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# Prevalence of Hepatitis E Virus in Pigs and Wild Boars in Lithuania

**Master's Thesis**

Molecular Biotechnology study program

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# Abbreviations

CI – confidence interval

DNA – deoxyribonucleic acid

ELISA – enzyme-linked immunosorbent assay

HEV – hepatitis E virus

HEV-3 – hepatitis E virus genotype 3

ORF – open reading frame

RdRp – RNA-dependent RNA polymerase

RNA – ribonucleic acid

RT-nPCR - multiplex reverse transcription-nested polymerase chain reaction

RT-PCR – reverse transcription polymerase chain reaction

# Introduction

Hepatitis E virus (HEV) is a member of the *Orthohepevirus* genus of RNA viruses in the *Hepeviridae* family. Along with hepatitis A, B, C, and D, Hepatitis E is one of the five human hepatotropic viruses, however, it is the only zoonotic one (Pavio et al., 2010). The main animal reservoirs of HEV that can transmit the infection to humans are domestic pigs (*Sus domesticus*), wild boars (*Sus scrofa*) and spotted deer (*Cervus nippon*) (Sridhar et al., 2015). The main ways humans can get infected by HEV include consumption of raw or undercooked meat, fecal-oral transmission through contaminated water, transfusions and organ transplantation from an infected donor, transmission to the fetus transplacentally and zoonotic transmission (Khuroo et al., 2016). Most of HEV infections tend to be asymptomatic or mildly symptomatic, however pregnant women and immunocompromised patients and patients with chronic liver diseases are at risk of severe hepatitis and hepatic failure (Aslan & Balaban, 2020).

HEV genotypes 1 (HEV-1) and 2 (HEV-2) have been reported in endemic regions of Southeast and Central Asia, the Middle East, and parts of West and North Africa, India (Aggarwal, 2011). In industrialized regions in Europe, the United States, Japan, Australia, and South Korea, HEV-3, HEV-4, and HEV-7 have been observed (Songtanin et al., 2023). In Europe, the factors associated with the highest risk of HEV infection include consumption of undercooked pork, game meat and occupational contact with pigs and wild boar (Said et al., 2014). Infection via contaminated food products (mostly pork) has been confirmed by molecular studies (Riveiro Barciela et al., 2015; Pavio et al., 2015).

In Lithuania Spancernienė and colleagues investigated the seroprevalence and genetic diversity of Hepatitis E virus in domestic pigs and several wildlife species including wild boar (*Sus scrofa*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and moose (*Alces alces*) in 2016 and 2018. The highest seroprevalence was identified in adult wild boars (80%) and weaned pigs (53.66%) (Spancernienė et al., 2016, 2018). All obtained sequences detected in Lithuanian domestic pigs and wildlife belonged to genotype 3. The research conducted in Lithuania showed that HEV is prevalent in Lithuanian domestic pigs and wildlife, making these animals HEV reservoirs that may transmit the zoonosis to humans. The present work aimed to contribute to knowledge about the prevalence of HEV in domestic pig and wild boar populations, as well as pork food products in Lithuania.

**Aim**

To determine the prevalence of Hepatitis E virus (HEV) in domestic pigs, wild boars and pork food products in Lithuania.

## Objectives

1. To determine HEV prevalence in domestic pig population in Lithuania using quantitative reverse transcription polymerase chain reaction (RT-qPCR) for analysis of liver samples.
2. To observe HEV prevalence in domestic pig population in Lithuania using RT-qPCR to analyze blood serum and effluent water samples.
3. To determine HEV prevalence in wild boar population in Lithuania using RT-qPCR for analysis of blood serum samples.
4. To investigate HEV prevalence in pork food products from Lithuanian supermarkets by analyzing pork pâté samples using RT-qPCR.
5. To assess the current HEV prevalence among animals considered to be virus reservoirs in Lithuania by interpreting molecular analysis results.

# 1. Literature Review

## 1.1. Hepatitis E virus infection epidemiology

According to World Health Organization (WHO, 2023), an estimated 20 million hepatitis E virus (HEV) infections occur annually, with more than 3 million symptomatic cases and more than 60,000 fatalities. Symptoms include fever, fatigue, stomach pain, loss of appetite, joint pain and jaundice. Hepatitis E is self-limiting, with a case fatality rate of 0.5–3% in young adults (Nan, 2014). However, pregnant women are at risk for up to 30% mortality in the third semester of pregnancy (Urooj et al., 2023) (Figure 1).

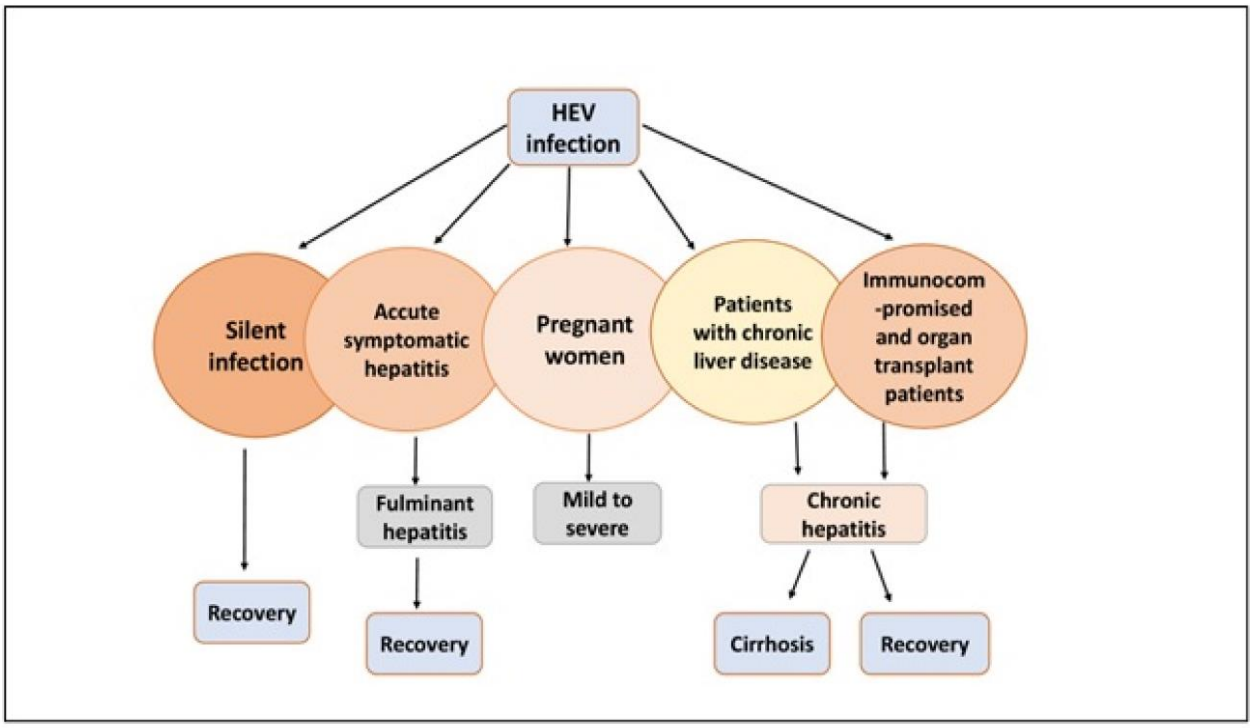


**Figure 1.** Diagrammatic presentation of HEV infection outcomes (Urooj et al., 2023).

In immunocompromised individuals such as organ transplant patients, patients receiving chemotherapy, and individuals with HIV infection or chronic liver disease Hepatitis E can develop into chronic hepatitis, which can further develop into cirrhosis if patients do not recover (Nimgaonkar et al., 2017; Urooj et al., 2023).

## 1.2. Hepatitis E virus zoonotic transmission

The main sources of Hepatitis E virus transmission to humans are pigs and pig products, as HEV RNA has been often detected in raw and undercooked pork liver, ground pork, and sausages (Treagus et al., 2021) (Figure 2).

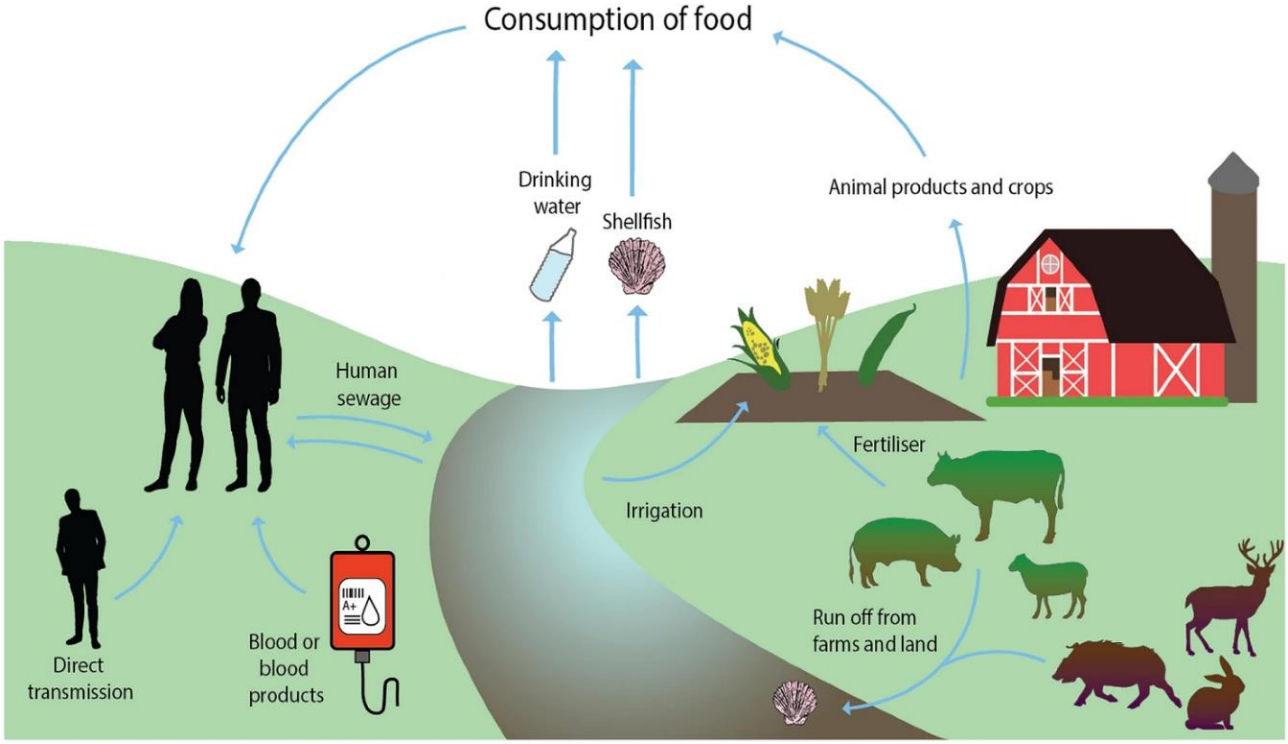


**Figure 2.** Theoretical and confirmed transmission routes of HEV. The theoretical transmission routes include HEV infections contracted from the consumption of shellfish, sheep, and cows, as well as crops and drinking water, as no confirmed outbreaks from these sources have yet been identified (Treagus et al., 2021).

HEV RNA has also been found in pig livers, feces, and bile, with high contamination in slaughterhouses and meat processing facilities (Wilhelm et al., 2017). Studies conducted in Spain (Kukielka et al., 2016), Brazil (da Silva et al., 2018) and Italy (Montone et al., 2019), also identified backyard pigs and wild boars as important reservoirs by HEV detected in both animals and ready-to-eat meat products.

In developed countries where HEV-3 is common, cross-species transmission occurs from infections in wild boars and red deer (Boxman et al., 2020). Pig slurry and porcine blood products pose a risk for transmission through environmental contamination, bringing awareness to have improved safety standards in farming and surveillance of meat products (Kantala et al., 2015).

## 1.3. Hepatitis E virus classification

The HEV virus has been classified in the Hepeviridae family, and most human pathogenic strains belong to the species *Orthohepevirus A* (Ahmad et al., 2022). These species are classified into 8 genotypes with genotypes 1 and 2 occurring in humans and transmitted via the fecal-oral route, through contaminated drinking water (Figure 3). Transmission of HEV genotypes 3 and 4 occurs through the consumption of undercooked pork, wild boar, and deer meat, it circulates in rabbits, goats, sheep, mongooses, Bottlenose dolphins, and swine and is known to cause zoonotic infections. Genotypes 5 and 6 of HEV are found in wild boar, genotypes 7 and 8 infect dromedary and Bactrian camels.

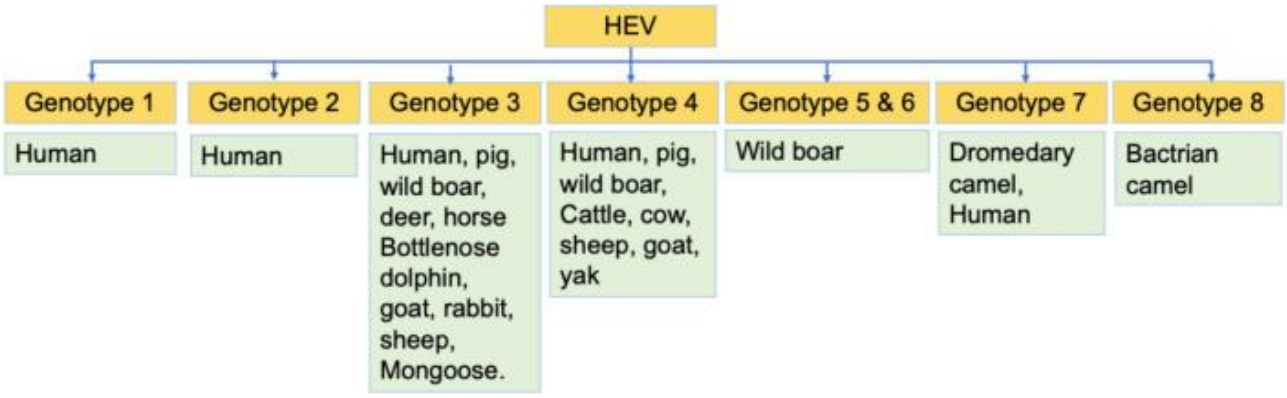


**Figure 3.** Orthohepevirus A genotypes and hosts (Ahmad et al., 2022).

In an immunosuppressed patient, genotype 7 HEV was identified after consuming camel milk and meat. However, for genotypes 5, 6 and 8 there have been no reports of transmission of the virus to humans (Ahmad et al., 2022).

## 1.4. Hepatitis E virus structure

HEV virus is a small (with a diameter of 27–34 nm), non-enveloped, icosahedral virus (Guu et al., 2009). Its 7.2 kb single-stranded, positive-sense RNA genome has a 5′ 7-methylguanosine cap structure, a 5′ untranslated region (UTR) consisting of 26 nucleotides, three open reading frames (ORFs) and a 3′UTR (Fig. 4A) (Zhang et al., 2001; Oechslin et al., 2020). The junction region between ORF1 and ORF3 with a stem-loop structure has cis-active elements which can control the expression of a subgenomic bicistronic messenger RNA (mRNA) (Huang et al. 2007). ORF3 overlaps the 5′ coding sequence of ORF2, whereas ORF1 is not overlapped by ORF3 or ORF2. Along ORF2, there is 3′UTR, terminating in a 3′ polyadenylated tail. A necessary for viral RNA replication 3′ cis-active element is in the 3′UTR, overlapping the carboxy-terminal sequence of the ORF2 (Agrawal et al. 2001). The 3′ cis-active element binds to the ORF1 polyprotein RNA-dependent RNA polymerase (RdRp) domain (Kenney & Meng, 2019).

ORF1 is the largest of the three ORFs with the length of 5109 nucleotides and its function is encoding a non-structural polyprotein (Sayed et al., 2022). ORF1 has essential for viral life cycle motifs and domains, including RNA dependent RNA polymerase (RdRp), methyltransferase (MTase), papain-like cysteine protease (PCP), Y-domain, hypervariable region (HVR), Xdomain and RNA helicase (Chandra et al., 2008).

ORF2 encodes the 1982 nucleotides long viral capsid protein, which is comprised of S, M, and P domains (van Tong et al., 2016). The main function of ORF2 capsid protein is virion assembly by binding to HEV RNA for viral packaging and auto-assembly of the glycosylated capsid proteins (Cao & Meng, 2012).

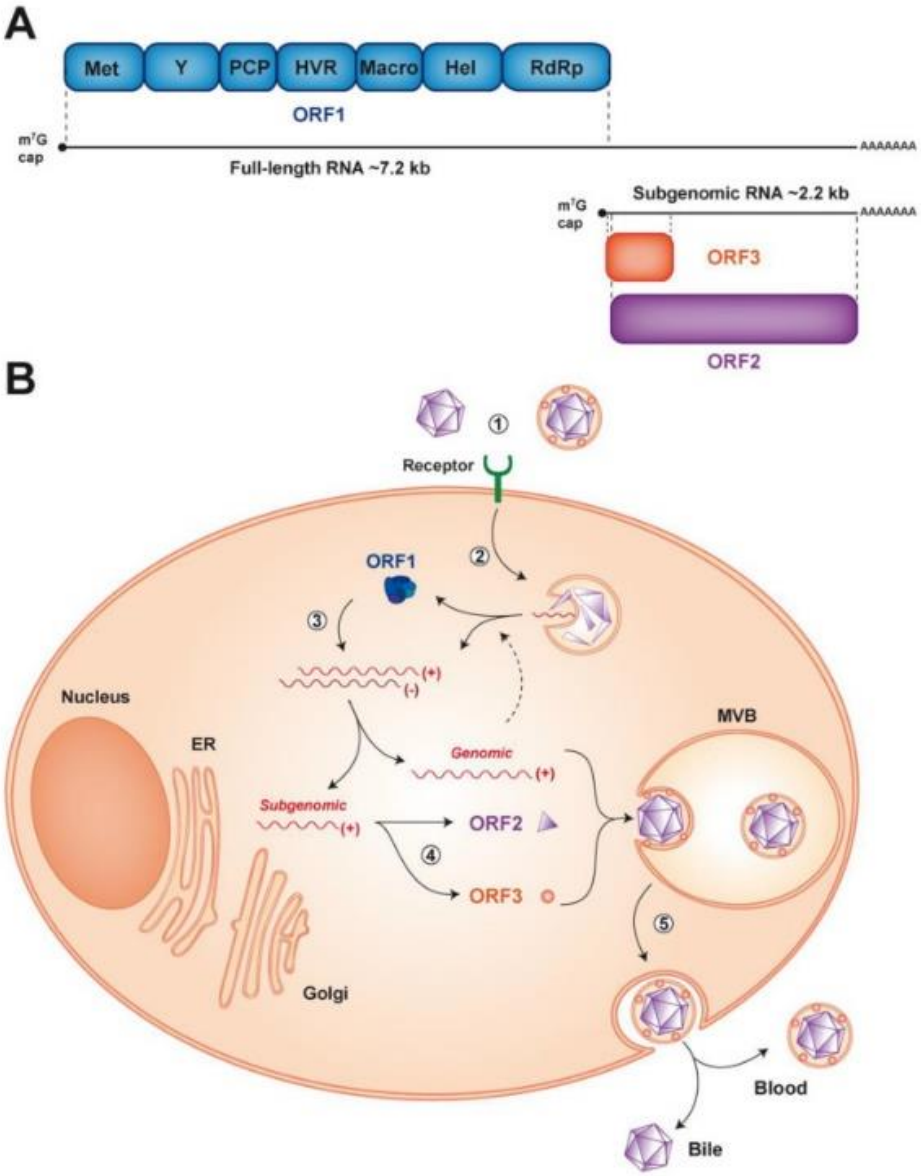


**Figure 4**. Genome organization (A) and life cycle (B) of hepatitis E virus (HEV) (Oechslin et al., 2020).

ORF3 is the smallest protein from the three ORFs, involved in virion release from infected cells and the formation of the quasi-envelope (Nagashima et al., 2014). Another function to assist HEV

replication is encoding a multifunctional phosphoprotein, which is involved in modulating cell signaling (Kamar et al., 2011). ORF3 disrupts the production of cytokines and causes host's immunosuppression (Yang & Nan, 2021).

## 1.5. Hepatitis E virus life cycle

The HEV life cycle can be characterized by five key steps (Figure 4B). Viral entry through unidentified receptors and endocytosis takes place during the first step of the HEV life cycle, followed by the viral positive-strand RNA genome being released into the cytosol (Oechslin et al., 2020). After the RNA is translated into the ORF1 protein, it proceeds with replicating the genome and via a negative-strand RNA intermediate generating the subgenomic RNA. To make the ORF2 and ORF3 proteins, the subgenomic RNA goes through the translation process (Oechslin et al., 2020). At the last step, during the genome packaging, the virus is released into the bloodstream and the bile after virion assembly. The endoplasmic reticulum (ER) and multivesicular bodies (MVB) are the main cellular structures involved in the process (Oechslin et al., 2020).

## 1.6. Hepatitis E virus risk factors on farms

HEV discharge mostly occurs through feces, making the fecal contaminated housing, feed, drinking water and manure storage likely infection sources, as well as porcine and other animals on farms (Meester et al., 2021) (Figure 5).

The infection stage of slaughtering pigs can indicate whether or not humans are susceptible to foodborne HEV. After eating pork, the risk is high if HEV can be identified in pigs' feces or liver, suggesting they have an active HEV infection (Meester et al., 2021).

HEV has been detected in different types of manure storages, such as manure pits below slatted floors, storages outside the barn, openings of slurry collecting channels leaving the barn, muddy lagoons, wetlands and pits (Fenaux et al., 2018). In one study HEV RNA was detected in 3% of the samples inside the farm buildings with samples from shovels, fans and feed tubes, while 11% of the samples had HEV RNA found outside the property (Nantel-Fortier et al., 2016). Polluted driving boards and paddles are also fomites capable of carrying HEV RNA (Souza et al., 2020).

For genotype 1 and 2 HEV infections drinking water is a frequent source. For pigs one study found HEV in water from feeding containers and identified one sample to be HEV RNA positive out of sixteen (Fernandez-Barredo et al., 2006). Water from hydrants or faucets from 28 farms tested negative in all cases (Kasorndorkbua et al., 2005). Additional elements that increase HEV-positive

samples are specific regions, whether the pigs live indoors or outdoors, and seasons when the samples are gathered.

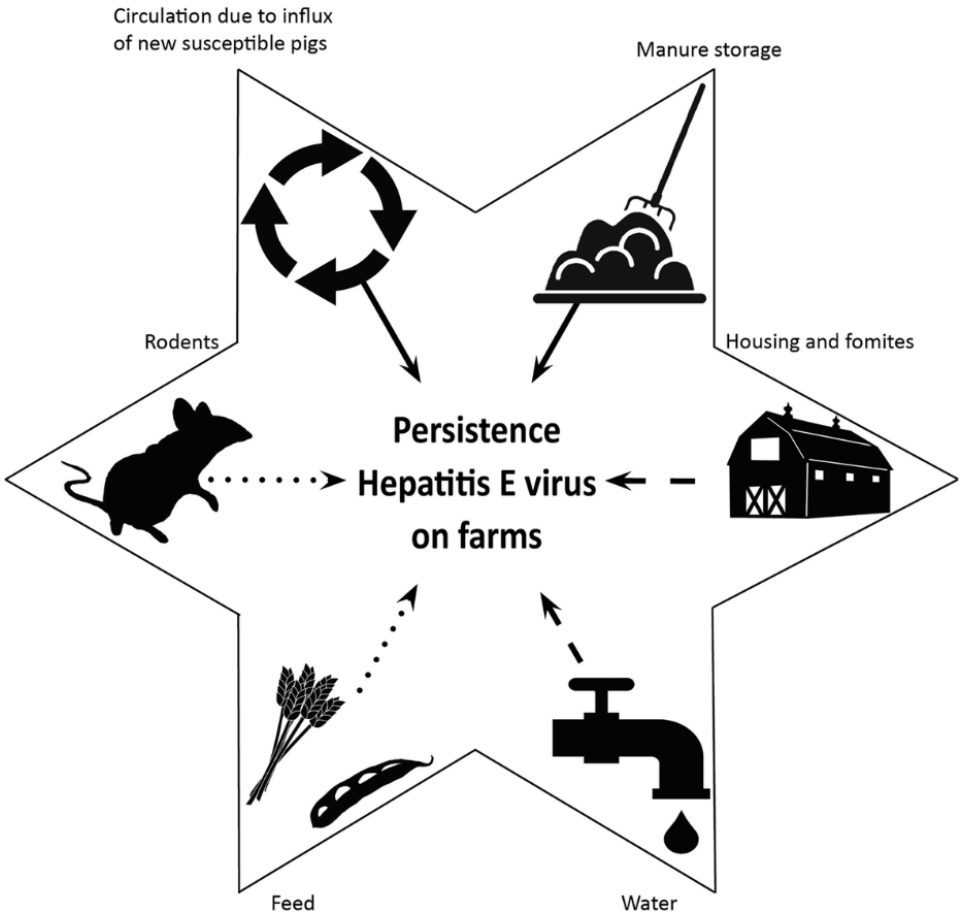


**Figure 5**. Potential mechanisms for HEV persistence on pig farms. Solid arrows represent mechanisms of persistence confirmed by literature, dashed arrows may be sources of persistence, yet more research is needed to conclude on these sources and dotted arrows are unlikely sources of persistence (Meester et al., 2021).

If pigs are fed non finely ground feed, it can be a way for them to get infected by HEV. Commercial pig feed tends to be free of HEV because of the heating process. During one study, HEV RNA was found in pig feed, as was the case in commercial spray-dried porcine plasma (SDPP), although the amount of HEV antibodies after feeding pigs with SDPP was not higher than a negative control group (Pujols et al., 2014). Additionally, in pig farms contaminated kitchen residues or crops fertilized with pig manure are sources of HEV and contribute to environmental exposure (Xiao et al., 2012).

Rodents also impact HEV infections on pig farms. HEV-3 RNA and a new genotype HEV genotype C1 (species *Orthohepevirus C*) has been detected in rats (Johne et al., 2010). In rodents, depending on the species (mice, *R. rattus* or *R. norvegicus* rats), the areas and what kind of samples were collected, the prevalence of both HEV genotypes can be identified from 0 to 18% (Grierson et al., 2018). In one study rats and mice found in pig farms tested positive for HEV-3 in spleens, but the samples mainly tested positive in the intestines (De Sabato et al., 2020). In a 2021 study by Meester

and colleagues it was discussed that rodents are accidental hosts of HEV-3, due to the low prevalence of HEV-3 found in them around farms and HEV-3 testing positive mostly in intestines. However, they can spread porcine fecal material, which leads to environmental contamination (Meester et al., 2021)

## 1.7. Hepatitis E virus prevention

The main methods for preventing HEV infections include immunity-increasing vaccines, appropriate hygiene, clean water, handling waste, cooking meat the required amount of time, and taking care of hygiene while handling raw meat (Aggarwal and Jameel, 2011).

Environmentally the key preventative practices are disposal of feces, community sanitation and sewage control, boiling and chlorination of water, as well as taking care of its supply and proper storage. In the case of the HEV outbreak, it is recommended to heat-treat imported water and chlorinate its reservoirs for disinfection (Khuroo, 2016). Although in 2004 in Darfur, Sudan during an HEV epidemic chlorination was identified as not effective towards recent contagions (Guthmann et al., 2006). When traveling to infected areas, avoiding water that has not been thermally treated, as well as raw shellfish, fruits and vegetables is one of the most important ways of decreasing risk of getting infected with HEV (Letafati et al., 2024).

## 1.8. Hepatitis E virus prevalence research in Lithuania

In 2016 Spancernienė and colleagues conducted a study in Lithuania, assessing the seroprevalence of Hepatitis E virus in domestic pigs and several wildlife species. Using an enzyme-linked immunosorbent assay (ELISA) serum samples from domestic pigs, wild boar, moose, roe deer, red deer, and European bison were tested for HEV antibodies (Spancernienė et al., 2016). Domestic pigs had a seroprevalence of 43.75%, wild boar 57.05%, moose 11.76%, and roe deer 1.20%, while red deer and European bison were found to have no HEV seroprevalence (Figure 6).

| Animal species | Number of tested / positive serum samples | % | 95 CI, % | OR | *P*-value |
|---|---|---|---|---|---|
| Domestic pig *(Sus scrofa domestica)* | 384/168 | 43.75 | 38.9-48.7 | 0.78 | < 0.05 |
| Wild boar *(Sus scrofa)* | 312/178 | 57.05 | 51.5-62.4 | 1.33 | < 0.05 |
| Moose *(Alces alces)* | 34/4 | 11.76 | 4.7-26.6 | 0.13 | < 0.001 |
| Roe deer *(Capreolus capreolus)* | 166/2 | 1.20 | 0.3-4.3 | 0.01 | < 0.001 |
| Red deer *(Cervus elaphus)* | 108/0 | 0.00 | 0-3.4 | 0.00 | < 0.001 |
| European bison *(Bison bonasus)* | 3/0 | 0.00 | 0-56.1 | 0.00 | |

HEV - hepatitis E virus; CI - confidence interval; OR - odds ratio

**Figure 6.** HEV seroprevalence in domestic pigs and wildlife samples in Lithuania during 2014–2015 (Spancernienė et al., 2016).

The highest seroprevalence was identified in adult wild boars (80%) and weaned pigs (53.66%). Larger pig herds of 15,001–30,000 pigs had a 2.4 times higher prevalence than smaller herds. Wild boar population density showed a moderate correlation with seropositivity but inconclusive significance. The moose, roe deer, and red deer had lower or no seroprevalence, these species being possibly accidental hosts of HEV. In wild boar, adult animals had significantly higher seroprevalence than younger boars. In the study, especially in regions with high wild boar populations, potential risks of zoonotic transmission of HEV through domestic pigs and wild boar was observed, with implications for public health (Spancernienė et al., 2016).

In 2018, Spancernienė and colleagues continued the 2016 study on the prevalence and genetic diversity of Hepatitis E virus in domestic pigs, wild boars, roe deer, red deer, and moose. This study differs from the previous one by the use of nested reverse transcription polymerase chain reaction (RT-nPCR) to detect HEV RNA. This method added more specificity to the serological results from the earlier study, which used enzyme-linked immunosorbent assay (ELISA). The prevalence of HEV in domestic pigs was highest from all observed species in both studies, although in the 2018 study it was between 22.55% and 32.97% (Figure 7), in 2016 the seroprevalence was higher (43.75%).

| Investigated host | ORF1 targeting primers | | | ORF2 targeting primers | | |
|---|---|---|---|---|---|---|
| | Sample type (number of HEV positive/tested samples (%)) | | All types of samples (number of HEV positive/tested (%, 95% CI)) | Sample type (number of HEV positive/tested samples (%)) | | All types of samples(number of HEV positive/tested (%, 95% CI)) |
| | Serum | Liver | | Serum | Liver | |
| Domestic pigs (*Sus scrofa domesticus*) | 155/470 (32.98) | – | 155/470 (32.98%, 28.88–37.35) | 106/470 (22.55) | – | 106/470 (22.55%, 19.01–26.55) |
| Wild boars (*Sus scrofa*) | 62/235 (26.38) | 69/270 (25.56) | 131/505 (25.94%, 22.31–29.93) | 41/235 (17.44) | 45/270 (16.67) | 86/505 (17.03%, 14.00–20.55) |
| Roe deer (*Capreolus capreolus*) | 10/45 (22.22) | 11/48 (22.92) | 21/93 (22.58%, 15.27–32.07) | 7/45 (15.56) | 5/48 (10.42) | 12/93 (12.90%, 7.54–21.21) |
| Red deer (*Cervus elaphus*) | 1/13 (7.69) | 0/2 (0) | 1/15 (6.67%, 1.19–29.82) | 0/13 (0) | 0/2 (0) | 0/15 (0%, 0.00–20.39) |
| Moose (*Alces alces*) | 1/13 (7.69) | – | 1/13 (7.69%, 1.37–33.31) | 0/13 | – | 0/13 (0%, 0.00–22.81) |

**Figure 7**. Prevalence of HEV in domestic pigs and wild animal species using RT-nPCR assay (Spancernienė et al., 2018)

The prevalence of wild boars decreased more than twice from the 2016 study, when it was 57.05% to 25.94% in 2018. Roe deer was found to have a significantly higher commonness of HEV infection (22.58%) in the more recent study compared to the 2016 study (1.20%), while moose were infected less in the newer study (7.69%) compared to the previous one (11.76%). The seroprevalence of red deer increased from none in 2016 to 6.67% in 2018. In the 2018 research an additional method was included and a phylogenetic analysis was performed. The results of the two studies had similarities, including that HEV sequences from wild boars and pigs clustered genotypes 3i and 3h. Additional findings in the 2018 study are the HEV subtype 3f in pigs from two counties and for the first time in this species the subtype 3i in roe deer. During this research it was noted that domestic pigs are more at risk for HEV infection than wild boars because of the limited space in farms, which was already observed in the 2016 study with large pig groups being most likely to get infected with HEV (Spancernienė et al., 2018).

## 1.9. Hepatitis E virus prevalence research worldwide

In 2020, Hepatitis E virus infection in domestic pigs and wild boars was researched in Bulgaria (Takova et al., 2020). The method used to test blood samples from pigs on farms and a slaughterhouse and hunted wild boars was ELISA to identify anti-HEV IgG antibodies. Seroprevalence in pigs was analyzed by age with slaughter-aged (6 months old) pigs showing a very high rate (73.65%) while none of the younger pigs' (3 months old) samples were positive. The general infection rate was 60.05% and rose between 2017 (45.33 %) and 2019 (98%) (Figure 8).

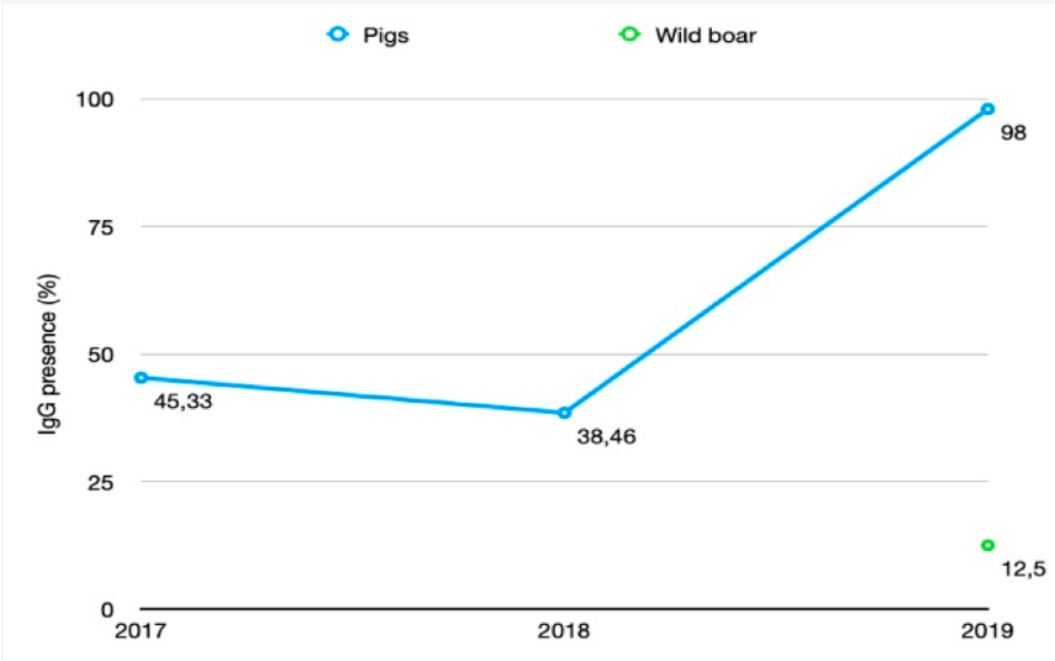


**Figure 8.** HEV IgG positive test results by year (Takova et al., 2020).

The infected samples (12,5 %) of wild boars were observed as potential hepatitis E virus reservoirs due to their scattering across four areas (Takova et al., 2020). The study placed focus on infection rate differences among certain regions in Bulgaria and found that northern and southern Bulgaria's HEV seroprevalence did not differ vastly (Takova et al., 2020).

| Farm | Age category | | | | | |
|---|---|---|---|---|---|---|
| | Suckling piglets (%) | Weaned pigs (%) | Fattening pigs (%) | Sows (%) | Boar (%) | Total (%) |
| A | 3/10 (30) | 4/10 (40) | 8/10 (80) | 9/10 (90) | 5/10 (50) | 29/50 (58) |
| B | 2/10 (20) | 5/10 (50) | 8/10 (80) | 7/10 (70) | 5/10 (50) | 27/50 (54) |
| C | 0/10 (0) | 7/10 (70) | 9/10 (90) | 7/10 (70) | 4/10 (40) | 27/50 (54) |
| Total | 5/30 (16.67) | 16/30 (53.33) | 25/30 (83.33) | 23/30 (76.67) | 14/30 (46.67) | 83/150 (55.33) |

**Figure 9**. Number of anti-HEV antibodies positive samples in different age categories in commercial pig farms (Kureljušić et al., 2020).

A study in Serbia tested commercial pigs, backyard pigs, slaughtered pigs and wild boars for HEV infection in the Belgrade region (Kureljušić et al., 2020). Serum samples were collected between 2016 and 2018 and analyzed for anti-HEV antibodies using ELISA. The research contributed to marking the differences between HEV seroprevalence in commercial farms and individual farms,

with commercial farms having a 55.33% rate, while individual ones had from 54% to 58% (Figure 9).

Individual species rates were observed as 75.71% in backyard pigs, 52.25% in wild boars and 22.58% in slaughtered pigs. The HEV seroprevalence was additionally compared between weaned piglets, which showed a 25% infection rate and fattening pigs with lower results at 20.69% (Kureljušić et al., 2020).

In Italy's Marche region, a study published in 2022 by Ferri and colleagues observed wild boars for Hepatitis E virus infection during the 2019–2020 hunting season. Liver and muscle tissue samples were taken from 312 wild boars and tested for infection using nested RT-PCR (Figure 10).

| Samples ID | Sex of positive animals | Estimated age* | Geographical localization |
|---|---|---|---|
| 2 | F | A | Vena Piccola (AP) |
| 3 | M | P | Rotella (AP) |
| 5 | F | A | Vena Piccola (AP) |
| 7 | F | A | Roccafluvione (AP) |
| 8 | F | A | Venarotta (AP) |
| 12 | F | A | Vena Piccola (AP) |
| 13 | M | P | Venarotta (AP) |
| 14 | F | P | Roccafluvione (AP) |

F: Female; M: Male. Age estimation was based on extent of tooth eruption and weight. *From this estimation animals were classified as: P = pre and puberal: weight between 15-40 Kg and estimated age between 13-24 months. A = adult: weight > 40 Kg and estimated age between 24-48 months.

**Figure 10**. Positive animals to the molecular screening for HEV RNA detection (Ferri et al., 2022).

The results showed HEV RNA was found in 5.45% of liver samples and in 1.35% of muscle samples (Figure 11), which was significantly lower than in the studies conducted in Bulgaria, Serbia and Lithuania.

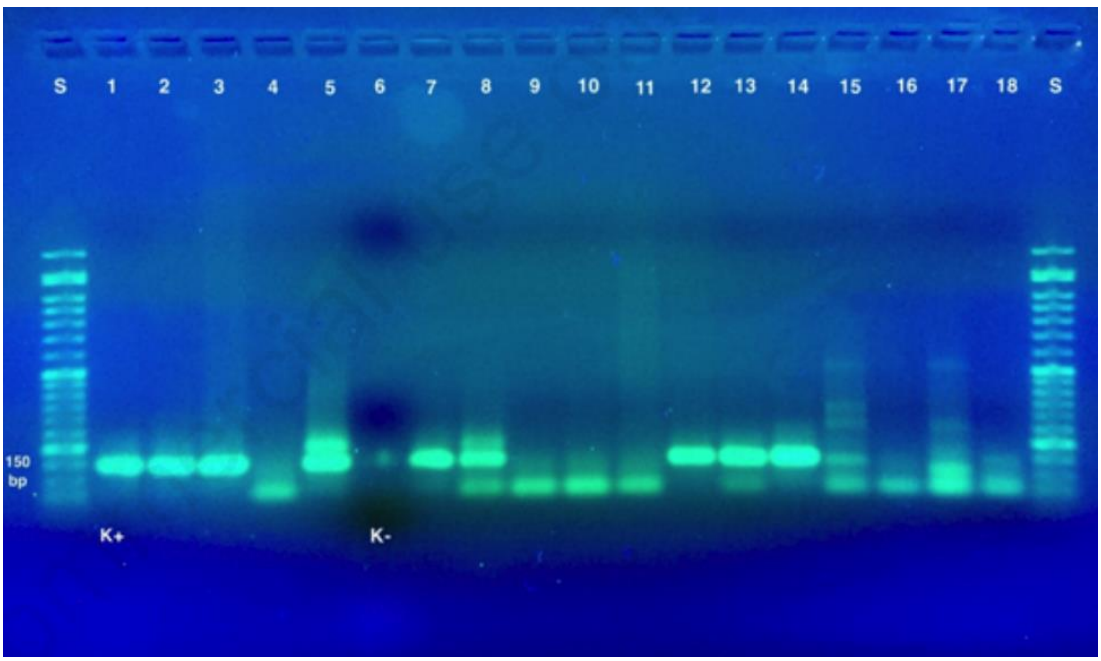


**Figure 11**. Electrophoresis gel (agarose 2%) in which it is possible to observe nitid positive amplicons: nested PCR products (145 bp): ORF2 gene. Wells loading: S = DNA ladder50 bp (Genetics® FastGene 50 bp DNA Marker) loaded into the first and last wells. Each line corresponds to 50 bp. 1 = K+ / Positive control (ATCC®

VR-3258SD RNA frag-ment). 6 = K- / Negative control. 2, 3, 5, 7, 8, 12, 13, 14 = Positive samples (samples ID and respective information are reported in Table 3). 4, 9, 10, 11, 15, 16, 17, 18 = Negative samples. [Italian Journal of Food Safety 2022; 11:9979][page 88]

Additionally, a phylogenetic analysis was performed and the genotype 3 subtype c was observed as the main strain (Ferri et al., 2022).

In 2024 a study in China tested HEV seroprevalence in domestic pigs in Guangdong Province, where HEV genotype 4 is commonly observed (Liu et al., 2024). The blood samples were taken from 25 farms in 16 cities between 2022 and 2024. After the samples were tested for anti-HEV IgG antibodies using ELISA, results showed an overall seroprevalence of 57.53%. Seroprevalence was observed comparing different years of testing, 2022 having the lowest HEV infection rate of 52.52% (Figure 12).

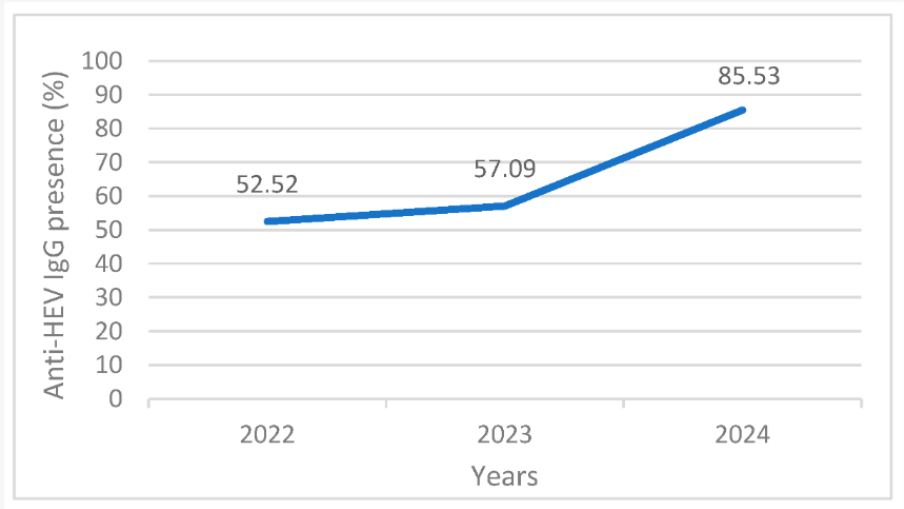


**Figure 12**. Anti-HEV IgG positive test results by year (Liu et al., 2024).

In 2023 it was higher at 57.09% and has increased significantly in 2024 at 85.53%. The results also differed in different seasons, with the highest infection rate of 75.91% observed in autumn and the lowest in spring at 33.93%. HEV Seroprevalence was compared by regions and eastern Guangdong had a more than two times higher infection rate (77.82%) than western Guangdong (40.96%). Additionally, infection by age groups was observed and was found to be more present in gilts and sows while infection rates were lowest in piglets. The study concluded that environmental factors such as regions and seasons have an impact on HEV dynamics and present different results accordingly (Liu et al., 2024).

The Lithuanian studies and the studies from Bulgaria, Serbia, Italy, and China all used ELISA for testing HEV seroprevalence. Research conducted in Italy and Lithuania (in 2018) also implemented nested RT-PCR to detect HEV RNA. Domestic pigs were found to have a higher seroprevalence than wild boars in Lithuania (32.97%-43.75%), Bulgaria (60.05%), and Serbia (up to 75.71%) (Spancernienė et al., 2016, 2018; Takova et al., 2020; Kureljušić et al., 2020). Studies in

Italy and China included differences in HEV infection rates based on regions or seasons. Italy's low wild boar prevalence (5.45%) contrasted with China's seroprevalence of up to 85.53%, impacted by genotype-specific dynamics and environmental factors like seasonality (Ferri et al., 2022; Liu et al., 2024). All reviewed studies concluded that domestic pigs and wild boars are the main reservoirs of HEV infection and raised awareness of the zoonotic risks related to Hepatitis E virus transmission (Spancernienė et al., 2016, 2018; Takova et al., 2020; Kureljušić et al., 2020; Ferri et al., 2022; Liu et al., 2024).

# 2. Materials and Methods

## 2.1. Sample collection

The study was carried out using a total of 407 samples collected in 10 districts in Lithuania during 2024−2025. Blood serum, liver samples from domestic pigs and effluent water were collected in farms and slaughterhouses. Wild boar blood serum was collected by hunters. Samples of pork pâté were collected from supermarkets in Vilnius.

The samples were received by and analyzed in the National Food and Veterinary Risk Assessment Institute, a subordinate body of the State Food and Veterinary Service of the Republic of Lithuania. Blood samples delivered to the laboratory were centrifuged and the serum was frozen at –20°C.

## 2.2. DNA Extraction

**Spin-Column DNA extraction from liver using Qiagen Rneasy Purification Kit:**

1. Homogenization of tissue using Qiagen Retsch TissueLyser II in a 2 ml tube with a bead and 500 µl Lysis Buffer for 3 minutes. Lysis Buffer contains guanidine thiocyanate, which protects RNA from ribonucleases.
2. Centrifugation of the sample for 1 minute in the Hettich MIKRO 200 centrifuge at the maximum speed of 13,500 rpm to separate the precipitate and to avoid clogging the column.
3. 500 µl of the sample is mixed with 500 µl of 70% ethanol and 5 µl of RNA internal extraction control is added. Guanidine thiocyanate mixed with ethanol causes the RNA to settle on the membrane while the lysate flows through it.
4. The solution is transferred into a spin column tube and is centrifuged at maximum speed. The collection tube is discarded and replaced with a new one and the the solution is transferred until there is no more solution present in the mixing tube.
5. 500 µl of RW1 Wash Buffer is added to the column and centrifuged.
6. 500 µl of RPE Wash Buffer is used to wash the extract twice, centrifuging each time.
7. For 1-2 minutes the spin column tube is centrifuged at maximum speed without added solutions in order to extract any leftover ethanol, which can inhibit PCR.
8. The column is placed in a 1,5 ml tube and 125 µl of Elution Buffer is added. The sample is centrifuged at 7000 rpm for 1 minute to collect the pure elution of the DNA.

**Spin-column DNA extraction from blood serum and effluent water using the QIAamp Viral RNA Mini Kit:**

1. 560 µl of a heated Lysis Buffer is mixed with 140 µl of the sample.

2. Into the solution 560 µl of 96% ethanol is added and transferred to a spin column tube to centrifuge for 1 minute at 13,500 rpm.
3. The solution is added to the spin column column and centrifuged repeatedly, until it is all transferred. The collection tube is discarded after each centrifugation.
4. 500 µl of AW1 Wash Buffer is added to the column and centrifuged for 1 minute.
5. 500 µl of AW2 Wash Buffer is added to the spin column tube and centrifuged at maximum speed.
6. For 1-2 minutes the spin column tube is centrifuged with an empty collection tube at maximum speed to extract leftover ethanol.
7. The spin column tube is placed in a 1,5 ml tube and 125 µl of Elution Buffer is added. The sample is centrifuged at 7000 rpm for 1 minute to extract the pure DNA.

Both DNA extractions were performed according to the manufacturer's recommendations.

## 2.3. Molecular analysis

Viral RNA was isolated from pig (n=194) and wild boar (n=99) blood serum, pig liver (n=70), effluent water (n=32) and pork pâté (n=12) samples using the Qiagen Rneasy Purification Kit for liver and QIAamp Viral RNA Mini Kit for blood serum and affluent water according to the manufacturer's recommendations.

The extracted RNA was analyzed by RT-PCR using AgPath-ID™ One-Step RT-PCR Kit (Thermo Fisher Scientific). The sample DNA was mixed with the RT-PCR Master Mix and loaded onto the StepOnePlus™ Real-Time PCR System. Reverse transcription and amplification were performed in order to detect HEV-specific RNA through fluorescence-based quantification. Viral RNA was first transcribed into complementary DNA (cDNA) and amplified using repeated thermal cycling. While analyzing the amplification plot, cycle threshold (Ct) value was observed and compared to a standard curve from known RNA standards.

## 2.4. Statistical analysis

Statistical analysis was performed using Microsoft Excel 2010. The HEV prevalence was calculated with a 95% confidence interval (CI). The $\chi^2$ test was used to determine differences in HEV prevalence across different regions. The results were considered statistically significant if the calculated value was $p<0.05$.

Using Fisher's exact test the reliability of the $\chi^2$ test outcome was checked and the results of both tests were assessed and compared.

# 3. Results

The samples were collected in ten regions in Lithuania: Panevėžys, Šiauliai, Tauragė, Kaunas, Utena, Vilnius, Alytus, Marijampolė, Telšiai and Klaipėda (Fig. 13).

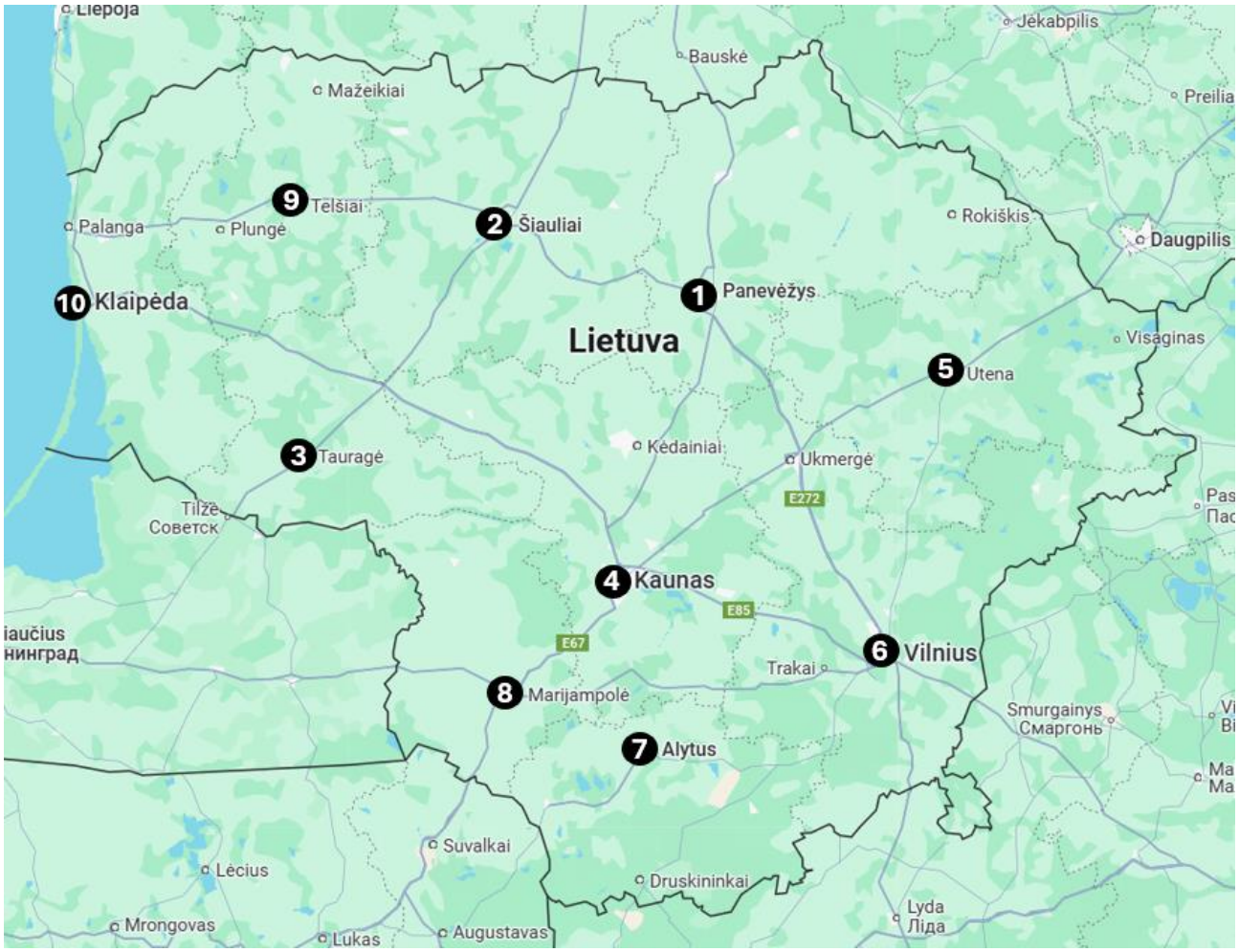


**Figure 13**. Sample collection regions in Lithuania (Google Maps, 2025).

From 407 samples including pig and wild boar blood serum, pig liver, effluent water and pork pâté, 16 were tested HEV-positive.

## 3.1. HEV prevalence in blood serum samples from domestic pigs

No blood serum samples from domestic pigs were tested positive for Hepatitis E virus, with most samples collected in Šiauliai (61) and the least in Vilnius (1). No pig blood serum samples were collected in Alytus and Marijampolė (Table 1).

Statistical analysis was not performed due to the absence of positive samples across all regions.

**Table 1**. Prevalence of hepatitis E virus antigen positive blood serum samples from pigs in different regions of Lithuania.

| District | Pig blood serum | |
|---|---|---|
| | **Positive samples/Total samples** | **Prevalence (%) [PI 95%]** |
| 1. Panevėžys | 0/8 | 0 [0 – 37.5]* |
| 2. Šiauliai | 0/61 | 0 [0 – 4.9]* |
| 3. Tauragė | 0/22 | 0 [0 – 13.6]* |
| 4. Kaunas | 0/33 | 0 [0 – 9.1]* |
| 5. Utena | 0/6 | 0 [0 – 50.0]* |
| 6. Vilnius | 0/1 | 0** |
| 7. Alytus | - | - |
| 8. Marijampolė | - | - |
| 9. Telšiai | 0/38 | 0 [0 – 7.9]* |
| 10. Klaipėda | 0/25 | 0 [0 – 12.0]* |
| **Total** | 0/194 | 0 [0 – 1.6]* |

*The rule of three was applied
**The number of samples is too small to apply the confidence interval and rule of three

## 3.2. HEV prevalence in liver samples from domestic pigs

In liver samples from pigs a 7.1% (95% PI: 3.1 – 15.7) HEV prevalence was observed, with 5 positive samples out of 70. Hepatitis E virus was most prevalent in samples from Panevėžys (15.4%; 95% PI: 4.3 – 42.2) and Utena (13.3%; 95% PI: 3.7 – 37.9), both having the most pig liver samples in total collected. Samples from Kaunas had a prevalence of 10% (95% PI: 1.8 – 40.4), while in the rest of the regions no samples were tested positive for HEV (Table 2).

Statistical analysis was performed and based on the chi-square test no statistically significant difference ($p>0,05$) in HEV prevalence between regions in liver samples was determined ($\chi^2 = 4.783$, $df = 9$, $p = 0.8528$). To confirm the statistical insignificance, Fisher's exact test was used to compare hepatitis E prevalence between pairs of regions with different numbers of positive samples. HEV prevalence between regions was observed as not statistically significant (all p-values > 0.05).

**Table 2**. Prevalence of hepatitis E virus antigen positive liver samples from pigs in different regions of Lithuania.

| District | Pig liver | |
|---|---|---|
| | **Positive samples/Total samples** | **Prevalence (%) [PI 95%]** |
| 1. Panevėžys | 2/13 | 15.4 [4.3 – 42.2] |
| 2. Šiauliai | 0/9 | 0 [0 – 33.3]* |
| 3. Tauragė | 0/7 | 0 [0 – 42.9]* |
| 4. Kaunas | 1/10 | 10 [1.8 – 40.4] |
| 5. Utena | 2/15 | 13. 3 [3.7 – 37.9] |
| 6. Vilnius | 0/4 | 0 [0 – 75.0]* |
| 7. Alytus | 0/4 | 0 [0 – 75.0]* |
| 8. Marijampolė | 0/2 | 0** |
| 9. Telšiai | 0/4 | 0 [0 – 75.0]* |
| 10. Klaipėda | 0/2 | 0** |
| **Total** | 5/70 | 7.1 [3.1 – 15.7] |

*The rule of three was applied
**The number of samples is too small to apply the confidence interval and rule of three

## 3.3. HEV prevalence in effluent water samples from pig farms

The total prevalence out of 32 samples of effluent water with 1 tested positive was 3.1% (95% PI: 0.6 – 15.7). The highest HEV prevalence (20%; 95% PI: 3.6 – 62.5) in effluent water was observed in samples from Panevėžys (Table 3). No samples from other regions were tested HEV-positive, therefore having a prevalence of 0%.

The dependence of the percentage of samples positive for HEV on different regions was not statistically significant ($\chi^2 = 5.574$, degrees of freedom = 9, $p = 0.7817$). Fisher's exact test confirmed the results of chi-square test, showing no significant differences in HEV prevalence between Panevėžys and other regions (all p-values > 0.05).

**Table 3.** Prevalence of hepatitis E virus antigen positive effluent water samples in different regions of Lithuania.

| District | Effluent water | |
|---|---|---|
| | **Positive samples/Total samples** | **Prevalence (%) [PI 95%]** |
| 1. Panevėžys | 1/5 | 20 [3.6 – 62.5] |
| 2. Šiauliai | 0/1 | 0** |
| 3. Tauragė | 0/5 | 0 [0 – 60.0]* |
| 4. Kaunas | 0/8 | 0 [0 – 37.5]* |
| 5. Utena | 0/2 | 0** |
| 6. Vilnius | 0/4 | 0 [0 – 75.0]* |
| 7. Alytus | 0/1 | 0** |
| 8. Marijampolė | 0/3 | 0 [0 – 100.0]* |
| 9. Telšiai | 0/2 | 0** |
| 10. Klaipėda | 0/1 | 0** |
| **Total** | 1/32 | 3.1 [0.6 – 15.7] |

*The rule of three was applied
**The number of samples is too small to apply the confidence interval and rule of three

## 3.4. HEV prevalence in blood serum samples from wild boars

The total prevalence of hepatitis E virus in samples from wild boars was observed at 9.1% (95% PI: 4.9 – 16.4), with 9 positive samples out of 99 (Table 4). The region with the highest HEV prevalence (33.3%; 95% PI: 12.1 – 64.6) found in the collected samples was Utena. Serum samples from Panevėžys had the second highest prevalence (16.7%; 95% PI: 4.7 – 44.8) and several samples were positive from Šiauliai (8%; 95% PI: 2.2 – 25.0) and Kaunas (6.3%; 95% PI: 1.7 – 20.2). However, 0% prevalence was observed in wild boar blood serum from Tauragė (95% PI: 0 – 75.0), Utena (95% PI: 0 – 60.0), Alytus (95% PI: 0 – 60.0), Telšiai (95% PI: 0 – 60.0), and Klaipėda (the number of samples was too small to apply the confidence interval and rule of three). There were no samples collected in Marijampolė.

No significant difference in HEV prevalence was found between regions in the wild boar sample group ($\chi^2 = 9.682$, df = 8, p = 0.2881) and no statistically significant differences were observed between pairs of regions (all p-values > 0.05).

**Table 4**. Prevalence of hepatitis E virus antigen positive blood serum samples from wild boars in different regions of Lithuania.

| District | Wild boar blood serum | |
|---|---|---|
| | **Positive samples/Total samples** | **Prevalence (%) [PI 95%]** |
| 1. Panevėžys | 2/12 | 16.7 [4.7 – 44.8] |
| 2. Šiauliai | 2/25 | 8 [2.2 – 25.0] |
| 3. Tauragė | 0/4 | 0 [0 – 75.0]* |
| 4. Kaunas | 2/32 | 6.3 [1.7 – 20.2]* |
| 5. Utena | 0/5 | 0 [0 – 60.0]* |
| 6. Vilnius | 3/9 | 33.3 [12.1 – 64.6] |
| 7. Alytus | 0/5 | 0 [0 – 60.0]* |
| 8. Marijampolė | - | - |
| 9. Telšiai | 0/5 | 0 [0 – 60.0]* |
| 10. Klaipėda | 0/2 | 0** |
| **Total** | 9/99 | 9.1 [4.9 – 16.4] |

*The rule of three was applied
**The number of samples is too small to apply the confidence interval and rule of three

## 3.5. HEV prevalence in food samples from pork products

12 samples of pork liver pâté were obtained from Lithuanian supermarket chains in Vilnius. HEV RNA was detected in 1 of them with 8.3 % determined prevalence (95% PI: 1.5 – 35.4) (Table 5).

Samples from pork products were collected from one region only, therefore no other regions were available for comparison statistically.

**Table 5**. Prevalence of hepatitis E virus antigen positive samples from pork food products in Lithuania.

| District | Food products from pork | |
|---|---|---|
| | **Positive samples/Total samples** | **Prevalence (%) [PI 95%]** |
| 1. Panevėžys | - | - |
| 2. Šiauliai | - | - |
| 3. Tauragė | - | - |
| 4. Kaunas | - | - |
| 5. Utena | - | - |
| 6. Vilnius | 1/12 | 8.3 [1.5 – 35.4] |
| 7. Alytus | - | - |
| 8. Marijampolė | - | - |
| 9. Telšiai | - | - |
| 10. Klaipėda | - | - |
| **Total** | 1/12 | 8.3 [1.5 – 35.4] |

HEV was found prevalent in pig liver (7.1%), effluent water (3.1%), wild boar blood serum (9.1%), and pork food products (8.3%) in 10 regions in Lithuania, however no statistically significant difference across regions was observed, as all values were $p>0,05$.

## Discussion

In the present study, HEV prevalence in domestic pigs, wild boars and pork food products in Lithuania was observed.

The overall prevalence determined in pig liver was 7.1%, with HEV most prevalent in Utena region (15.4%). HEV can be found in liver because the virus is transmitted by the fecal-oral route, and the focus of pathological signs is the liver, as it is the main site of viral replication (Schlosser et al., 2014). Infected pigs having frequent contact in confined spaces allows for a more effective spread of HEV. Compared to the infection rate observed in pig liver samples other European countries, such as Serbia (44%) (Milojević et al., 2024), the prevalence in pig liver is lower in Lithuania.

Pig blood serum samples showed 0% HEV prevalence, which may be due to HEV being present in blood for only a few weeks after infection in the case of acute disease, or during a narrow viremia window (Nan & Zhang, 2016). Such prevalence can be considered very low compared to the 2018 study in Lithuania, during which pig blood serum was infected by 22.55% - 32.98% (Spancernienė et al., 2018).

Effluent water from pig farms had an HEV prevalence of 3.1% across all regions but the region with the most HEV-positive samples detected (20%) was Panevėžys. HEV prevalence in effluent water in Lithuania is significantly lower than in endemic regions, such as South Africa (74.4%) (Salemane et al., 2024). High HEV (Hepatitis E Virus) prevalence in effluent water from pig farms is mostly due to pigs shedding the virus in their feces resulting in a fecal-oral transmission. HEV contaminates water via runoff, percolation, or the use of pig slurry, which leads to higher prevalence of HEV in effluent water (Ahmad et al., 2022).

In the present study, the highest HEV prevalence was detected in blood serum samples from wild boar (33.3%) collected in Utena region. It was higher than the HEV prevalence of wild boar blood serum (25.94%) observed in the 2018 study in Lithuania (Spancernienė et al., 2018). The overall HEV prevalence in wild boar was 9.1%, which is in line with the 9.5% prevalence observed in research conducted in Italy (De Massis et al., 2022). Wild boars are HEV reservoirs due to the faecal-oral transmission route, mostly via contaminated water and food, as well as possible contact with domestic pigs (Ahmed & Nasheri, 2023).

Hepatitis E can be transmitted through the consumption of contaminated pork products, originating from pigs infected with HEV or game meat from infected wild boar. To determine the risk of HEV infection to humans who consume such products, twelve samples of pork liver pâté were obtained from Lithuanian supermarket chains in Vilnius. HEV RNA was detected in 1/12 (8.3%)

samples, showing HEV at points of sale. In Lithuania, no studies have been conducted on HEV prevalence in pork and game meat products. In Belgium, HEV RNA was found in 65 % of the pork liver pâtés (Locus et al., 2023), which is significantly higher prevalence. However, results of the research conducted in Germany, during which 10% of the samples were positive for HEV, including liver pâté samples (15%) (Pallerla et al., 2021), are similar to the 8.3% prevalence determined in the present study

Some of the highest HEV prevalence in the present study (33.3% from wild boars and 15.4% from pigs) was detected in samples from Utena region but without a statistically significant difference across regions ($p>0,05$).

# Conclusions

1. In Lithuanian pig population 7.1% liver samples were tested positive for HEV using RT-qPCR, most prevalent in Panevėžys (15.4%) and Utena (13.3%), without a statistically significant difference across regions ($p>0,05$).
2. In Lithuanian pig population 0% blood serum samples were tested positive for HEV, however effluent water samples showed an overall 3,1% HEV prevalence, tested positive in Panevėžys (20%), without a statistically significant difference across regions ($p>0,05$).
3. In Lithuanian wild boar population 9.1 % blood serum samples were tested positive for HEV, most prevalent in Vilnius (33.3%), without a statistically significant difference across regions ($p>0,05$).
4. HEV prevalence in pork food products from Lithuanian supermarkets was observed at 8.3%.
5. The results of the detection of HEV in pig and wild boar populations, and pork food products, have proven that this zoonosis is relevant in Lithuania and may affect not only animals but humans as well, especially through consumption of raw or undercooked pork and game meat products.

## Recommendations

The conducted studies have revealed that HEV viral prevalence exists in populations of domestic pigs and wild boars in Lithuania and humans are susceptible to the infection as it is zoonosis, therefore:

1. The risk of HEV exposure can be reduced by maintaining hygienic practices, avoiding raw or not fully heat-treated meat, especially pork and game meat, and water from unknown sources.
2. Performing HEV testing in immunosuppressed patients with symptoms consistent with acute hepatitis is important, as they are more susceptible to a possible fatal outcome.
3. Improving epidemiologic and diagnostic tools, implementing routine vaccination programs, improving hygiene, water and sanitation is essential to stop HEV contribution to mortality in endemic regions.

## Author’s Personal Contribution

Dr. Simona Pilevičienė: Conceptualization, Methodology, Validation, Resources, Supervision, Project administration, Funding acquisition.

Rita Vorobjovienė: Methodology, Validation, Resources, Supervision, Project administration.

Ieva Beitnaraitė: Formal analysis, Investigation, Data curation, Writing- Original draft preparation, Writing - Review & Editing, Visualization.

VILNIAUS UNIVERSITETAS

GYVYBĖS MOKSLŲ CENTRAS

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Magistro baigiamasis darbas

# Hepatito E viruso paplitimas kiaulėse ir laukiniuose šernuose Lietuvoje

## SANTRAUKA

Hepatito E virusas (HEV) yra labiausiai paplitusi ūminio virusinio hepatito priežastis ir gali būti perduodama per maistą, vandenį ir zoonozinius perdavimo būdus. Siekiant nustatyti hepatito E viruso paplitimą Lietuvos naminių kiaulių (*Sus domesticus*), šernų (*Sus scrofa*) populiacijose ir maisto produktuose (kiaulienos paštetuose), atlikta ealaus laiko kiekybinė atvirkštinės transkripcijos polimerazės grandininė reakcija (RT-kPGR) analizė. Kiaulių kraujo serumo (n=194), kiaulių kepenų (n=70), nuotekų vandens (n=32), šerno kraujo serumo (n=99) ir kiaulienos pašteto (n=12) mėginiai 2024–2025 m. buvo paimti iš skirtingų Lietuvos rajonų. Siekiant nustatyti specifinį HEV genotipą mėginiuose, atlikta filogenetinė analizė. HEV paplitimas nustatytas 7,1 % kiaulių kepenų, 3,1 % nuotekų vandens, 9,1 % šernų kraujo serumo mėginių ir 8,3 % kiaulienos kepenėlių paštetų mėginiuose. Aptiktas kiaulių ir šernų užsikrėtimas HEV įrodo, kad šie gyvūnai yra virusų rezervuarai Lietuvoje ir kiaulienos bei žvėrienos produktų vartojimas padidina Hepatito E infekcijos riziką.

**Raktažodžiai:** hepatitas E; Paslahepevirus balayani; zoonozė; hepatito E virusas; RNR; kiaulė; Sus domesticus; šernas; Sus scrofa; Lietuva.

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Master's Thesis

**Prevalence of Hepatitis E virus in pigs and wild boars in Lithuania**

ABSTRACT

Hepatitis E virus (HEV) is the most prevalent cause of acute viral hepatitis and can be transmitted through foodborne, waterborne, and zoonotic transmission routes. Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis was performed to determine the prevalence of hepatitis E virus in Lithuanian domestic pig (*Sus domesticus*), wild boar (*Sus scrofa*) populations and food products (pork pâtés). Pig blood serum (n=194), pig liver (n=70), effluent water (n=32), wild boar blood serum (n=99) and pork pâté (n=12) samples were collected from different districts of Lithuania during 2024–2025. Phylogenetic analysis was performed to determine the specific genotype of HEV in samples. HEV prevalence was detected in 7.1% percent of pig liver, 3.1 % of effluent water, 9.1 % of wild boar blood serum samples and 8.3% of pork liver pâtés. The detected infection of pigs and wild boars with HEV showed that these animals can be HEV virus reservoirs in Lithuania. Consumption of pork and game meat products was shown to pose a risk for HEV infection in humans.

**Keywords:** hepatitis E; Paslahepevirus balayani; zoonosis; hepatitis E virus; RNA; pig; Sus domesticus; wild boar; Sus scrofa; Lithuania.

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