OTKA 130215

The animal health and food-safety significance of hepatitis E virus in swine and rabbit

Final report

About 75% of the new infectious diseases that have affected humans over the past 10 years have been caused by pathogens originating from animals or from products of animal origin. Approximately 60% of these diseases are zoonoses, the investigation and follow up of them is a matter of utmost importance (Aluwong and Bello, 2010). The European Food Safety Authority (EFSA), besides Noroviruses and hepatitis A virus, has described Hepatitis E virus (HEV) as one of the most important foodborne viruses (Andreoletti et al., 2011). However, several studies have been done in the recent past to learn about the epidemiology, zoonotic potential and food safety risk of HEV, there are still unknown details that warrants the further investigation of the virus (Yugo and Meng, 2013).

HEV causes an acute, self-limiting illness in humans that resolves within 4–6 weeks. Occasionally, a fulminant form of hepatitis develops (acute liver failure), which can lead to death. Infections can be asymptomatic; when they occur, the clinical signs are similar to those of other types of acute viral hepatitis and can include fever, loss of appetite, nausea, abdominal pain and jaundice. The overall case-fatality rate is about 1%; however, among pregnant women in their third trimester of pregnancy the mortality rate can exceed 20%. Developed countries until recently were regarded as “non-endemic”, since cases of clinical hepatitis E were sporadic, mainly imported infections occurred and only a few autochthonous cases were published (Aggarwal and Krawczynski, 2000; Worm et al., 2002; Reuter and Szűcs, 2004). At present these areas are reckoned as “endemic”, because the presence of HEV has become persistent, however the prevalence is less than 25% among the non-A non-B acute hepatitis cases (Teo, 2009; Teo, 2010). In Hungary the incidence of the cases has been followed up since 2007: the number of cases has been continuously increasing (from 12 cases in 2007 to 90 cases in 2012) with 0-1 fatal case per year (OEK, 2008; 2009; 2010; 2011; 2012; 2013).

HEV is a non-enveloped virus with 32-34 nm of diameter and icosahedral symmetry. The viral capsid contains a positive sense single stranded RNA. It belongs to the *Hepeviridae* family *Orthohepevirus* genus (Smith and Simmonds, 2018). The virus-variants detected in several countries are classified into 4 genogroups. HEV strains of genotype 1 and 2 are detected exclusively in human, while viruses of the 3rd and 4th genotype can infect both humans and animals (Xia et al., 2011). The zoonotic character and food-borne spread of HEV is suggested

by investigations of experimental cross-species infections and clinical cases occurred after consumption of HEV-infected meat and meat products (Meng et al., 1998; Yazaki et al., 2003; Tamada et al., 2004; Meng, 2005; Reuter et al., 2009a and 2009b).

In endemic areas animal reservoirs are considered to play an important role in the maintenance of the virus and in the spread of HEV to humans (Yazaki et al., 2003; Widdowson et al., 2003; Wichmann et al., 2008). HEV-induced seroconversion was described in several species, however clinical hepatitis in animals has not been observed to date. Besides genetic and epidemiological studies, the zoonotic nature of the virus was proven by experimental infections. The phylogenetic analyses indicated close genetic relationships between hepatitis E viruses of human and animal origins, which further support the theories about the zoonotic character of the virus (Xia et al., 2011). HEV infection of animals has both food-hygiene and occupational health importance (Meng et al., 1998; Yazaki et al., 2003; Tamada et al., 2004; Galiana et al., 2008; Chaussade et al., 2013).

Besides domestic swine, wild boar and several deer species were found to be the potential reservoir hosts of HEV (Meng, 2005; Forgách et al., 2010a and 2010b). Serological evidences of the susceptibility of domestic ruminants (cattle, sheep, and goat) were published; however, it was unsuccessful to prove the HEV-reservoir role of these animals (Arankalle et al., 2001; Wang et al., 2002; Meng, 2005; Peralta et al., 2009; Meng, 2011). In case of goat, a survey based on enzymelinked immunosorbant assay (ELISA) and reverse-transcription polymerase chain reaction (RT-PCR), revealed, that a HEV-related agent was circulating and maintained in the goat population in Virginia and that the detected “goat HEV” were likely genetically very divergent from the known HEV strains (Sanford et al., 2012). Samples of rats and mice were also investigated in order to prove the suspected reservoir role of these animals in the epidemiology of HEV (Kabrane-Lazizi et al., 1999; Favorov et al., 2000; Arankalle et al., 2001; Hirano et al., 2003; Johne et al., 2010). Their results indicate that HEV strains of rat-origin are restricted to the natural host (Guan et al., 2013; Li et al., 2013). In addition, hepeviruses were recently isolated from bats, ferrets and cutthroat trout, and in consequence, the revision of hepevirus taxonomy was suggested (Batts et al., 2011; Drexler et al., 2012; Raj et al., 2012; Oliveira-Filho et al., 2013).

In the recent years HEV infection of rabbits has drawn attention to researchers. Studies in China, USA and France found domestic and wild rabbits susceptible to HEV. Serological and genetic investigations revealed that the rabbit-derived viruses are closely related to viruses detected in human (Zhao et al., 2009; Cossaboom et al., 2011; Geng et al., 2011, Wang et al.,

2013). Successful crosspecies infection of swine and cynomolgus macaques suggests the zoonotic potential of rabbit HEV (Lhomme et al., 2013; Liu et al., 2013).

Between 2004 and 2010 a survey was done to figure out the incidence of HEV in domestic and wild animals in Hungary. Among the 41 investigated swine farms, the presence of HEV was detected in 16 (39%). The incidence of infection was highest among the 11-16-week-old pigs (37%), however 9% of the swine over 17 weeks were also found HEV-positive. Eight out of 75 (9%) wild boar, 9 out of 41 (22%) roe deer and 3 out of 30 (10%) red deer liver samples contained HEV RNA. Animal samples positive for HEV were found in the whole territory of Hungary (Reuter et al., 2009b; Forgách et al., 2010a and 2010b). In our previous study we attempted to explore the infection of domestic ruminants, rodents and shrews by investigation of faeces, liver or intestine samples. Neither the faeces samples of cattle and small ruminants nor the investigated organ samples of rat, mouse, hamster and shrew samples contained HEV RNA.

The phylogenetic analysis revealed that the viruses detected in Hungarian animal samples belong to 3 subgroups (3a, 3e and 3h) of the 3rd genotype of HEV showing high similarity to HEV sequences of human and animal origin detected in other countries. High variability of the nucleotide sequence of the virus was also observed (Forgách et al., 2010b).

Based on the study a new investigation was started in 2014, focusing animal health, food safety and occupational health significance of HEV infection in on food producing animals.

1. SURVEY ON THE PREVALENCE OF HEV-INFECTION IN POPULATIONS OF RESERVOIR ANIMALS

In the first year we focused on the overall prevalence of the virus in populations of food producing animals. As the presence of the virus can be most easily investigated serologically, our aim was to survey domestic swine and rabbit farms as well as to test goat samples; on one hand to produce results to be compared to the results of 2004-2010 investigation on the spread of HEV, and on the other hand to recognise the presence of the virus in populations of species proven or suspected to be reservoir of HEV.

1.1 Materials and methods

1.1.1 Blood (serum) samples

Serum samples were collected from 14 swine farms in Hungary; 10 regular swine farms, and 4 imported-fattening swine farms. The imported-fattening farms buy weaned piglets approximately 28 days old from different countries and import them to Hungary. The imported-fattening piglets are kept in quarantine for 40 days, before sampling, so they will be around 70 days old when sampled. Altogether 473 serum samples were collected from swine from different age groups: older sow (174 samples), 1st pregnancy sow (22 samples), 24 weeks old piglets (from slaughterhouse, 80 samples), 18-20 weeks (51 samples), 16 weeks (10 samples), 12 weeks (10 samples) 6-10 weeks (30 samples), 3 weeks old (10 samples) and imported-fattening piglets (86 samples).

Serum samples of domestic rabbit were collected from 11 farms belonging to 2 production integration. The 231 sampled animals were mainly 6 months old rabbits, either still in production (Integration N) or sent to be slaughtered because of infertility (Integration P). At Integration N, the samples were collected by the practicing veterinarian at the farm, samples originated from Integration P were collected during the slaughter.

Goat samples originally arrived to the laboratory in order to perform caprine arthritis-encephalitis investigation as part of the eradication program. All 333 samples originated from goats of different age groups between 0.5 and 7 years of age and were collected at the same farm. However, some of the animals originated from another farm, previously bought by the owner.

The coagulated blood samples arrived at our laboratory within 24 hours after sampling. The samples were centrifuged at 1860 × g for 10 min, to separate the serum. The serum was then stored at minus 20 °C until we analysed it with ELISA.

1.1.2 Laboratory analysis – ELISA

For the serological investigations, ID Screen® Hepatitis E Indirect Multi-species ELISA kit was used. The method was validated on swine serum samples as a competitive ELISA test, but it also enables the testing of serum and juice samples of different animal species. The plate is coated with genotype 3 antigens, the genotype most commonly affecting swine and other animal species, as well as humans in Europe. The kit applies a bi-well method, allowing each sample to be tested twice; once in a coated well (even columns) and once in a non-coated well (odd columns). The results will then be calculated according to the corrected sample OD (optical density): OD = even well OD – odd well OD.

The dilution of the serum samples was done according to the manufacturer's recommendations.

1.2 RESULTS

1.2.1 Serum samples

The swine results are listed in table 1 and 2. Of the 473 samples in total, more than 50 % of the samples were positive to IgG. All of the regular farms were positive (100 %), and all but 1 of the imported-fattener farms was positive (75 %). In total 9 of the samples were measured to be doubtful.

Table 1. Total domestic swine serum ELISA results, and the number of farms participating in this study.

Swine total samples	Total	Positive	% Positive	Negative	Doubtful
Serum	473	240	51 %	227	9
Regular farms	10	10	100 %	0	0
Imported fattener farms	4	3	75 %	1	0

In the younger piglets, aged 3 weeks, 6-10 weeks and 12 weeks, the presence of anti-HEV IgG is decreasing from 20 % to 7 % to 0 % respectively. Piglets aged 18-20 weeks, 24 weeks, 1st pregnancy sows and older sows had relatively high levels of anti-HEV IgG, 61 %, 78 %, 68 % and 60 % respectively. The imported-fattener piglets had a seropositive level of 16 % at the age of approximately 70 days.

Table 2. Domestic swine serum ELISA results according to the age groups of the swine tested.

Swine age groups	Total	Positive	% Positive	Negative	Doubtful
Older sow	174	104	60 %	68	2
1st pregnancy sow	22	15	68 %	7	0
3 weeks	10	2	20 %	7	1
6-10 weeks from regular farms	30	2	7 %	28	0
10 weeks from imported fattener farms	86	14	16 %	70	2
12 weeks	10	0	0 %	10	0
16 weeks	10	10	100 %	0	0
18-20 weeks	51	31	61 %	19	1

The rabbit serum ELISA results are listed in table 3. Of the 230 samples from rabbits, 11 % were positive. All the positive farms come from the same integration (Integration P) of farms, i.e. 1 of the integrations was positive to anti-HEV IgG. However, from 5 of the sampled farms the samples arrived rotten (3 week's storage of whole blood at 4°C before submitting to laboratory). Some of the samples arriving rotten were positive, so we know the farms of origin to be positive to HEV infection, but the negative result in case of these samples is not reliable.

Table 3. Rabbit serum ELISA results.

Rabbit samples	Total	Positive	% Positive	Negative
Samples	230	25	11 %	205
Farms	11	5	45 %	6

The goat serum ELISA results are listed in table 4. In total 333 samples were collected from goats in Hungary. All of the 333 samples collected were negative to anti-HEV IgG antibodies.

Table 4. The goat serum ELISA results.

Goat samples	Total	Positive	Negative
Samples farm	333	0	333

2. THE TIME COURSE OF INFECTION IN A SWINE HERD FOLLOWED BY SEROLOGICAL AND PCR INVESTIGATION

Based on the results of the first stage, the aim of the 2nd stage of the study was to follow the course of HEV infection at Hungarian pig farm in order to figure out, on one hand the length of the virus shedding, on the other hand whether the finishing pigs are infected at the time when they depart for slaughter, therefore estimating mean potential food hygiene threat to the consumer. The investigation was conducted by serological test (ELISA) from serum samples of different age groups in the herd and real-time RT-PCR (qRT-PCR) from faeces samples. The goal of the serological investigation was to determine, by the appearance of antibodies, at what stage of pig production the infection happens. The collection of faeces samples began in the age group in which the infection was most likely to have occurred and continued until around the time of slaughter. The investigation was done with the consent of the owner and with the help of the practicing veterinarian.

2.1 MATERIALS AND METHODS

2.1.1 Serum samples

Altogether 100 serum samples were collected from the farm in different age groups. The investigated age groups were the following: 3 weeks old piglets or weaners (10 samples), 10 weeks old piglets or growers (10 samples), 12 weeks old (35-40 kg) growers (10 samples), 16 weeks old (60 kg) fatteners (10 samples), 20 weeks old (100-105 kg) fatteners (10 samples), first-time-pregnant sows (50 days old pregnancy, 10 samples), pregnant sows (20 samples), 24 weeks old, slaughtered pigs (from slaughterhouse, 20 samples). The coagulated blood samples arrived to our laboratory within 24 hours after sample taking and were centrifuged at 1860×g for 10 minutes. The serum was separated and was kept at a -20 °C until they were analysed in the ELISA test.

2.1.1.1 *Serological investigations*

The ELISA test was performed using the ID Screen® Hepatitis E Indirect Multi-species ELISA kit as described in 1.1.3.

2.1.2 Faeces samples

Based on the results of the serological tests, the collection of the faeces samples began after the piglets were moved to the nursery (about 35 kg weight, 12 weeks old piglets). By the number of pigs in the production round (cca. 800 animals grouped in about 30 cages) we chose 6 groups

of pigs for the follow-up: group no.1 was raised to become breeding animals, groups no.2-6 were fattening pigs. In each of the groups about 30 animals were grouped, therefore the sampling method was to take about 20 grams of 4 randomly chosen, possibly fresh faeces from the floor of the cages in each cage at every sampling date. The 4 samples per cage were handled as separate samples altogether characterizing the virus shedding status of the entire group.

2.1.2.1 *Sampling dates*

The first sampling was conducted a day after the piglets were moved from the nursery into the barn of the first fattening stage. For 3 weeks 2 samplings (on Tuesday and Thursday) each week, after that one sampling (on Wednesday) per week was performed. Until the end of the investigation, samples were collected at 19 sampling dates, of which the last two (18th and 19th), since group 2-6 animals had been slaughtered, was done only in group no. 1 Altogether the sample collection lasted for 13 weeks (91 days). The sampling schedule and the technological steps are summarized in *table 5*.

Table 5. Sampling schedule, technological steps at the investigated farm.

week of age	**study day**	**sampling**	**note / technological step**
week 12	*Day 0*	-	move from nursery to 1st stage of fattening
	Day 1	sample 1	Fattening stage 1. feed change: 2 weeks after move from nursery
	Day 6	sample 2	
week 13	Day 8	sample 3	
	Day 13	sample 4	
week 14	Day 15	sample 5	
	Day 20	sample 6	
week 15	Day 22	sample 7	
	Day 27	sample 8	
week 16	Day 29	sample 9	
	Day 34	sample 10	
week 17	Day 36	sample 11	
week 18	Day 42	sample 12	
week 19	Day 49	sample 13	
week 20	*Day 50*	-	move to 2nd stage of fattening
	Day 56	sample 14	Fattening stage 2.
week 21	Day 63	sample 15	
week 22	Day 70	sample 16	
week 23	Day 77	sample 17	
week 24	*Day 78*	-	move to the slaughterhouse
	Day 84	sample 18	sampling only in group 1.
week 25	Day 91	sample 19	

2.1.2.2 *Handling of samples*

The samples arrived at our laboratory within 3 hours after sample taking, and were chilled during transportation. About 1 gram of faeces from each sample was diluted in 10 ml of PBS and homogenized using vortex. The diluted samples were centrifuged at 1860×g for 10 minutes and the supernatant was used for RNA extraction. The remaining faeces samples, both diluted and original, as well as the supernatant separated after centrifugation were kept at a temperature -70 °C for later use.

2.1.2.3 *RNA extraction and real-time RT-PCR*

For the RNA extraction the QIAmp Viral RNA mini kit (Qiagen, Germany) was used by the manufacturer's instructions.

The extracted viral RNA was further used in a single tube real-time RT-PCR by using Verso 1-step qRT-PCR kit (Qiagen, Hilden, Germany), where reverse transcription, which produce cDNA, and real-time PCR reaction were combined in the same reaction mixture. In a 1-step approach to real-time RT-PCR, both the reverse transcription (RT) step and real-time polymerase chain reaction (qPCR) steps are performed in a single tube. Adopting a 1-step approach is ideal when no cDNA storage is necessary for subsequent experiments. The approach minimizes the risk of contamination and improves reproducibility by reducing sample handling.

Amplification of the RNA was performed in a total reaction mixture volume of 25 µl, which contained reagents listed in table 6.

Reaction mixtures with verified positive samples served as positive controls, whereas sterile nuclease-free water was used instead of RNA in the negative controls.

Table 6. Reaction mixture applied in the qRT-PCR reactions.

Reagent	Volume (µL)	Final concentration
Water, nuclease free	5.5	
1Step qPCR ROX Mix (2X)	12.5	1×
RT Enhancer	1.25	
Verso Enzyme Mix	0.25	
Forward primer (10 µM)	1	400 nM
Reverse primer (10 µM)	1	400 nM
Probe (10 µM)	1	100-250 nM
Template RNA	2.5	1 pg-100 ng

Hepatitis E virus specific oligonucleotide primers and dual-labelled probe (table 7) were used for the amplification, in which the specific primers anneal only to defined sequences of particular mRNA and can be used to synthesize cDNA. The PCR target approximately position 5293 to 5362 in the SwX07-E1 sequence (accession no. EU360977; Xia et al., 2008).

Table 7. Primer and probe sequences (Jothikumar et al. 2006).

Primer/Probe		**Sequence (5' – 3')**
Forward primer	HEVORF3-S	GGTGGTTTCTGGGGTGAC
Reverse primer	HEVORF3-AS	AGGGGTTGGTTGGATGAA
Probe	HEVORFprobe	C5-TGATTCTCAGCCCTTCGC-BHQ2

The qRT-PCR reactions were performed at the PCR Laboratory of the Food Hygiene Department applying the SLAN real-time PCR machine following the reaction protocol summarized in table 8.

Table 8. Real-time RT-PCR protocol.

Step	**^{0}C**	**Time**
cDNA synthesis (RT)	50	15 min
Thermo-Start Activation	95	15 min
Denaturation	*95*	*15 sec*
Annealing-Extension	*53*	*60 sec*

The amplification cycle (denaturation and annealing-extensions steps) were repeated 45 times. Detection of fluorescence emission was performed at the end of the annealing-extension step.

The qRT-PCR reaction was used to determine the positivity of the samples, virus (RNA) content of the samples was expressed and compared by only the Ct values.

2.2 RESULTS

As statistical analysis the positive % was calculated by the number of positive samples in correlation with the number of collected samples. In the comparison of virus shedding, the mean Ct values of the groups per samplings were taken into consideration.

2.2.1 Serological investigations

The results of the ELISA tests performed using the serum samples are summarized in table 9.

Table 9. Results of the ELISA tests of pig rera samples collected at the investigated farm.

Age groups	No. of samples	No. of positives	% of positives	No. of negatives	No. of suspect samples
3 weeks	10	2	20 %	7	1
10 weeks	10	1	10 %	9	0
12 weeks (35-40 kg)	10	0	0 %	10	0
16 weeks (60 kg)	10	10	100 %	0	0
20 weeks (100 kg)	10	9	90 %	0	1
24 weeks (120 kg, slaughtered)	20	20	100 %	0	0
50-day-old-pregnant sow	10	8	80 %	2	0
pregnant sow	20	17	85 %	3	0

As the table above shows in the age groups of 3-weeks-old, 10-weeks-old and 12-weeks-old piglets the presence of presumably maternal IgG antibodies was detected, decreasing by age from 20 % to 0. In the 16-week-old age group the presence of antibodies suddenly increased to 100 % and remained high till slaughter, suggesting that the infection has occurred between 12 to 14 weeks of age. Anti-HEV antibodies were detected in 80 % of the samples taken from first time pregnant sows and 85 % of older sows.

2.2.2 qRT-PCR

The results of the qRT-PCR tests performed using the faeces samples are summarized in table 10.

Based on the results of the ELISA test, the start of sampling for qRT-PCR investigation was scheduled a day after the piglets were moved from nursery to the first fattening stage. The investigated groups were followed for about 3 months, from 12-week-old age to 23- or 25-week-old age. Group 1 contained sows for breeding while group 2-6 were fattening pigs which were sent to slaughter at their 24th week of age, therefore they were not included in the last two samplings.

A day after the animals were moved (Study Day 1) from nursery to the 1st fattening stage we detected a 79 % positivity of the samples. At the next two samplings (Day 6 and Day 8) only 2 and 4 samples were found positive, respectively. At the next two saplings, a marked increase in positivity was observed with 16 and 23 out of 24 samples being positive, while between Day 20 and 49 (14-19-week-old pigs) all samples were positive.

Table 10. Results of the qRT-PCR tests performed on the faeces samples.

Sampling schedule			**qRT-PCR results**		**Positive samples in groups (Gr)**					
week of age	*study day*	*sampling*	*positives*	*positive %*	*Gr 1*	*Gr 2*	*Gr 3*	*Gr 4*	*Gr 5*	*Gr 6*
week 12	Day 0	move from nursery to 1st stage of fattening								
	Day 1	sample 1	19/24	79	1	4	4	3	3	4
	Day 6	sample 2	2/24	8	0	1	1	0	0	0
week 13	Day 8	sample 3	4/24	17	0	1	3	0	0	0
	Day 13	sample 4	16/24	67	0	3	4	4	1	4
week 14	Day 15	sample 5	23/24	96	3	4	4	4	4	4
	Day 20	sample 6	24/24	100	4	4	4	4	4	4
week 15	Day 22	sample 7	24/24	100	4	4	4	4	4	4
	Day 27	sample 8	24/24	100	4	4	4	4	4	4
week 16	Day 29	sample 9	24/24	100	4	4	4	4	4	4
	Day 34	sample 10	24/24	100	4	4	4	4	4	4
week 17	Day 36	sample 11	24/24	100	4	4	4	4	4	4
week 18	Day 42	sample 12	23/23	100	4	4	4	4	4	4
week 19	Day 49	sample 13	24/24	100	4	4	4	4	4	4
week 20	Day 50	move to 2nd stage of fattening								
	Day 56	sample 14	17/24	71	4	1	3	1	4	4
week 21	Day 63	sample 15	12/24	50	4	0	3	0	2	3
week 22	Day 70	sample 16	9/24	38	4	1	2	0	1	1
week 23	Day 77	sample 17	6/24	25	4	0	1	0	0	1
week 24	Day 78	move to the slaughterhouse								
	Day 84	sample 18	4/4	100	4	no sampling				
week 25	Day 91	sample 19	2/4	50	2	no sampling				

Although an exact virus titre (ge/ml) was not determined, the changes of the mean Ct values of which lower number mean higher viral RNA concentration suggest the changes of the virus load in the faeces. Based on these data a diagram of the results of 3 representative groups (Figure 1) shows a fluctuation of the virus concentration in the faeces samples, indicating that the virus slowly spread among the animals time to time infecting new susceptible pigs, and between sample 1 and sample 17 there were always some animals in the cages shedding high number of virus particles.

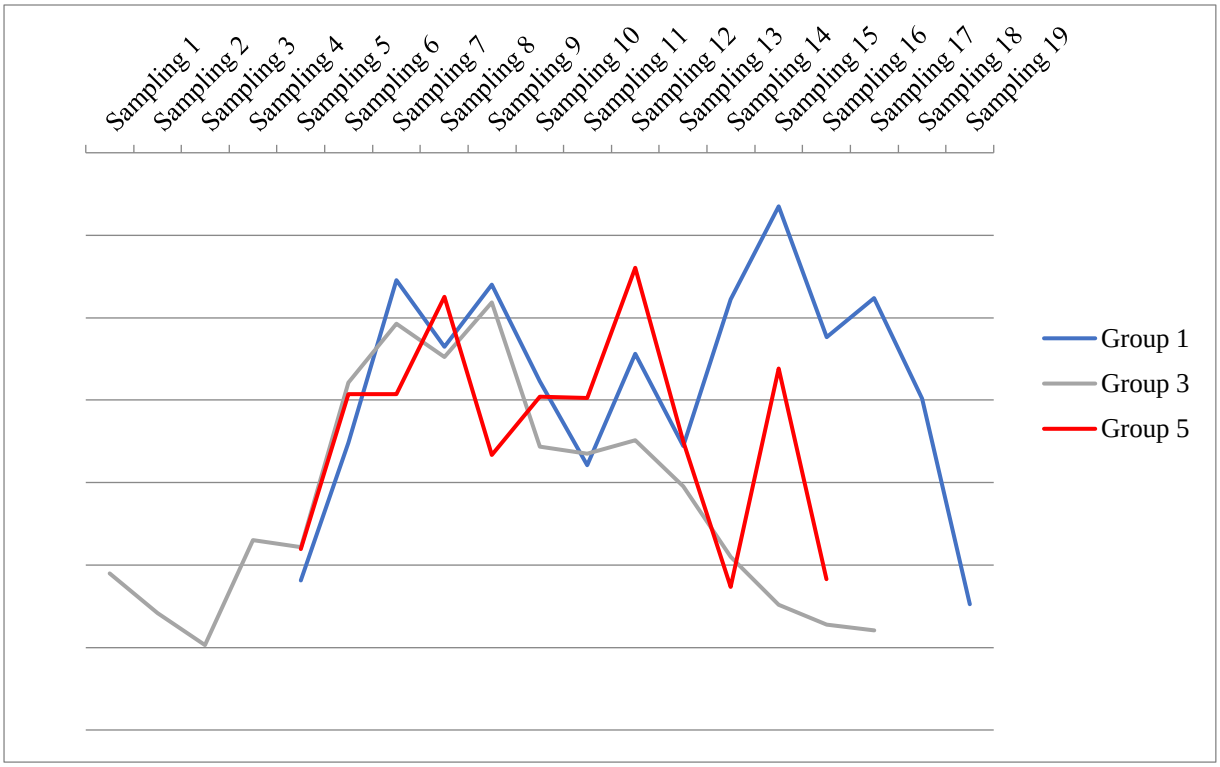

Figure 1. Changes of the virus concentration in the faeces samples of 3 representative groups of investigated animals.

Generally, virus shedding was detected to decrease from Day 56 (14th sampling date, 20-week-old age) after movement of animals to the 2nd stage of fattening. However, at the time of slaughter, animals in group 1 (sows for breeding) were still shedding the virus in their faeces; all 4 samples were positive. A week later the virus shedding started to decrease in this group as well.

3. THE FOOD SAFETY AND OCCUPATIONAL HEALTH SIGNIFICANCE OF HEV-INFECTION IN SWINE

After observing a slow spread of the infection during the fattening stage of production, we decided to continue the study with the more detailed investigation of food safety significance of HEV. In the project application we planned the following activities for the 2nd year:

- Collection of meat and meat product samples at slaughterhouses, meat-processing plants and local markets.

- Virus quantification by qRT-PCR in meat and meat product samples.

- Determination of the infectivity of the viruses detected in meat samples using virus isolation, viability dyes combined with qRT-PCR and RNase-treatment combined with qRT-PCR, comparison of the methods.

- Genetic analysis of the detected viruses.

3.1 MATERIALS AND METHODS

3.1.1 Serum samples

Serum samples of 80 animals originated from 4 farms were collected at slaughter. The handling and procession of samples was the same as described in 1.1.

3.1.2 Juice samples

Fluid drained from a muscle tissue or sample was used as an alternative to serum for the detection of specific anti-*Salmonella* antibodies by ELISA (Nielsen et al., 1998). Based on this, several investigations were done on the application of the method for the detection of anti-HEV antibodies in juice separated from meat and liver of swine. The detection of antibodies in juice requires lower dilution than in serum, therefore higher volume of the test material is needed compared to serum. The results of juice samples are statistically not concordant with the results of the serum samples, however in case of necropsied or slaughtered animals, and/or in lack of blood (serum), it is a suitable test material for serological investigation (Nielsen et al., 1998, Casas et al., 2011, Wachek et al., 2012).

Simultaneously with the serum samples, swine liver and meat samples were collected from animals originated from 4 farms (20 animals from each). From the altogether 80 animals sampled at the slaughterhouse, 65 liver juice samples and 37 meat juice samples could be collected.

After arrival to the laboratory liver and meat samples were frozen at -70 °C. During thawing, the fluid (so-called "juice") drained from the pieces of liver and meat were collected by micropipette and were stored in microcentrifuge tubes at -20 °C until the ELISA investigation. Depending on the dryness of the samples, more or less juice could be separated. We investigated only the juice samples where at least 200 µl volume was available.

3.1.3 Serological investigations

The ELISA test was performed using the ID Screen® Hepatitis E Indirect Multi-species ELISA kit as described in 1.1.3.

In case of juice samples, the applied dilution is partially based on our own experimental results, which were confirmed by data from the literature or by the manual of another ELISA kit, which suggest the dilution of the juice to be l log lower than the dilution of the serum (Nielsen et al., 1998; Casas et al., 2011).

3.2 RESULTS

As statistical analysis the positive % was calculated by the number of positive samples in correlation with the number of collected samples. In the comparison of virus shedding, the mean Ct values of the groups per samplings were taken into consideration.

3.2.1 ELISA tests

The result of the serum samples of 80 pigs are summarized in table 11.

Table 11. ELISA results of juice samples from slaughtered pigs seroconverted until the time of slaughter. S: serum; LJ: liver juice; MJ: meat juice.

Swine age groups	**Total**	**Positive**	**% Positive**	**Negative**	**Doubtful**
24 weeks (slaughterhouse)	80	62	78 %	17	1

Results of ELISA tests performed on juice samples are summarized in tables 12–14.

Table 12. ELISA results of juice samples from slaughtered pigs seroconverted until the time of slaughter. S: serum; LJ: liver juice; MJ: meat juice.

Animals with ELISA-positive serum	**In total: 62 out of 80 swine**	**77.5 %**
S positive, LJ & MJ not available	5	6.25 %
S positive, LJ not available, MJ negative	7	8.75 %
S positive, LJ not available, MJ doubtful	6	7.5 %
S positive, LJ not available, MJ positive	18	22.5 %

S positive, LJ positive, MJ not available	3	3.75 %
S positive, LJ positive, MJ negative	4	5.0 %
All 3 samples (S, LJ, MJ) positive	19	23.75 %

Table 13. ELISA results of juice samples from slaughtered pigs with no seroconversion until the time of slaughter. S: serum; LJ: liver juice; MJ: meat juice.

Animals with ELISA-negative serum	**In total: 11 out of 80 swine**	**13.75 %**
S negative, LJ & MJ not available	3	3.75 %
S negative, LJ negative, MJ not available	1	1.25 %
S negative, LJ not available, MJ negative	5	6.25 %
All 3 samples (S, LJ, MJ) negative	1	1.25 %
S doubtful, LJ not available, MJ negative	1	1.25 %

Table 14. ELISA results of juice samples from slaughtered pigs showing incongruent results in samples. S: serum; LJ: liver juice; MJ: meat juice.

Animals with incongruent results	**In total: 7 out of 80 swine**	**8.75 %**
S negative, LJ positive, MJ doubtful	1	1.25 %
S negative, LJ positive, MJ negative	3	3.75 %
S negative, LJ positive, MJ positive	2	2.5 %
S negative, LJ not available, MJ doubtful	1	1.25 %

3.3 Attempts on the isolation of HEV in cell culture

Although the presence of a virus in an animal population can be surveyed by the detection of antiviral antibodies, the presence of a virus in a sample can be proven by the detection of the viral nucleic acid, the infectivity of the detected virus can be investigated only either by experimental infection of laboratory animals or the isolation of the virus in cell culture. Because of animal welfare reasons and costs, the in vitro virus propagation is feasible.

In case of HEV, although several publications (Okamoto, 2013) describe the methods of virus isolation that promise success, there only a few laboratories which could manage the propagation of the virus in cell culture without genetic engineering, for which our laboratory has no licence. One of the original goals of the project was to estimate the food safety significance of HEV infection in animals by the investigation of the infectivity of the viruses

detected in meat and meat products, we attempted the isolation of HEV following the suggestions of publications available.

For the virus isolation several cell lines were purchased, among them human hepatocellular carcinoma cells (HepG2 and PLC/PRF/5), A549 from human lung cancer, as well as porcine and rabbit kidney (PK-15 and RK-13, respectively). In all attempts we used samples in which we detected the highest concentration of viruses by real-time RT-PCR.

Several protocols were tested, such as suspension method (mixing the sample with cell suspension providing young, dividing cells for the virus replication), adsorption method (inoculation of the sample to 80% confluent monolayer cell culture) in different lengths of incubation time, at different temperatures, and even repeated inoculation of the sample to the same cell culture. Since HEV is known to not cause obvious cytopathic effects, the virus replication was followed by sampling the medium covering the cell culture in different time periods and testing it by real-time RT-PCR to detect the expected increase of the virus nucleic acid.

Several publications suggest, that for the successful infection of a cell culture high concentration of infective virion is needed (Okamoto, 2013). As the RT-PCR detects the nucleic acid of both infective and inactivated virus particles, despite the high concentration of viral RNA in the sample it can happen, that the titer of the infective virus particles is not enough to successfully infect the cells culture. It is already known about HEV that despite being quite resistant to heat, the storage of the samples at -70-80 °C inactivates the virus in relatively short period of time (Bradley, 1990). Therefore, the virus isolation may be successful only if fresh sample containing high concentration of infective virions is inoculated to the susceptible cell cultures.

It seems our practice of handling the samples did not support our goal to isolate the virus in cell culture. After several failed attempts being afraid of wasting the time and money, although it meant we had to skip the investigation of meat and meat products that was originally planned, we decided to drop the virus isolation part of the study and instead focus on the epidemiology of HEV, i.e. by the investigation on the incidence of HEV in populations of wild reservoirs (chapter 5).

4. THE FOOD SAFETY AND OCCUPATIONAL HEALTH SIGNIFICANCE OF HEV-INFECTION IN RABBIT

The detailed investigation of HEV infection in rabbits was based on the survey on the presence of HEV in rabbit farms (see chapter 1.). The aim of the study was to survey the presence and estimate the food safety and occupational health risks of HEV infection in farmed rabbit and hare populations of Hungary.

4.1 MATERIALS AND METHODS

4.1.1 Collection and processing of samples

Based on the results of the serological survey described in chapter 1.2, farms P/1 and P/2 were chosen for further investigations. Focusing on the food safety and occupational health significance of HEV infection in rabbits, we targeted the age groups in which we suspected the occurrence of the infection, i.e. slightly before the weaning (6-8 weeks of age) and after the slaughtering age (11 weeks). Therefore, the samples were taken from 30 animals approx. 4–14 weeks (0.8–2.85 kg) sent for pathological investigations to the University of Veterinary Medicine Department of Pathology and from 100 animals sent for slaughter.

As serum samples were not available from dead animals, based on the findings of previous investigations (Nielsen et al., 1998, Casas et al., 2011, Wachek et al., 2012) juice samples were collected for serological investigations. Altogether 88 liver juices samples (27 of necropsied, 61 of slaughtered rabbits) and 39 meat juice (7 of necropsied, 32 of slaughtered animals) were investigated. The collection of juice samples was the same as described in chapter 3.1.2.

On the detection of HEV RNA by real-time RT-PCR liver, faeces and meat samples were collected either from the necropsied or the slaughtered animals. The samples were processed as follows: approximately 1 g of tissue/faeces was homogenized using sterile quartz sand in ceramic mortar and was diluted with 3 mL of sterile phosphate buffered saline (PBS). From the homogenized samples 1.5 mL was collected in microcentrifuge tubes and were frozen at -70 °C for the further destruction of the cells. After thawing, the samples were centrifuged for 10 minutes at 1500×g speed. RNA was extracted from the supernatant using QIAamp Viral RNA Mini Kit (Qiagen, cat. no.: 52906) by the manufacturer's instructions. Purified viral RNA was collected in microcentrifuge tubes and stored at -70 °C until the RT-PCR investigations.

4.1.2 Serological investigations of juice samples

ELISA was conducted applying ID Screen® Hepatitis E Indirect Multi-species ELISA kit (ID-Vet; cat. no.: HEVB-MS-4P) with the modification related to juice samples as described in chapter 3.1.3.

4.1.3 Detection and analysis of viral nucleic acid

Single-tube one-step qRT-PCR was performed using Verso 1-step qRT-PCR kit (Thermo Fischer Scientific Inc. cat no.: AB4101A) by the manufacturer's instructions regarding both the master mix and the reaction protocol applied, in 25 μL volume. The reactions were performed in Qiagen Rotor-GeneQ 5plex HRM Platform instrument at the virology laboratory of Department of Microbiology and Infectious Disases of University of Veterinary Medicine, Budapest. Primers and probe were described previously by Jothikumar et al. (2006), the amplified 69 bp long product fits between the 5293–5362 nt positions of SwX07-E1 strain (GeneBank accession number EU360977; Xia et al., 2008). Reaction mixture without template RNA was used as no-template negative control, while RNA extracted from previously tested and sequenced sample of swine origin was used as positive control.

For genome equivalence investigations only the positive samples were used. The reaction mixture, primers and probes were the same as described above. Reaction mixture without template RNA was used as no-template negative control. Standard curve was determined by the 10-fold dilution (10-2–10-6) of the PCR product of a sequenced sample containing 2.06×1011 copies per reaction.

In statistical analysis the ratio of positive samples among the investigated samples were considered. Samples were accepted as "positive" if fluorescence was detected before the 40th cycle out of 45, and the curve indicated the specificity of the product. In the determination of genome-equivalence the detected copy number was calculated by QRex v1.0.0 software (Qiagen) based on the standard curve.

For sequencing, primer set described by Izopet et al. (2012), which is a slight, rabbit HEV-specified modification of the primer set designed by van der Poel et al. (2001) for the ORF2 (capsid region) was used in a single-tube one-step RT-PCR applying Qiagen OneStep RT-PCR Kit (Qiagen, cat. no.: 210212), according to the manufacturer's instructions and the primer characters. However these primers amplify an only 189 bp genome fragment, as the same fragment was targeted in our previous investigations conducted between 2004 and 2010, and

the genome region was described as suitable for genotyping, as well as a number of sequences in the GenBank are available, this region was considered useful in phylogenetic investigations (Lu et al., 2006; Forgách et al., 2010b). One serum sample from P/2 farm, 5 liver samples of P/1 farm from the pathological investigations and 42 liver samples of P/1 farm from the slaughterhouse were chosen for sequence analysis.

The reactions were performed in the Applied Biosystems™ 2720 Thermal Cycler instrument, the temperature and time conditions were as described by Izopet et al. (2012).

Specific amplification products were excised from the agarose gel, and DNA was extracted using QiAQuick Gel Extraction Kit (Qiagen GmbH, cat. no.: 28206), according to the manufacturer's instructions. The nucleotide sequences were determined in fluorescence-based direct sequencing reactions, in two directions, using the previously described primers at the Molecular Taxonomy Laboratory of the Hungarian Natural History Museum, Budapest. The sequences were identified by the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) search against the GeneBank database. The sequence corrections, multiple alignments and the phylogenetic analysis on the 148 bp long sequences of the ORF2 regions were performed applying Mega X software (Kumar et al., 2018).

4.2 RESULTS

4.2.1 Serological investigations

Anti-HEV antibodies were detected in 2 and 5 animals out of 27 (7.4%) and 61 (6.8%) necropsied and slaughtered animals, respectively (table 15).

Table 15. ELISA results of juice samples from necropsied and slaughtered rabbits.

Origin	Sample	No. of samples	Positive (%)	Negative (%)
necropsied animals	Liver juice	27	2 (7,4 %)	25 (92,6 %)
	Meat juice	7	0 (0 %)	7 (100 %)
slaughtered animals	Liver juice	61	5 (8 %)	56 (92 %)
	Meat juice	32	1 (3 %)	31 (97 %)

4.2.2 Nucleic acid detection

HEV RNA was detected in both the liver (bile) and faeces samples of 7 rabbits out of the 30 necropsied animals. In case of 2 rabbits, the meat samples were positive as well. Among the slaughtered animals, by the results of qRT-PCR performed on the liver samples, 42 rabbits were found to have active HEV infection at the time of slaughter. Of them, 32 animals shed the virus

in their faeces as well, while viral RNA was detected in the faeces of one animal of which the liver was found to be negative. HEV nucleic acid was detected in the meat of 8 rabbits with positive liver samples. The results of the qRT-PCR investigations of necropsied and slaughtered animals are summarized in table 16.

By the genome-equivalence investigations, 2.5 log difference was detected in the number of copies comparing the liver (more) and the meat (less) in both the necropsied and slaughtered animals.

Table 16. RT-PCR results of samples from necropsied and slaughtered rabbits.

Origin	Sample	No. of samples	Positive (%)	Negative (%)
necropsied animals	Liver (bile)	30	7 (23 %)	23 (77 %)
	Faeces	30	7 (23 %)	23 (77 %)
	Meat	30	2 (7 %)	28 (93 %)
slaughtered animals	Liver (bile)	100	42 (42%)	58 (58%)
	Faeces	88	32 (36%)	56 (64%)
	Meat	39	8 (20,5%)	31 (79,5%)

4.2.3 Sequence analysis

Of the positive samples, in 48 (1 serum, 5 liver from necropsied and 42 liver from slaughtered animals) a 197 bp long fragment of the ORF2 (capsid) region was sequenced and without the primer sequences, the 148 bp long genome fragment was analysed. We have identified 14 different sequence variants, and detected 1–2 point mutations in the different strains comparing to the chosen reference.

We found all 14 virus variants to be rabbit-specific HEV. In the phylogenetic tree the variants detected in Hungary form a distinct branch of the rabbit-specific HEV sequences, showing the highest similarity to a strain detected in the Netherlands in 2013 (Figure 2).

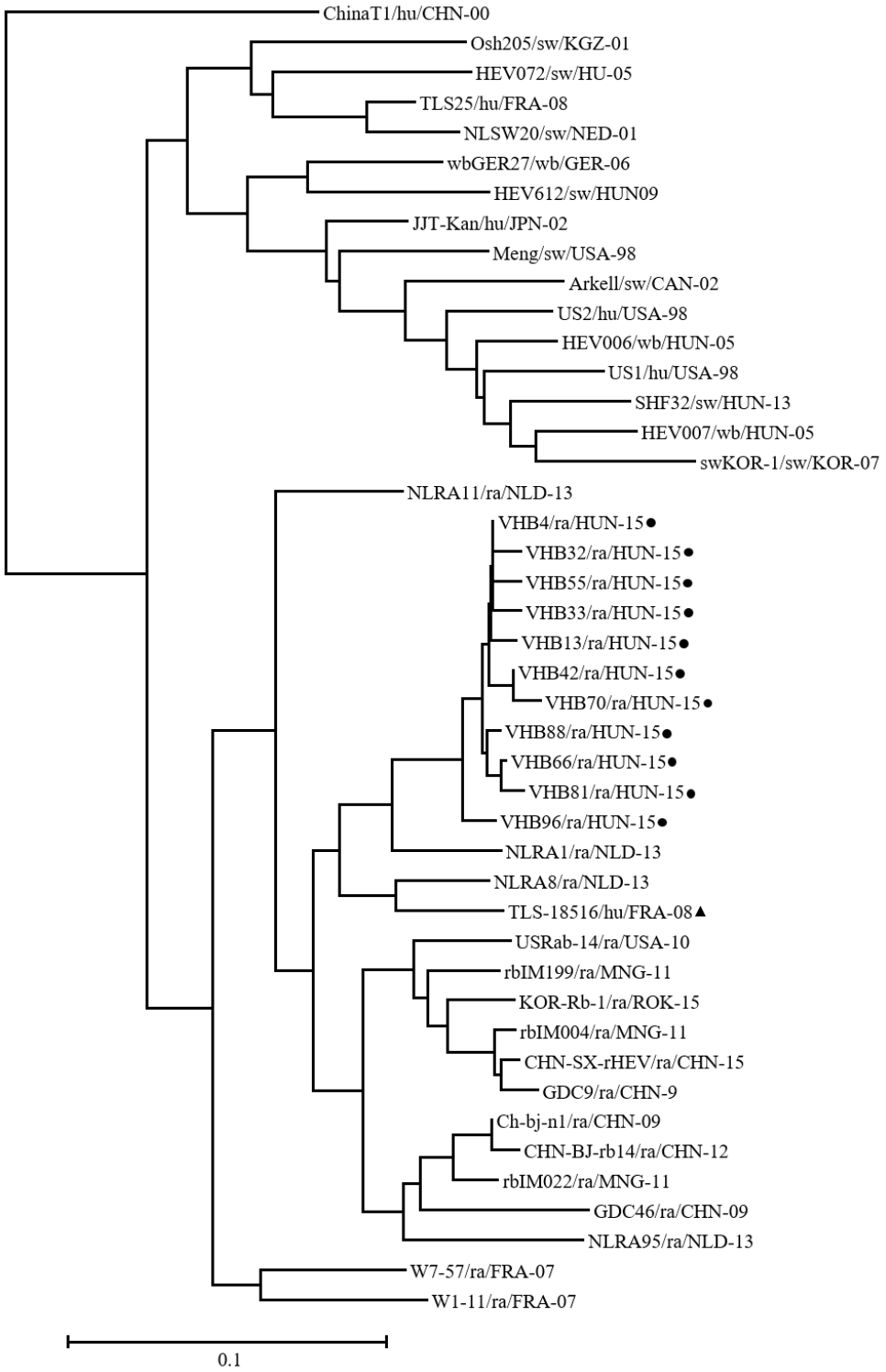

Figure 2. Phylogenetic analysis of the rabbit-origin sequences (ORF2). Naming of sequences: code of strain/host (hu: human; sw: swine; wb: wild boar; ra: rabbit)/place of detection (country's 3-letter code)-year of detection (2 digits). Black dot indicates the viruses detected in this study. Black triangle indicates the virus sequence of human origin showing high similarity to rabbit-origin virus sequences (Izopet et al., 2012).

5. THE MAINTENANCE OF HEV INFECTION IN RESERVOIR ANIMALS LIVING IN THE WILD

As it is well known by the recent spread of African swine fever (ASF) in Europe, despite efforts on the eradication of the infection in domestic swine, viruses of suids can be maintained by wild boars. The prevalence of the infection in wild boars on one hand proves that a certain virus is endemic in a geographic area, on the other hand means a risk that in certain epidemiological situations the infection spreads from wild boar to domestic swine (Chenais et al., 2018).

However, nowadays HEV infection of swine is not taken as seriously as ASF, we cannot leave the wild boars out of consideration when we investigate the epidemiology of HEV. Wild boars are not only reservoirs of HEV maintaining the infection, but being food-producing animals, can transmit the disease to human by contact with either their faeces or raw meat.

The aims of the investigation of wild boar samples collected in Hungary, Austria and Serbia were to survey the incidence of HEV infection in wild boar populations in endemic countries, to recognise the epidemiological connection spanning borders of countries and also to detect new virus genotypes entering Hungary.

During the study we also had the chance to test hare samples kindly provided by the Veterinary Diagnostic Directorate of the National Food Chain Safety Office. The heart blood samples were originally collected for testing the hunted animals before export as described by the regulation (Reg. (EC) 854/2004) “laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption”. The 44 samples were collected at different dates from 3 different hunting areas; Dombiratos, Gyomaendrőd and Szarvas. The aim of the tests was to survey whether the HEV infection spreads among populations of wild animals, as well as to recognise, whether hare serves as potential reservoir of HEV.

5.1 MATERIALS AND METHODS

The wild boar samples were originated from Hungary, Austria and Serbia.

In Hungary 382 blood samples from wild boars living in the wild and 35 blood samples from farmed wild boar farm located in Érsekcsanád (Bács-Kiskun county) were collected. Apart from blood, 17 faecal samples of living animals and 3 liver, muscle and intestines (faeces) samples from dead wild boar piglets were collected in the previously mentioned farm. From Austria National Park Donau-Auen we received 48 serum samples, while from Novi Sad, Subotica and Gornji Milanovi regions of Serbia 63 frozen wild boar liver tissue samples were sent.

Samples of wild boar were collected at a breeding farm located at the southern part of Hungary and designated to produce wild boar to be released into hunting areas. Blood samples were taken from 7-10 months old piglets (23 samples) and older sows (12 samples).

The blood, faeces and liver samples were processed, as well as liver juice was obtained as described previously. In case of juice only samples with more than 200ul exudate were investigated. The blood and juice samples were tested applying the ID Screen® Hepatitis E Indirect Multi-species ELISA kit (ID-Vet; cat. no.: HEVB-MS-4P) as described in the previous chapters. From blood, tissue and faeces samples viral RNA was extracted the same way as previously explained. The detection of RNA, sequencing and genome analysis also followed the same protocols as used in the other parts of the project.

5.2 RESULTS

5.2.1 Wild boar

The results of ELISA tests and detection of viral RNA is summarized in table 17.

Table 17. Results of ELISA tests and detection of viral RNA in wild boar samples

Country of origin	Sample	No. of samples	ELISA positive no.	ELISA positive %	PCR positive no.	PCR positive %
Hungary	Wild blood	382	214	56%	12	3%
	Farmed blood	35	35	100%	3	9%
	Farmed faeces	20			19	95%
Austria	Wild blood	48	35	73%	4	8%
Serbia	Liver juice	63	38	60%	5	8%

5.2.2 Hare

All 44 hare samples collected were negative to HEV.

5.2.3 Sequence analysis

Out of all positive samples, the sequence of the RT-PCR products 10 of the wild and 10 of the farmed wild boars originated from Hungary, 3 samples collected in Austrian and 3 from Serbia were analysed. We found all detected viruses to belong the 3rd genotype of Orthohepevirus A. By the phylogenetic analysis we recognised, that the viruses detected in samples from Serbia form a distinct group, while the viruses detected in Hungary and Austria are placed mixed on the phylogenetic tree indicating the epidemiological connection between the 3 countries' wild boar populations (Figure 3).

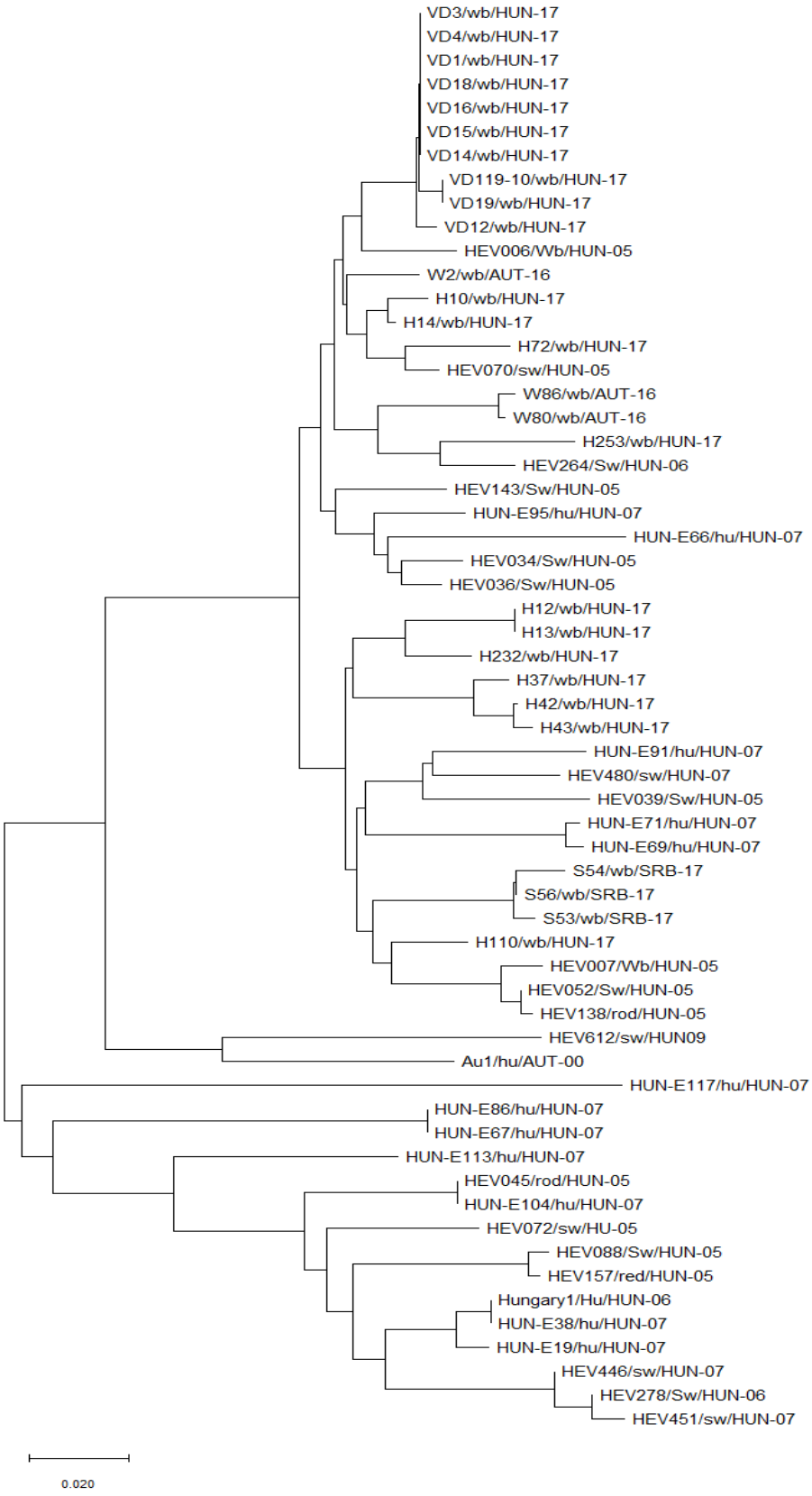

Figure 3. Phylogenetic analysis of the wild boar-origin sequences (ORF2). Naming of sequences: code of strain/host (hu: human; sw: swine; wb: wild boar)/place of detection (country's 3-letter code)-year of detection (2 digits). The viruses detected in this study were collected in 2016 (Austria) and 2017 (Hungary, Serbia).

6. CONCLUSIONS

6.1 Swine

The results of the studies suggest the following:

- HEV infection has become endemic in the investigated swine farms.
- Due to the presence of maternal antibodies the infection presumably occurs between 12 and 16 weeks of age.
- The re-grouping and the change of feed facilitates the spread of the infection among the animals.
- The infection spreads slowly, therefore at the time of slaughter in 24 weeks of age there are still virus shedding animals, which means food safety and occupational health risks.

6.2 Rabbit

Based on the findings we drew the following conclusions:

- HEV infection is present in rabbit farms located in Hungary.
- Hygiene conditions at the farm may have role In the spread and maintenance of the infection.
- The infection in rabbits occur around the 9th week of age.
- As the slaughter of rabbits happens at 11th week of age, high number of animals with active HEV infection (viraemia, virus shedding) are slaughtered, which means food safety and occupational health risks.
- The food safety and occupational health risks of the HEV infection of rabbits are supported by the results of phylogenetic investigation, i.e., that there are genetic similarities between the virus strains causing infection in rabbits and human.

6.3 Goat

Despite literature data suggesting that goats are susceptible to HEV infection, in our study we did not detect anti-HEV antibodies in the investigated animals. Although we tested high number of samples, all of them were collected at the same farm. Therefore, we can only state, that the investigated goat farm was free from HEV infection at the time of sampling, and regarding the role of the goats in the epidemiology pf HEV more investigations are needed.

6.4 Wild reservoirs (wild boar and hare)

The test performed on wild boar and hare samples, led to the conclusion of the following:

- Comparing to the previous studies between 2004 and 2010, the HEV infection has been spreading among the wild boar populations and the virus has become endemic in Hungary, Serbia and Austria, therefore wild boars can serve as the main reservoirs of HEV.
- Detecting similar incidence of HEV infection in East and West or North and South Hungary, it seems, the natural and artificial hurdles, such as rivers (Danube, Tisza) or highways cannot stop the migration of wild boars and therefore the spread of the viral diseases.
- By the phylogenetic analysis it seems, Hungary and Austria forms one epidemiological unit, while Serbia, due to the fence established at the border in order to handle the migration crisis, forms a separate epidemiological unit in case of HEV.
- HEV infection has not been detected in hare, however, due to the relatively low number of tested samples, we cannot state, hares are not susceptible to HEV infection.
- Combining the results of the study conducted in 2004 and 2010 and the present investigation, further research targeting the food producing animal species living in the wild, such as wild boar, hare, deer and roe deer is needed.

6.5 Food safety

Since the main goal of the project was to estimate the animal health and food safety significance of HEV infection of food producing animals, by the results of the studies we conclude the following:

- Due to presence of maternal antibodies, therefore infection that occurs in relatively late age (around 12^{th} weeks of age) and spreads slowly among the fattening pigs, some of the animals are slaughtered at 24^{th} weeks of age shedding the virus (i.e. with active HEV infection.
- As in rabbits the infection occurs 2 weeks before slaughter, comparing to swine higher number of animals are slaughtered with active HEV infection (viraemia, virus shedding).

- We detected viral RNA in the blood of shot wild boars that indicates viraemia (i.e. active HEV infection) at the time of hunting.
- By the analysis of RT-PCR products genetic similarities between virus strains of animal and human origin were detected.
- The results of our investigations suggest that the HEV infection of food producing animals pose a risk on the consumers and workers handling infected animals, their faces or get into contact with row meat and meat products. Although to estimate the severity of this risk further investigations are needed, our results support the need of food safety regulations regarding HEV.

6.6 Further investigations

Although the present project answered many questions concerning the food safety aspects of HEV infection in food producing animals, based on the results we figured out new ideas for further investigations.

- In vitro propagation of the virus is essential in the investigation of food safety concerns of HEV.
- Animals are slaughtered with active HEV infection – investigations are needed on the presence of infective virus particles in marketed raw meat and meat products.
- Due to the global trade of livestock, raw meat and meat products, taking the "One World – One Health" approach into consideration investigations are needed on the global epidemiology of HEV by international joint research.

7. LIST OF PUBLICATIONS RELATED TO THE STUDY

7.1 Thesis works

Sam Gallagher (2015) – Hepatitis E virus: the time course of infection in a swine herd followed by serological and PCR investigation

Helle Hagenlund (2016) – Serological investigation on the prevalence of hepatitis E virus infection in food-producing animals

Kanizsai Krisztián (2016) – Házinyulak hepatitis E vírus fertőzöttsége Magyarországon [Hepatitis E infection of domestic rabbit in Hungary]

Michelle Troye-White (under submission) – Hepatitis E infection in wild boar populations of Hungary, Austria and Serbia

7.2 Congress presentations and posters

K. Kanizsai, P. Forgách, P. Laczay, Z.L. Német, M. Rusvai, Á. Hornyák, T. Bakonyi (2017): Nyulak hepatitis E vírus fertőzöttsége Magyarországon. [Hepatitis E virus infection of rabbits in Hungary] Akadémiai beszámolók, Budapest, Magyarország

S. Gallagher, D. Ferenczy, T. Bakonyi P. Laczay, P. Forgách (2017): A hepatitis E vírus fertőzés időbeli lefolyása egy magyarországi sertéstelepen [Time course of hepatitis E virus infection in a Hungarian swine herd]. Akadémiai beszámolók, Budapest, Magyarország

P. Forgach (2017): A Hepatitis E vírus okozta fertőzés állategészségügyi és élelmiszer-biztonsági jelentősége [The animal health and food safety significance of hepatitis E virus infection]. Magyar Zoonózis Társaság Rudnai-Kemenes Nap, Budapest, Magyarország

P. Forgách, P. Laczay, H. Hagenlund, Z.L. Német, M. Rusvai, T. Bakonyi (2017): Serological Investigation on the Prevalence of Hepatitis E Virus Infection in Food-producing Animals. poster at 33th World Veterinary Congress, Incheon, Republic of Korea

P. Forgách, P. Laczay, K. Kanizsai, Z.L. Német, M. Rusvai, T. Bakonyi (2017): Food Safety and Occupational Health Significance of Hepatitis E Virus infection in Rabbit and Hare. poster at 33th World Veterinary Congress, Incheon, Republic of Korea

P. Forgách, P. Laczay, S. Gallagher, D. Ferenczy, T. Bakonyi (2017): Hepatitis E Virus: the Time Course of Infection in a Swine Herd Followed by Serological and PCR Investigation. poster at 33th World Veterinary Congress, Incheon, Republic of Korea

P. Forgách (2017): The presence and food safety significance of hepatitis E virus in food producing animals in Hungary. seminar at the Seoul National University College of Veterinary Medicine, Seoul, Republic of Korea

P. Forgách (2018): The presence and food safety significance of hepatitis E virus in food producing animals in Hungary. Presentation at the 2018 International Symposium of Research Institute of Veterinary Medicine, Chungnam National University, Daejeon, Republic of Korea

8. CLOSING WORDS

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