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1 **Detection of Hepatitis E virus in samples of animal origin collected in**
2 **Hungary**

3

4 Petra Forgách^{1*}, Norbert Nowotny², Károly Erdélyi³, Attila Boncz⁴, János Zentai⁵, György
5 Szűcs⁶, Gábor Reuter⁶, Tamás Bakonyi^{1,2}

6

7 ¹Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent
8 István University, Hungária krt. 23-25., H-1143 Budapest, Hungary

9 ²Zoonoses and Emerging Infections Group, Clinical Virology, Clinical Department of
10 Diagnostic Imaging, Infectious Diseases and Clinical Pathology, University of Veterinary
11 Medicine, Vienna, Veterinärplatz 1, A-1210 Vienna, Austria

12 ³Central Agriculture Office, Veterinary Diagnostic Directorate, Tábornok u. 2., H-1149,
13 Budapest, Hungary

14 ⁴Zala County Agricultural Office Directorate of Food Chain Safety and Animal Health,
15 Göcseji út 18., H-8900 Zalaegerszeg, Hungary

16 ⁵MAVAD-Vecsés Game Meat Processing Ltd., Lőrinci út 45. H-2220 Vecsés, Hungary

17 ⁶Regional Laboratory of Virology, Regional Institute of State Public Health Service, National
18 Reference Laboratory of Gastroenteric Viruses, Szabadság út 7., H-7623 Pécs, Hungary

19

20 *Corresponding author

21 Address: Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science,
22 Szent István University

23 Hungária krt. 23-25.

24 Budapest, Hungary

25 H-1143

26 Tel.: 003612519900

27 Fax: 003612519260

28 E-mail: Forgach.Petra@aotk.szie.hu

29

1 **Abstract**

2 Hepatitis E Virus (HEV) is an enterically transmitted human pathogen. HEV infections
3 are mainly associated with acute, self-limited, icteric hepatitis with an average mortality rate
4 of 1%. Animal reservoirs are considered to play an important role in the maintenance of the
5 virus and in the spread of HEV to humans. HEV-induced seroconversion was described in
6 several species, however clinical hepatitis in animals has not been observed to date. HEV
7 strains from animals are genetically closely related to human HEV isolates, which supports
8 the opinions on the zoonotic transmission of the virus.

9 In this expansive study the occurrence of HEV was investigated in Hungarian wild and
10 domesticated animal samples. HEV RNA was detected by reverse transcription-polymerase
11 chain reaction in liver samples of wild boars, roe deer, and deer. The investigations of
12 domestic swine samples detected HEV in 39% of the investigated Hungarian pig farms.
13 Simultaneous investigation revealed no definite difference between liver and faeces samples
14 of domestic pigs in the frequency of HEV positivity. The highest (36%) incidence of HEV
15 infection was found among the eleven to sixteen week old pigs. Samples from domestic cattle
16 and rodents collected in pig farms, forests and meadows were tested negative for HEV RNA.

17 Phylogenetic analysis of partial sequences amplified within the ORF 1 and ORF 2
18 regions of selected strains revealed that the detected viruses belong to three subgroups of the
19 third genogroup of HEV, and are closely related to human and swine HEV strains detected in
20 different countries. The investigations revealed widespread distribution of HEV in Hungarian
21 wild ungulate and domesticated swine populations, with considerable genetic diversity
22 amongst the strains.

23

24 **Keywords:** viral hepatitis, hepatitis E virus (HEV), zoonosis, RT-PCR, phylogenetic
25 analysis

26

1 **Introduction**

2 Human health is inextricably linked to animal health and food production. A number of
3 communicable diseases (known as zoonoses) are transmitted from animals to humans. Among
4 them, Hepatitis E, as an emerging disease, is of high priority, and has been intensively
5 investigated worldwide.

6 Hepatitis E virus (HEV) was detected first in 1983 in a stool sample of a volunteer infected
7 with a virus isolate from a previous non-A non-B hepatitis epidemic (Balayan *et al.*, 1983,
8 Sreenivasan *et al.*, 1984). Since that time, the knowledge on this virus had been increasing,
9 but still there are several unknown details and presumptions about the characteristics of the
10 virus.

11 It has icosahedral capsid symmetry with a diameter of around 27-30 nm. The non-enveloped
12 virion contains a positive-sense, single-stranded, 7.2 kilobase long RNA with three
13 overlapping open reading frames (ORFs). ORF1 encodes the replication proteins, ORF3 is the
14 most variable part of the genome and codes for a protein with unknown function, and ORF2
15 encodes the capsid proteins (Emerson and Purcell, 2003). The virus, which was previously
16 classified into the *Picornaviridae*, later into the *Caliciviridae* family, now belongs to the
17 unassigned *Hepevirus* genus of +ss RNA viruses (Fauquet *et al.*, 2005).

18 HEV is detected all around the world, and classified into four genotypes, named after the
19 place of isolation of the reference strains. Two of the genotypes contain strains which infect
20 only humans (genotype-1 "Burma", genotype-2 "Mexico"); the other two genotypes
21 (genotype-3 "USA", genotype-4 "China") contain viruses isolated from both human and
22 animal samples (Worm *et al.*, 2002). Since the cultivation of HEV in cell culture is difficult,
23 the virus is usually detected by reverse transcription-polymerase chain reaction (RT-PCR) or
24 electron microscopy; anti-HEV antibodies are detected using commercial ELISA or
25 immunoblot kits (Vasickova *et al.*, 2007).

1 HEV causes acute hepatitis syndrome in humans with mild clinical symptoms of icterus,
2 anorexia, hepatomegalia, rarely abdominal sensitivity or pain, nausea, vomiting and fever.
3 The mortality rate is around 1% in the general population, but amongst pregnant women,
4 especially if the infection occurred during the third trimester it can reach 20% (Worm *et al.*,
5 2002). The virus is transferred by fecal-oral route, by contaminated water; and possibly with
6 raw meat (Meng *et al.*, 1997). In many areas of Asia, Africa, and Central America, HEV
7 infection is a major cause of epidemic and acute sporadic hepatitis, therefore these areas of
8 the world are considered endemic. North America and Europe have traditionally been
9 considered non-endemic for HEV; the majority of the hepatitis E cases were imported, the
10 seroprevalence ranges from 1% to 5% (Clemente-Cesares *et al.*, 2003), as compared to
11 endemic areas, where the seroprevalence is 7-55% (Worm *et al.*, 2002).

12 Several studies, even successful cross-species experimental infections (Meng *et al.*, 1998)
13 suggest that hepatitis E is a zoonotic disease. Swine are suspected to be one of the main
14 reservoirs of the virus (van der Poel *et al.*, 2001, Yazaki *et al.*, 2003), however, consumption
15 of game infected with HEV also results in acute hepatitis (Tamada *et al.*, 2004, Tei *et al.*,
16 2003). Clinical illness was not observed in animals, the blood-parameters remained normal,
17 only laboratory investigations (serology, PCR and histopathology investigation of liver) could
18 prove the infection (Halbour *et al.*, 2001). As a result of the asymptomatic animal infection is
19 that people who work with swine (i.e. veterinarians, slaughterhouse workers, and pig-farm
20 workers), are almost continuously exposed to HEV infections as indicated by higher anti-
21 HEV seroprevalance (Olsen *et al.*, 2006). Also HEV can reach the market and the consumers
22 via infected pork, especially liver (Yazaki *et al.*, 2003).

23 The aim of this study was to detect HEV in different animal samples from Hungary, and gain
24 an insight into the infection levels and genetic diversity of the virus in different animal
25 populations.

1 **Materials and methods**

2

3 **Samples and sample processing**

4 Between January 2005 and May 2009, 687 samples were collected in Hungarian animal farms
5 in order to detect HEV in the suspected reservoir species. From domesticated pigs (*Sus scrofa*
6 *domestica*) forty five liver and two hundred and forty eight faeces samples were collected
7 from four age groups according to the breeding and fattening program generally used in
8 Hungarian pig farms. From forty one animals faeces and liver samples were investigated
9 simultaneously in order to compare the occurrence of the virus in different organ samples.
10 From cattle (*Bos primigenius taurus*), one hundred and twenty five faeces samples were
11 collected from adult and young animals. Wild animal samples were collected in a game
12 processing slaughterhouse (Vecsés, Hungary), which purchases, processes and stores hunted
13 game carcasses from all around the country. Altogether seventy five liver samples of wild
14 boars (*Sus scrofa*), seven faeces and thirty four liver samples of roe deer (*Capreolus*
15 *capreolus*) and thirty liver samples of red deer (*Cervus elaphus*) were investigated. The only
16 data available is the geographical origin of the shot animals, and the date of death. In order to
17 investigate the subsistence of the infection, we also investigated liver and faeces samples of
18 rats (*Rattus norvegicus*) and mice (*Mus musculus*) collected in pig farms as well as European
19 hamster (*Cricetus cricetus*) and common shrew (*Sorex araneus*), collected in forests and
20 meadows.

21

22 Approximately one gram of samples were homogenized in ceramic mortars using sterile quartz
23 sand, and were diluted in 10 ml phosphate buffered saline. The homogenized samples were
24 centrifuged at $1,500 \times g$ for 10 min, and then the supernatants were transferred into sterile tubes
25 and centrifuged again at $12,000 \times g$ for 15 min to clear them from cell debris and bacteria. The

1 viral RNA was isolated from the supernatants using QIAamp viral RNA mini kit (Qiagen, Hilden,
2 Germany). Homogenized samples and extracted RNAs were stored at -80 °C.

3

4 **Reverse transcription-polymerase chain reaction**

5 Reverse transcription was performed on the extracted RNA and amplified in a single tube RT-
6 PCR assays using Qiagen OneStep RT PCR kit (Qiagen, Germany), according to the
7 manufacturer's instructions. The reaction mixture contained the following ingredients: 10 µl of 5×
8 reaction buffer, 0.4 mM of deoxynucleoside triphosphate (dNTP) mix, 1-1 µM of appropriate
9 genomic and complementary primers, 2 µl of enzyme-mix (containing Omniscript and Sensiscript
10 reverse transcriptases and HotStarTaq DNA polymerase), 20 U of RiboLok RNase inhibitor
11 (Fermentas, Vilnius, Lithuania), 5 µl of template RNA, and distilled water to make up the final
12 volume of 50 µl. As positive control, RNA of a reference HEV positive sample (kindly provided
13 by Marion Koopmans DVM, Department of Virology, Diagnostic Laboratory for Infectious
14 Diseases, National Institute of Public Health, the Netherlands), or Hungarian HEV-positive
15 samples (which were confirmed by partial sequencing) were used. Reaction mixtures without
16 template RNA were used as negative controls.

17

18 The samples were tested first with a primer-pair, which anneal within the ORF2 region, and
19 positive results were confirmed by a second RT-PCR with primers annealing the ORF1. The
20 HEV-specific, degenerated primer-pairs were published by van der Poel *et al.* (2001). For the
21 amplification of a 196 bp long sequence of the ORF2 region, ORF 2-s1 (sequence: 5'-
22 GACAGAATTRATTTTCGTCGGCTGG-3', position on M73298 Burmese strain: 6298-6321)
23 and ORF2-a1 (sequence: 5'-CTTGTCRTGYTGGTTRTCATAATC-3', position on M73298
24 Burmese strain: 6494-6470) primers were applied. To amplify a 417 bp long sequence of the

1 ORF1 region, ORF1-s1 (sequence: 5'-CTGGCATYACTACTGCYATTGGC-3', position on
2 M73298 Burmese strain: 56-79) and ORF2-a1 (sequence 5'-
3 CCATCRARRCAGTAAGTGCGGTC-3', position on M73298 Burmese strain: 473-451)
4 primers were used.

5
6 The thermal profile of the amplifications contained a reverse transcription step at 50 °C for 30
7 minutes, and a HotStarTaq activation at 95 °C for 15 min, which was followed by 40 cycles of
8 denaturation at 94 °C for 1 minute, annealing at 55 °C for 1.5 minutes, and primer extension at 72
9 °C for 1 min. The amplifications were finished by a final extension at 72 °C for 10 min.
10 Eventually, the reaction mixtures were maintained at 4 °C until they were removed from the
11 device.

13 **Gel-electrophoresis**

14 Following the RT-PCRs 5µl of the amplification products were electrophoresed in 2% Tris-
15 borate-EDTA agarose gel (Seakem, Rockland Main, USA) containing 0.5 µg/ml ethidium
16 bromide, at 8 V/gelcm for 60 min. The bands were visualized by UV transillumination and
17 photographed by Kodak DS Electrophoresis Documentation and Analysis System. Product sizes
18 were determined with reference to Gene Ruler 100 bp DNA Ladder (Fermentas, Vilnius,
19 Lithuania).

21 **Nucleotide sequencing and computer analysis**

22 Samples were selected for sequencing by the following conditions: host species; intensity of
23 the amplified DNA in the gel; expected epidemiologic results (samples from neighbouring pig
24 farms; or sequential samples from the same farm from different years). Specific amplification

1 products were excised from the agarose gel, and DNA was extracted using QIAquick Gel
2 Extraction Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The
3 nucleotide sequences of selected amplification products were determined in fluorescence
4 based direct sequencing reactions, in two directions, using the previously described primers.
5 Sequences were read in an ABI PRISM 310 Genetic Analyzer (Applied Biosystem, Stafford,
6 USA). The sequences were identified by the Basic Local Alignment Search Tool (BLAST,
7 <http://www.ncbi.nlm.nih.gov/BLAST>) search against the gene bank databases. The sequence
8 corrections and multiple alignments were made with the help of BioEdit 4.7.8 and Clustal X
9 programs. Primer sequences were removed; therefore 371 bp long sequences of ORF1 and
10 148 bp long sequences of ORF2 regions were used in the phylogenetic analyses. The analyses
11 were performed using the Phylogeny Inference Program Package (PHYLIP) version 3.6b. The
12 sequences determined in this study were compared to HEV genotypes deposited in the
13 GenBank database. Bootstrap resampling analyses of 1000 replicates were generated with the
14 seqboot program to test the stability of the trees. Neighbor-Joining distance matrices were
15 generated by the dnadist and Fitch programs, using a transition/transversion ratio of 2.0.
16 Phylogenetic trees were delineated with the help of the TreeView program. Sequences were
17 submitted to GenBank under accession numbers from EU718629 to EU718650, EU530671 to
18 EU530676, EU057982 GQ468295 és GQ468296. To exclude possible laboratory
19 contaminations, the sequences of the samples frequently used as positive controls (HEV006,
20 HEV007, HEV072, HEV264) were also included in the analysis.

21

22 **Results**

23 The amplifications of HEV genome regions from animal samples resulted clear DNA
24 products with the previously expected molecular size. The samples which were positive in the
25 first reactions (detecting the partial ORF-2 region) always gave positive reactions in the

1 second confirmatory RT-PCR (detecting the partial ORF-1 region). Amplifications were
2 never detected in the negative controls. HEV RNA was detected in fifty two (21%) of 248
3 faeces and in fourteen (31%) out of forty five liver samples of domesticated pigs. The samples
4 were collected in forty one Hungarian pig farms, and sixteen (39%) of these farms were found
5 to have HEV-positive swine. The cattle faeces samples, the rat, mice, hamster and shrew
6 samples, as well as the faeces samples of wild ruminants and wild boar did not contain HEV
7 RNA. Eight out of seventy five wild boar, nine out of forty one roe deer and three out of thirty
8 red deer liver samples contained HEV RNA. The geographical origins of the positive samples
9 are demonstrated in Figure 1.

10 Information on the age of 204 pigs of the two hundred and fifty one investigated domestic
11 pigs was available. These animals were classified into four age groups: 1-4 week old piglets,
12 5-10 week old pigs, 11-16 week old pigs, swine over 17 weeks; 9%, 27%, 36%, and 10%
13 were found positive for HEV nucleic acid, respectively (Figure 2.).

14 Among the forty two animals, from which faeces and liver samples were investigated
15 simultaneously, thirteen animals were found, in which HEV RNA was detected in the liver.
16 Among them ten animals were found shedding HEV RNA in their faeces, and in the case of
17 three animals, the faeces were found HEV negative. Liver samples of the other twenty nine
18 pigs were found HEV negative. From these animals, the faeces sample of twenty five pigs
19 was found HEV negative, and HEV RNA was detected in the faeces sample of four animals
20 (Table I.).

21 Twenty four amplification products of the partial ORF-2 region and five selected products of
22 the partial ORF-1 region of the HEV-positive animal samples were sequenced. All sequences
23 showed the highest similarity to HEV sequences.

24 The phylogenetic analysis of the partial ORF-2 region revealed that the viruses detected in
25 Hungarian animal samples belong to the third genogroup of HEV. Although the one hundred

1 and ninty six base-pair sequences are relatively short for reliable phylogenetic analysis (as
2 indicated by the low bootstrap values), the genetic relatedness between the Hungarian viruses
3 are well illustrated in the phylogenetic tree (Figure 3.). The Hungarian viruses belong to three
4 subgroups of the third genogroup of HEV according to the system described by Lu *et al.*
5 (2006). Five Hungarian viruses of domesticated swine, roe deer and red deer belong to the “e”
6 subgroup, where they form four different separate branches, however, within the subgroup,
7 these branches are mixed with other branches composed by HEV of human origin. The “e”
8 subgroup contains viruses detected in Hungary, Greece, Great Britain and Japan. Two viruses
9 of swine origin are more different from the other Hungarian viruses of the “e” subgroup.
10 HEV072 and HEV278 form two separate branches. HEV278 was detected in the same farm
11 with eight other swine viruses (279, 281-285, 288 and 290) in 2006. These viruses are
12 identical at the sequenced region, therefore only the sequence of HEV278 was selected to
13 present on the tree. However, during a repeated sampling in 2007 two other viruses were
14 detected in this farm: HEV446 and HEV451. These viruses are different from the original
15 (HEV278) one, and also from each other. The difference between the viruses is only one
16 nucleotide, and this is a silent mutation in the sequence, the amino acid sequences of the three
17 viruses are identical. Sample HEV088, a swine origin virus is very closely related to
18 HEV157, which was detected in a deer liver sample. The two samples were collected in the
19 same year, but about three hundred km far from each other. HEV045 is a virus of roe deer
20 origin, and it is identical to another Hungarian virus of human origin; however
21 epidemiological connection between the two samples were not found. In the “e” subgroup the
22 Hungarian viruses of both human and animal origin form a separate cluster, except for one
23 virus of human origin, which is more closely related to the Japanese sequence.

24 One Hungarian virus of swine origin, detected in 2009, belongs to the “h” subgroup of
25 genogroup 3. HEV612 virus is closely related to viruses of human and animal origin, detected

1 in the Netherlands, Germany, Italy and Mongolia. The virus was detected in a swine farm
2 located in North-East Hungary, and it is identical with two other viruses detected at the same
3 farm. HEV612 was selected to represent the three identical viruses on the phylogenetic tree.
4 Thirteen other Hungarian HEV sequences belong to the “a” subgroup of genogroup 3.
5 HEV264, a virus of swine origin forms a separate branch with two other Hungarian viruses,
6 one of them is detected in humans, the other one, HEV480 represents two other viruses
7 (HEV478, HEV479) which were collected in the same pigfarm in 2007, and with which
8 HEV480 is identical. Four other viruses, HEV 143, HEV070, HEV006 and HEV034 of swine
9 and wild boar origin form another separate branch within the “a” subgroup. The samples of
10 swine origin presented in this branch were collected in three different pig farms,
11 geographically far from the forest, where the wild boar (HEV006) was shot. Although
12 HEV034 and 036 were detected in the same farm and the same time, the nucleotide sequences
13 slightly differ from each other, therefore HEV036 forms a different branch with a Hungarian
14 virus of human origin. The next Hungarian branch within the “a” subgroup is composed by
15 three viruses detected in three different species. HEV007 is of wild boar origin, HEV052 was
16 detected in domesticated swine, and HEV138 is of roe deer origin. These viruses are from
17 three different regions of Hungary; epizootiological connection between them was not found,
18 and they are closely related to a virus of human origin, detected in Japan. HEV039 is a virus
19 of domesticated swine origin, which forms a cluster with another Hungarian HEV sequence of
20 human origin, and a virus detected in swine in the Netherlands.

21 The nucleotide sequences of five selected Hungarian animal HEV viruses were determined on
22 the partial ORF-1 region. The topology of the ORF-1 derived tree (Figure 4.) is basically
23 similar to the ORF-2-based tree; however the separation of 3h and 3i subgroups is collapsed.
24 HEV072 is closely related to human HEV isolates from Greece, Great Britain and Japan,
25 viruses of the “e” subgroup of genogroup 3. The other four sequenced viruses (HEV006 and

1 HEV034, HEV007 and HEV039), which are members of the “a” subgroup of the ORF-2 tree
2 are clustered together in the same, “a” subgroup in the ORF-1 tree, and show the closest
3 relationship to a swine HEV isolate from the USA.

4 The partial ORF-1 and ORF-2 genome regions were translated to putative amino acid
5 sequences. The alignment of the amino acid sequences of the viruses detected in Hungary
6 revealed a few, single amino acid substitutions within the coded proteins (Table II. and III.).

7

8 **Discussion**

9 The Hepatitis E has been investigated keenly since it was first detected. Although nowadays
10 several methods of investigation are available, some of the reasons for the spreading of the
11 infection are unclear. In the developing countries, humans are mainly infected via
12 contaminated water, food and environment; the transmission of the virus by meat of infected
13 animals or direct connection with infected animals is also suspected (Worm *et al.*, 2002).
14 Several studies suggest, that HEV is transmitted to human from domesticated swine (Yazaki
15 *et al.*, 2003., Meng *et al.*, 2005., van der Poel *et al.*, 2001), and also there are some reports of
16 human infection presumably caused by consumption of undercooked game, such as wild boar
17 and deer (Tei *et al.*, 2003, Tamada *et al.*, 2004). Therefore in this survey, we investigated
18 samples from domesticated swine, wild boar, red deer and roe deer as species, because they
19 are subject to human consumption. Besides samples of these documented reservoir species,
20 samples of domesticated cattle were also investigated, to establish whether they play a role in
21 the transmission of HEV from animals to humans. Samples of rats, mice, hamsters and
22 common shrews were investigated in order to figure out, whether these animals have a role in
23 the spread of HEV among wild animals.

24 Hungary belongs to that region of the world, which was previously categorized as “non
25 endemic” with respect to HEV infection (Worm *et al.*, 2002). A clinical HEV case (Reuter *et*

1 *al.*, 2006) and further one hundred and sixteen asymptomatic HEV infections have been
2 reported in humans in Hungary (Reuter *et al.*, 2009) between 2001 and 2006. The occurrence
3 of HEV infection amongst domestic swine appears to be quite high, indicated by the detection
4 of HEV by RT-PCR in 39% of the investigated swine farms. By PCR investigations, in the
5 Netherlands 22% and 55% of the investigated pig farms were found HEV positive in 1999
6 and 2005 respectively (Rutjes *et al.*, 2007). In the USA 54% and 40% of the investigated pig
7 farms were found HEV positive in 2002 and 2005 respectively (Huang *et al.*, 2002,
8 Kasorndorkbua *et al.*, 2005). HEV RNA was detected in 100% of the six investigated pig
9 farms located in Northern Italy (di Bartolo *et al.*, 2008); in 73% of pig farms investigated in
10 Sanghaj (Ning *et al.*, 2008); and in Spain the prevalence of HEV among investigated pig
11 farms increased from 38% in 2006 to 76% in 2007 (Fernandez-Barredo *et al.*, 2006 and
12 2007). Besides PCR-positivity, also the high seroprevalance indicates the presence of the
13 infection in pigs. In 2008 the investigations of forty one pig farms revealed 97% IgG and 83%
14 IgM positivity in Spain (Seminati *et al.*, 2008); in Canada, the seropositivity varied between
15 0-100% among pig farms (Leblanc *et al.*, 2007). The presence of the virus, apart from the
16 food-hygiene aspects (Yazaki *et al.*, 2003) is of significance as a hygienic risk for the people,
17 working at the swine farms and slaughterhouses, since they can become infected by direct
18 contact with the reservoir animals.

19 The purpose of investigating HEV in different pig age groups was to determine the
20 course of infection in HEV infected pig farms. Several studies describe, that the spread of
21 the virus among pigs can happen through contact infection. The source of the infection is
22 usually the infected sow, or after weaning, infected pigs with which the susceptible
23 animals are grouped together (Bouwknegt *et al.*, 2009, Tanaka *et al.*, 2004, Kasorndorkbua
24 *et al.*, 2004, de Deus *et al.*, 2008b). Maternal antibodies can prevent the infection of
25 suckling pigs (Kasorndorkbua *et al.*, 2003), however virus shedding sows can infect the

1 piglets during the suckling period (de Deus *et al.*, 2008b). Later stress factors, such as
2 mixing, re-grouping and changes in the types of feeding can facilitate the infection (Kelley
3 1980). Direct contact between susceptible pigs with virus-shedding pigs after weaning,
4 may result in the subsistence HEV infection in pig farms (Bouwknegt *et al.*, 2008, Casas *et al.*,
5 2009). In Hungary, the highest (36%) HEV positivity was observed amongst the eleven
6 to sixteen week old pigs, and the presence of HEV RNA was detected in 27% of samples
7 collected in the five to ten week old age group. Low virus-shedding was detected among
8 the suckling pigs (9%), and also in the group of pigs over seventeen weeks old age (10%),
9 among which finishing pigs and sows or boars used in breeding were also tested. The age
10 distribution of the HEV infection amongst domestic pigs in Hungary is in line with the
11 results detected in other countries. In Taiwan the highest prevalence of HEV was detected
12 amongst the eight week old pigs (4.5%). Lower, 1.3% and 0.8% positivity was found
13 amongst the two to five month old and over five month old pigs respectively (Wu *et al.*,
14 2002). In Japan, high (75-100%) shedding frequency was observed in pigs at one to three
15 months of age, and at the finishing stage, 9-14% of the pigs in two of the investigated pig
16 farms shed viral particles in their faeces (Nakai *et al.*, 2006). In Spain the highest
17 prevalence of HEV infection (60%) was detected amongst the thirteen to sixteen week old
18 pigs, however also high, 42% HEV positivity was found amongst the five to twelve week
19 old pigs. In the youngest age-group (1-4 weeks old pigs) the RT-PCR positivity was only
20 11%, higher than our result in the same age group. Surprisingly, 21% of the investigated
21 Spanish sows shed the virus in their faeces (Fernandez-Barredo *et al.*, 2006). In Canada
22 HEV RNA was detected in the faeces samples in the 86% of the eighteen week old pigs,
23 and amongst the finishing pigs, the RT-PCR positivity was 41% (LeBlanc *et al.*, 2007).
24 However the majority of the pigs become infected after weaning, during the first months of
25 feeding (Fernandez-Barredo *et al.*, 2006). Infected pigs can be found in the finishing age

1 groups too, leading to the likely possibility of slaughter and consumption of HEV infected
2 pig meat (Bouwknegt *et al.*, 2007).

3 To investigate which sample is more suitable for the detection of HEV RNA in
4 infected animals, faeces and liver samples were simultaneously investigated in the case of
5 forty one domestic pigs; and the data were compared with the results of similar studies. By
6 studies of experimental infections, it is known that after contact infection, HEV RNA can
7 be detected in the faeces around the seventh day of infection, and viraemia develops
8 around the twentieth day of infection; virus shedding in the faeces for about three weeks,
9 and the viraemia lasts for about ten days (Bouwknegt *et al.*, 2009, Kasorndorkbua *et al.*,
10 2004). In the faeces HEV RNA can also be detected at the initial period, when virus
11 particles enter the large intestines directly after ingestion. Virus multiplication in the liver
12 is followed by shedding of virions in the faeces through the bile (Lee *et al.*, 2009). In our
13 study, we found the virus in fourteen (33%) of forty two faeces samples and in thirteen
14 (31%) of forty two liver samples, that means no definite difference among the samples.
15 Both faeces and liver samples of twenty five from the investigated forty one animals were
16 found negative for HEV, and viral RNA was detected in the samples of seventeen pigs.
17 Among the infected pigs, in the case of ten animals (59%) both the liver and faeces
18 samples were found positive. In three animals (18%) only the liver, in four animals (24%)
19 only the faeces was found positive (Table I.). In other studies, the difference between liver
20 and faecal samples on the frequency of detecting HEV RNA in samples of infected pigs
21 was also not significant. In the study done by de Deus *et al.* (2007), among twenty four
22 animals from which both liver and faeces samples were available, HEV RNA was detected
23 in nine of the liver and eight of the faeces samples. Both liver and faeces samples were
24 found HEV negative in eleven cases. Amongst the thirteen animals which were found HEV
25 infected by the positivity of the liver and/or the faeces sample, the distribution of the

1 positive results of the parallel samples were almost the same: 4 animals were declared
2 HEV infected by the positivity of both liver and faeces samples, and HEV RNA was
3 detected in the case of five animals only in the liver, four animals only in the faeces
4 samples. In another study of de Deus *et al.*, (2008b), from twenty animals in which HEV
5 RNA was detected in the liver and/or the faeces sample, in ten of the animals the virus was
6 found in the liver, while in thirteen animals, the virus was found in the faeces. By these
7 results, at least in the case of the domestic pig, both liver and faeces sample seems to be
8 suitable for the detection of HEV infection.

9 The consumption of game is more and more popular in Europe, however it is confined mainly
10 to the muscles of the animals, liver and other intestines are rarely eaten. Several cases are
11 described, when the consumption of the meat of infected animals lead to clinical disease in
12 humans (Takahashi *et al.*, 2004). These studies also suggest that wild animals also play a role
13 in the spread of the virus. By the investigation of samples from Hungarian wild animals, HEV
14 RNA was detected in 11% of the wild boar samples, 22% of the roe deer and 10% of the red
15 deer samples. Compared to Italy, where 25% of eighty eight investigated wild boars (bile
16 samples), and to Spain, where 20% of the investigated one hundred and fifty wild boars (sera
17 samples) found to be HEV positive in RT-PCR investigations (Martelli *et al.*, 2008, de Deus
18 *et al.*, 2008a), the HEV infection among wild boars is lower in Hungary. On the contrary,
19 only 3% of wild boar sera samples collected between 2001 and 2004 in the Ehime area of
20 Japan were found to be positive by RT-PCR (Michitaka *et al.*, 2007). Two surveys on the
21 occurrence of HEV infection in wild boars in Germany resulted two different results:
22 although among the sera samples collected in 1995-1996 only 5% were found to be positive
23 (Kaci *et al.*, 2008), HEV RNA was detected in 14.9% of the liver tissue samples of wild boars
24 collected between 2005 and 2008 (Schielke *et al.*, 2009). The reason of the difference in these
25 results can be the use of archived samples in the surveys done by Kaci *et al.*, and Michitaka *et*

1 *al.*: during the storage of the archived samples the HEV nucleic acid might decompose,
2 therefore the chance for the detection of HEV RNA is decreased. The type of the investigated
3 samples can also have effect on the PCR result. The differences in the prevalence of HEV
4 detected in different samples (faeces, bile, tissue, blood) are described in several papers
5 (Huang *et al.* 2002, Cooper *et al.*, 2005, Fernandez-Barredo *et al.*, 2006, Nakai *et al.*, 2006,
6 Rutjes *et al.*, 2007), as well as discussed above. Comparing to the period of virus shedding by
7 faeces or the presence of HEV in the liver, the duration of viraemia is relatively short
8 (Bouwknegt *et al.*, 2009, Kasorndorkbua *et al.*, 2004), therefore the detection of HEV RNA in
9 sera is less frequent than in faeces or tissue samples.

10 The prevalence of HEV infection among deer is not deeply investigated. In Japan, where the
11 consumption of undercooked deer meet is frequent, HEV RNA was detected in only 1% of
12 one hundred deer which lived in the same forest, whereas three out of the seven investigated
13 wild boars were found to be HEV positive (Takahashi *et al.*, 2004). In another study, only 4%
14 of sera samples of deer collected in Japan contained anti-HEV antibody, and HEV RNA in the
15 sera and liver samples was not detected. Unfortunately wildlife management data from Japan
16 and other European countries was not available to us; therefore the comparison of the results
17 is difficult. The investigated species were found in the whole territory of Hungary, the
18 estimated size of the wildlife population contained 95582 wild boars, 85081 red deer and
19 340384 roe deer in 2008 (Csányi *et al.*, 2008). The investigated samples were collected in
20 animals shot in four regions of Hungary: Western Hungary, near to the Austrian border;
21 Southern Hungary near to the Serbian border; Central-Hungary, area around Budapest; and
22 Northern Hungary, near to the Slovakian border. Generally, HEV was found in all four
23 investigated areas in wild boar, and in the Western and Eastern areas also in roe deer and red
24 deer. Since samples were not collected in all areas of Hungary, our results do not support the
25 Japanese theory, that there are “hot spots” in the country, infected forests, in which the virus

1 is circulating between wild boars, red deer and roe deer. However, it seems that where HEV
2 positivity was found in wild boars, infection in deer and roe deer was also detected. The
3 results of the investigation of hamster and shrew samples does not support that these rodents
4 have a role in the spread of the virus. Instead of rodents as reservoir, the supplementary
5 winter-feeding of the wild animals might play role in the spread of HEV among wild boars
6 and wild ruminants. Grouping behaviour of wild animals are characteristic in the winter (Bao
7 *et al.*, 2005), and around the supplementary feeding sites wild boars and also wild ruminants
8 can be found together. The infected animals can contaminate the feed, especially when the
9 feed is served on the ground; therefore - although the coprophagic behaviour is not
10 characteristic for ruminants (Lescano *et al.*, 2007) - red deer and roe deer can become infected
11 by the consumption of the feed contaminated by virus-shedding wild boars. This theory is
12 supported by the collection date of the samples: all positive samples of wild boar and red
13 deer, and five positive samples of roe deer were collected February and four positive samples
14 of roe deer were collected in April. All samples (thirteen samples of wild boar and one sample
15 of roe deer) collected in October were HEV negative. The spread of hepatitis E virus between
16 wild animals, the course of infection in different age groups and species and the subsistence
17 of the HEV infection in forests and meadows are subjects of further investigations.

18 Besides swine, wild boar and deer, which are suggested to be the main reservoirs of HEV,
19 other animals can become infected by the virus (Meng, 2005). Studies presented, that
20 domestic ruminants are also susceptible for HEV. The percentage of seropositivity detected
21 amongst cattle in Somalia, Tajikistan and Turkmenistan was 29 to 62%, in Ukraine, 12%
22 (Favorov *et al.*, 1998), and in India 4.4% to 6.9% (Arankalle *et al.*, 2001). In this study one
23 hundred and twenty five faeces samples of cattle were investigated, but HEV RNA was not
24 detected. Nevertheless, when wild ruminant (roe deer) samples were investigated, only liver
25 samples gave positive reactions, although among the investigated roe deer samples seven

1 were faeces. Therefore, it is possible that infected ruminants shed the virus in the faeces in
2 lower titres or for shorter periods, which might not be detected by the diagnostic methods.
3 Further studies are planned to test cattle liver samples for HEV RNA.
4 Selected amplification products were sequenced and analyzed. The aim of the sequencings
5 was on one hand to prove the positive results and exclude any aspecific amplifications, on the
6 other hand, to infer the phylogenetic relations of Hungarian viruses with HEV strains detected
7 in other countries. The phylogenetic analyses show that in Hungary several genetic variants of
8 HEV are present including circulation of genetically different HEV strains on the same farm.
9 Those Hungarian HEV strains, which were investigated more detailed by sequencing, belong
10 to three subgroups of genotype 3 according to the system described by Lu *et al.* (2006).
11 Generally, the Hungarian viruses (both human and animal origin) form separate branches
12 within the subgroups. Within the “e” subgroup, seven Hungarian hepatitis E viruses of animal
13 origin can be found together with viruses of human origin, detected in Japan, and European
14 countries, such as Hungary, Greece, and the United Kingdom. The virus HEV045, which was
15 detected in roe deer, is identical with another Hungarian virus of human origin, however
16 epidemiological connection between the two samples is not proved. The virus HEV612 is
17 presented in the “h” subgroup of genotype 3. This virus was detected in May 2009, in the
18 Northern part of Hungary and it is unique in the country, hence similar viruses from other pig
19 farms or human were not detected. However the virus is similar to viruses detected in
20 Mongolia, Italy, Germany and the Netherlands. The viruses presented at the “a” subgroup of
21 genotype 3 are related to HEV, detected in the US (human origin), the Netherlands (swine
22 origin), Japan (human origin), and Korea (swine origin). The close genetic correlation
23 between the animal strains found in Hungary and human HEV strains from different countries
24 is in line with previous publications suggesting the zoonotic potential of HEV. This study also
25 reveals, that HEV strains, on one hand, show close genetic relationship to strains with which

1 they are not in known epidemiological relationship. High similarity was found between
2 Hungarian and Japanese strains, there are two identical viruses detected in a human and in an
3 animal sample without any epidemiological connection; and some of the Hungarian viruses
4 are also very similar, in spite of their differences in host species and geographical origin. On
5 the other hand, strains, detected at places geographically near to each other, are sometimes
6 less similar, for example HEV034 and HEV036 are viruses detected in the same pig farm in
7 the same time, but they form two different branches within the “a” subgroup. These two
8 viruses also differ in their amino-acid sequence of the investigated ORF2 region (Table III.).
9 Other example is the case of HEV264, HEV278, HEV446, HEV451 and HEV480.
10 Epidemiological connection is unknown between the three farms, where HEV264, HEV278
11 and HEV480 were collected, and which are geographically very close to each other in the
12 Western part of Hungary. The HEV480 and HEV264 are two very similar viruses detected in
13 2007 and 2006 respectively in two different farms located thirty eight km far from each other.
14 Both HEV264 and HEV278, two genetically different viruses detected in 2006 in two
15 different farms, with 7 km distance between them. HEV278 detected in January 2006 and
16 HEV446 and HEV451 collected in July 2007 are from the same pigfarm. The three viruses
17 differ from each other, however only in one nucleotide at three different positions of the
18 investigated nucleotide sequence. The amino acid sequences of HEV278, HEV446 and
19 HEV451 are identical.

20 Although the variability of the nucleotide sequence of the virus is quite high, the analysis of
21 the deduced amino acid sequences (Table II. and III.) indicates that the nucleotide-differences
22 are mainly silent mutations; only in a few cases there are single amino-acid substitutions in
23 the sequences. The similarities and differences between the viruses at nucleotide level,
24 however, are well illustrated in the amino-acid sequences of the ORF 1 region: there are two
25 characteristic substitutions which are consistently present in the viruses belonging to different

1 subgroups. In the case of ORF2 the substitutions are not parallel with the genetic
2 classification based on the nucleotide sequences: only the threonine in the amino acid position
3 no. 426 is characteristic to the viruses belonging to the 3a subgroup.

4 The variability of Hepatitis E Virus is well known; the analysis of the amplification products
5 of the ORF1 region as well as the ORF2 region - despite the relative short sequence - is a
6 reliable tool for the identification of the detected viruses, and also for phylogenetic analyses.
7 The background of the high variability is still the subject of further investigations.

8

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19

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1 **Legend of tables and figures**

2

3 Table I.: Results of simultaneously investigated liver and faeces samples.

4

5 Table II.: Amino acid substitutions at the ORF1 region of the Hepatitis E viruses detected in
6 Hungary. Positions by the GenBank reference ORF1 protein sequence, accession number
7 AAA03189.

8

9 Table III.: Amino acid substitutions at the ORF2 region of the Hepatitis E viruses detected in
10 Hungary. Positions by the GenBank reference ORF2 protein sequence, accession number
11 AAA03191.

12

13 Figure 1.: Geographical origin of the HEV-positive animal samples. Dots indicate the
14 domestic swine farms, triangles are wild boars, stars are roe deer and squares are red deer
15 samples. Positive samples are marked with filled; negative samples are marked with empty
16 shapes.

17

18 Figure 2.: Prevalence of HEV infection in different age groups of domesticated pigs.

19

20 Figure 3.: Neighbor-joining phylogenetic tree based on the partial ORF2 region of HEV
21 genotypes. The viruses are marked as follows: strain/host (Sw: domestic swine, Wb: wild
22 boar, Hu: human, Red: red deer, Rod: roe deer)/country (three letters sign) – year of detection.
23 Sequences determined in this study are indicated in bold. Internal labels indicate the bootstrap
24 resampling analysis of 1000 replicates. Only bootstrap values >500 are indicated on the tree.
25 Bar demonstrates the genetic distance.

1

2 Figure 4.: Neighbor-joining phylogenetic tree based on the partial ORF1 region of HEV
3 genotypes. The viruses are marked as follows: strain/host (sw: domestic swine, wb: wild boar,
4 hu: human)/country (three letters sign) – year of detection. Sequences determined in this study
5 are indicated in bold. Internal labels indicate the bootstrap resampling analysis of 1000
6 replicates. Only bootstrap values >500 are indicated on the tree. Bar demonstrates the genetic
7 distance.

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	Pig code	Weight	Liver	Faeces
1.	048/05/1	nd	-	-
2.	048/05/2	nd	-	-
3.	048/05/3	nd	-	-
4.	048/05/4	nd	-	-
5.	050/05	2 kg	-	-
6.	050-5-06	288 g	-	-
7.	065-1-05	31 kg	+	+
8.	065-2-05	29 kg	+	+
9.	065-3-05	17 kg	+	+
10.	077-1-06	nd	-	-
11.	080-2-05	14 kg	+	+
12.	080-3-05	47 kg	+	+
13.	088-06/3	nd	-	-
14.	103-05-2	27 kg	-	+
15.	103-05-3	nd	-	+
16.	103-05-4	16 kg	-	-
17.	106-1M-05	7 kg	-	-
18.	106-2M-05	19 kg	-	-
19.	108-1-05	5 kg	-	-
20.	108-2-05	10 kg	-	-
21.	108-3-05	12 kg	-	-
22.	108-4-05	12 kg	-	-
23.	108-5-05	11 kg	-	+
24.	118-4-05	36 kg	+	+
25.	118-5-05	75 kg	-	-
26.	119-05	3 kg	-	-
27.	126-05	nd	+	+
28.	128-3-05	nd	-	-
29.	133-4-06	nd	-	-
30.	149-03-05	nd	+	+
31.	172-1-05	nd	+	-
32.	177-1-05	nd	+	-
33.	192-1-05	nd	+	+
34.	198-1-05	nd	+	-
35.	255-1-06	nd	-	-
36.	299-7-06	nd	-	-
37.	542-05	nd	-	-
38.	576-05	nd	-	-
39.	E7	nd	-	-
40.	Ja2000-1	nd	-	+
41.	Ja2000-2	nd	-	-
42.	Ja2000-3	nd	+	+

Sequences	Amino acid positions		Subgroup
	41	43	
HEV072	t	i	<i>3e</i>
HEV006	v	t	<i>3a</i>
HEV007	v	t	<i>3a</i>
HEV034	v	t	<i>3a</i>
HEV039	v	t	<i>3a</i>

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Sequences	Amino acid positions						Subgroup
	394	395	409	421	426	435	
<i>HEV072</i>	<i>q</i>	<i>l</i>	<i>t</i>	<i>q</i>	<i>a</i>	<i>d</i>	<i>3e</i>
HEV088	d	<i>3e</i>
HEV045	d	<i>3e</i>
HEV157	d	<i>3e</i>
HEV278	.	.	a	.	.	d	<i>3e</i>
HEV446	.	.	a	.	.	d	<i>3e</i>
HEV451	.	.	a	.	.	d	<i>3e</i>
HEV612	.	.	a	.	.	d	<i>3h</i>
<i>HEV039</i>	<i>t</i>	<i>d</i>	<i>3a</i>
HEV036	t	d	<i>3a</i>
HEV143	t	d	<i>3a</i>
HEV052	t	d	<i>3a</i>
HEV138	t	d	<i>3a</i>
HEV480	t	d	<i>3a</i>
HEV264	.	m	.	.	t	d	<i>3a</i>
<i>HEV034</i>	<i>r</i>	.	.	.	<i>t</i>	<i>d</i>	<i>3a</i>
<i>HEV007</i>	<i>r</i>	.	.	.	<i>t</i>	<i>d</i>	<i>3a</i>
<i>HEV006</i>	<i>r</i>	.	.	.	<i>t</i>	<i>d</i>	<i>3a</i>
HEV070	.	.	.	h	t	d	<i>3a</i>

Figure 1

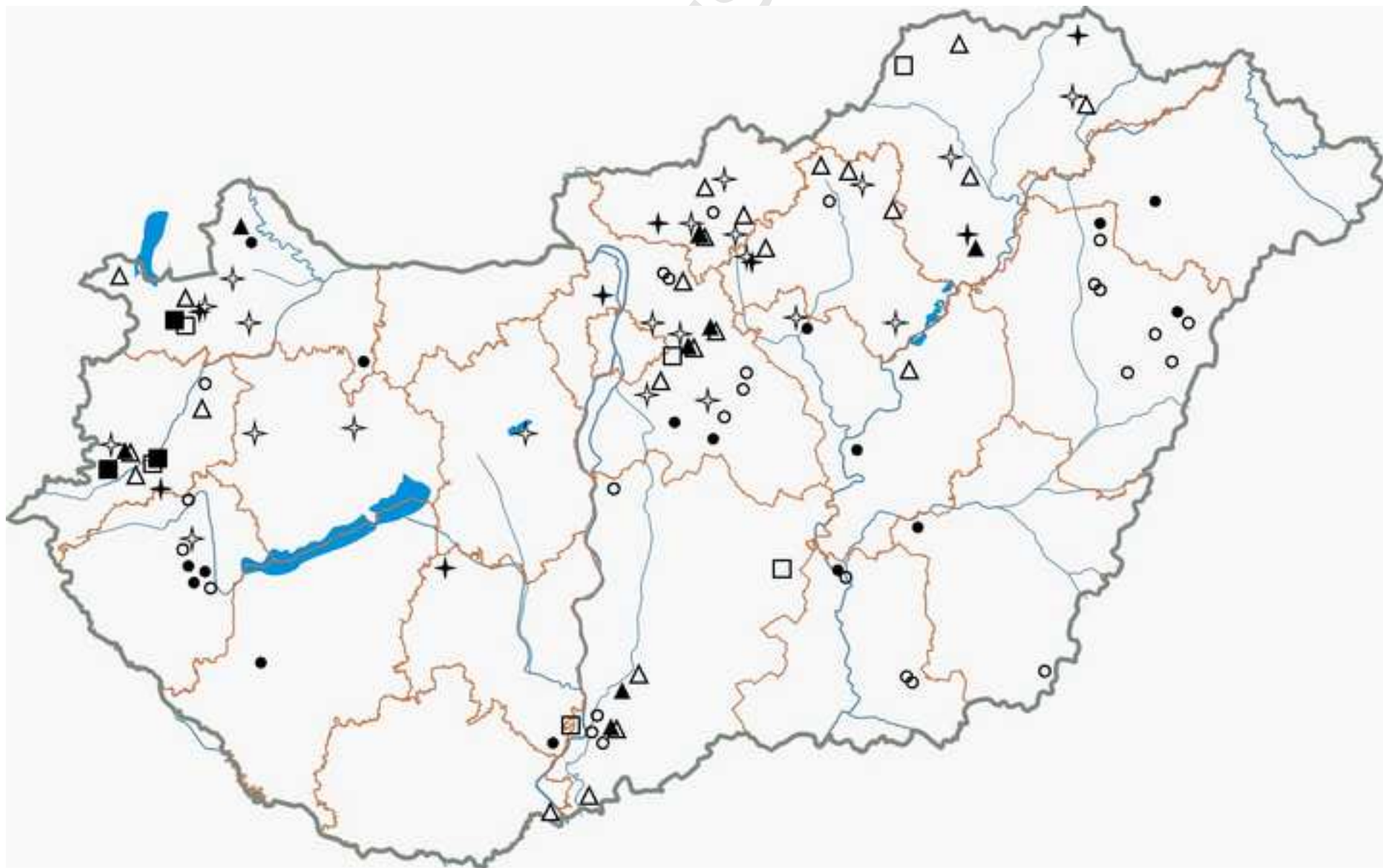


Figure 2

Script

