DETECTION OF HEPATITIS E VIRUS IN PATIENTS SERA IN SOUTHERN SPAIN

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Summary. – The aim of the present study was to detect acute Hepatitis E virus (HEV) infection in patients with abnormal alanine transaminase (ALT) in which other viral hepatitis infections had been excluded in southern Spain, an area adjacent to regions where this disease is endemic. Of 336 sera tested 30 (8.92%) were positive for IgM antibodies against HEV (anti-HEV IgM) and 7 (2.08%) were negative in a repeated assay. Immunoblot analysis (IBA) was applied to the 37 positive sera in the first assay; its results were positivity for 26 (7.73%), ambiguous for 5 and negative for 6 sera. Amplification of ORF1 and ORF2 of HEV by means of nested RT-PCR was carried out with the 37 sera that were either positive or ambiguous by ELISA; a positive result was obtained only with one serum for the ORF2 protein. IgM antibodies against the HEV ORF2 protein could be a useful marker in the diagnosis of acute infection and a substitute for the determination of viral RNA in serum; this is of both diagnostic and epidemiological importance as it would allow the patients transmitting the infection to be recognized by means of a simple determination of antibodies. The sequence of the ORF2 fragment of HEV occurring in samples taken from both humans and animals amplified in this study has considerable homology with the sequences of HEV strains/isolates of European origin. These results demonstrate that an autochthonous HEV circulates in Spain.

Key words: Hepatitis E virus; IgM antibodies against HEV; ELISA; immunoblot analysis; nested RT-PCR

Introduction

Historically, HEV has been referred to as enterically transmitted non-A, non-B hepatitis. HEV is a major cause of waterborne epidemics of hepatitis in various regions of the developing countries (Skidmore *et al*., 1992). Sporadic cases of hepatitis E occur in these as well as developed countries where large-scale outbreaks have not been reported. The prevalence of antibody positivity in the populations of these countries implies that subclinical infections are not uncommon (Zafrullah *et al*., 1997). HEV was initially considered to be a member of the family *Caliciviridae*. However, on the basis of comparative phylogenetic analysis, it was recently removed from the family *Caliciviridae* and assigned as an unclassified genus "*Hepatitis E-like virus*" (van Regenmortel *et al*., 2000). HEV is a non-enveloped single-stranded RNA virus. The positive sense viral RNA genome contains 3 ORFs. ORF1 almost certainly encodes non-structural gene products such as helicase, protease, and RNA-dependent RNA polymerase; ORF2 encodes the putative capsid protein; ORF3 encodes a cytoskeleton-associated phosphoprotein (Huang *et al*., 1992). Initially, it has been believed that HEV does not occur in industrialized countries in which an occasional diagnosis of hepatitis E is usually associated with a travel of a patient to an endemic area. Thus, in developed countries, there has been a low index of suspected hepatitis E in acute cases of sporadic, presumed non-A, non-B, non-C viral hepatitis, in the absence of travel as a risk factor. It has now become

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Abbreviations: ALT = alanine aminotransferase; anti-HEV IgM = IgM antibodies against HEV; HEV = Hepatitis E virus; IBA = immunoblot analysis

clear that hepatitis E may be acquired in Europe, where recently indigenous cases have been reported (Schlauder *et al*., 1999) and in the USA (Kwo *et al*., 1997; Zanetti *et al*., 1999). The aim of the present study was to detect acute cases of HEV infection in southern Spain, an area adjacent to the HEV-endemic regions, in patients with abnormal ALT, in which other viral hepatitis infections had been excluded.

Materials and Methods

Patients. A total of 336 serum samples were obtained from non-A, non-D acute hepatitis patients. Acute viral hepatitis was defined by the presence of specific clinical symptoms and serum ALT levels 10 times higher than the upper normal values and by exclusion of other causes of hepatocellular injury, including medications, alcohol, congestive heart failure, biliary disease, autoimmune hepatitis and other infections (cytomegalovirus, Epstein-Barr virus and herpes simplex viruses). All the serum samples were collected beetween January 1999 and August 2001 in the Puerto Real University Hospital, Cádiz, Spain and were stored at -80ºC until the detection of HEV antibodies and HEV genomic sequences.

ELISA. Commercially available ELISA was performed to detect anti-HEV IgM (Biokit, Spain) using a recombinant HEV antigen corresponding to the structural region. If the specimen absorbance value was greater than or equal to the cut-off value, the assay was repeated.

IBA. ELISA-positive IgM sera were subjected to IBA using the HEV IgM Blot (Genelabs Diagnostics, Singapore) with a nitrocellulose strip with bands corresponding to ORF3-Mexico, ORF2, ORF3-Burma, and GST control. This test was performed directly by the manufacturer in Singapore.

Nested RT-PCR. RNA was extracted from a 140 µl serum sample by using the QIAmp Viral RNA kit (Qiagen, Germany) according to the manufacturer's instructions. The RNA was subjected to reverse transcription (RT) using random hexamers and the GeneAmp RNA PCR kit (Perkin-Elmer, USA). The RT reaction mixture (25 µl) consisted of 10x PCR II buffer, 10 mmol/l dNTPs, 20 mmol/l random hexamers, 2 U of RNase inhibitor and 10 U of MuLV reverse transcriptase. The reaction was run at a Thermal Cycler model 9600 (Perkin-Elmer, USA) at 20ºC/10 mins, 42ºC/30 mins, and 99ºC/5 mins.

Table 1. Primers used for nested-PCR

Primer	Size/position (nt)	Sequence (5'–3')
ORF1		
HEVORF1con-s1	418 (56–79)	CTGGCATYACTACTGCYATTGAGC
HEVORF1con-a1	418 (473–4510	CCATTCRARRCAGTAAGTGCGGTC
HEVORF1con-s2	287 (101–124)	CTGCCYTKGCGAATGCTGTGG
HEVORF1con-a2	287 (389–367)	GGCAGWRTACCARCGCTGAACATC
ORF2		
HEVORF2con-s1	197 (6298–6321)	GACAGAATTRATTTCGTCGGCTGG
HEVORF2con-a1	197 (6494–6470)	TTGTTCRTGYTGGTTRTCATAATC
HEVORF2con-s2	145	GTYGTCTCRGCCAATGGCGAGC
HEVORF2con-a2	145	GTTCRTGYTGGTTRTCATAATCCTG

Two 5 µl aliquots of the RT mixture were transferred into two tubes for two different PCRs:

ORF1 HEV-PCR1 with the ORF1 primer set and ORF2 HEV-PCR1 with the ORF2 primer set. The degenerate PCR primers were designed with regional identities from among Burmese, Mexican and US HEV isolates (Wang *et al.,* 1999; Schlauder *et al.,* 1999) (Table1). The ORF1 PCR1 reaction mixture consisted of 10x PCR II buffer, 10 mmol/l dNTPs, 10 µmol/l HEVORF1con-s1, 10 µmol/l HEVORF1con-a1, and 1 U of Ampli-Taq Polymerase (all chemicals from Perkin Elmer, USA). Similarly, the ORF2 PCR1 reaction mixture was set up by substituting HEVORF2con-s1 and HEVORF2con-a1 for the ORF1 consensus primers, respectively. The first round PCR1 cycling conditions consisted of 1 cycle of 94ºC/1 min, 35 cycles 94ºC/20 secs, 55ºC/30 secs, and 72ºC/30 secs, and 1 cycle of 72ºC/10 mins. The second round PCR1 was performed by adding 5 µl of the first round PCR1 products to 20 µl of PCR mixture containing 10x PCR II buffer, 10 mmol/l dNTPs, 10 µmol/l HEVORF1con-s2, 10 µmol/l HEVORF1con-a2, and 1U of Ampli-Taq Polymerase. The PCR2 ORF2 procedure was the same as the PCR2 ORF1 except using the primers HEVORF2con-s2 and HEVORF2con-a2 in place of the ORF1 primers. The cycling conditions were the same as those used in the first round of amplification.

Nucleotide sequencing. The PCR products were separated by electrophoresis in 2% agarose gel and detected by staining with ethidium bromide. The amplicon was purified and both strands were sequenced with the ABIPRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, USA). The nucleotide sequence reported in this study has been deposited in the GenBank database under Acc. No. AF448920. The names and GenBank accession numbers of the HEV isolates utilized in this study are: Swine isolate NLSW85 (AF336010); Italian isolate It1 (AF110390); Austrian isolate Au1 (AF279123); Greek isolates G1 (AF110391); and G2 (AF110392).

Results

Of the 336 samples analyzed, 30 (8.92%) were repeatedly positive for anti-HEV IgM and 7 (2.08%) were negative in the repeated assay. IBA was applied to the 37 positive samples in the first assay, confirming the positivity in 26 patients (7.73%); it was ambiguous in 5 and negative in 6 samples.

Of the 30 anti-VHE IgM serum samples that were positive by ELISA, 26 were confirmed positive by IBA, and of the remaining 4 samples 3 were IBA-negative and 1 was ambiguous. The 7 serum samples ambiguous by ELISA were IBA-negative in 3 and ambiguous in 4 cases.

The IBA pattern was variable. There were 24 samples positive for ORF3-Mexico, 5 samples reactive with ORF2, and 17 samples were positive for ORF3-Burma. Of the 5 ORF2-positive samples only 1 was clearly positive by giving the three bands (Table 2), while the remaining 4 were ORF2-weakly positive and ambiguous for ORF3-Mexico and ORF3-Burma.

Table 2. Immunoblot pattern

No.	ELISA			IBA: HEV IgM[a]				Result	RT-PCR
				Band 1	Band 2	Band 3	Band 4		
	Sample	A	Result	ORF3	ORF2	ORF3 B	GST		ORF2
1	2075/00	2.01	+	3+	+/-	3+	–	+	–
2	19609/99	3.13	+	3+	–	–	–	+	–
3	18915/99	1.15	+	2+	–	2+	–	+	–
4	18807/99	1.03	+/-	2+	–	2+	–	+	–
5	18079/99	1.36	+	2+	–	+	–	+	–
6	17417/99	1.69	+	+	–	+	–	+	–
7	14083/99	1.16	+	2+	–	–	–	+	–
8	10179/99	0.61	–	2+	–	3+	–	+	–
9	9534/99	1.68	+	2+	–	2+	–	+	–
10	9299/99	0.52	–	+	+/-	+	–	+	–
11	9087/99	1.40	+	+/-	–	+	–	+	–
12	8997/99	1.15	+	+/-	–	+	–	+	–
13	7522/99	>9.50	+	4+	3+	2+	–	+	–
14	7463/99	2.16	+	+	+	–	–	+	–
15	879/99	0.85	–	+	+	2+	–	+	–
16	326/99	1.18	+	+	–	–	–	+	–
17	17995/98	2.32	+	2+	–	–	–	+	–
18	13879/98	1.37	+	+	–	–	–	+	–
19	12544/98	1.46	+	2+	–	2+	–	+	–
20	11242/98	1.28	+	+	–	+	–	+	–
21	10610/00	1.02	+/-	+	–	–	–	+	–
22	10535/00	1.05	+/-	+/-	–	+	–	+	–
23	10361/00	0.36	–	–	–	–	–	–	–
24	6557/00	0.45	–	+/-	–	–	–	+/-	–
25	3615/00	0.07	–	–	–	–	–	–	–
26	13012/00	0.38	–	–	–	–	–	–	–
27	4871/00	5.92	+	2+	–	–	–	+	–
28	3944/00	1.77	+	+	+/-	2+	–	+	–
29	7657/00	9.50	+	3+	–	4+	–	+	–
30	8341/00	0.12	–	–	–	–	–	–	–
31	7204/00	0.49	–	–	–	+/-	–	+/-	–
32	3234/00	0.03	–	–	–	–	–	–	–
33	13321/00	0.49	–	+/-	+/-	–	–	+/-	–
34	7363/00	0.58	–	+	+	–	+/-	indeter.	–
35	8899/00	0.26	–	–	–	–	–	–	–
36	9696/00	0.67	–	–	+/-	–	–	+/-	–
37	8654/00	1.17	+	+	–	–	–	+	–

[a] The intensity of HEV IgM bands on blots is graded from 2+ to 4+. A = absorbance; (+) = positive; (–) = negative; (+/-) = ambiguous.

The amplification of ORF1 and ORF2 by the nested RT-PCR was successful with the 37 samples that were ELISA-positive or ambiguous, while only 1 sample was found positive for ORF2. This particular serum was the same one that presented the three positive bands in IBA. This amplicon was purified and sequenced for both chains; a fragment of 169 bp was obtained, which was utilized for phylogenetic comparison with other ORF2 HEV sequences. The latter revealed nucleotidic identity values of 92%, 91%, 86%, 88% and 85% for G1, NLSW85, G2, It1 and Au1, respectively.

Discussion

The incidence of hepatitis E in Europe appears to be higher than it has been recognized until only a few years ago (Clemente-Casares *et al.,* 2003). Sporadic cases of hepatitis E of autochthonous origin are frequently being described (Widdowson *et al.,* 2003). In our study, more than 7% of sera were found positive for anti-HEV IgM by IBA, and amongst them, one serum was viremic. The presence of anti-HEV IgM should signify a recent infection; however, the absence of virus in the blood indicates a certain phase

of the infection. In studying the reactive bands in the IBA a correlation between the presence of the virus (viremia) and the reactivity in the band of ORF2 was observed, which was not the case of the non-viremic sera. If this correlation will be confirmed, the presence of anti-ORF2 IgM could be a useful marker in the diagnosis of acute HEV infection and a substitute for the determination of viral RNA in serum; this is of both diagnostic and epidemiological importance, as it would allow the patients transmitting the infection to be identified by a simple determination of antibodies.

The phylogenetic study of the HEV genome demonstrated the presence of diverse interrelated genotypes. The sequence of the ORF2 fragment amplified in this study presents considerable homology with the corresponding sequences of HEV strains/isolates of European origin, obtained from humans or animals. This demonstrates that an autochthonous HEV circulates in Spain, which is not related to nearby endemic regions like North Africa, and that an evident relationship exists between human and animal (namely porcine) forms of the virus (Pina *et al*., 2000). The importance of the pig as a reservoir of the infection should be studied in depth, since the two viruses are very similar (Drobeniuc *et al*., 2001; Nishizawa *et al.,* 2003). More complete studies are necessary to resolve definitively the epidemiology of hepatitis E in developed countries, in order to establish adequate measures for prevention and control.

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