Acta Veterinaria (Beograd), Vol. 59, No. 2-3, 205-211, 2009.

DOI: 10.2298/AVB0903205S UDK 619:616.36-002

FIRST REPORT OF SEROLOGICAL EVIDENCE OF HEPATITIS E VIRUS INFECTION IN SWINE IN NORTHERN GREECE

SIOCHU ASIMOULA*, TZIKA ELENA**, ALEXOPOULOS C**, KYRIAKIS SC*** and FROESNER G****

**Department of Medical Laboratories, Technological Institution of Thessaloniki, Greece; **Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece; ***National Agricultural Research Foundation, Athens, Greece; ****Max Von Pettenkofer Institute, Medical Faculty, Ludwig-Maximilians-University, Munich, Germany*

(Received 14. October 2008)

Hepatitis E virus (HEV) infection is responsible for large epidemics of acute hepatitis and sporadic cases in developing countries. Nowadays, there is an implication of viral transmission from pigs to humans assuming that hepatitis E might be a zoonosis, while swine is considered as a reservoir of HEV. The results of the present study have shown that all the tested swine farms from Northern Greece were positive for anti-HEV IgG (the percentage of positive anti-HEV IgG samples in tested farms ranged between 67 to 90%). Moreover, 80% of the tested serum samples from healthy pigs aged 5-6 months were found positive. Additionally, a low rate of anti-HEV antibodies against ORF3 peptide in pigs aged 5-6 months was revealed. Consequently, it seems that the HEV infection is relented in older pigs and that after the age of 5-6 months pigs are less hazardous as far as human infection is considered.

Key words: hepatitis E virus, IgG, swine, Greece

INTRODUCTION

Hepatitis E is responsible for large epidemics of acute hepatitis in the human population in many developing countries. Hepatitis E Virus (HEV) is transmitted by the faecal-oral route, often causing water-born epidemics. HEV the causative agent of hepatitis E, is a positive single-stranded RNA virus without an envelope with a diameter from 27 to 38 nm (Reyes, 1997). The genome, which is about 7.25 kb in size, contains three partially overlapping open reading frames (ORF's) and a short 5' and 3' noncoding region (Tam *et al.*, 1991). HEV, on the basis of comparative phylogenetic analysis, was recently reclassified as the sole member of the family *Hepeviridae* of the genus *Hepevirus*, which is related to caliciviruses (Emmerson *et al*., 2004).

In developed countries clinical cases of hepatitis E are rare and most often have been associated with visits to endemic areas. However, sporadic cases of acute hepatitis E have also been reported in individuals, with not any known

epidemiological risk factors, in countries such as USA, the Netherlands, Germany and New Zealand (Worm *et al*., 1998; Preiss *et al.*, 2006). Lately, novel strains of HEV have been isolated in USA and Europe from patients without a history of visiting regions endemic for HEV, considering that these new strains must be of indigenous origin. Serological studies in industrialized countries have shown that the prevalence of anti-HEV antibodies in humans is quite high (up to 21%) (Drobeniuc *et al*., 2001; Meng *et al*., 2002).

The cause of this relative high prevalence is not fully clarified yet. However, there is evidence that the virus may be a zoonotic agent. Interspecies transmission of HEV has been already demonstrated after the experimental infection of non-human primates by swine HEV strain and vice versa (Meng *et al*., 1998). The comparison between human and swine strains originated from the same country, even in non-endemic regions, revealed a high degree of homology in the nucleotide sequence (Meng *et al.*, 1997; Schlauder *et al*., 1998; Schlauder *et al*., 1999; Zanetti *et al*., 1999). Consequently, there is the implication of a possible transmission of the virus from pigs to humans assuming that hepatitis E might be a potential zoonosis.

The existence of anti-HEV antibodies in the swine population has been reported in various countries (Froesner *et al.*, 1998; van der Poel *et al*., 2001). Referring to the European swine population, during the last 5 years, HEV has been detected in Spain, the UK, the Netherlands and in Italy. The aim of the present study was to investigate the presence of anti-HEV antibodies in northern Greek swine farms.

MATERIALS AND METHODS

Swine serum samples

A total of 96 blood serum samples were collected from apparently healthy market-age pigs (5-6 months of age) from four slaughterhouses. The study was performed during February and March 2002. The sampled pigs were derived from 10 different swine farrowing to finishing farms located in Northern Greece. All blood samples included in this study were centrifuged (3 000 g per min) and the serum was harvested and stored at –20 ^{o}C (for 10 days) until assayed.

Method of anti-HEV antibodies detection

The assessment of anti-HEV IgG in serum was performed with the use of a commercially available Western blot method (recomBlot HEV, Mikrogen GmbH, Munich, Germany), a qualitative *in vitro* test which is based on electrophoresis in order to separate antigens. Thus, it allows a secure detection of special antibodies against specific HEV peptides and it is characterized by a great sensitivity and especially a great specificity.

Instead of HRP-conjugated human IgG antibodies a HRP-conjucated swine antibody (Sigma Corporation) was used in dilution of 1:1000. In this blood antibodies four recombinant HEV-proteins were used: the ORF 2 N-terminal peptide, 5220-5820 nucleotide, the ORF 2 M peptide, 5804-6390 nucleotide, the ORF 2 C peptide, 6350-7130 nucleotide and the ORF 3 peptide, 5100-7130

nucleotide. These four peptides derived from a completely sequenced HEV strain of genotype 1 (Mandras/India HEV strain genome/Gen Bank accession number: X 99441) (Froesner *et al*., 1999).

The purified, recombinant antigens were separated according to their molecular weight by SDS polyacrylamide gel electrophoresis. Thereafter, the HEV peptides were transferred electrophoretically to strips of nitrocellulose membrane (western blotting). In order to detect the anti-HEV IgG, these strips were incubated with the diluted serum samples for 2 hours. After washing the strips with the recomBlot ready-to-use buffer and adding of peroxidase conjucated anti-HEV IgG, a second incubation period of 1 hour followed. Lastly, the strips were washed again with the buffer. After the addition of a ready-to-use substrate solution to each one and incubation for 10-15 minutes the strips were washed with deionized water and evaluated.

The evaluation of the intensity of the reaction bands was accomplished with the aid of Table 1, according to the manufacturer's instructions (Mikrogen GmbH, Munich, Germany). The sum of points for each test strip was calculated in order to determine the positive samples. Thus, the samples with two or more bands presented (sum of points ≥4) in the test strip characterised as anti-HEV IgG positive. Correspondingly, the samples with the sum of 3 points defined as equivocal and those with points 0-2 as negative.

Table 1. Intensity of antigen band in the test strip of recomBlod HEV IgG method

Reaction	Intensity	Points
No reaction	-	0
Very weak reaction	+/-	1
Weak reaction (Corresponds with the most upper band of the triple band (O2-C) in the weak positive serum control IgG)	+	2
Normal and strong reaction	++ or (+++)	3

Data were subjected to descriptive analysis using the SAS Statistical Package ("The SAS® System", release 8.1 for WINDOWS - 2002/ SAS Institute Inc., Cary, NC 27513, USA).

RESULTS AND DISCUSSION

In cases where anti-HEV IgG were present, immunocomplexes were formulated, and presented in the strips after implementation of the immunoenzyme reaction. The evaluation of the test trips was performed regarding to the appearance or not of bands at the corresponding positions above the control bands of the test strip.

Healthy pigs aged 5-6 months from Northern Greece, deriving from 10 different herds, were tested for the prevalence of anti-HEV IgG. All ten herds were positive for anti-HEV IgG (the percentage of positive anti-HEV IgG samples in tested farms ranged between 67 to 90%). From the total of 96 serum samples collected, 76 (80%) were found to be positive.

Moreover, it was revealed that in 84% of the positive samples one of the bands corresponded in peptide ORF2N, 97% in peptide ORF2C, 63% in peptide ORF2M and 13% serum samples in peptide ORF 3 (Table 2).

Table 2: Anti-HEV antibodies against HEV peptides (%)

Positive samples	ORF2N	ORF2C	ORF2M	ORF3
76/96	64/76 (84.2%)	74/76 (97.4%)	48/76 (63.2%)	10/76 (13.2%)

The noticeable difference between high anti-HEV IgG prevalence and low percentage of cases with clinical disease in industrialized countries comprised a riddle for several years. Recently, cases of acute forms of hepatitis E have been reported in the population with no travelling history in endemic countries (Thomas *et al.*, 1997; Schlauder *et al*., 1998; Worm *et al*., 1998; Schlauder *et al*., 1999; Zanetti *et al*., 1999).

Anti-HEV antibodies have been detected in animals such as cows, goats, sheep, rodents, dogs and *Rhesus macaques* in endemic regions. As a consequence, it has been considerated that animals could be a reservoir of the virus. Recent studies from all over the world encourage this hypothesis (Favorov *et al*., 1998; Maneerat *et al*., 1996).

Already from 1990 Balayan *et al*. ascertained that pigs can be infected by human HEV strains. In Nepal, an endemic region, anti-HEV antibodies were detected in 30% of the swine population. Further studies refer high rates of anti-HEV IgG in pigs all over the world thus HEV is widespread in the swine population (Froesner *et al.*, 1998; Chandler *et al*., 1999; Wu *et al*., 2002). These data corroborate the thesis that HEV is enzootic in swine independently from its presence in humans.

After challenge exposure of pigs with HEV, viral RNA was detected in faecal samples for 2-6 weeks and in serum samples for 4-6 weeks. The transmission of HEV through the faecal-oral route makes possible human infection from pigs. The significant genetic similarity of the genome between swine and human HEV strains in the same geographic regions and the aspect that there might be a potential cross reaction between these strains, supports the hypothesis that pigs likely constitute a "depository" for HEV. As a consequence, pigs could be a source of human infection with HEV.

Anti-HEV rarely detected in pigs aged less than 3 months of age anti-HEV. On the contrary anti-HEV have been detected in almost 85% of pigs older than 5 months of age. Antibodies against HEV have been found in pigs which were originally negative in hepatitis E infection, after their close contact with

seropositive pigs. The virus' RNA has been also found in faeces of animals that were previously challenged to exposure to the virus strain derived from animals suffering from natural infection. Likewise, anti-HEV in serum and RNA of SwUS-HEV in faeces have been found in pigs inoculated with the SwUS HEV strain, while histological examination of liver samples revealed the existence of hepatitis lesions. Additionally, the virus' RNA has also been detected in slaughterhouses' manure (Pina *et al*., 2000; Kasorndorbua *et al*., 2002).

The existence of HEV in Greece remained unclear until now. Thus, the purpose of the present study was to detect the presence of anti-HEV antibodies in blood serum samples of clinically healthy pigs. The assessment of anti-HEV IgG in serum was performed with the use of a commercially available Western blot method (recomb HEV, Mikrogen GmbH, Munich, Germany). The method proved very successful and showed that the epitope of human strains can be identified from swine anti-HEV antibodies also, a fact indicating the possibility of the existence of a cross reaction.

For the first time, the results of the present study showed, a very high prevalence of anti-HEV IgG in healthy pigs (80%) of the targeted aged group (5-6 months of age), a result congruent to those of previous studies in other countries. Thereby, it could be thought that the virus is widespread, at least in the Northern Greek, swine population. Moreover, a low rate of anti-HEV antibodies against ORF3 peptide in pigs aged 5-6 months was revealed. It should be noted that the ORF3 peptide has been postulated to be a viral regulatory protein involved in the modulation of cell signalling. The ORF3 protein of HEV appears to be the first example of tyrosine kinase homology 3 domain-blinding protein encoded by a virus that causes an acute infection and self-limited infection (Korkaya *et al*., 2001). ORF3 could be accounted as an indicator of a recent infection, rarely detected in older infections. Consequently, it seems that the HEV infection is relented in older pigs and that after the age of 5-6 months pigs are less hazardous for human infection.

Apart from the assumption that hepatitis E could be a zoonosis that can be transmitted from pigs to humans and has relative consequences for public health, the possibility of being a xenoanthropozoonosos must be put also into consideration. At the xenotransplantation procedure organs derived mainly from swine, because of their small size, which approximates that of human organs. Additionally pig production is relatively simple, with high expectation of life. However, individuals are under immunosuppresion during the transplantation procedure, so there are propitious circumstances for the occurrence of hepatitis E, if the transplanted organ of swine origin is contaminated by the virus. The fact that anti-HEV antibodies against ORF3 peptide is found in low rates in pigs aged 5-6 months should be under consideration in these cases.

Address for correspondence:
Asimoula Siochu
Kazazi 31
55133 Thessaloniki
Greece
E-mail: siochu@otenet.gr

REFERENCES:

1. *Balayan MS, Usmanov RK, Zamyatina DI, Karas FR*, 1990, Experimental hepatitis E infection in domestic pigs, *J Med Virol*, 32, 58-9.
2. *Chandler JD, Riddell MA, Li F, Love RJ, Anderson DA*, 1999, Serological evidence for swine hepatitis E virus infection in Australian pig herds, *Vet Microbiol,* 68, 95-105.
3. *Drobeniuc J, Favorov MO, Shapiro CN, Bell BP, Mast EE, Dadu A et al,* 2001, Hepatitis E virus antibody prevalence among persons who work with swine, *J Infect Dis*, 84, 12, 1594-7.
4. *Emerson SU, Anderson D, Arankalle A, Meng XJ, Purdy M, Schlauder GG et al,* 2004, Hepevirus. In: Faquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, editors, Virus taxonomy. VIIIth Report of the ICTV, London, United Kingdom: Elsevier/Academic Press; 851-5.
5. *Favorov MO, Nazarova O, Margolis HS*, 1998, Is hepatitis E an emerging zoonotic disease? *Am J Trop Med Hyg*, 59, 242.
6. *Froesner GG, Seebach J, Kraemer B*, 1999, Hepatitis E Serodiagnostik mit einem neuen Western Blot auf der Basis rekombinanter Antigene HEV. Poster in the Annual Meeting of the German Society for Hygiene and Microbiology (DGHM), Regensburg, Germany, Oct 1999.
7. *Froesner GG, Subburayalu A, Nitschko H,* 1998, High prevalence of hepatitis E related virus in German pigs, but no indication of transmission to humans. Poster in the "Progress in Clinical Virology IV" Symposium of the "European Society for Clinical Virology", Hamburg, Germany.
8. *Kasorndorkbua C, Halbur PG, Thomas PJ, Guenette DK, Toth TE, Meng XJ*, 2002, Use of a swine bioassay and a RT-PCR assay to assess the risk of transmission of swine hepatitis E virus in pigs, *J Virol Meth*, 101, 1-2, 71-8.
9. *Korkaya H, Jameel S, Gupta D, Tyagi S, Kumar R, Zafrullah M et al.,* 2001, The ORF3 Protein of Hepatitis E Virus Binds to Src Homology 3 Domains and Activates, *J Biol Chem*, 276, 42389-400.
10. *Maneerat Y, Clayson ET, Myint KS, Young GD, Innis BL,* 1996, Experimental infection of the laboratory rat with the hepatitis E virus, *J Med Virol*, 48, 121-8.
11. *Meng XJ, Purcell RH, Halbur PG, Lehman JR, Webb DM, Tsareva TS et al.,* 1997, A novel virus in swine is closely related to the human hepatitis E virus. Proc Natl Acad Sci USA, 94, 9860-5.
12. *Meng XJ, Halbur PG, Shapiro MS, Govindarajan S, Bruna JD, Mushahwar IK et al*, 1998, Genetic and experimental evidence for cross-species infection by swine hepatitis E virus, *J Virol*, 72, 9714-21.
13. *Meng XJ, Wiseman B, Elvinger F, Guenette DK, Toth TE, Engle RE et al,* 2002, Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries, *J Clin Microbiol*, 40, 1, 117-2.
14. *Pina S, Buti M, Cotrina M, Piella J, Girones R*, 2000, HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain, *J Hepatol*, 33, 826-33.
15. *Preiss JC, Plentz A, Engelmann E, Schneider T, Jilg W, Zeitz M et al.,* 2006, Autochthonous hepatitis E virus infection in Germany with sequence similarities to other European isolates. *Infection*, 34, 173-5.
16. *Reyes GR,* 1997, Overview of the epidemiology and biology of the hepatitis E virus. In: Willson RA, editor, Viral hepatitis, NY: Marcel Dekker Inc, 239-58.
17. *Schlauder GG, Dawson GJ, Erker JC, Kwo PY, Knigge MF, Smalley DL et al.,* 1997, The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States, *J Gen Virol*, 79, 447-56.
18. *Schlauder GG, Dawson GJ, Erker JC, Kwo PY, Knigge MF, Smalley DL et al*., 1998, The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States, *J Gen Vir*, 79, 447-56.
19. *Schlauder GG, Desai SM, Zanetti AR, Tassopoulos NC, Mushahwar IK*, 1999, Novel hepatitis E virus (HEV) isolates from Europe: evidence for additional genotypes of HEV, *J Med Virol*, 57, 243-51.
20. *Tam AW, Smith MM, Guerra ME, Huang CC, Bradley DW, Fry KE et al.,* 1991, Hepatitis E virus: molecular cloning and sequencing of the full-length viral genome, *Virology*, 185, 120-31.

21. *Thomas DL, Yarbough PO, Vlahov D, Tsarev SA, Nelson KE, Saah AJ et al.,* 1997, Seroreactivity to hepatitis E virus in areas where the disease is not endemic, *J Clin Microbiol*, 35, 1244-7.
22. *van der Poel WHM, Verschoor F, van der Heide R, Herrera M-I, Vivo A, Kooreman M et al.*, 2001, Hepatitis E virus sequences in swine related to sequences in humans, the Netherlands, *Emerg Infect Dis*, 7, 970-6.
23. *Worm HC, Wurzer H, Froesner GG,* 1998, Sporadic hepatitis in Austria, *N Engl J Med,* 339, 1554-5.
24. *Wu JC, Chen CM, Chiang TY, Tsai WH, Jeng WJ, Sheen IJ et al,* 2002, Spread of hepatitis E virus among different-aged pigs: two-year survey in Taiwan, *J Med Virol*, 66, 4, 488-92.
25. *Zanetti AR, Schlauder GG, Romano L, Tanzi E, Fabris P, Dawson GJ et al,* 1999, Identification of a novel variant of hepatitis E virus in Italy, *J Med Virol,* 57, 356-60.

PRVA SEROLOŠKA POTVRDA HEPATITISA TIPA E KOD SVINJA U SEVERNOJ GRČKOJ

SIOCHU A, TZIKA ED, ALEXOPOULOS C, KYRIAKIS SC i FROESNER G

SADRŽAJ

Infekcije virusom hepatitisa tipa E (HEV) mogu da izazovu epidemije širokih razmera ili samo sporadične slučajeve oboljevanja u zemljama u razvoju. Postoje implikacije da je moguća transmisija ovog virusa sa svinja na ljude i da hepatitis E predstavlja zoonozu, sa svinjama kao rezervoarima HEV. Rezulati naših ispitivanja ukazuju da su sve ispitivane farme u severnoj Grčkoj bile pozitivne na anti-HEV Ig G antitela (procenat pozitivnih uzoraka je varirao od 67-90). Dodatno, više od 80% uzoraka poreklom od zdravih svinja starih 5-6 meseci je takođe bilo pozitivno. U ovoj grupi životinja je bio nizak nivo pozitivnih jedinki na anti-HEV antitela protiv ORF3 peptida. Izgleda da je kod starijih jedinki infekcija blaga i da one nose manji rizik za zdravlje ljudi.