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To cite this article: L Milojevic *et al* 2021 *IOP Conf. Ser.: Earth Environ. Sci.* **854** 012059

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Detection and quantification of hepatitis E virus genome in pig liver samples originating from Serbian retail establishments

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Abstract. Hepatitis E is considered an emerging human viral disease with a zoonotic nature, and domestic and wild pigs are the main reservoirs of hepatitis E virus (HEV) among animals. Pork liver is the target tissue of this virus. This study aimed to investigate the presence of HEV in commercial pig liver samples. Sixty samples were collected during one year from different retail outlets in Serbia. Furthermore, the collected samples were separated by four seasons, and every season included three months. The presence of HEV in the livers was examined by molecular analysis using RT-qPCR. The overall prevalence of the virus in analysed pig livers was 5%. HEV was detected in three livers, two in the first season and one in the second, while in the third and fourth season, no positive livers were detected. However, there were no statistically significant differences between the surveyed seasons. HEV was quantified in positive livers. Among positive livers, HEV concentrations ranged between 8×10^1 and 1.9×10^4 genome copies of the virus per gram. The presence of HEV in commercial pig livers indicates a potential risk for consumers. Appropriate heat treatment of meals during preparation is essential to eliminate the potential risk of developing the illness.

1. Introduction

Hepatitis E virus (HEV) is a pathogen that causes Hepatitis E disease. In addition to humans, HEV infection has been confirmed in several animal species, while the prime route of transmission is faecal-oral [1]. Furthermore, zoonotic HEV transmission was also confirmed by Meng et al. [1]. This epidemic is increasing worldwide and presents public health concerns in developing countries and developed countries [1]. During the previous decade, an increase in HEV confirmed cases were recorded in developed countries [3].

This virus is a small, non-enveloped icosahedral virus with a single-stranded, positive-sense RNA genome approximately 6.6-7.2 kb in size. It belongs to the *Hepeviridae* family and the *Orthohepevirus* genus [4]. Further, HEV has been classified as the *Orthohepevirus A* species, encompassing seven genotypes [4]. Only the first four (1-4) can infect humans [5]. Generally, it has been recognised that HEV genotypes 1 and 2 are specific for waterborne transmission, while genotypes 3 and 4 are common to humans and other animal species [6]. Endemic regions for genotypes 1 and 2 are Asia, Africa, and Central America, where the water is a fundamental reservoir of the virus.

On the contrary, in the developed countries, HEV transmission primarily occurs through food. The domestic pig (*Sus scrofa domestica*), wild boar (*Sus scrofa*) and deer (*Cervidae*) are considered the essential animal reservoirs of HEV [7]. Meat consumption is increasing worldwide due to rapid population growth and urbanisation [8][9]. Consequently, the risk of contamination increases since meat,



tissues and meat products from these animal species are the primary sources of HEV[7]. In the modern world of hedonism, “new” food or locally specific food characteristic of specific regions is increasingly attractive [10]. For instance, *Figatellu*, a customary type of sausage made from raw pork liver, was the primary source of some acute hepatitis E cases[11]. The liver is the target organ for HEV, and because of that, this organ and its products are the most apparent sources of the virus. The presence of HEV in liver and liver products has been confirmed by many studies, while the prevalence of HEV has varied from study to study[12][13][14]. In Serbia, according to Milojević et al., HEV was detected in 34% of liver samples of pigs younger than three months, while in retail establishments, it was not detected[14].

The food-borne route is specific because most pigs infected with HEV do not have visible symptoms and enter the abattoir as healthy animals[15]. As a result, their tissues and meat go into production, posing a risk to human consumers. Usually, humans infected with HEV are asymptomatic[16]. However, after an incubation period between 2 and 8 weeks, a certain percentage of patients have symptoms such as abdominal pain, vomiting, icterus with nausea, fever and hepatomegaly[1]. Approximately 2% of human cases are lethal[1]. Like other diseases, immunocompromised persons are particularly at risk, especially pregnant women, with fatal outcomes rising to 25%[17].

Given these facts, this study’s objectives were to detect and quantify HEV from pig livers originating from retail establishments on the territory of Serbia.

2. Materials and methods

2.1. Commercial pig liver samples

The sampling was performed between January and December 2019 from various retail establishments in Serbia. The one-year period was divided into four seasons, while each season included three months. A total of 60 pig livers were sampled, enclosed in sterile containers, stored in an insulated icebox and transferred in the shortest possible time to the laboratory. All pig liver samples were then collected in sterile 50 mL Falcon centrifuge tubes and stored in a deep freezer at -20°C until further processing.

2.2. RNA extraction

An amount (100 mg) of each liver sample was homogenised in 1 mL of Trizol (Invitrogen, USA) and 600 µg of zirconia beads using a BeadBeater homogeniser (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 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.

2.3. Detection of HEV RNA

A real-time PCR (RT-qPCR) assay was developed to detect HEV3 genotype, using previously published but modified primers and probe[18]. Primer HEV3-f was partially changed with the replacement of guanine (G) with the degenerate nucleotide “R” at position 5323 (nucleotide position determined based on the HEV genome registered in GenBank under accession number AF060669). The HEV3-r primer and HEV3 probe were not changed. Primers and probe were developed in the highly conserved, overlapping ORF2/3 region of the HEV genome. TaqMan probes were labelled with the fluorophore and quencher molecules FAM/Blackhole Quencher 2 (Microsynth, Switzerland). TaqMan RT-qPCR (RNA UltraSense One-Step Quantitative RT-PCR System, Invitrogen, USA) was performed in 20 µL reaction volumes (Table 1) and 5 µL of total RNA was extracted from each sample.

Table 1. TaqMan RT-qPCR master mix

Ingredients	Volumes
5× Reaction Mix	5 μ L
Enzyme mixture	1.25 μ L
Forward primer	0.5 μ M
Reverse primer	0.9 μ M
Probe	0.25 μ M

The reactions were carried out in 96-well optical reaction microplates (Agilent, USA) in an AriaMX RT-qPCR 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. Positive and negative controls were included in each run. All samples with a cycle threshold value (Ct) for detecting HEV lower than 40 were interpreted as HEV-positive, and all other samples were interpreted as HEV-negative.

2.4. Quantification of HEV in the positive samples

To quantify genome copies of the virus per gram (g.c./g) in the positive liver samples, a synthetic molecule (RNA transcript) was constructed. Complementary DNA to hepatitis E virus (GenBank acc. No. MG051653), 71 nucleotides long, was cloned into pEXA2 vector (Eurofins, Germany), then cloned into *E. coli* One Shot aTOP10F (Invitrogen, USA). The target sequence was used to generate the standard curve; curves with a slope lying between -3.1 and -3.6 and an $R^2 \geq 0.98$ were used for quantification.

2.5. Statistical analysis

Statistical analysis of the performed experiment was done in the statistical package GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, California, USA), www.graphpad.com, and MS Excel.

3. Results and discussion

During a one-year period, sixty liver samples from different retail stores were examined for the presence of HEV using RT-qPCR (Figure 1). Over the year, the prevalence of HEV in all liver samples examined was 5 % (3/60).

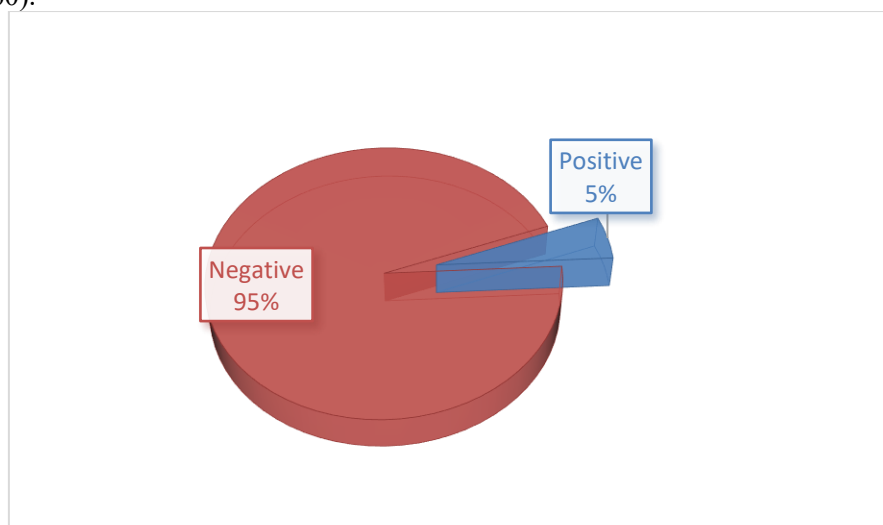
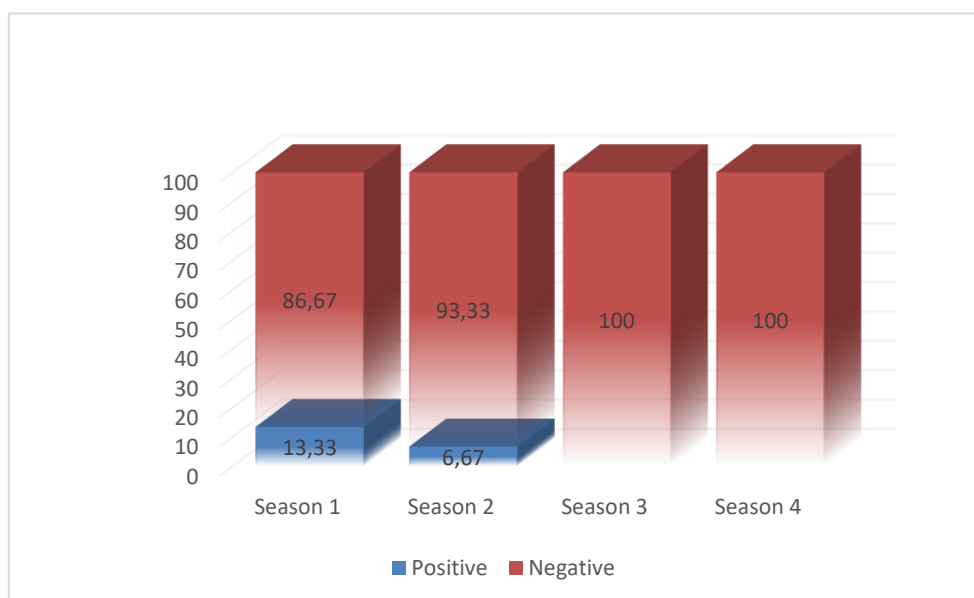


Figure 1. Percentage of HEV-positive pig livers in the examined samples

When we analysed the obtained data by seasons, we determined that during the first season, of the 15 samples tested, two (13.33%) were positive (Figure 2). Among 15 liver samples examined in the second season, 6.67% (1) was HEV-positive. During the third and fourth seasons, HEV was not detected in the examined pig livers. Therefore, statistically significant differences in HEV prevalence between surveyed seasons were not confirmed. Furthermore, HEV was quantified in the three positive pig livers. Among positive pig liver samples, the HEV concentration ranged between 8×10^1 and 1.9×10^4 HEV g.c./g.

**Figure 2.** Percentage of HEV-positive pig liver samples according to annual season

The percentage of HEV-positive pig livers originating from Serbian retail establishments in this research was similar to the results of testing commercial liver samples published so far. Wenzel et al. published results wherein the HEV prevalence in liver samples from German retail stores was 4% [19]. Also, in Germany, approximately the same prevalence (5%) for HEV in commercial pig liver samples was reported [19]. Similarly, results obtained by Bouwknecht et al. showed that among tested commercial liver samples in the Netherlands, 6.5% of them were positive for this virus [20]. Besides that, in the United Kingdom, among 80 tested liver samples, HEV was not detected [21]. In Canada, a group reported that 10.5% of tested pork products were HEV positive [22]. Overall, the HEV prevalence was approximately the same in all studies, regardless of country. However, some differences in prevalence were observed for some studies (countries) compared to others. These differences in HEV prevalence between studies can be because of a different national prevalence of HEV among pigs or because of differences in detection procedures. For example, differing RT-qPCR protocols can have different sensitivity levels. Still, in most of the studies, HEV was detected in pig-origin samples, and HEV presence in commercial pig livers was confirmed.

The exact infectious dose of HEV required to infect humans has not yet been determined; however, according to the French Agency for Food, Environmental and Occupational Health and Safety (ANSES), the infective dose for the oral route in humans is approximately $10^{5.5}$ g.c./g. **Error! Reference source not found.** The results obtained in our research show that the average HEV concentration in positive pig livers was 6.7×10^3 g.c./g. Feurer et al. published similar results to ours, in which the average HEV

concentration in HEV-positive pig liver samples was 1.3×10^5 g.c./g[23]. Boxman et al. reported HEV concentrations in positive pork sausages ranged between 2.1×10^2 and 1.2×10^6 g.c./g[24]. Quantitative data are an essential parameter in risk assessment, especially in ready-to-eat products. For raw meat products that are cooked, adequate heat treatment is the most critical process to reduce HEV numbers, because heating for 5 min at 71 °C is enough for to inactivate the virus. This type of heat treatment will reduce the risk and enable safe food to be consumed.

4. Conclusion

The presence of HEV in liver samples from retail establishments can be an essential risk to food safety for consumers, especially for immunocompromised groups, including pregnant women as the primary at-risk group. The results of this study warrant the start of monitoring to follow HEV RNA levels in pig livers and pork products over time for risk assessments and risk management purposes. Routine coordinated surveillance of viral epidemics and surveillance of HEV in food products, combined with systematic of typing strains, and joint expertise of veterinarians, food and clinical microbiologists is recommended to help study source and identify risk prevention measures. Furthermore, strict implementation of good hygienic practices at all stages of the food chain and hazard analysis and critical control point (HACCP) procedures is required to decrease the HEV concentration in food and prevent further dissemination of this pathogen. In regions with high HEV prevalence among pigs, vaccination of pig herds could be an excellent measure in combating the spread of this zoonosis.

Acknowledgment

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, according to the provisions of the Contract on research financing in 2021 (No 451-03-9/2021-14/200050 dated 05.02.2021).

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