Article
https://doi.org/**10.61900/SPJVS.2023.03.19**

ASSESSMENT OF THE CONTRIBUTION OF WILDLIFE AND DOMESTIC PIGS IN HEPATITIS E VIRUS TRANSMISSION AND ZOONOTIC POTENTIAL IN EASTERN ROMANIA

Adriana ANIȚĂ[1], Dragoș ANIȚĂ[1],

Iași University of Life Sciences, Faculty of Veterinary Medicine,
Regional Center of Advanced Research for Emerging Diseases Zoonoses and Food Safety
e-mail:danita@uaiasi.ro

Abstract

Hepatitis E virus (HEV) has been confirmed within the landscape of the European food industry, representing a significant factor in the dissemination of HEV among European citizens. Food-borne transmission of HEV appears to be a major route in Europe, with pigs and wild boars being the main source. The results of this study highlight an overall HEV seroprevalence of 12.8% (95%CI: 7.95-17.75) in wild boars and the detection of HEV RNA in all three fresh pig liver batches sampled from a slaughterhouse in Iași County. Given the prevalent dietary preferences in Romania, pork stands out as a highly favored food choice among the populace. However, the popularity of pork also raises concerns, as there exists the occasional risk of contamination with HEV, presenting a potential threat to consumer health. Ongoing surveillance, regulatory measures, and public awareness initiatives collectively may represent a comprehensive strategy to protect the consumers and ensure the safety of pork products in the market.

Keywords: hepatitis E virus, swine, wild boar

INTRODUCTION

Hepatitis E virus stands out as a prominent global contributor to acute hepatitis, holding the leading position among viral causes. The family *Hepeviridae, Orthohepevirinae* subfamily comprises single-stranded RNA viruses organized into four distinct genera. Among these, only strains within the *Paslahepevirus* (HEV) and *Rocahepevirus* (RHEV) genera have exhibited zoonotic potential, posing a significant health threat (Purdy et al, 2022). Within the Paslahepevirus genus, two species, *P. balayani* and *P. alci,* exist. The *P. balayani* species further delineates into eight genotypes (HEV A1-8) based on the host species it infects. Genotypes 3 and 4 are identified in pigs; 3, 4, 5, 6 in wild boars; 3 in rabbits, mongooses, and deer; 4 in yaks; and 7 and 8 in camels (Ahmed et al, 2023). The expanded host range indicates the high variability of these HEV strains and their zoonotic potential.

Five HEV genotypes (HEV1-4 and HEV-7) are recognized to induce hepatitis in humans. In low-income countries, during epidemics, the fecal contamination of water reservoirs serves as the source of infection for genotypes 1 and 2.

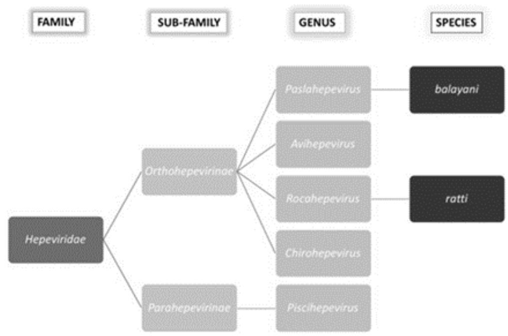

Fig. 1 Taxonomy and structure of *Hepeviridae* family

Autochthonous hepatitis E in the developed world primarily results from HEV-3 transmission occurring through the consumption of undercooked pork or cervid meat, especially the liver of these animals, and also through contact with contaminated animals. More recently, it has been demonstrated that the environment can be contaminated by zoonotic sources. Other foods such as salads, field raspberries, or shellfish (mussels, oysters) are potential sources of contamination. The frequency of human clinical forms associated with HEV infection is not

[1]ROVETEMERG Research Center, Faculty of Veterinary Medicine, University of Life Sciences “Ion Ionescu de la Brad” Iași, Romania

known, but it does not appear to depend on the route of contamination (contact or food-borne) (Geng et al, 2023). There are clustered cases of exposure to HEV through food, but not all infected individuals (showing seroconversion) have reported acute hepatitis.

The present study was designed to provide data from a selected group of hunting founds and a slaughter house from Iași County, aiming to highlight the presence of the hepatitis E virus in the pig reservoir. The investigation framework encompasses a seroprevalence survey, delving into the prevalence of antibodies against HEV in wild boars and a molecular investigation focusing on the characteristics of the present viral strains in offal's of domestic swine. Through these multifaceted analyses, the study seeks to contribute valuable insights into the dynamics and potential pathways of zoonotic transmission of hepatitis E infection.

MATERIAL AND METHOD

The investigations war carried out between 2021 and 2022, involving the sampling of 179 wild boar sera from various hunting founds in Iași County. Additionally, 16 liver tissue samples were collected from two distinct slaughtering batches of pigs aged 5-6 months. The domestic pigs weighed approximately 100 kg at the moment of slaughtering.

The PrioCHECK® VHE Ab porcine kit (Applied Biosystems) was employed for the detection of HEV antibodies in serum samples from wild boars. The protocol followed a four-step protocol, including sample preparation, sample incubation, conjugate incubation, and detection. The ELISA plate is coated with recombinant hepatitis E virus (HEV) antigens from the ORF 2 and 3 of genotypes 1 and 3, being used for the purposes of monitoring and surveillance of potential zoonotic infection in swine.

For fresh liver samples, the protocol consisted first of RNA extraction and reverse transcription. Nested PCR was employed to identify HEV RNA in pig liver samples, utilizing a modified PCR technique aimed at enhancing the sensitivity and specificity of the assay. The method incorporates two sets of primers and two consecutive PCR reactions. Following the initial PCR reaction, the resulting amplicon served as a template for a second set of primers and an additional amplification step. The targeted region corresponds to a fragment of the ORF2 from the HEV genome. The specific primers employed in the reaction are detailed in figure 3.

RNA Extraction
↓
Revers Transcription
↓
Nested-PCR for detection of HEV RNA
↓
Data Analysis

3156N	AATTATGCYCAGTAYCGRGTTG	ORF2
3157N	CCCTTRTCYTGCTGMGCATTCTC	
3158N	GTWATGCTYTGCATWCATGGCT	
3159N	AGCCGACGAAATCAATTCTGTC	

Figure 2. Steps of the molecular diagnosis protocol and the primers used for HEV detection

RESULTS AND DISCUSSIONS

Pigs and wild boars are recognized as reservoirs for infections in humans in European countries. The transmission of these infections to humans occurs through various means, including direct contact, the consumption of raw or undercooked pork meat, and the ingestion of products derived from wild boars, such as sausages (Renou et al, 2014). Notably, several field studies have underscored the prevalence of HEV, revealing that between 2% and 11% of pork livers available in supermarkets across Japan, Europe, and the United States have tested positive for HEV RNA. Moreover, the infectivity of HEV has been corroborated through experiments using animal models.

In addition to direct transmission, there exists the potential for indirect transmission through contact with the manure of pigs infected with HEV (De Schryver et al, 2015). Interestingly, HEV has demonstrated its adaptability beyond traditional sources. It has been detected in unexpected places, such as strawberries cultivated in Canada, frozen raspberries, and salad vegetables in Europe. Furthermore, the versatility of HEV extends to seafood, where genotypes 3 and 4 of the virus are frequently identified. Oysters, flat oysters, mussels, and clams, among other seafood, have been found to harbor HEV. This occurrence is attributed to the bioaccumulation of the virus from water, resulting in its concentration within the digestive tissues of these marine organisms (Takuissu et al, 2022).

Hepatitis E has become the leading cause of acute viral hepatitis in Europe. Recent studies on blood donor populations, conducted using the same serological test with validated analytical performance in terms of sensitivity and specificity, reveal anti-HEV IgG seroprevalence rates exceeding 20% in countries such as France (22%) (Gallian et al, 2014), Germany (29%), and the Netherlands (27%) (van Gageldonk-Lafeber et al, 2017). The seroprevalence is 16% in the United Kingdom (Hewitt et al, 2014). The rise in seroprevalence with age reflects cumulative exposure to the virus. The prevalence of asymptomatic forms contributes to its relatively low awareness. At the other end of the pathological spectrum, one can observe deadly fulminant hepatitis and chronic forms, especially in transplant recipients (Kamar et al, 2012).

The natural infection kinetics of pigs in farming settings reveal a predominant susceptibility among the animals during their early stages, specifically at the juncture of weaning and the decline in maternal immunity transfer. The transmission pathway primarily involves oro-fecal transmission, typically manifesting around the 10th week of age. Subsequently, the virus undergoes active replication within the liver, with substantial excretion observed in feces between the ages of 12 and 18 weeks before eventual elimination.

2021

Hunting Found	No. of samples
1. Poieni	8
2. Cătălina	1
3. Sineşti	1
4. Criveşti	6
5. Grajduri	45
6. Schitu-Duca	7
7. Pietrosu	22

2022

Hunting Found	No.of samples	Hunting Found	No.of samples
1. Bivolari	1	17. Corneşti	1
2. Horleşti	5	18. Prisecani	2
3. Ţibăneşti	8	19. Gropniţa	1
4. Stolniceni-Prăjescu	4	20. Valea Lupului	1
5. Stroieşti	1	21. Larga-Jijia	1
6. Victoria	5	22. Gorban	2
7.Ţuţora	1	23. Moţca	2
8. Popeşti	1	24. Pocreaca	2
9. Brădiceşti	4	25. Fermă de suine (Gorban)	13
10. Mogoşeşti	3	26. Buneşti	1
11. Hărmăneşti	3	27. Bârnova	1
12. Gheorghiţoaia	10	28. Strunga	1
13. Turia Perieni	4	29. Tătăruşi	1
14. Dagâţa	3	30. Minosloveşti	2
15. Criveşti	2	31. Brăieşti	1
16. Grajduri	1	32. Poieni	1

Fig. 3. Details of wild boar samples tested for HEV antibodies

It's noteworthy that during the viremic phase, the virus may extend its presence to other organs, including muscles such as the longissimus, biceps femoris, and iliopsoas (Bouwknegt et al, 2009). In certain instances, infections may occur later in the animals' development, leading to the arrival of individuals in the active phase of viral multiplication in the liver and viremic animals at the abattoir. This scenario poses a pronounced risk of exposure to Hepatitis E Virus (HEV) through the consumption of meat products. Turning attention to wildlife, wild boars and deer are identified as carriers of the virus, although the specifics of HEV dissemination within these reservoirs are not yet fully elucidated. Nonetheless, these species remain crucial reservoirs, with HEV prevalence reaching 40% in wild boars and 34% in deer within specific European regions (Pavio Net al, 2010). The evidence of the Hepatitis E Virus (HEV) in food products originating from pork, particularly pork liver, has been substantiated through comprehensive investigations across diverse regions. Five distinct studies conducted in Japan, the Netherlands, India, South Korea, and the United States have collectively shed light on the prevalence of HEV in these consumables. Notably, these studies disclosed that a range of 1% to 11% of commercially available pork livers tested positive for HEV (Di Cola et al, 2021). It's essential to highlight that certain liver-based preparations, such as semi-dry liver sausages, pose a potential risk when consumed in their raw state. These products, as recently underscored in a report from France (Colson et al, 2010) may harbor the virus and could be implicated in human cases. This emphasizes the need for continued vigilance and research to comprehend and mitigate the risks associated with HEV transmission through pork-derived food items.

In the present study, HEV circulation in suid populations in Iasi County was asses through the detection of HEV antibodies in wild boars and evaluation of the presence of HEV RNA in commercial pig fresh liver.

Following a meticulous analysis of the results derived from serological testing aimed at detecting antibodies against the hepatitis E virus in samples collected from wild boars throughout the year 2021, 19 seropositive animals were identified, with a seropositivity rate of 21.11% (95% CI: 12.68-29.54%). The wild boars that tested positive for HEV hailed from four distinct hunting founds: Poieni, Crivești, Grajduri, and Pietrosu. The scrutiny of results stemming from serological testing for HEV antibodies in samples collected from wild boars throughout the year 2022 highlighted four seropositive animals out of 90 wild boars tested. The seroprevalence for the samples examined in the year 2022 stands at 4.4% (95% CI: 0.9 – 8.70%). Noteworthy is the fact that the wild boars positive for HEV antibodies originated from four distinct hunting founds: Poieni, Stolniceni-Prăjescu, Gheorghițoaia, and Mogoșești (Figure 4).

2021	Hunting Found	No. of positive samples	%	CI 95%
	Poieni	4	50%	15.35-84.65
	Crivești	1	16.7%	-13.15-46.49
	Grajduri	2	1.44%	-1.58-10.47
	Pietrosu	12	54.5%	33.74-75.35
	Total	**19**	**21.11%**	12,68-29,54

2022	Hunting Found	No. of positive samples	%	CI 95%
	Stolniceni-Prăjescu	1	25%	-17.44 - 67.44
	Gheorghițoaia	1	9%	-8.59 – 28.59
	Mogoșești	1	33.3%	-20 – 86.68
	Poieni	1	100%	100
	Total	**4**	**4.4.%**	0.9 – 8.70

Fig.4 Results of the detection of HEV antibodies in wild boar population

Similar results have been reported in two previous studies conducted in the Eastern region of Romania, highlighting the HEV seroprevalence at values of 10.29% and 9.61% in wild boar populations (Porea et al, 2018). Two studies from France and the Netherlands have also shown that HEV is endemic in these territories, with HEV seroprevalence in wild boars reaching values of 14% and 12%, respectively (Carpentier et al, 2012; Rutjes et al, 2010). A recent study conducted in Spain reported a 23% prevalence of HEV RNA and suggested that the dynamics of hepatitis E virus in wild boars may be seasonal, peaking at the beginning of the hunting season in late October and November (Rivero-Juarez et al., 2018). Furthermore, individuals with professional exposure, such as hunters and forestry workers, show an increased risk of the presence of specific anti-HEV antibodies, indicating a heightened risk of hepatitis E virus infection among them (Dremsek et al, 2012).

In the study involving domestic pigs, the results of the molecular analysis of the 16 liver samples, highlighted that all samples were

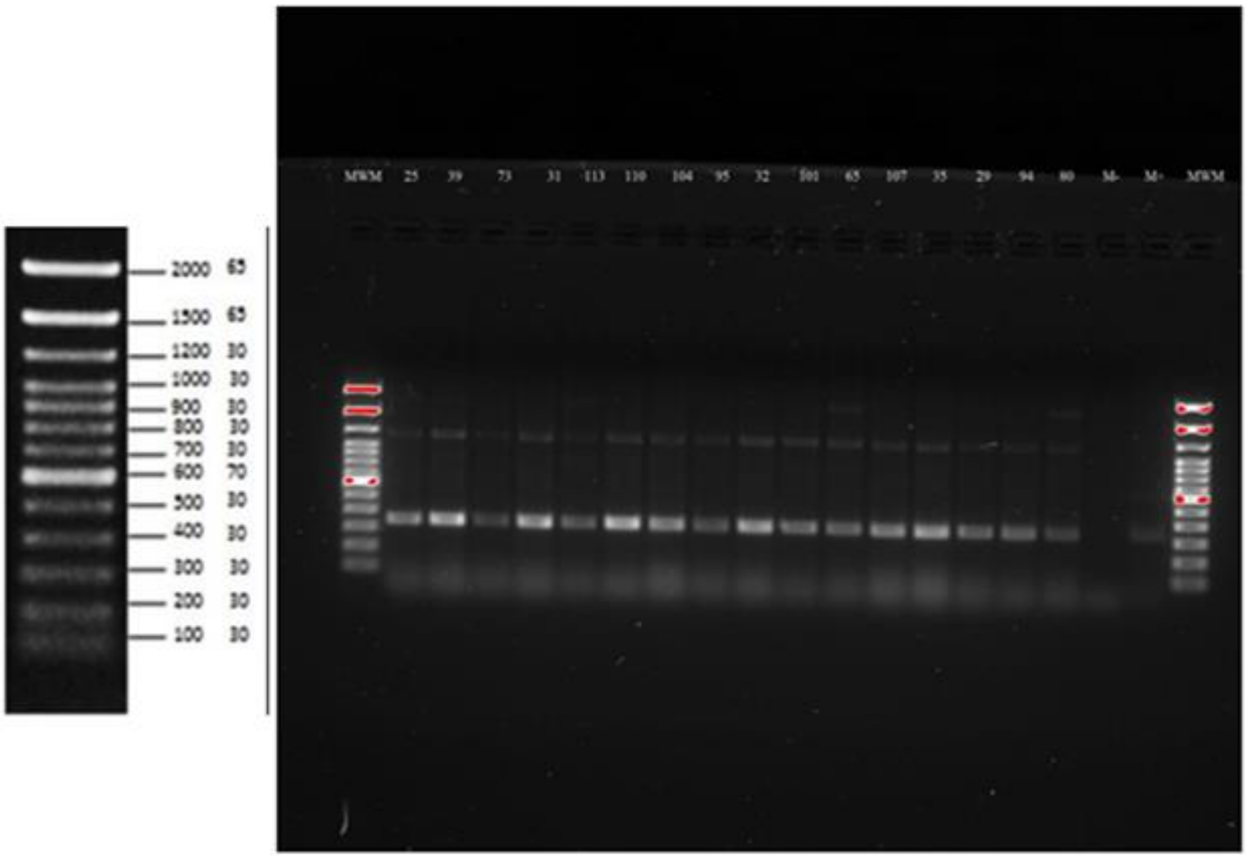

Figure 5. The results of the nested-PCR for detection of HEV RNA in pig liver samples. The expected positive PCR product length: 347 nt

positive for HEV RNA (Figure 5). This noteworthy finding underscores a uniform positivity across all three slaughtering batches, irrespective of the particular sample under examination. This consistent positivity across different batches strongly reinforces the assertion that HEV is a zoonotic disease, capable of transmission through improperly cooked pig meat.

In this context, the farm environment has been identified as a potential source of HEV in pigs. However, understanding the specific risk factors associated with on-farm and between-farm transmission of HEV remains elusive for interpretation. The last research studies highlighted the fact that HEV infections are not uncommon among pigs, the virus being detected in pig liver around four months old (Widen, 2016). Pork and liver undergo diverse industrial food-processing techniques to enhance their safety, flavor, and shelf life. These methods encompass a spectrum of approaches, including fermentation, acidification, high-pressure processing steps, and thermal treatments. Despite the application of thermal processing to pork meat and products, there remains a noteworthy concern regarding the stability of the hepatitis E virus. Published literature indicates that certain temperatures commonly employed in cooking may not effectively inactivate the virus, even when subjected to thermal treatment (EFSA Panel on Biological Hazards, 2017).

CONCLUSIONS

Presently, the substantial prevalence of the hepatitis E virus in animal reservoirs, particularly in pigs, underlines its status as a significant source of contamination for humans. To effectively mitigate the risk of zoonotic human infection, a comprehensive approach is essential. This involves not only the continued monitoring of pigs but also the active surveillance of all animal reservoirs. Recognizing the presence of HEV in animal offal's emphasizes the critical need for advancing diagnostic methodologies within food products. Implementing rigorous processing procedures in the food industry is imperative to curtail the potential for contamination. Furthermore, practical cooking methods should be emphasized to inactivate the virus, thereby enhancing overall food safety and minimizing the risk of transmission to humans during consumption.

These multifaceted measures, encompassing surveillance, diagnostics, and processing protocols, collectively contribute to a comprehensive strategy aimed at reducing the incidence of zoonotic transmission of HEV to humans through the food chain.

REFERENCES

1. **Ahmed R, Nasheri N.** Animal reservoirs for hepatitis E virus within the Paslahepevirus genus. Vet Microbiol. 2023; 278:109618.

2. **Bouwknegt M, Rutjes SA, Reusken CB, Stockhofe-Zurwieden N, Frankena K, de Jong MC, et al.** The course of HepatitisEvirus infection in pigs after contact-infection and intravenous inoculation. Vet Res. 2009; 5:7.

3. **Carpentier A, Chaussade H, Rigaud E, Rodriguez J, Berthault C, Boué F, Tognon M, Touzé A, Garcia-Bonnet N, Choutet P, Coursaget P**. High hepatitis E virus seroprevalence in forestry workers and in wild boars in France. J Clin Microbiol. 2012; 50(9):2888-93.

4. **Colson P, Borentain P, Queyriaux B, Kaba M, Moal V, Gallian P, et al.** Pig liver sausage asasource of Hepatitis E virus transmission to humans. J Infect Dis. 2010. 15 ;202(6):825-34.

5. **De Schryver A, De Schrijver K, François G, Hambach R, van Sprundel M, Tabibi R et al.** Hepatitis E virus infection: an emerging occupational risk? Occup Med (Lond) 2015, 65(8):667–672.

6. **Di Cola, G.; Fantilli, A.C.; Pisano, M.B.; Re, V.E.** Foodborne transmission of hepatitis A and hepatitis E viruses: A literature review. Int. J. Food Microbiol. 2021, 338, 108986.

7. **Dremsek P, Wenzel JJ, Johne R, Ziller M, Hofmann J, Groschup MH, Werdermann S, Mohn U, Dorn S, Motz M, Mertens M, Jilg W, Ulrich RG.** Seroprevalence study in forestry workers from eastern Germany using novel genotype 3- and rat hepatitis E virus-specific immunoglobulin G ELISAs. Med Microbiol Immunol. 2012; 201(2):189-200.

8. **Gallian P, Lhomme S, Piquet Y, et al.** Hepatitis E virus infections in blood donors, France. Emerg Infect Dis, 2014; 20:1914-7.

9. **Geng Y, Shi T, Wang Y.** Transmission of Hepatitis E Virus. Adv Exp Med Biol. 2023; 1417:73-92.

10. **EFSA Panel on Biological Hazards (BIOHAZ); Ricci A, Allende A, Bolton D, Chemaly M, Davies R, Fernandez Escamez PS, Herman L, Koutsoumanis K, Lindqvist R, Nørrung B, Robertson L, Ru G, Sanaa M, Simmons M, Skandamis P, Snary E, Speybroeck N, Ter Kuile B, Threlfall J, Wahlström H, Di Bartolo I, Johne R, Pavio N, Rutjes S, van der Poel W, Vasickova P, Hempen M, Messens W, Rizzi V, Latronico F, Girones R.** Public health risks associated with hepatitis E virus (HEV) as a food-borne pathogen. EFSA J. 2017, 11;15(7):e04886.

11. **Hewitt PE, Ijaz S, Brailsford SR, et al.** Hepatitis E virus in blood components: a prevalence and transmission study in southeast England. Lancet. 2014; 384:1766-73.

12. **Kamar N, Garrouste C, Haagsma EB, et al.** Factors associated with chronic hepatitis in patients with hepatitis E virus infection who have received solid organ transplants. Gastroenterology 2012; 140:1481-9.

13. **Pavio N, Meng XJ, Renou C**. Zoonotic Hepatitis E: Animal reservoirs and emerging risks. Vet Res. 2010; 41:46.

14. **Porea D, Anita A, Demange A, Raileanu C, Oslobanu Ludu L, Anita D, Savuta G, Pavio N.** Molecular detection of hepatitis E virus in wild boar population in eastern Romania. Transbound Emerg Dis. 2018; 65(2):527-533.

15. **Purdy MA, Drexler JF, Meng XJ, Meng XJ, Norder H, Okamoto H, Van der Poel** WHM et al. ICTV virus taxonomy profile: Hepeviridae 2022. J Gen Virol, 2022, 103(9).

16. **Renou C, Afonso A-MR, Pavio N.** Foodborne transmission of hepatitis E virus from raw pork liver sausage, France. Emerg Infect Dis, 2014, 20:1945–1947.

17. **Rivero-Juarez A, Risalde MA, Frias M, García-Bocanegra I, Lopez-Lopez P, Cano-Terriza D, Camacho A, Jimenez-Ruiz S, Gomez-Villamandos JC, Rivero A.** Prevalence of hepatitis E virus infection in wild boars from Spain: a possible seasonal pattern? BMC Vet Res. 2018;14(1):54.

18. **Rutjes SA, Lodder-Verschoor F, Lodder WJ, van der Giessen J, Reesink H, Bouwknegt M, de Roda Husman AM.** Seroprevalence and molecular detection of hepatitis E virus in wild boar and red deer in The Netherlands. J Virol Methods. 2010; 168(1-2):197-206.

19. **Takuissu GR, Kenmoe S, Ndip L, Ebogo-Belobo JT, Kengne-Ndé C, Mbaga DS, Bowo-Ngandji A, Oyono MG, Kenfack-Momo R, Tchatchouang S, Kenfack-Zanguim J, Lontuo Fogang R, Zeuko'o Menkem E, Kame-Ngasse GI, Magoudjou-Pekam JN, Nkie Esemu S, Veneri C, Mancini P, Bonanno Ferraro G, Iaconelli M, Suffredini E, La Rosa G.** Hepatitis E Virus in Water Environments: A Systematic Review and Meta-analysis. Food Environ Virol. 2022; 14(3):223-235.

20. **van Gageldonk-Lafeber AB, van der Hoek W, Borlée F, Heederik DJ, Mooi SH, Maassen CB et al.** Hepatitis E virus seroprevalence among the general population in a livestock-dense area in the Netherlands: a cross-sectional population-based serological survey. BMC Infect Dis 2017, 17(1):21.

21. **Widen F.** Hepatitis E as a zoonosis. Adv. Exp. Med. Biol., 2016, 948, 61-71.