such as maintaining water temperature of at least 82 °C for utensils during slaughtering and evisceration, prevention of bile waste dispersion, selection of virucidal reagents for cleaning-in-place, etc. Special attention to the level of hygiene and staff training should be paid in the slaughterhouse in Syrmia County, where all the swabs were positive, and which also had a high HEV prevalence in liver samples from pigs.

One of the main purposes of this work was to investigate the molecular epidemiology of HEV in slaughter pigs in Serbia, by sequence analysis and subtyping of viral strains. In this sense, phylogenetic reconstructions of the hypervariable region of ORF1 allowed us to determine the viral subtypes in the samples. A region corresponding to the RNA-dependent RNA polymerase gene within the 5′-end of the ORF1 gene was analyzed to this purpose. A preliminary phylogenetic analysis of the 26 Serbian HEV strains, together with reference isolates of most of the subtypes in genotype 3, clustered the majority of them with very consistent bootstrap values in a phylogenetical group that comprised sequences of subtype 3a.

The study revealed that our Serbian HEV sequences have high genetic diversity and also revealed the existence of four clusters that were classified as subtype 3a. However, genetic homogeneity among sequences from Syrmia and Kolubara shows us that there is a connection between these counties, probably with constant pig trade between these two adjacent counties or by some other routes of HEV transmission between them. The lower number of samples sequenced may also contribute to the lower heterogeneity. In contrast with these two counties, Šumadija County is a relatively

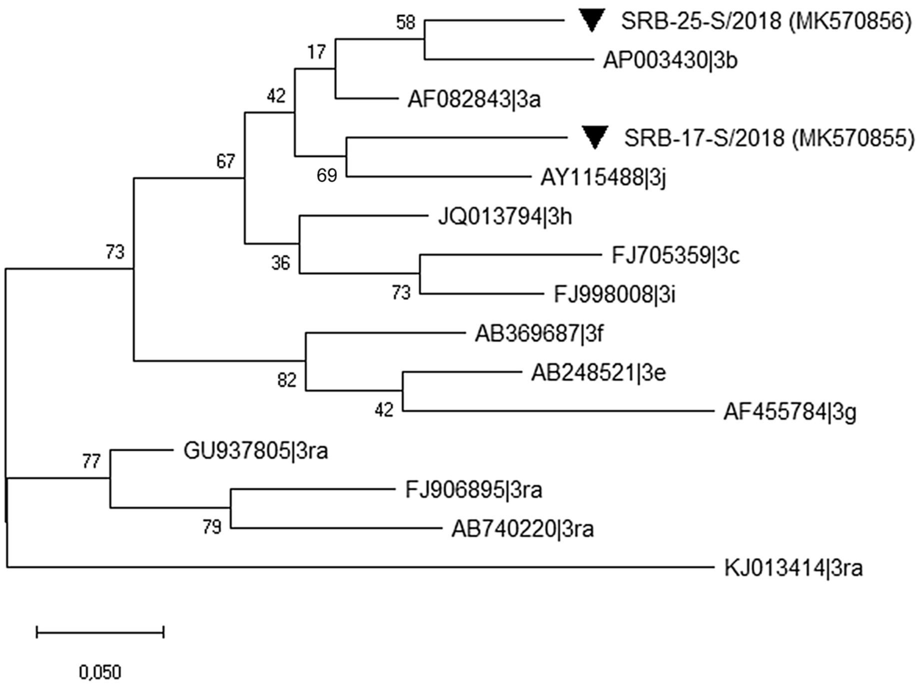

Fig. 3 ORF1-based phylogenetic tree. Rooted phylogenetic tree, constructed using HEV ORF1 334 bp-length partial sequences, and based on a maximum likelihood analysis employing the General Time Reversible model (Nei and Kumar 2000). The tree with the highest log likelihood (− 1908.65) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the MCL approach and then selecting the topology with superior log likelihood value. This analysis involved 15 nucleotide sequences. There were a total of 259 positions in the final dataset. Bootstrap values acquired after 1000 replications are shown (branch lengths measured in the number of substitutions per site). Serbian sequences were compared only to reference sequences of sub-genotype 3, as proposed by Smith et al. (2016)

geographically self-contained area, and we assume that there is not much exchange of pigs with other regions, but only within the county itself. However, we stress out that in this study no high fidelity proof-reading enzyme was used and possible nucleotide mismatching events could arise.

The topology of the ORF1 tree showed that the four well-supported groups within sub-genotype 3a from Serbia clustered together with sequences from Germany, The Netherlands, Japan, the United States, France, and Italy. Sub-genotype 3a has also been detected in Central and South Eastern European countries such as Slovenia, Croatia, and Hungary. However, a direct and current relationship with these sub-genotypes in other countries is unlikely. It is likely, although we speculate, that the viral variants in Serbian pigs described in this study could originate from pigs imported from Germany and/or The Netherlands. This hypothesis is based on fact that significant majority of piglets and gilts are imported from Germany and/or The Netherlands to former Yugoslavia, at least 500,000 pigs per year (European Commission 2013) and probably transmission of German/Dutch HEV-3a to Serbia occurred leading to multiplication at pig farm level. Yet, to confirm this hypothesis, it would require more in-depth phylogenetic investigation on much larger number of sequences.

Single finding of Serbian HEV-3b is quite interesting since HEV-3b, which is a common subtype in Japan, has been reported in animals in Europe just recently (Vina-Rodriguez et al. 2015). Also, single finding of Serbian HEV-3j was unexpected, because the first report that HEV-3j has been detected in French pigs occurred in 2018 (Feurer et al. 2018). The HEV-3j subtype (reference sequence AY115488) has only been described previously in North America (Canada) (Pei and Yoo 2002).

In Syrmia County, the liver sequence obtained from one particular pig was not equal to the swab sequence from the same pig. As suggested by other authors (Gardinali et al. 2012), this inconsistency could be explained by a co-infection with two variants circulating around hog production sites in this county.

We would also like to address problem of topology trees constructed by derivation with the relatively short ORF1 sequences deposited in NCBI GenBank, since these show low bootstrap values and identification of HEV subtypes can be fairly confusing. A complete genome sequence would be required to confirm the taxonomic position of an HEV RNA isolate (Smith et al. 2014).

Conclusions

The presence of HEV variants in livers of pigs at slaughter is a potential infection risk for consumers of this type of animal tissue when it is not properly thermally treated, as well as for slaughterhouse staff (Gardinali et al. 2012; Mughini-Gras et al. 2017; Huang et al. 2019). This study provides the first insight into the existence of HEV sub-genotype 3a, but also sub-genotypes 3b and 3j, as these variants circulate without geographical restriction in pigs in Serbia. This phylogenetic analysis of HEV genotypes and subtypes that exist in Serbian pig populations will be helpful in building a sequence database for correlating both zoonotic and foodborne HEV transmission pathways, tracing pigs imported from other countries and planning risk mitigation measures regarding HEV infection in the country (Mulder et al. 2019).

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