Risk Factors and Immune Response to Hepatitis E viral Infection among Acute Hepatitis Patients in Assiut, Egypt

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Hepatitis E virus (HEV) infection is a common cause of acute viral hepatitis (AVH) in Egypt. We aimed to identify risk factors of HEV among acute hepatitis cases, measure HEV specific immune response to differentiate between symptomatic and asymptomatic infections. The study included symptomatic acute hepatitis (AH) patients (n=235) and asymptomatic contacts (n=200) to HEV cases. They completed a lifestyle questionnaire, screened for common hepatotropic viruses. Blood and serum samples were collected from patients and contacts after onset of disease and follow-up samples collected until convalescence. PBMC were separated and tested for specific HEV T-cell response by INF-γ ELISPOT assay. Serum samples were tested for IgM and IgG anti-hepatitis E virus by ELISA. IgM antibodies to HAV were detected in 19 patients (8.1%), 37 (15.7%) with HBV, 10 (4.3%) with HCV. HEV infection was identified in 42 (16%) patients with AVH. Of the 200 contacts, 14 (7%) had serological evidence of recent HEV asymptomatic infection, showed stronger CMI responses than HEV infected subjects (2540 ± 28 and 182 ± 389 ISCs /106 cells, respectively; *P* <0.05). In conclusion, HEV is a major cause of AVH in Egypt. Asymptomatic HEV patients are likely to have stronger immune responses including CMI responses, than symptomatic cases.

In Egypt, community-based studies showed that up to 80% of the inhabitants of rural villages have Hepatitis E Virus (HEV) antibodies with very little or no evidence that the infection causes acute hepatitis in these subjects (Fix *et al.,* 2000; Meky *et al.,* 2006; Stoszek *et al.,* 2006), and the reasons for this discrepancy are unclear. A study, (Abdel Aziz *et al.,* 1999) of the prevalence of HEV antibodies among apparently healthy Egyptians with different age groups, reported that HEV is endemic in Egypt, especially in rural areas. There was no significant relation of HEV to gender, but there was significant relation to residence, crowded living conditions and poor sanitation which is important in spreading of the virus.

Acute HEV infection is usually self limited with no chronic sequelae, however the disease can lead to fulminant hepatic failure. HEV first appears in the liver and is followed by viraemia. Viraemia and faecal shedding are detected prior to liver abnormalities, which usually appear with the development of humoral immune response (Jameel, 1999). Anti-HEV IgM appears first, followed by IgG anti-HEV. Immunologic events observed in patients and in experimentally infected primates suggest that the liver pathology in HEV infection is immune mediated rather than the direct cytopathic effect of HEV (Purcell & Tichehurst, 1997). Lymphoproliferative assays carried out in HEV infected patients indicate involvement of HEV-specific cellular immunity in the pathogenetic events, however direct ex vivo assay data of the HEV-specific T cell response in hepatitis E (HE) patients is still limited (Naik *et al.,* 2002).

Humoral immune responses used either for the diagnosis of acute infection or for detection of prior exposure to HEV have poor

sensitivity and specificity (Lin *et al.,* 2000). In acute HEV infection, the anti-HEV IgM levels do not correlate completely with HEV viremia. This might be attributed to infection by different HEV genotypes (Wu *et al.,* 2009).

Cell-mediated immune (CMI) responses are highly sensitive and long lasting after sub-clinical infections as shown in HCV (Shata *et al.,* 2003, Al-Sherbiny *et al.,* 2005) and HIV (Kaul *et al.,* 2004; Alimonti *et al.,* 2006). Memory CMI responses are also long lasting and could be detected up to 20–30 years after exposure despite the decrease of humoral immune responses (Takaki *et al.,* 2000). Many reports examined T cell proliferation (Pal *et al.,* 2005; Aggarwal *et al.,* 2007; Srivastava *et al.,* 2007) or flow cytometry (Srivastava *et al.,* 2007) as a surrogate marker for CMI responses to HEV, however, the sensitivity and specificity of these assays were low.

An HEV-specific interferon-gamma (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay was optimized to analyze HEV-specific CMI responses by Shata *et al.,* (2007). They used peripheral blood mononuclear cells (PBMC) and sera from experimentally infected chimpanzees and from seroconverted and control human subjects to validate the IFN-γ ELISPOT assay and to quantitatively evaluate CMI responses in HEV infection. They noticed that CMI responses to HEV in the convalescent chimpanzees were still detectable 3– 4 years after infection despite the absence of chronicity in HEV infection.

The aims of this study were to identity risk factors of HEV infection among acute hepatitis patients admitted to Assiut fever and University hospitals, to measure HEV specific humoral and cell mediated immune responses and to determine their association with disease morbidity.

Patients, Materials and Methods

Patients

This prospective study was carried out on patients who suffered from acute hepatitis at Assiut fever and university hospitals through the interval from March, 2007 to August, 2008. Two hundred and thirty five patients were included as acute hepatitis cases, (136 males and 99 females), with age ranged from 1-65 years old. Acute hepatitis cases (symptomatic patients) were febrile patients of any age with duration of illness less than 2 weeks, alanine aminotransferase levels that were 2 times the upper limit of normal and clinical signs that were compatible with AH cases as, jaundice, dark urine, pale stool, yellow sclera and tender liver. Patients with known preexisting chronic liver disease were excluded from the study. All patients were subjected to full medical history, clinical examination, liver function tests and abdominal sonography. Two hundred contacts and family members to patients suffering from acute HEV infection (111 male and 89 female) were also enrolled as the asymptomatic group. The Ethical Committee of the Faculty of Medicine, Assiut University approved the study. Informed oral consent was taken from patients and their relatives.

Sample collection

All patients with clinical AH infection were subsequently screened for the common hepatotropic viruses at the Medical Microbiology and Immunology department, Assiut University, Faculty of Medicine. Serum samples were tested for HAV using HAV rapid test for anti-HAV IgM (CTK Biotech, Inc, San Diego, CA), HBVsAg ((ACON laboratories, Inc., USA), anti HBV core IgM (IND Diagnostic, Canada), and anti-HCV IgG test using the fourth generation HCV Tri-Dot test control (Atlas Link, USA). Patients with no serological evidence for HAV, HBV and HCV infection were further tested for HEV IgM using ELISA.

For patients symptomatic and asymptomatic, who were anti HEV IgM positive 15 ml of whole blood were collected in vacutainers that contain ethylene diamine tetracetic acid (EDTA) and 3 ml were collected for serum separation. Sera were aliquoted and stored at -20 °C. For patients with non HAV, non HBV, non HCV, and non HEV IgM, a second serum sample was collected within 20 days, in order to detect anti-HEV IgG rising titre (as single testing of anti-HEV IgG can not separate prior exposure from current infection with HEV). Hepatitis E patients were followed up clinically after recovery from acute illness, follow up whole blood and serum samples were also collected.

Sera were tested for HEV IgM, HEV IgG while blood samples were further tested for HEV IFN-γ ELISPOT. Stool samples were also collected from HEV cohort.

HEV Virological Testing

Serologic testing for HEV infection

a. Detection of HEV IgM

Detection of HEV IgM was performed using the commercial anti-HEV IgM kit (HEV IgM ELISA, MP Diagnostics (MPD), formely Genelabs Diagnostic, Singapore). The presence or absence of IgM antibodies specific for HEV was determined by relating the absorbance of the specimens to the cut-off value of the plate. The cut-off value was calculated as: 0.4 + the mean absorbance of the non reactive control. Specimens with absorbance values greater than or equal to the cut-off value were considered reactive.

b. Detection of HEV IgG

Some samples that were Non HAV, Non HBV, Non HCV and were HEV IgM negative, were further tested for HEV IgG rising titer after collection of a second serum sample within 2 weeks from the first sample using in-house ELISA according to Engle *et al.* (2002) and Shata *et al.* (2007). Polystyrene microtitre plates (catalog no. 468667; Nunc Maxisorp) were incubated with HEV ORF2 antigen diluted in a carbonate-bicarbonate (pH 9.6) buffer for 18 h at room temperature then blocked with bovine serum albumin. Ten micro-liters of each test and control sample was diluted at a ratio of 1:10. The sample was further diluted at a ratio of 1:10 into an antigen-coated test plate (final test dilution, 1:100) and incubated for 30 min at 37°C. Wells were washed five times, and 100 µl of horseradish peroxidase-labeled anti-IgG was added to each well. The horseradish peroxidase-labeled secondary antibodies were species-specific anti-IgG (heavy and light chain) and were used at a concentration of 1.0 µg/ml. Following a 30-min incubation at 37°C, unbound conjugate was removed by washing five times. Hundered microlitres of Sigma fast substrate solution O-Phenylenediamine dihydrochloride (Cat No P 9187, 0.4 mg/ml, one OPD tablet and one urea hydrogen peroxide/buffer tablet, dissolved in 20 ml of distilled H_2O) were added to each well. The plate was incubated, in the dark, for 30 min. at room temperature. Then, 50 µ1 of stop solution (3M HCL or 3 M H2S04) were applied to each well when the lowest dilution or no sera began to show color, the OD at 492 nm was read. The cut off for the assay was established for each test from internal controls: Cut-off = average of negative controls (NR) + 0.4. Specimens with absorbance values greater than the cut-off value were considered HEV IgG positive. To consider the specimen HEV IgG rising titre positive, the first and second specimens for the same patient were positive HEV IgG with rising titre more than 1.2. The Signal/Cut off ratio was calculated as following:

$$S/C = \frac{\text{OD of the tested sample}}{\text{OD of Cut off}}$$

Cell Mediated Immunity for HEV

Peripheral blood mononuclear cells (PBMC) drawn from 42 symptomatic cases and 14 asymptomatic contacts were purified by Ficoll-Hypaque density centrifugation and either used fresh or cryopreserved at -80°C in 90% serum and 10% dimethyl sulfoxide (DMSO). Before performing any assay, cryopreserved PBMC were thawed, washed three times, and counted. HEV-specific T cell responses were quantified using an IFN-γ ELISPOT assay (Shata *et al.,* 2007). IFN-γ ELISPOT assay was performed with IFN-γ ELISPOT Kit (MABTECH, Sweden) according to the manufacturer's instructions. Briefly, 96 well plates with polyvinylidene difluoride membrane (Millipore MAIPS 4510) were coated with mouse anti-human IFN-γ monoclonal antibody (MAbTech clone 7-B6-1) at a concentration of 10µg/ml of phosphate buffered saline (PBS). Plates were incubated overnight at 4°C for 24 hours then washed and blocked with complete RPMI medium containing 10% fetal bovine serum. Synthetic peptides containing pooled HEV ORF2 peptides (Robinson *et al.,* 1998) at a concentration equivalent to 1-5 µg/ml of individual peptide were incubated with PBMC at 5% of humidified CO2 at 37°C for 18 hours. Phytohemagglutinin-L (PHA-L) (Sigma, MO) was used as a positive control at a concentration of 1–5 µg/ ml, anti-CD3 or SEB (Staph Toxin as superantigen) stimulation was also used as a positive control. Plates were washed and incubated with biotinylated mouse anti-human IFN-gamma monoclonal antibody (MAbTech clone 7-B6-1, catalog 3420-6) at a concentration of 1 µg/ml in dilution buffer (PBS containing 1% (w/v) bovine serum albumin, Sigma) for 2 hours. Plates were washed, and alkaline phosphatase-conjugated anti-biotin monoclonal antibody (Vector Laboratories, cat. # SP-3020) was added for 30 minutes followed by washing for 5 times. The substrate solution of 5-bromo, 4-chloro, 3-indolylphosphate /nitro blue tetrazolium chloride (BCIP/NBT), (Pierce) was added and data were presented as the increase in the number of IFN γ producing spot forming cells (ISC) in the presence of HEV ORF2 peptides after subtraction of the background (no stimulus) and expressed per 10^6

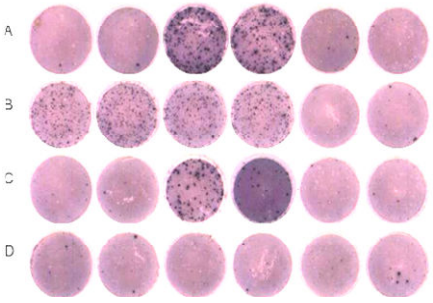
cells. Spots were counted in each well using ELISPOT 3B reader (CTL, Cleveland), digital images of each well were recorded and the images used to count spots on a computer with appropriate software (Figure 1).

Virological diagnosis: Stool samples from HEV cohort were resuspended in phosphate-buffered saline and utilized for detection of viral RNA as described in Blackard *et al.,* (2009). Figure (2) summarizes the different assays performed on the HEV cohort whether symptomatic or asymptomatic.

Statistical Analysis

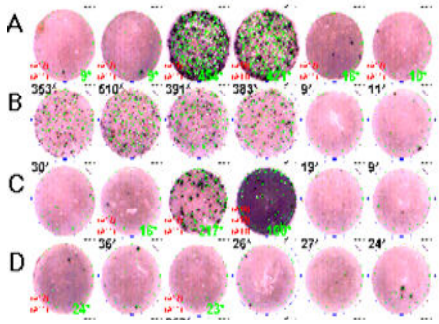
Statistical analysis was performed with SPSS statistical software (Scientific Package for Social Sciences). Descriptive statistics were reported. Continuous variables were summarized as mean ± SD. The chi-square test $\chi 2$, Fisher's exact test were used to compare the proportions between groups. *P* values <0.05 were considered to be statistically significant. Graphs were done using Microsoft excel 2003.

Protocol for ELISPOT of PBMC from HEV-infected subject 83



Patient ID 83				
	A	Medium	SEB	Medium
	B	Anti CD3 0.1 ug/ml	Anti CD3 0.03 ug/ml	Medium
	C	Rec. protein 5 ug/ml	Rec. HEV protein 3 ug/ml	Rec. HEV protein 1 ug/ml
	D	Pool HEV peptides 5 ug/ml	Pool HEV peptides 3 ug/ml	Pool HEV peptides 1 ug/ml

Reading of the No of spots using ELISPOT reader



A	**9**	**9**	**454**	**421**	**16**	**10**
B	353	510	391	383	9	11
C	30	**16**	**217**	**100**	19	9
D	**24**	36	**23**	26	27	24

Figure 1. A plate scan readout of a typical IFN-γ ELISPOT assay. In this assay, $1x10^5$ cells were plated per well (duplicate wells) and stimulated for 18 hours with each of a pools of HEV peptides or HEV protein (ORF2), and positive controls, (Anti-CD3, SEB (Staph B toxin as super-antigen), or no stimulus (media alone). The method followed was described in our IFN-γ ELISPOT protocol, using BCIP/NBT as the coloured substrate. Cells from sample number 83 (HEV-caused AVH subjects) were in wells according to the protocol.

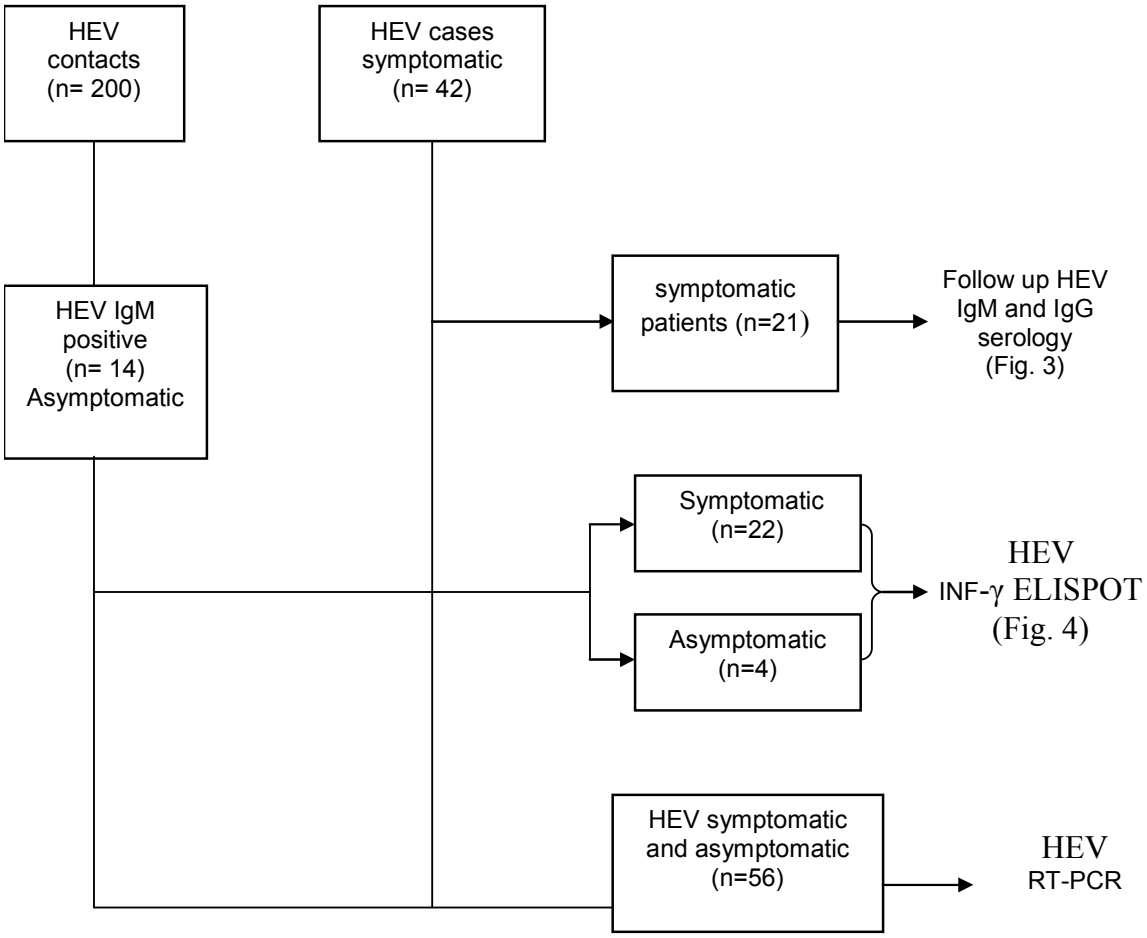


Figure 2. Follow up of the HEV Cohorts and Different Assays.

Results

Clinical and Laboratory Data of Acute Hepatitis Patients

The mean of age ± SE of all 235 subjects with acute hepatitis (AH) was 15.9 ± 1.0 years old. They were 136 males (57.9%) and 99 females (42.1%). Patients with age less than 10 years represented 123 (52.3%) of the study group. Suspected acute hepatitis patients (235) were serologically screened for HAV, HBV, and HCV, it was found that 19 (8.1%) acute hepatitis patients suffering from HAV, 37 (15.7%) with HBV, and 10 (4.3%) were HCV sero-positive. Three patients had mixed HAV and HBV antibodies, two patients had mixed HBV and HCV antibodies, and one patient had mixed HAV and HCV.

Anti HEV-IgM were found in 37 AH patients (37/235, 15.7%). Out of 138 negative anti-HEV IgM patients, 43 patients were tested for HEV IgG after collection of second sample within 15 days in order to detect the HEV IgG rising titre. Only five patients

(5/235, 2.1%) were found anti-HEV IgG rising titre

Epidemiology of Acute HEV Patients

Infection with HEV accounted for 42 (17.9%) patients who had serological evidence of acute HEV, while one hundred thirty three (56.6%) were negative for hepatotropic viral infection A -E (other etiology). Both male and female groups were equally (50%) affected by HEV. A higher prevalence of anti-HEV positivity was seen in the children less than 10 years age group (59.5%). Only 9.5% were 10 to < 20, 14.3% were 20 < 30, and 16.7% were 30 years or more. However no significant association was found between anti-HEV positivity with respect to age and sex. Ten acute hepatitis E patients were resident in urban areas (23.8%), while 32 residents in rural areas (76.2%). Out of the 32 rural resident patients, eighteen HEV patients were from Abnob Center including 13 (46.4%) patients were from Kom Elmansora village, 2 (7.1%) from Abnob center, 2 (7.1%) from Arab Elshanablla village, and 1 (3.6%) from Kom Abo- Sheel village.

Table (1) demonstrates the habitual risk factors associated with acute HEV patients. Out of the 42 patients suffering from acute HEV, there was a significant association between presence anti-HEV antibodies and contact of patients with animals and patients who drink water from underground sources (*P*-value< 0.001). Thirty patients (71.4%) used underground water using superficial water pump and 17 patients (40.5%) gave history of contact with animals (odds ratio = 21.25 and 3.84 respectively).

Table 1. Habitual Risk Factor Associated With Acute Hepatitis E Patients

Habits	HEV Positive (n=42)		HEV Negative (n=133)	
	No.	%	No.	%
Contact with animals:				
Yes	17	40.5	20	15.0
No	25	59.5	113	85.0
P-value (O.R)*	<0.001 (3.84)			
Source of drinking water:				
Public	12	28.6	114	85.7
In home	0	0.0	5	3.8
Underground	30	71.4	14	10.5
P-value (O.R)*	<0.001 (21.25)			
Washing vegetables and fruits before eating:				
Yes	20	47.6	70	52.6
No	1	2.4	7	5.3
Occasionally	21	50.0	56	42.1
P-value	NS			

(O.R*): odds ratio. *P* <0.05 is significant. NS= not significant

Table (2) shows comparison of the clinical characteristics of HEV and HAV- caused acute hepatitis. Comparing HEV (42 patients) and HAV (19 patients) infected patients, patients with HEV were older, and among children less than 10 years old, there was a significantly higher frequency of acute HAV infection in comparison to HEV infection (94.7% versus 59.5%, *P*= 0.005). HEV infected patients were more likely used underground water sources using superficial water pump (71.4% versus 10.5%, *P*=0.001), and have contact with animals (40.5% versus 10.5%, *P*= 0.019) in comparison to HAV-infected patients (odds ratio = 1.9 and 5.3 respectively).

Table 2. Comparison Of The Clinical Characteristics Of HEV And HAV- Caused Acute Hepatitis

Variables	HEV (n=42)		HAV (n=19)		*P*-value
	No.	%	No.	%	
Characteristics:					
Age (< 10 years)	25	59.5	18	94.7	0.005
Male	21	50.0	14	73.7	0.083
Liver enzymes:					
Total bilirubin (> 2mg/dL)*	36	85.7	17	89.5	NS
ALT fold (≥ 3)*	34	81.0	18	94.7	NS
AST fold (≥ 3)*	33	78.6	16	84.2	NS
Habits:					
Contact with animals	17	40.5	2	10.5	0.019
Source of drinking water (Underground)	30	71.4	2	10.5	<0.001
Main symptoms:					
Dark urine*	39	92.9	19	100.0	NS
Yellow sclera*	35	83.3	19	100.0	NS
Fever	28	66.7	14	73.7	NS
Jaundice	28	66.7	12	63.2	NS
Abdominal pain	26	61.9	9	47.4	NS
Weight loss	23	54.8	8	42.1	NS

Chi-square test * Fisher exact test

Serological Assays To Symptomatic and Asymptomatic HEV Patients

Out of the 42 HEV symptomatic patients, 21 patients were followed up with another serum samples. Anti-HEV IgG and anti-HEV IgM ELISA assays performed on paired 21 sera from acutely HEV-infected symptomatic subjects (6 days after the onset of illness) and convalescent subjects (3-6 months after the onset). The 21 patients with paired serum samples showed a typical serological pattern of an acute HEV infection in their first samples, both IgM and IgG detected, while at the convalescence state 85.7% becomes HEV IgM negative and 100% were become HEV IgG positive. There were significant differences between anti-IgM signal/cut off (S/C) ratio, and anti-IgG S/C in acute and

convalescent subjects (4.01 ± 1.1 and 0.5 ± 0.2, $P = 0.004$, and 10.5 ± 0.85 and 6.9 ± 3.4, $P = 0.003$, respectively) (Figure 3).

A total of 200 serum samples obtained from apparently healthy asymptomatic household members in contact to symptomatic acute HEV patients (111 male and 89 female) were tested for anti-HEV IgM using ELISA as control group. None of the contacts were clinically diagnosed as having AH or reported jaundice or symptoms compatible with AH but upon screening for anti-HEV IgM, 14 patients (7%) of the tested population were anti-HEV IgM positive including 4 male (28.6%) and 10 female (71.4%). All the 14 asymptomatic HEV IgM seroconverting subjects were HAV IgM, HBV core IgM and HCV IgG negative. All the asymptomatic subjects shared the same locality, source of water, and habits and were from Abnob- Kom El mansora village- Assiut Governorate. Among 235 acute hepatitis patients, HEV prevalence of 17.90% was found as opposed to 7.0% in a control group (200 asymptomatic subjects).

Cell Mediated Immune Response to HEV

The cell mediated immunity using IFN-γ ELISPOT test data were analyzed for 22 symptomatic acute HEV patients and 4 asymptomatic HEV positive subjects who have valid positive control responses (PHA responses >1000 ISC/106 cells). Data were presented as the increase in the number of ISC in the presence of HEV ORF2 peptides after subtraction of the background (no stimulus). The HEV specific CMI responses in asymptomatic subjects were significantly higher ($P<0.05$) than in acute HEV hepatitis symptomatic subjects (2540 ± 28 and 182 ± 389 ISCs /106 cells, respectively) Figure 4.

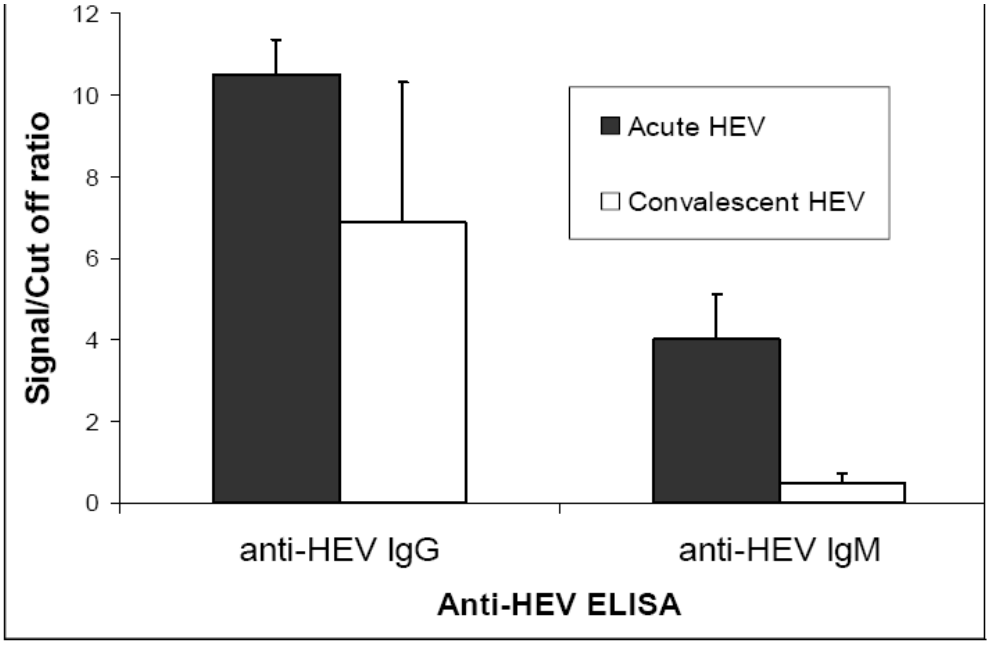


Figure 3. Serological Markers for 21 Symptomatic HEV Infection. There were Significant Differences between Anti-IgM signal/cut off (S/C) Ratio in Acute and Convalescent Subjects (4.01 ± 1.1 and 0.5 ± 0.2, $P = 0.004$), and also anti-IgG S/C in Acute and Convalescent Subjects (10.5 ± 0.85 and 6.9 ± 3.4, $P = 0.003$).

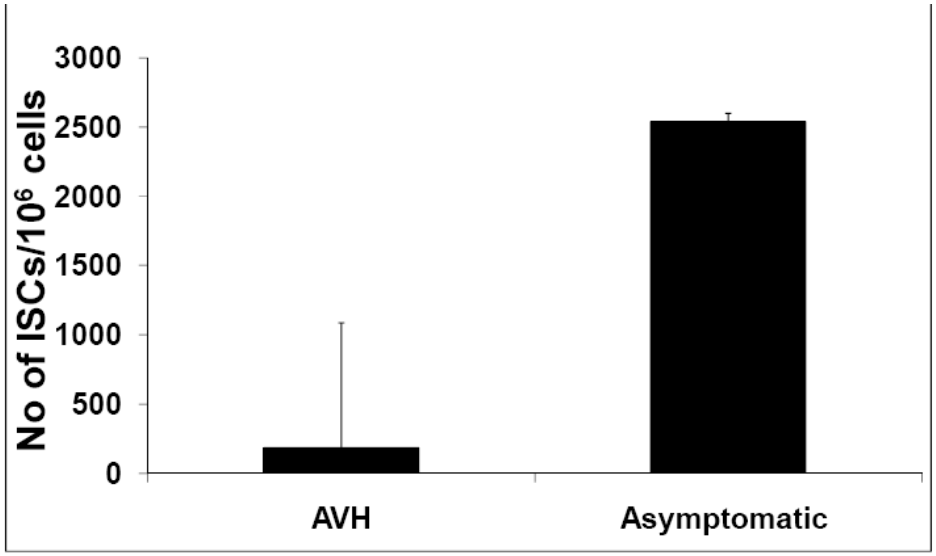


Figure 4. Interferon Gamma Cell Mediated Immune Responses in HEV-infected 22 Symptomatic and 4 Asymptomatic Subjects. The HEV Specific CMI responses in Asymptomatic Subjects were Significantly Higher (P<0.05) than in Acute HEV hepatitis Symptomatic Subjects (2540 ± 28 and 182 ± 389 ISCs /106 cells, respectively)

Discussion

Viral hepatitis continues to be the major health threat in both developed and developing nations. This occurs despite, the recognition over the past few decades of the viral agents responsible for this disease, and despite development of methods to detect these viruses and vaccines to prevent the spread of some of them. Nevertheless, the number of viruses that are known to cause acute hepatitis continues to increase (Aggarwal & Naik, 2009). In Egypt, acute viral hepatitis (AVH) remains a serious problem and a public health priority. Most cases of AVH that result in hospitalization are caused by the enteric hepatitis viruses, HAV; 0.7%–44% and HEV (12%–42%) (Bassily *et al.,* 1986; Darwish *et al,.* 1992; El-Zimaity *et al.,* 1993; Divizia *et al.,* 1999)

The aims of the present work were to understand the reasons behind the high seroprevalence of HEV and relative low incidence of HEV-caused AVH in upper Egypt. Also to evaluate the role of the immune responses in HEV infection, and the differences between symptomatic and asymptomatic infection. Humoral immune responses have been used for the diagnosis of acute infection or prior exposure to HEV. Markers for prior exposure, or current infection, with HEV include enzyme immunoassay testing for anti-HEV IgG and IgM and RT-PCR detection of HEV-RNA. In AVH cases due to HEV, anti-HEV IgM is usually positive for few weeks. Additionally, HEV-RNA could be detected in the blood or stool from about 50% of anti-HEV IgM positive cases (El-Sayed Zaki *et al.,* 2006). Anti-IgM peaks up to 4-weeks after onset of AVH and is no longer detectable in half of the cases after 3- months (Arankalle *et al.,* 1994; Bryan *et al.,* 1994).

Due to the high sensitivity and specificity of IgM anti HEV for detection of acute HEV infection, it was used successfully as a marker for detection in early stage of the disease in many studies (Liu *et al.,* 2003 and Wong *et*

al., 2004), However, in other studies, it has poor specificity (Li *et al.,* 1999). The reasons for these differences in the specificity are not clear, but the types of kits used and the reagents used in the study may influence the results (Fix *et al.,* 2000). Recently a commercially available assay for anti-HEV IgM (HEV-IgM ELISA 3.0, MP Diagnostics, formerly and Genelabs Diagnostic, Singapore), used in the current study, appears promising for screening for acute HEV infections (Bendall *et al.,* 2008). In this study we identified HEV-caused AVH by positive anti-HEV IgM, rising titer of anti-HEV IgG and negative for other viral hepatitis markers.

In this study, we measured anti-HEV IgM and IgG in acute and convalescent HEV infected subjects (4-6 months after seroconversion). The S/C ratio of anti-HEV IgM > 2, and the S/C ratio of rising titer of anti-HEV IgG >10 within 1-2 months could be used as markers for detection of acute HEV infection. Combinations of both markers may increase the specificity of the assays. All the samples in acute stage were anti-HEV IgM and IgG positive, while after a period of time 85.7% of the samples in the convalescent stage were anti-HEV IgM negative and 100% were anti-HEV IgG positive and there were significant differences between anti-IgM S/C in acute and convalescent subjects (P= 0.004), and also anti-IgG S/C in acute and convalescent subjects (p= 0.003). Anti-HEV IgM appears early in the blood than anti-HEV IgG during the course of acute HEV infection. Then anti-HEV IgM declines after 40-60 days while IgG anti-HEV level increases for months then decline and lasts for as long as 13-14 years after the HEV infection (Khuroo *et al.,* 1993). This course of anti-HEV antibodies explains why follow up serum samples for HEV cases becomes anti-HEV IgM negative but still anti-HEV IgG positive. It also explains the IgG-positive, IgM negative cases which indicate past infection to HEV. Comparable data had been reported in other studies (Anderson & Shrestha, 2002; Liu *et al.,* 2003; Anwar *et al.,* 2005). It is not known how long anti-IgG persists because of differences in sensitivity of the EIA. However, there are data suggested that anti-HEV IgG could be detected 14 years after infections during an Indian outbreak (Khuroo et al., 1993).

Enterically transmitted acute viral hepatitis is caused predominantly by HAV and HEV. Both agents are comparable in size, produce clinically similar uncomplicated disease, and result in no chronic carrier state or sequelae. However, the epidemiology of HAV and HEV differ in a number of important ways. In areas endemic for both viruses, HAV is primarily a disease of children while HEV predominantly affects young adults (Divizia *et al.,* 1999). This study demonstrates that HEV infection is more common among young adult while HAV infected mainly young children (94.7% of anti-HAV in age group less than 10 years old versus 59.5% of anti- HEV) and this was statistically significant (P= 0.005). The reasons for the age difference in susceptibility to HEV and HAV infections, despite similar mode of transmission, are not clear but such differences have been described in other studies (Sainokami *et al.,* 2004; Wong *et al.,* 2004).

Fix *et al.* (2000) reported that HAV infection tends to be asymptomatic in children <5 years of age and imparts lifelong immunity. Thus, in high-prevalence areas where most of the population is exposed at a very young age and develops immunity without symptoms, fewer symptomatic cases will be expected. If exposure to infection occurs for the first time at older ages, patients are more likely to develop symptomatic disease. This phenomenon is observed in countries with transitional economies, where improved sanitation in more affluent areas

tends to delay an individual's first exposure to HAV until older age.

Exposure to enteric infections is generally increased by poor sanitation and contamination of the water supply. In this study out of 42 the HEV patients, 30 subjects depended on underground water using superficial water pump. A significant difference in prevalence of HEV patients was found between underground water, and public water source with 71.4% higher risk for HEV infection in subjects using underground water sources than water from public sources (odds ratio= 21.25). El-Farrash *et al.* (2005) reported that the main source of HEV infection in their study in Northern Egypt through contaminated drinking water. This is in agreement with other studies reported that contaminated water, especially slowly moving water, with high viral concentration plays a major role in the ecology of HEV transmission (Zhuang, 1992; Bile *et al.,* 1994). HEV outbreaks have often linked to fecal contamination of municipal water systems which, when contaminated, may spread pathogens to a large number of people (Stoszek *et al.,* 2006). As HEV is shed into feces of infected patients, several studies have reported the detection of HEV in sewage water, suggesting a possibility of HEV contamination of aquatic environments (Aggarwal & Naik, 2009; Kitajima *et al.,* 2009).

In the present study anti-HEV patients live and work in densely populated areas where the sanitary conditions are very deplorable and also where animals, such as, sheep, goats, cows, dogs, rats, and cats share their habitat with humans. A significant association ($P< 0.001$) has been found between anti-HEV antibodies and contact of patients with animals (40.5%, Odds ratio= 3.84). However, transmission of HEV viruses from these animals to human has not been confirmed.

The asymptomatic HEV subjects are considered as potential reservoir of the virus may be a continuously circulating pool of individuals with subclinical HEV infection. In an experimental cynomolgus macaque model, HEV-infected animals that lacked biochemical evidence of liver injury were found to excrete large amounts of viable and infectious HEV (Aggarwal *et al.,* 2001). Similar fecal shedding of the virus by persons with subclinical HEV infection could lead to continuous maintenance of a source of infection in a disease-endemic area. This pool of infection could, in turn, lead to periodic contamination of drinking water supplies. Thus, it appears that in this region, the infection is acquired from either an environmental or a human reservoir through poor general sanitation, contaminated drinking water supplies and lack of attention to personal hygiene.

During the last ten years, highly sensitive IFN-γ ELISPOT assays have been used successfully for measuring the CMI responses in different diseases. Quantitative assessment of the CMI responses in HEV will also help us to evaluate the role of CMI in HEV morbidity. HEV specific CMI responses in this study did not correlate with age, duration of illness, and anti-HEV IgM S/C ratio. However, Shata et al. (2007) studied the HEV specific CMI responses using IFN-γ ELISPOT assay. They used PBMC and sera from experimentally infected chimpanzees and from seroconverted and control human subjects and reported that the HEV-specific IFN-γ ELISPOT responses correlated strongly and significantly with anti-HEV ELISA positive/negative results.

In the current study, we used IFN-γ ELISPOT assays, developed by (Shata *et al.,* 2007) for measuring the CMI responses in HEV-infected patients whether symptomatic or asymptomatic. CMI responses to HEV in the asymptomatic HEV infected patients were

significantly higher compared to patients with acute HEV-caused AVH. These data suggested that protective immune responses against HEV may play a role in HEV morbidity and may explain some of the mechanisms for discrepancy in areas with high HEV morbidity (e.g. India), and in area of low HEV morbidity despite high seroprevalence (e.g. Egypt, and the developed countries). However, other potential mechanisms may also explain this discrepancy including exposure to avirulent strain, infection with zoonotic strains of HEV with low virulence, or the presence of protective cross-reactive immune responses to virulent HEV strains. However, there are limited data to support any of these hypotheses.

Although inhabitants of rural communities in Egypt and the Indian subcontinent should have similar high levels of exposure to fecal-oral transmitted viruses, HEV exposures in Egypt may be more universal (endemic transmission) than in South Asia (epidemic transmission). The alternative explanation is that HEV strains in Egypt are generally less virulent, and, thus, are less likely to cause epidemics than those in South Asia as reported by Mitsui *et al.* (2005) and Herremans *et al. (*2007). It is concluded that the outcome of the interaction between HEV and the immune system is variable depending on previous exposure to the virus and the predominant immune response of the host.

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