

ORIGINAL ARTICLE

Evidence of Hepatitis E Virus (HEV) infection in human and pigs in Sardinia, Italy

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Key words

HEV Prevalence • Zoonosis • Swine

Summary

Introduction. The aim of this study was to determine the seroprevalence of anti-HEV antibodies in humans sera and to study HEV prevalence in swine from different Sardinian farms, testing viral HEV-RNA in bile samples.

Methods. In the first six months of 2008, 532 subjects of whom 402 blood donors and 130 workers at zoonotic risk, were enrolled. Anti-HEV were determined with an enzyme linked immunosorbent assay (ELISA). In positive subjects, RNA was extracted and tested by RT-Nested-PCR.

From July 2006 to March 2007, 95 bile samples were collected from randomly selected pigs. RNA was extracted from 250 µl of bile and tested by RT-Nested-PCR.

Results. The overall prevalence of anti-HEV antibodies was 4.3%; 5.0% among blood donors and 2.3% among workers at

zoonotic risk, with no statistically significant differences between sex, age classes and occupation. The search for HEV-RNA in the subjects positive for antibodies, gave negative results.

HEV genome was detected in 6 of the 95 swine bile samples tested. Sequences were clustered within the genotype 3 and are edited on GenBank under accession number: from FJ850960 to FJ850962 and from FJ883000 to FJ883002.

Discussion. The overall prevalence of anti-HEV shows that the virus circulates without giving origin to cases of acute hepatitis. The low prevalence value found in workers at zoonotic risk do not apparently support the hypothesis of professional risk. In this study, HEV-RNA was isolated from pigs in Sardinia for the first time confirming the role of swine as HEV reservoir and the possibility of virus transmission to humans.

Introduction

Hepatitis E virus (HEV) is responsible for a faecal-oral transmitted disease with the clinical characteristics of acute hepatitis, already known as non-A, non-B and non-C hepatitis. Hepatitis E was not recognized as a distinct human disease until 1980; the highest rates of infection occur in regions where low standards of hygiene promote virus transmission, especially where faecal contamination in drinking water or food is common [1]. However, sporadic cases of hepatitis E have also been reported in industrialized countries, including Italy, and serological surveys suggest the diffusion of strains of hepatitis E of low pathogenicity [2-6]. The worldwide confirmation of anti-HEV in the sera of different animal species, (non-human primates, pigs, bovines, sheep, poultry, dogs, cats and wild animals), has suggested the hypothesis that these animals could be infected by HEV-like viruses and that they may act as a reservoir for the infection [3, 4, 7, 8]; at the same time another hypothesis suggests that some human cases reported in literature are to be considered of zoonotic origin [9]. This hypothesis is reinforced by the evidence of cases of food-borne HEV transmission described in humans after consuming raw or undercooked meat from wild boars or organs from pigs [10-12] and also from a recent case of human hepatitis E transmitted

via contact with a pet pig in Vietnam [13]. This disease is today considered an emergent zoonosis and could, in the future, be treated as a professional disease. The aim of this study was to determine the prevalence of HEV immunoglobulin (IgG anti-HEV) in blood donors and workers at zoonotic risk, in order to evaluate whether occupation may be a potential risk of infection, and to study HEV-RNA prevalence in swine from different Sardinian farms for evaluating any possible involvement of these animals in transmission of the virus.

Material and methods

HUMAN SAMPLES

In the first six months of 2008, 532 persons (389 males and 143 females) of whom 402 blood donors and 130 workers at zoonotic risk (35 abattoir workers; 95 laboratory workers exposed to biological swine material) were enrolled.

All the subjects had given informed consent to take part in this work. The study was conducted in compliance with the Helsinki Declaration and with Law Decree n. 196/2003, article 24 (Code for the protection of personal data).

Samples were divided into 4 age classes (20-29, 30-39, 40-49 and ≥ 50 years). The occupations of the blood donors are shown in Table I.

A commercially available diagnostic anti-HEV ELISA kit (Human anti-HEV ELISA) manufactured by Diagnostic Bioprobes Srl, Milan, Italy was used to detect anti-HEV antibodies in the serum samples. The ELISA test is based on the use of synthetic antigens corresponding to the immunodominant epitopes found in ORF2 and ORF3 of the Mexico strain and the Burma strain. The assay, a third-generation ELISA for the qualitative determination of IgG antibodies to HEV in human serum, was performed according to the manufacturer's instructions where it presents 99.5% specificity and 100% sensitivity.

In positive subjects, RNA was extracted from 200 μ l of sera by using High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer's instructions.

SWINE SAMPLES

From July 2006 to March 2007, 95 bile samples were collected from randomly selected pigs, aged 8-9 months (fatteners) during the slaughtering process; a bile sample was withdrawn from each animal through the gallbladder wall using a sterile syringe (used once and then discarded), and stored at -20°C until processing. Animals belonged to six different Sardinian intensive breeding farms. All the sampled pigs appeared to be clinically healthy. RNA was extracted from 250 μ l of bile using TRIZOL LS reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions [14].

RT-NESTED-PCR

RNA reverse transcription (RT) and first PCR reaction were conducted using a Superscript III Onestep RT-PCR System with Platinum Taq DNA polymerase (Invitrogen) according to the manufacturer's instructions. The RT-PCR reaction was conducted in a MyCycler (Bio-Rad, Hemel Hempstead, UK) thermal cycler under the following conditions: 50°C for 30 min for RT, followed by 25 cycles of denaturation at 94°C for 10 s, annealing at 49°C for 30 s, elongation at 68°C for 1 min and a final elongation at 68°C for 7 min. Nested PCR was conducted using a Platinum PCR Supermix (Invitrogen) according to the manufacturer's instructions, following the subsequent thermal conditions: initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 2 min and a final elongation at 72°C for 7 min.

For RT-PCR and Nested PCR, sets of degenerate primers, HEVORF2con-a1/HEVORF2-con-S1 and HEVORF2con-a2/HEVORF2con-S2 [15], amplifying a 154 bp region of the HEV open reading frame 2 (ORF2) were used. Amplified products were visualized in a 2% agarose gel stained with SybrSafe DNA Gel Stain (Invitrogen) and visualized by a blue-light transilluminator. Positive samples were tested with a different

Tab. I. Blood donors occupations.

Occupation	N. subjects
1. Clerk workers	220
2. Health workers	20
3. Military personnel	30
4. Stud/housewives/senior citizens	40
5. Freelance	37
6. Unknown	54

set of degenerate primers;3156N, 3157N, 3158N and 3159N [16], amplifying a 348 bp sequence under the same run conditions.

CAPILLARY SEQUENCING PROCEDURE

PCR products were sequenced by a conventional capillary sequencing procedure [17]. The sequence reaction was performed in 10 μ l volumes using a Big Dye chemistry Kit (Perkin-Elmer Applied Biosystems Division, Foster City, Calif) according to the manufacturer's instructions. The mixture contained: 5 μM of each primer, 2 mM MgCl_2 , and 1,5 μ l of purified PCR products. The cycling parameters were 25 cycles consisting of denaturation at 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 sec. The sequences were determined with an ABI Prism 310 automatic sequencer (Applied Biosystems Foster City, Calif.). The results were edited and analyzed by the Basic Local Alignment Search Tool (<http://www.ncbi.nlm.nih.gov/BLAST/>).

SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS

HEV capsid protein gene sequences of all the isolates were compared with those of previously described strains of HEV deposited in the NCBI GenBank. Nucleotide sequences were aligned using CLUSTAL X version 1.83.9 A phylogenetic tree was constructed by GeneBee-NET:Internet-based server with TreeTop-Phylogenetic Tree Prediction [18, 19]. A sequence variation analysis was performed using the MegAlign program from DNASTAR. A helpful motif sequence, for a comparative clear phylogenetic analysis, was only obtained for 3/6 samples, corresponding to GenBank Accession numbers from FJ850960 to FJ850962. The remaining samples showed a scant DNA fragment size by sequencing for comparative analysis.

STATISTICAL ANALYSIS

Frequencies for the different groups were expressed with the respective confidence intervals at 95% (CI 95%) according to a Poisson distribution, and average values with relative standard errors (ES). Significance of differences of anti-HEV between the groups' age and sex was calculated by the chi square test. Statistical significance was established at $p < 0.05$. The Mantel-Haenszel test was used to assess the association of HEV between blood donors and workers at zoonotic risk, adjusted for the potential confounding effect of gender and age.

Results

HUMAN SUBJECTS

The overall prevalence of anti-HEV was 4.3% (23/532 CI 95% 2.7-5.8); 5.0% (20/402 CI 95% 2.3-5.9) among blood donors and 2.3% (3/130, CI 95% 0.2-5.0) among abattoir workers and /or laboratory workers exposed to biological swine materials (Fig. 1).

No statistically significant differences ($p > 0.05$) in anti-HEV prevalence were detected between sex and age classes; similarly, we did not find any differences in anti-HEV prevalence according to occupation (Tab. II). Finally, the search for the genome of the virus in the 23 subjects, testing positive for antibodies, gave negative results.

HEV PREVALENCE IN SWINES

HEV genome was detected in 6 of the 95 bile samples tested (6.3% CI 95% 1.4-11.2).

Sequences obtained from these RT-PCR products, have been deposited with *Genbank* accession numbers from FJ850960 to FJ850962 and from FJ883000 to FJ883002.

PHYLOGENETIC ANALYSIS

The phylogenetic tree (on 3 samples) was generated using GeneBee software from HEV capsid protein gene

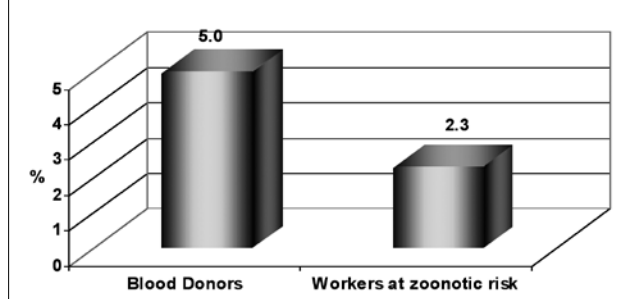
sequences obtained with swine positive samples. These results (Fig. 2) showed that at least 2 Sardinian isolates (isolates n. 5 and n. 10) belonged to a unique lineage and similar comparative distances were found with European (France, Netherlands) strains, thus indicating a possible virus spread between these areas. Swine sequences were clustered within the genotype 3.

Discussion

The overall prevalence of anti-HEV antibodies appears to be in agreement with that noticed in a previous study conducted in Italy in the '90s (4.3% vs 5.3% $p > 0.05$) [20].

However these values are still lower compared to prevalence found in other countries with a similar socio-economic and health status, in which Hepatitis E is generally a sporadic disease but where sometimes lethal cases of fulminant Hepatitis have also been reported [21, 22]. Furthermore, the fact that a number of European countries, such as Germany, have classified Hepatitis E as a notifiable infectious disease for several years, means that they have well defined case records [23] whereas Italy only started to do so in 2008. The SEIEVA (Integrated Epidemiological system for Acute Viral Hepatitis), coordinated by the *Istituto Superiore di Sanità*

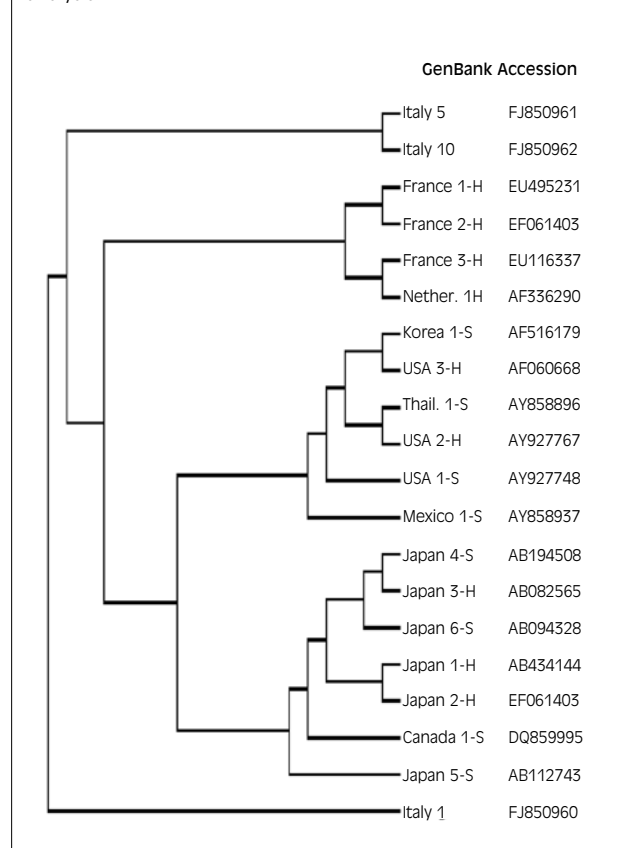
Fig. 1. Prevalence of anti-HEV among blood donors and workers at zoonotic risk.



Tab. II. Anti-HEV prevalence between age classes, sex and subject category.

Age group	pos	No. subjects	% pos	CI _{95%}
20-29	2	69	2.9	1.9-7.0
30-39	6	170	3.5	0.7-6.5
40-49	9	193	4.7	1.7-8.0
≥ 50	6	100	6.0	1.4-11.3
Total	23	532	4.3	2.7-6.3
P = 0.72				
Gender				
females	5	143	3.5	0.5-6.5
males	18	389	4.6	2.5-6.7
P = 0.57				
Subject category				
Blood Donor	20	402	5.0	2.3-5.9
Workers at zoonotic risk	3	130	2.3	0.2-5.0
P = 0.31				

Fig. 2. Phylogenetic tree showing the relationship of 3/6 HEV isolates from Italy (Sardinia) and others capsid sequences described in DNA Data Bank (GenBank). For the remaining 3 isolates, it was not possible to obtain a sufficient DNA sequence for comparative analysis.



(Italy's National Health Service) has included Hepatitis E among notifiable infectious diseases. Cases are reported on the basis of the presence of the serological marker for Hepatitis E identified in the IgM anti-HEV. To the present day, the cases of HEV reported in the period between 2007 and 2009 have been 37, equal to 1% of the total cases of acute Hepatitis. The main reported risk factor was travel to endemic areas, in particular Bangladesh (11/20) and India (7/20), and the consumption of water from wells or springs (SEIEVA 2009).

The rate of 4.8% in blood donors is, in any case, lower than data reported by European and international literature which counts frequencies from 16% up to 20.6% [24, 25.] In this category defined as a healthy population par excellence, it is difficult to identify any risk factor for the infection: none of the 20 anti-HEV positive subjects carry out a working activity which could be defined as "risky" (handling animals or contact with animals), even though domestic handling of meat could be a risk factor through secondary contamination of food consumed raw (vegetables, fruit). The source of the relatively high anti-HEV prevalence in blood donors is still difficult to investigate, unless a study is made of common practices such as the use of water from natural sources (uncontrolled) and the simultaneous use of the same utensils (chopping boards, knives) in the kitchen in the preparation of raw meat and other foods. The prevalence of 2.3% found in the group of people working with biological material of animal origin including swine, is low compared to the anti-HEV frequency reported in other studies carried out in veterinarians and farmers working with pigs in other countries [26, 27]. The data obtained in Sardinia seems to suggest that handling meat from pigs and diagnostic work on biological matrices from the same species does not apparently represent an evident risk of infection: this evidence must however be associated with the rather low percent of HEV RNA positive bile samples observed in Sardinian swine, which might suggest a reduced circulation of virus in these animals as opposed to other studies in both continental Italy and other European countries [28-30].

In any case, the detection of the virus genome in Sardinian pig bile confirms the role of swine as an HEV reservoir and the possibility of virus transmission to humans, probably in different ways, as a result of different territorial realities. This datum is enforced by a study carried out in 2004 on swine sera coming from different Sardinian pig farms showing a prevalence of anti-HEV antibodies of 27% (41/152) (results not published), thus enforcing the zoonosis hypothesis.

The epidemical situation is not of easy reading; it could be motivated by environmental contamination by refluents in some rural areas near swine farms associated with a low virulence of HEV virus.

As regards the risk of virus transmission through the consumption of raw or undercooked foods, numerous cases have been reported in literature, confined to countries where HEV is present in an endemic form and can result in acute hepatitis [11, 12]. Sardinian food habits are somewhat different: in fact although there is a large consumption of pork, it is always eaten well cooked.

In this study, HEV RNA was isolated from pigs in Sardinia for the first time and the capsid sequences were characterized. Phylogenetic analysis suggests that at least 2 Sardinian isolates belong to a unique cluster characterized by frequencies phylogenetically correlated with European strains (France and Holland), highlighting possible virus diffusion between these geographic areas (Fig. 2).

Breeding stock from these European regions are frequently imported into Sardinian pig farms and commercial exchanges with European farms are also common practice. All frequencies belonged to genotype 3 which is prevalent in Europe [31, 32]. It is now common knowledge that both pigs and wild boars represent the most important reservoir of HEV virus in the European and North-American epidemical situation [33, 34]. These results, together with the fact that the pigs appeared clinically healthy, raise the hypothesis that HEV infection may be subclinical for them.

This is the first description of HEV infection in Sardinian swine and further studies are needed to better assess their role in the circulation of the virus.

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