International Journal of Infectious Diseases 29 (2014) 232–237

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Evidence of hepatitis E infection in swine and humans in the East Region of Romania

Adriana Aniţă [a], Lucian Gorgan [b], Dragoş Aniţă [a], Luanda Oşlobanu [a], Nicole Pavio [c], Gheorghe Savuţa [a,*]

[a] Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, 8 Mihail Sadoveanu Alley, 700489 Iaşi, Romania
[b] Faculty of Biology, Alexandru Ioan Cuza University, Iaşi, Romania
[c] UMR 1161 Virology, Anses, Animal Health Laboratory, Maisons-Alfort, France

ARTICLE INFO

Article history:
Received 4 July 2014
Received in revised form 29 August 2014
Accepted 2 October 2014

Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Hepatitis E virus
Swine
PCR
Human
Seroprevalence

SUMMARY

Objective: Swine hepatitis E virus (HEV) is considered to be a new zoonotic agent due to its close genomic resemblance to the human HEV. The aim of this study was to determine human HEV seroprevalence in eastern Romania and to characterize circulating swine HEV sequences.
Methods: Serological investigations of human serum samples were done using a commercial ELISA kit (MP Biomedicals). Swine faecal samples were tested to detect the HEV ORF2 sequence by nested reverse transcription PCR.
Results: One hundred and forty-eight human serum samples were tested for anti-HEV IgG of which 22 were found to be positive. Fresh swine faeces (pools) were collected from five farms in eastern Romania. Six out of 19 pooled samples were positive for HEV RNA. Phylogenetic analysis based on alignment of the ORF2 sequence indicated that the Romanian swine HEV isolates belonged to genotype 3.
Conclusions: This is the first study showing HEV to be present in Romanian pig herds and that the human population is exposed.

1. Introduction

The hepatitis E virus (HEV) is a major pathogen causing acute hepatitis in young people in developing countries where there is poor sanitation and a high population density. HEV infection is often asymptomatic but can induce a self-limited acute hepatitis.[1] Hepatitis E has been diagnosed in industrialized countries, and it has been demonstrated that the genomic sequence of isolated HEV strains is most related to swine HEV prevalent in the swine population from the same region.[2] The existence of a zoonotic reservoir has also been revealed, as the virus has been isolated from wild and domestic animals including swine, cattle, chickens, sheep, goats, and rodents.[3,4] The first animal virus strain was isolated from a pig in the USA.[5] Interspecific infections with hepatitis E among pigs and non-human primates have been demonstrated experimentally, suggesting the zoonotic nature of the disease.[6]

Human and swine HEV are classified in the *Hepeviridae* family as a separate *Orthohepevirus* genus *Orthohepevirus A* species.[7] Like most enteric viruses, HEV is a relatively stable particle, resistant to gastric secretions and bile salts, which explains its survival in the intestinal environment.[8] It is a small virus with a diameter between 27 and 33 nm, with an icosahedral structure without an envelope, which has shaped bumps visible on its surface.[9] The genome is represented by a single-stranded positive-sense RNA molecule of 7.2 kb in length, which contains three open reading frames (ORF).[10] Four major genotypes have been defined in mammals based on ORF2 nucleotide sequence analysis.[11] The four genotypes are classified into 24 subtypes, based on a sequence located at the 5′ end of the ORF2 region, which is the best conserved among all HEV isolates.[12] Genotypes 1 and 2 do not infect swine, while genotypes 3 and 4 are zoonotic.[13]

The natural transmission of swine HEV in pigs is via the faecal–oral route. HEV infection in swine usually remains asymptomatic. HEV replication has been demonstrated to occur in the liver and in the intestines.[14] Viraemia is transient and lasts 1 to 2 weeks. In infected pigs, faecal virus shedding may persist for up to 7 weeks.[15] Serological studies reveal a worldwide

* Corresponding author. Tel.: +40 74 4228143; fax: +40 232 407318.
E-mail address: epirovet@yahoo.com (G. Savuţa).

http://dx.doi.org/10.1016/j.ijid.2014.10.018

distribution of HEV infection in pigs, and the prevalence varies from 15% to 75%.[16–18] In Europe, HEV has been detected in swine in several countries; the isolated swine HEV has been characterized as genotype 3[19] and 4.[20] In Spain, HEV RNA detected in slaughterhouse sewage presented 92–94% nucleotide similarity with virus strains detected in humans.[21] The prevalence of swine HEV in France was estimated at 31% at the individual pig level and 65% at the farm level.[22] In the Netherlands, 97 pig farms were screened in 2005 and the prevalence rate of swine HEV was estimated at 55%.[23] Investigations of domestic swine in Hungary detected HEV in 39% of the pig farms involved.[24]

The purpose of this investigation was to assess the HEV seroprevalence in human samples and to detect the presence of swine HEV RNA in farm pigs from Romania, with genetic characterization and phylogenetic analysis.

2. Materials and methods

2.1. Sample collection

Human serum from anonymous donors was provided by hospitals in the North-eastern Region of Romania. The donors were undergoing routine haematological tests and had no hepatitis symptomatology. Serum from a total of 148 people aged between 10 and 90 years was evaluated between 2011 and 2012.

Pig stool samples (n = 19) were collected from five swine farms in the eastern part of Romania in 2009 and 2010. Faecal pools were sampled from shelters where the 2- to 4-month-old pigs were housed. All samples were transported at refrigeration temperature and stored at −80 °C until testing.

2.2. Anti-HEV serology

Tests for anti-HEV immunoglobulin (Ig) G were performed using a commercial ELISA kit (MP Biomedicals; catalogue number 0721150096T) in accordance with the manufacturer's instructions. This assay uses three recombinant polypeptides derived from the 3′ end of ORF2 (42 amino acids) and ORF3 (33 amino acids) from Burmese and Mexican prototype sequences (genotypes 1 and 2, respectively). For each determination, positive and negative controls, provided with the kit, were used. The cut-off value was calculated as 0.500 plus the mean absorbance of the non-reactive controls. IgG-reactive samples were retested using the same commercial kit.

2.3. Processing of stool samples

Pooled samples were resuspended in 10% calcium- and magnesium-free phosphate-buffered saline and centrifuged at 3000 rpm.

2.4. RNA extraction and nested RT-PCR

The RNA extraction was performed from 200 μl stool suspension supernatant using the QIAamp Viral RNA Mini Kit (Qiagen; catalogue number 52904). The extracted RNAs were stored at −80 °C until tested. Reverse transcription was performed using a random hexamer primer (Promega; catalogue number C1181).

For the detection of HEV RNA, a nested RT-PCR was performed using the degenerate primers.[13] All samples were tested for HEV RNA, targeting an ORF2 348-bp fragment. For the nested RT-PCR assay, two sets of primers were used: external primer set 3156N (5663 to 5684) and 3157N (6371 to 6393), and internal primer set 3158N (5948 to 5969) and 3159N (6274 to 6295). Negative and positive controls were included in each set of PCRs. Negative control (ultrapure water) was included in every reverse transcription, first PCR, and nested PCR. An extract from a positive HEV genotype 3 swine faecal sample was used as positive control.

The PCR parameters for the first-round PCR with primers 3156N and 3157N included a denaturation step at 94 °C for 1 min, followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C, extension for 30 s at 72 °C, and 10 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 40 °C, extension for 30 s at 72 °C, followed by a final incubation at 72 °C for 5 min.

At the end of the first-round PCR, 5 μl of the amplification product and an internal primer set were used to prepare a 50-μl reaction mix for the second-round PCR. Ten microlitres of DNA were analysed by agarose gel electrophoresis.

2.5. Nucleotide sequencing and sequence analyses

The amplified 348-bp fragments were excised from the gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega; catalogue number A9281). The purified products were directly sequenced for both strands with nested PCR primers using the CEQ 8000 Genetic Analysis System and dye-labelled dideoxy termination method (DTCS QuickStart sequencing reagent; Beckman Coulter, Fullerton, CA, USA).

Individual sequences were proofread and edited using CEQ 8000 software (Beckman Coulter), and merged into composite sequences with SeqMan software (Lasergene v. 7; DNASTAR Inc., Madison, WI, USA). Different sequences were used to root the phylogeny and were chosen as the best hits to our sequences in GenBank. Phylogenetic inference was conducted for the combined concatenated dataset. jModelTest version 2.0[25] was used to infer the best-fit model of evolution, based on hierarchical log-likelihood ratio tests comparing successively complex models. The evolutionary history was inferred using the maximum likelihood method based on the generalized time-reversible model (GTR+G). The tree topology search was done using the Best of NNIs and SPRs algorithm, over 100 bootstrapped replications of the dataset. The analysis involved 260 nucleotide sequences. Evolutionary analyses were conducted in PhyML v3.0,[26] run on the Phylemon 2.0 platform.[27]

3. Results

3.1. Serological results

Out of 148 human sera tested, 22 were positive for anti-HEV IgG. In sera analysed in 2011 (first study) a 17.14% seroprevalence (12/70) was estimated, whereas in 2012 (second study) a 12.82% seroprevalence (10/78) was recorded (Tables 1–3). Anti-HEV IgG antibody in subjects without clinical signs of hepatitis was used as an epidemiological tool to measure exposure to this virus. Our results revealed that HEV infections in the eastern area of Romania affect middle-aged adults.

3.2. Swine HEV detection

Faecal samples (19 pools) collected from five swine farms with pigs aged between 2 and 4 months were subsequently analysed by RT-PCR to detect the presence of HEV. The analysis of nested PCR

Table 1
Results of the serological study on human sera in 2011

	Patient age, years	Number of samples tested	Anti-HEV IgG-positive samples	Seroprevalence (%)	95% CI
Group 1	9–20	11	0	0%	0
Group 2	21–40	28	8	28.6%	11.8–45.3
Group 3	41–62	31	4	12.9%	1.1–24.7
Total		70	12	17.1%	8.3–26

CI, confidence interval.

Table 2
Results of the serological study on human sera in 2012: distribution of the tested sera by patient gender

Patient gender	No. of tested samples	Anti-HEV IgG-positive samples	Percent of positivity (%)
Female	38	6	15.78%
Male	40	4	10%
Total	78	10	12.82%

Table 3
Results of the serological study on human sera in 2012: distribution of the tested sera by patient age

	Patient age, years	Number of samples tested	Anti-HEV IgG-positive samples	Seroprevalence (%)	95% CI
Group 1	18–45	32	2	6.25%	−2.1–14.6
Group 2	46–65	25	7	28%	10.4–45.6
Group 3	66–90	21	1	4.76%	−4.3–13.9
Total		78	10	12.82%	5.4–20.2

products revealed six positive stool samples (Table 4). The virus was detected in pooled samples collected from pigs of different ages: 2.5 months old (farms B and E), 3 months old (farms C and A), and 4 months old (farm D). To characterize the viral strains circulating in the farm-pig populations, two of the positive PCR products were sequenced and analysed. A 347-nucleotide sequence (roEF5; GenBank accession number **KM058114**) and a 341-nucleotide sequence (roFPR4, GenBank accession number **KM042904**) were obtained.

3.3. Phylogenetic analysis

The two Romanian sequences were found to be distributed in different clades. According to the nucleotide sequence similarities analysis there was a 16.2% difference between the two newly identified swine HEV sequences.

4. Discussion

Autochthonous HEV infection, caused by genotype 3, is recognized as an emerging infectious disease in developed countries. Our previous investigation on HEV infection in eastern Romania revealed an anti-HEV IgG prevalence of 12% (3/25) in patients diagnosed with hepatitis B or C,[28] and a 5.9% anti-HEV IgG seroprevalence (4/67) in the general population.[29] In 2010, Voiculescu et al. reported an HEV seroprevalence of 12.5% in the very low risk population and 13.98% in the low risk population.[30]

In this study the presence of anti-HEV IgG was evaluated in 148 anonymous donors aged between 10 and 90 years. Eleven serum samples collected from children and adolescent patients (9–20 years old) were identified as negative for anti-HEV IgG. All infections seem to occur among middle-aged persons. These observations agree with the data recorded from patients with locally acquired hepatitis E in non-endemic countries.[31] The HEV antigens used in this kit included an ORF3 antigen, making it possible to detect a class of IgG antibody that is present early in infection.[32] The commercially available anti-HEV ELISA test (MP Biomedicals) uses antigens derived from genotypes 1 and 2 of HEV and may underestimate the HEV seroprevalence in non-endemic regions where genotype 3 HEV is the most prevalent.

In various studies, the IgG antibodies have proved to persist for 2–13 years.[33] The results of studies done in developed countries have shown that seropositivity increases in linear proportion with increasing age. In certain developed countries, the seroprevalence of anti-HEV IgG in the general population has been shown to be generally much higher than would be expected given the low prevalence of acute symptomatic hepatitis E cases. The prevalence of anti-HEV in human populations suggests the possibility of subclinical HEV infections and/or under-diagnosis. Autochthonous hepatitis E in developed regions is frequently misdiagnosed as drug-induced liver injury, a common problem that occurs at an increased frequency in elderly people.[34] The prevalence of HEV IgG antibodies in low-incidence populations in the developed world ranges from 3.2% in central France,[35] 4.9% in southwest Switzerland,[36] and 16% in southwest England,[31] to 21% in residents of the USA.[37] In Spain, the prevalence of IgG antibodies against HEV, which indicates exposure to this virus, varies from 0.6% to 7.3% in the general population, reaching 19% in individuals with risk factors such as exposure to pigs.[38] Our results on random serum samples demonstrated an overall seropositivity of 14.86%. The age-dependent seroprevalence could only be explained by age-dependent exposure or age-dependent susceptibility, which is difficult to consider for a food-borne pathogen.[39]

It has been reported that a large proportion of pigs are infected with HEV in the world. Our previous investigations regarding swine HEV infection in Romania consisted of serological testing for the detection of anti-HEV IgG in farm and backyard pigs. The immunoblot results of our investigations of farm pigs revealed 37 anti-HEV IgG-positive serum samples out of 95 tested (38.94%).[40] For the backyard pigs, we detected 34 positive serum samples out of 69 (49.27%) tested by immunoblot.[29]

In this study, two swine HEV strains isolated from farm pigs in Romania were characterized. To our knowledge, this is the first study to confirm the presence of swine HEV in Romania. All faecal samples were collected from swine without clinical symptoms. Swine HEV RNA was isolated from faecal samples collected from 2- to 4-month-old pigs. These results are similar to previously published data, confirming that HEV is circulating in all farms and in all age groups, from weaners to fatteners, and that pigs that are close to slaughter age can still be HEV-infected.[41] Romanian swine HEV isolates were grouped into genotype 3 by phylogenetic tree analysis and were closely related to the swine and human HEV isolates identified in European countries.

Analysis of the sequences (Fig. 1) revealed that, at the amino acid level, the partial capsid protein sequence (ORF2) of strain roEF5 presented a maximum 92% identity with a French human isolate (GenBank accession number **EU495231**) and 89% identity with a swine HEV isolate from wild boar in Germany (GenBank accession number **KF303501.1**), a French human strain (GenBank

Table 4
Results of swine hepatitis E virus detection

Farm	Pool sample	Pig age, months	N-PCR result
A	IS1	4	Negative
	IS4	3	Positive
B	IS2	2.5	Positive
	IS3	2	Negative
C	EF1	3	Negative
	EF2	3	Negative
	EF3	3.5	Negative
	EF4	3.5	Negative
	EF5	3	Positive
	EF6	3	Positive
D	FPR1	3	Negative
	FPR2	3	Negative
	FPR3	3.5	Negative
	FPR4	4	Positive
E	FM1	2.5	Negative
	FM2	2.5	Positive
	FM3	2	Negative
	FM4	2	Negative
	FM5	2	Negative

N-PCR, nested PCR.

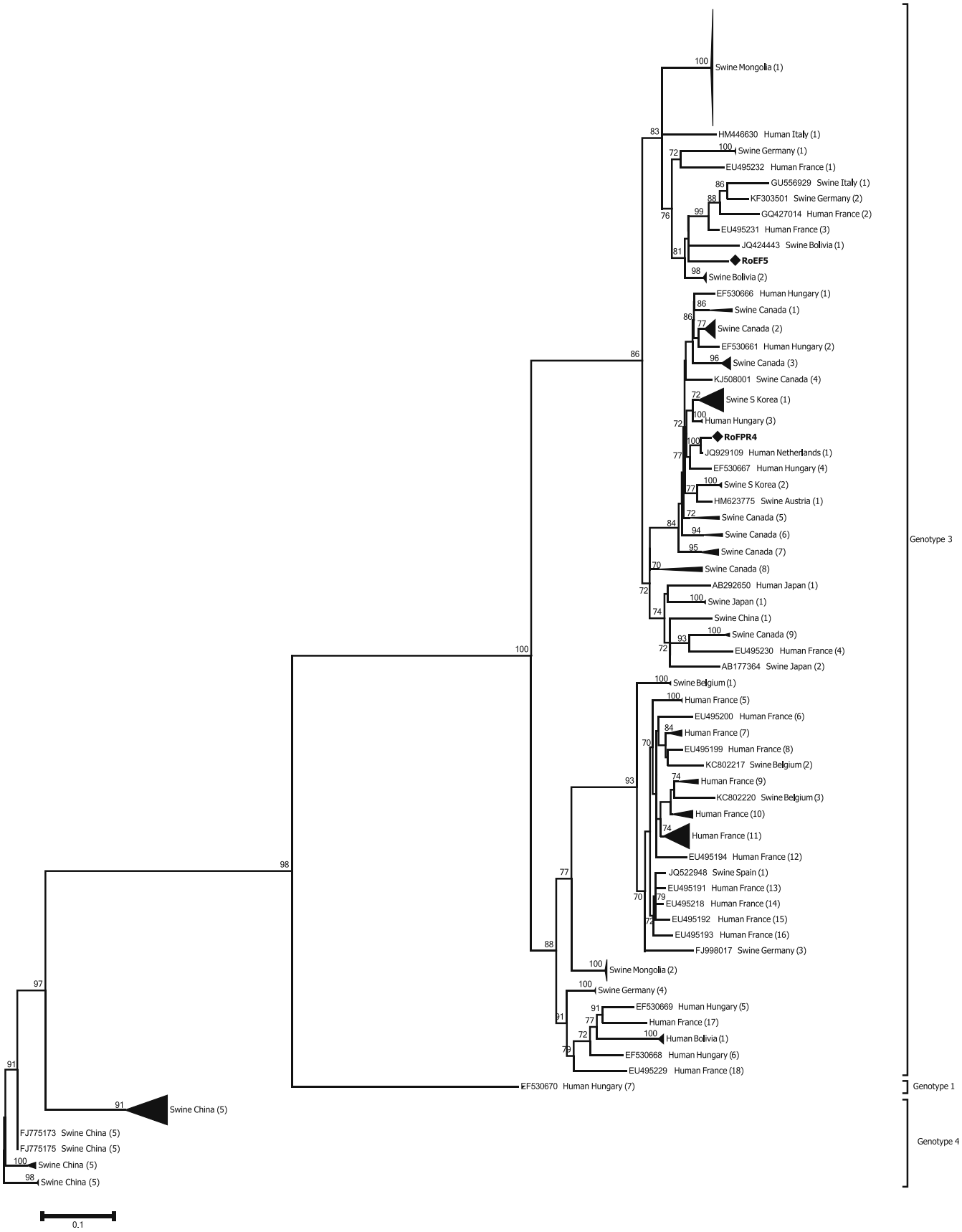

Figure 1. Phylogenetic tree analysis of the Romanian isolates of swine HEV using the maximum likelihood method based on the generalized time-reversible model. The analysis involved 260 nucleotide sequences. Evolutionary analyses were conducted in PhyML v3.0 run on the Phylemon 2.0 platform. Human and swine HEV included for comparison are indicated with their GenBank accession number, host, and country where the strain was isolated. The final number included in parenthesis refers to strain identification from polyphyletic clades listed in the **Supplementary Material**.

accession number **GQ427014**), and a human strain isolated in Italy (GenBank accession number **HM446630.1**). Phylogenetic analysis revealed that this swine HEV had 91% identity with six swine strains isolated in Bolivia (GenBank accession numbers **JQ424439.1**, **JQ424440.1**, **JQ424437.1**, **JQ424442.1**, **JQ424441.1**, and **JQ424438.1**) and 90% identity with three swine strains isolated in Japan (GenBank accession numbers **AB471981.1**, **AB471980.1**, and **AB292650.1**).

Similar results were obtained for pig livers in Germany,[42] where 4% of the samples tested were found to be positive for HEV. Sequence determination and phylogenetic analysis classified the isolate strains as HEV genotype 3. In Italy, HEV RNA was detected in 31 out of 48 pigs (64.6%).[43] The virus was found at a statistically higher rate in the 3- to 4-month-old pigs (95.0%) than in the 9- to 10-month-old ones (42.9%).

According to the nucleotide sequence phylogenetic tree of the ORF2 region (341 nucleotides) of the other isolated strain roFPR4, it was very closely related, presenting a maximum identity of 97% with a human isolate from the Netherlands (GenBank accession number **JQ929109**) and an identity of 93% with one human isolate from Hungary (GenBank accession number EF530667). The sequence identity of the analysed ORF2 fragment within the roFPR4 and the other strains from GenBank was in the range of 91–97%. Because of the lack of published studies describing human HEV circulation in Romania, we used human and swine HEV isolated in neighbouring European countries in our phylogenetic analysis.

In the Netherlands, different swine HEV strains were characterized and typed as genotype 3, showing close genetic similarity to European human strains.[44] The same results were obtained in France after comparing the genetic identity of HEV strains found in humans and in swine.[45]

Our results confirm that domestic pigs may represent an important animal reservoir of HEV infection. Intensive pig farming may have become the major amplifier of the virus. In our study, we found positive pooled faecal samples for HEV RNA at each farm. Positive samples were detected in young pigs aged 2.5–4 months.

In conclusion, the prevalence of HEV in humans in Romania is comparable to that in other European countries. The detection of HEV RNA in pooled faecal samples indicates the presence of HEV in pig farms, and swine are considered a reservoir for HEV infection in humans. The detection of swine positive for HEV raises concerns about the potential zoonotic transmission of this virus, either through exposure to the environment or by consumption of contaminated pork products.

Acknowledgements

We would like to thank the Public Health District Authority Iaşi for cooperation and for providing the human serum samples.

Funding: This work was supported by CNCSIS–UEFISCSU, project number PNII–IDEI 1104/2008.

Conflict of interest: The authors have no conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2014.10.018.

References

1. Worm HC, Van der Poel WH, Brandstatter G. Hepatitis E: an overview. *Microbes Infect* 2002;**4**:657–66.
2. Wu JC, Chen CM, Chiang TY, Tsai WH, Jeng WJ, Sheen IJ, et al. Spread of hepatitis E virus among different-aged pigs: two-year survey in Taiwan. *J Med Virol* 2002;**66**:488–92.
3. Favorov MO, Kosoy MY, Tsarev SA. Prevalence of antibody to hepatitis E virus among rodents in the United States. *J Infect Dis* 2000;**181**:449–55.
4. Meng XJ. Novel strains of hepatitis E virus identified from humans and other animal species: is hepatitis E a zoonosis? *J Hepatol* 2000;**33**:842–5.
5. Meng XJ, Purcell RH, Halbur PG, Lehman JR, Webb DM, Tsareva TS, et al. A novel virus in swine is closely related to the human hepatitis E virus. *Proc Natl Acad Sci U S A* 1997;**94**:9860–5.
6. Meng XJ, Halbur PG, Shapiro MS, Govindarajan S, Bruna JD, Mushahwar IK, et al. Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. *J Virol* 1998;**72**:9714–21.
7. Smith DB, Simmonds P, Jameel S, Emerson SU, Harrison TJ, Meng XJ, et al. Consensus proposals for classification of the family *Hepeviridae*. *J Gen Virol* 2014. http://dx.doi.org/10.1099/vir.0.068429-0.
8. Emerson SU, Purcell RH. Recombinant vaccines for hepatitis E. *Trends Mol Med* 2001;**7**:462–6.
9. Balayan MS. Type E hepatitis: state of the art. *Int J Infect Dis* 1997;**2**:113–20.
10. Tam AW, Smith MM, Guerra ME, Huang CC, Bradley DW, Fry KE. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology* 1991;**185**:120–31.
11. Schlauder GG, Mushahwar IK. Genetic heterogeneity of hepatitis E virus. *J Med Virol* 2001;**65**:282–92.
12. Meng XJ. Hepatitis E virus: animal reservoirs and zoonotic risk. *Vet Microbiol* 2009;**140**:256–65.
13. Cooper K, Huang FF, Batiste L, Rayo CD, Bezanilla JC, Toth TE, et al. Identification of genotype 3 hepatitis E virus (HEV) in serum and fecal samples from pigs in Thailand and Mexico, where genotype 1 and 2 HEV strains are prevalent in the respective human population. *J Clin Microbiol* 2005;**43**:1684–8.
14. Ha SK, Chae C. Immunohistochemistry for the detection of swine hepatitis E virus in the liver. *J Viral Hepat* 2004;**11**:263–7.
15. Pavio N, Meng XJ, Renou C. Zoonotic hepatitis E: animal reservoirs and emerging risks. *Vet Res* 2010;**41**:46.
16. Yoo D, Willson P, Pei Y, Hayes MA, Deckert A, Dewey CE. Prevalence of hepatitis E virus antibodies in Canadian swine herds and identification of a novel variant of swine hepatitis E virus. *Clin Diagn Lab Immunol* 2001;**8**:1213–9.
17. Rutjes SA, Bouwknegt M, van der Giessen JW, De Roda Husman AM, Reusken CB. Seroprevalence of hepatitis E virus in pigs from different farming systems in the Netherlands. *J Food Prot* 2014;**4**:640–2.
18. Walachowski S, Dorenlor V, Lefevre J, Lunazzi A, Eono F, Merbah T, et al. Risk factors associated with the presence of hepatitis E virus in livers and seroprevalence in slaughter-age pigs: a retrospective study of 90 swine farms in France. *Epidemiol Infect* 2013;**28**:1–11.
19. Lu L, Li C, Hadedorn CH. Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. *Rev Med Virol* 2006;**16**:5–36.
20. Monne I, Ceglie L, Di Martino G, Natale A, Zamprogna S, Morreale A. Hepatitis E virus genotype 4 in a pig farm, Italy, 2013. *Epidemiol Infect* 2014 May;**15**:1–5 [Epub ahead of print].
21. Pina S, Buti M, Cotrina M, Piella J, Girones R. HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain. *J Hepatol* 2000;**33**:826–33.
22. Rose N, Lunazzi A, Dorenlor V, Merhab T, Eono F, Eloit M, et al. High prevalence of hepatitis E virus in French domestic pigs. *Comp Immunol Microbiol Infect Dis* 2011;**34**:419–27.
23. Rutjes SA, Lodder WJ, Bouwknegt M, De Roda Husman AM. Increased hepatitis E virus prevalence on Dutch pig farms from 33 to 55% by using appropriate internal quality controls for RT-PCR. *J Virol Methods* 2007;**143**:112–6.
24. Forgách P, Nowotny N, Erdélyi K, Boncz A, Zentai J, Szucs G, et al. Detection of hepatitis E virus in samples of animal origin collected in Hungary. *Vet Microbiol* 2011;**143**:106–16.
25. Posada D. jModelTest: phylogenetic model averaging. *Mol Biol Evol* 2008;**25**:1253–6.
26. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 2010;**59**:307–21.
27. Sánchez R, Serra F, Tárraga J, Medina I, Carbonell J, Pulido L, et al. Phylemon 2.0: a suite of web-tools for molecular evolution, phylogenetics, phylogenomics and hypotheses testing. *Nucl Acids Res* 2011;**39**(Suppl 2):W470–4. http://dx.doi.org/10.1093/nar/gkr408.
28. Aniţă A, Aniţă D, Ludu L, Savuţa G. Seroepidemiological investigation of human and swine hepatitis in Botoşani County. Bulletin of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. *Vet Med* 2010;**67**:19–22.
29. Savuţa G, Aniţă A, Aniţă D, Ludu L, Duca E, Pavio N. Seroepidemiological researches regarding swine and human hepatitis E in Romania. *Lucr Şt Med Vet USAMVB Timişoara* 2008;**XLI**:309–13.
30. Voiculescu M, Iliescu L, Ionescu C, Micu L, Ismail G, Zilisteanu D, et al. A cross-sectional epidemiological study of HBV, HCV, HDV and HEV prevalence in the Sub-Carpathian and South-Eastern regions of Romania. *J Gastrointestin Liver Dis* 2010;**19**:43–8.
31. Ijaz S, Vyse AJ, Morgan D, Pebody RG, Tedder RS, Brown D. Indigenous hepatitis E virus infection in England: more common than it seems. *J Clin Virol* 2009;**44**:272–6.
32. Bendall R, Ellis V, Ijaz S, Ali R, Dalton H. A comparison of two commercially available anti-HEV IgG kits and a re-evaluation of anti-HEV IgG seroprevalence data in developed countries. *J Med Virol* 2010;**82**:799–805.

33. Panda SK, Jameel S. Hepatitis E virus: from epidemiology to molecular biology. *Viral Hepat Rev* 1997;**3**:227–51.
34. Dalton HR, Stableforth W, Thurairajah P, Hazeldine S, Remnarace R, Usama W, et al. Autochthonous hepatitis E in southwest England: natural history, complications and seasonal variation, and hepatitis E virus IgG seroprevalence in blood donors, the elderly and patients with chronic liver disease. *Eur J Gastroenterol Hepatol* 2008;**20**:784–90.
35. Boutrouille A, Bakkali-Kassimi L, Cruciere C, Pavio N. Prevalence of anti-hepatitis E virus antibodies in French blood donors. *J Clin Microbiol* 2007;**45**: 2009–10.
36. Kaufman A, Kenfak-Foguena A, André C, Canellini G, Bürgisser P, Moradpour D, et al. Hepatitis E virus seroprevalence among blood donors in southwest Switzerland. *PLoS One* 2011;**6**:e21150. http://dx.doi.org/10.1371/journal.-pone.0021150.
37. Kuniholm MH, Purcell RH, McQuillan GM, Engle RE, Wasley A, Nelson KE. Epidemiology of hepatitis E virus in the United States: results from the Third National Health and Nutrition Examination Survey, 1988–1994. *J Infect Dis* 2009;**200**:48–56.
38. Riveiro-Barciela M, Rodríguez-Frías F, Buti M. Hepatitis E: scale of the problem in Spain. *Gastroenterol Hepatol* 2012;**35**:719–24. http://dx.doi.org/10.1016/j.gastrohep.2012.03.003.
39. Slot E, Hogema BM, Riezebos-Brilman A, Kok TM, Molier M, Zaaijer HL. Silent hepatitis E virus infection in Dutch blood donors, 2011 to 2012. *Euro Surveill* 2013;**18**. pii: 20550.
40. Savuţa G, Aniţă A, Aniţă D, Ludu L, Pavio N. Preliminary epidemiological investigations regarding hepatitis E virus infection in swine from the North-east of Romania. *Bulletin USAMV-CN* 2007;**64**:356–8.
41. Martelli F, Caprioli A, Zengarini M, Marata A, Fiegna C, Di Bartolo I, et al. Detection of hepatitis E virus (HEV) in a demographic managed wild boar (*Sus scrofa scrofa*) population in Italy. *Vet Microbiol* 2008;**126**:74–81.
42. Wenzel JJ, Preiss J, Schemmerer M, Huber B, Plentz A, Jilg W. Detection of hepatitis E virus (HEV) from porcine livers in Southeastern Germany and high sequence homology to human HEV isolates. *J Clin Virol* 2011;**52**:50–4.
43. Di Bartolo I, Ponterio E, Castellini L, Ostanello F, Ruggeri FM. Viral and antibody HEV prevalence in swine at slaughterhouse in Italy. *Vet Microbiol* 2011;**149**: 330–8.
44. Rutjes SA, Lodder WJ, Lodder-Verschoor F, van den Berg HH, Vennema H, Duizer E. Sources of hepatitis E virus genotype 3 in the Netherlands. *Emerg Infect Dis* 2009;**15**:381–7.
45. Bouquet J, Tessé S, Lunazzi A, Eloit M, Rose N, Nicand E, et al. Close similarity between sequences of hepatitis E virus recovered from humans and swine, France, 2008–2009. *Emerg Infect Dis* 2011;**17**:2018–25.