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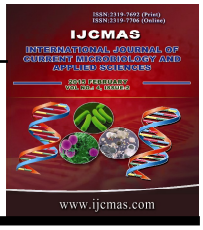

Original Research Article

Prevalence of Hepatitis E Virus Genotype(s) in Al-Muthanna Province/ Iraq Patients

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Keywords

Genotypes;
HE;
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A B S T R A C T

Hepatitis E (HE) is an inflammation of the liver caused by hepatitis E virus (HEV) infection. Iraq is one of the Asian countries with high incidence and prevalence of hepatitis. In this paper, the prevalence of HEV genotype(s) will be determined in Al-Muthanna province/ Iraq. Micro-ELISA kit was used to test 270 patients for HEV IgM antibodies. Among the 270 analyzed serum samples, a total of 72 samples (26.66%) showed positive results for anti-HEV IgM antibodies, and all these patients were tested for confirmatory test at central public health laboratories (CPHL) in Baghdad province. These patients consist of 45 females and 27 males with age ranged between (4-74) years old, were all negative for routinely screened markers of Hepatitis A, Hepatitis B and Hepatitis C. Ten normal healthy individuals, used as normal control in this study. This study showed that the HEV IgM is more common among younger age group (15-24), with a percentage of (41.67%), and it was higher in females (63%) than in males (37%). Highly significant differences ($\chi 2 = 10.271$, $p \leq 0.01$) appeared among age groups. The positive sera anti-HEV IgM also were tested for total serum bilirubin (TSB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) colorimetrically. The level of liver function enzymes were demonstrated significant differences ($p \leq 0.05$) in HEV patients as compared with healthy controls. Furthermore, RNA was extracted from Sera of positive anti-HEV IgM antibodies. High levels of RNA concentration were measured by Nanodrop. Forty-six samples have high RNA concentration out of 72 samples were detected for genotypes I, III & IV by Real time PCR technique. In this study, HEV type I and III were detected in 23 samples out of 46 samples by using Real Time PCR systems. Ten samples were positive for this test. Thirteen samples (56.52%) were negative for this test. The prevalence percentage of genotypes I & III was (43.48%). The results appeared significant difference ($P \leq 0.05$). Also by using the same technique (Real time PCR systems) the HEV type IV was detected in 23 samples out of 46 samples. Five samples were positive for HEV type IV and 18 samples (78.26%) were negative. The prevalence percentage of genotype IV was (21.74%). This study indicated that the HEV is highly endemic in Al-Muthanna province in Iraq. Genotypes I & III were the most prevalent than genotype IV in humans in Al-Muthanna province in Iraq. These results suggest that the genotypes I & III are the main causative agents of sporadic HEV infection in Al-Muthanna province.

Introduction

Hepatitis E virus (HEV), the etiological agent of hepatitis E (HE) infection. It was described for the first time by using electron microscopy in 1983 as a spherical viral particle non-enveloped being 27 to 30 nm in size. The HEV genome comprises a 7.2 kb

non-segmented single-stranded positive-sense RNA chain. Encoding three open reading frames (ORFs), with ORF 3 overlapping both ORF 1 and ORF 2 (Vasickova *et al.,* 2007).

The non-structural proteins, including the putative methyltransferase, helicase and RNA polymerase are encoded by ORF 1, while the major structural protein (PORF2) is encoded by ORF 2 (Tam *et al*., 1991). A hydrophobic stretch of 22 amino acids is present at the amino terminus of PORF2 and it has been suggested that this functions as a signal peptide (Tam *et al*., 1991; Jameel *et al*., 1996). And ORF3 encodes a small multifunctional protein. The ORF2 and ORF3 proteins are translated from a single, bicistronic mRNA (Dianjun *et al*., 2010). HEV was suggested to be classified in the Picornaviridae family (Balayan *et al*., 1983; Vasickova *et al*., 2007).

However, later studies showed that it does not belong to members of this family. Between 1988 and 1998, HEV was tentatively classified in the Caliciviridae family, based on virion morphology. This classification also was rejected after a phylogeny analysis of the HEV genome, and HEV was newly classified as an independent genus HEV-like virus, unassigned to any family (Berke and Matson, 2000; Acha and Szyfres, 2003). At present, HEV is the only member of the Hepevirus genus, Hepeviridae family (Emerson *et al*., 2004; Vasickova *et al*., 2007).

HEV causes acute sporadic and epidemic viral hepatitis worldwide. HEV infections are spread mainly by the faecal-oral route where as large epidemics are often associated with contaminated water (Ashbolt, 2004; Koopmans and Duizer, 2004; Vasickova *et al*., 2005). The incubation period after exposure ranges from 3 to 8 weeks (mean 40 days) and it is dose dependent (Krawczynski *et al.,* 2000). There is also a possibility of zoonotic transmission of the virus. Seroepidemiological studies revealed that anti-HEV antibodies are present in numerous animal species including pigs, rodents, chickens, dogs, cows, sheep and goats from developing and industrialized countries (Arankalle *et al*., 2001). There are four major genotypes of HEV: I, II, III and IV. Homology of members of the same genotype is presumed not to be less than 81% (Vasickova *et al*., 2007).

The phylogenetic analysis divided HEV genotype I into five subtypes, genotype II into 2 subtypes, whereas genotypes III and IV were divided into 10 and 7 subtypes, respectively (Lu *et al*., 2006). Genotypes I and II have been identified exclusively in humans, and genotypes III and IV have been found in humans and several animal species. Genotypes I and II have been isolated from Asia, Africa, North America while genotype IV has been identified only in Asia, and genotype III has been found in almost every country (Ning *et al.,* 2007). Various clinical manifestations of the disease have been observed, from more frequent subclinical forms to fulminant forms of hepatitis. HEV infection is most often seen in children, young to middle aged adults (15 to 40 years old) and might be serious in pregnant women. In most cases, the signs and symptoms of the disease include moderately severe hepatitis with concurrent signs of influenza-like symptoms, abdominal pain, fever tenderness, nausea, vomiting and with concurrent jaundice and dark urine, liver enzyme elevations, antibody seroconversion and clearing of the virus (Vasickova *et al*., 2007). Prolonged viraemia and viral shedding are unusual and chronic infection does not occur. Fulminant hepatitis occurs more frequently in pregnancy and induces a

mortality rate of 20% and can also cause premature births (Al-Naaimi *et al*., 2012). Hence the aim of this research is to identify the prevalence of HEV genotypes in Al-Muthanna Provence (Iraq) patients.

Materials and Methods

A total of 270 blood samples from jaundiced patients of both sexes from Al-Muthanna province/ Iraq were enrolled in this study and 10 samples from normal healthy individuals were used as normal control. Serum specimens were separated from all the samples.

Serology

Serum specimens were stored at −20°C until tested for HEV IgM antibody using commercially available Micro-ELISA for markers of hepatitis E (HEV IgM, Foresight, USA). This assay is based on synthetic immunodominant antigens derived from ORF2 and ORF3. specimens were tested accordance to the manufacturer's instruction, those with absorbance (S) value less than cut-off (CO) value were considered negative, whereas those with absorbance (S) value greater than or equal to CO value were considered positive; thereafter, They were re-tested in duplicate to confirm the result at central public health laboratories (CPHL) in Baghdad province.

Biochemical Tests

Sera of positive HEV IgM antibodies also were tested for total serum bilirubin (TSB), serum alanine aminotransferase(ALT), serum aspartate aminotransferase (AST), and serum alkaline phosphatase (ALP) by using a colorimetric method according to the guidelines mentioned in the leaflet supplied by the manufacturer (BioMerieux, France).

RNA Extraction

RNA was extracted from 140 µl of serum of acute hepatitis E cases by using a QIAamp® Viral RNA Mini Kit (Qiagen, USA), and total RNA was eluted in 60 µl of Buffer AVE (RNase-free water).

Real-Time RT- PCR

The HEV I, III and IV Real Time RT-PCR Kits (LiferiverTM , China) were used for the detection of HEV type I, III and IV in serum. The kit consists of 25 tests. The test includes an internal control (IC) to avoid false negative results. This assay is based on the fluorogenic 5'nuclease assay. During the PCR reaction, the DNA polymerase cleaves the probe at the 5' end and separates the reporter dye from the quencher dye only when the probe hybridizes to the target DNA. This cleavage results in the fluorescent signal generated by the cleaved reporter dye, which is monitored real-time by the PCR detection system. The PCR cycle at which an increase in the fluorescence signal is detected initially. Threshold cycle (Ct) is proportional to the amount of the specific PCR product. Monitoring the fluorescence intensities during real time allows the detection of the accumulating product. HEV I, III and IV real time RT-PCR kits contain a specific ready-to-use system for HEV detection (for genotype I, III and IV) by Reverse Transcription Polymerase Chain Reaction (RT-PCR) in the real-time PCR system. The master contains super Mix for the specific amplification of HEV RNA. The reaction is done in one step real time RT-PCR. The first step is a reverse transcription (RT) meaning HEV RNA is transcribed into cDNA. Then, a thermostable DNA polymerase is used to amplify the specific gene fragments by PCR. Fluorescence is emitted and measured by the real time systems´ optical unit. The

detection of amplified HEV DNA fragment is performed in fluorimeter channel 530nm with the fluorescent quencher. In addition, the kits can be used for identification of possible PCR inhibition by measuring the 560nm fluorescence of the internal control (IC). An external positive control defined as 1×10^7 copies/ml is supplied to allow the determination of the gene load.

Statistical Analysis

Chi-square test was used to significant compare between percentage, and Least significant difference (LSD) test was used to significant compare between means in this study. The statistical analysis were performed using the statistical analysis system- SAS (2012) program. Values were regarded as significantly different at the $p < 0.05$ level.

Result and Discussion

Out of 270 patients, 72 patients (26.66 %) (ranged between 4-74 years old) marked with appearance of anti-HEV IgM antibodies, and all these patients were tested for confirmatory test. These patients consist of 45 females and 27 males were all negative for routinely screened markers of Hepatitis A, Hepatitis B and Hepatitis C. This study demonstrates that the age groups of patients with HEV infection showed a wide spread and it is more common among younger age group(15-24), with a percentage of (41.67%) (Table 1). The statistical results showed highly significant differences ($\chi2 = 10.271$, $p \leq 0.01$) among age groups. This result agree with Chandra *et al.* (2012) from India who found the most common HEV infection among younger age group. Due to that this age group is the most contact with the environment and more tendencies to eat and drink at the outside of their home. The prevalence of HEV-IgM antibodies between patient groups from Al-Muthanna province in Iraq higher in females (63%) than in males (37%) (Figure 1). This indicats that exposure to HEV might be more frequent in females than males. This was probably due to poor nutrition and pregnancy converged, and works up the energy. The influence of each of these problems in public health for women, and strain the body becomes more susceptible to the disease. Elevated levels of (TSB), (ALT), (AST), and (ALP) (Table 2) show the significant differences ($P \leq 0.05$) between patients and healthy controls.

The increase in bilirubin levels reflect the deficiencies in bilirubin metabolism caused by viral hepatitis. Several studies suggest that elevated serum (ALT), (AST), and (ALP) may be marker of HEV infection Chandra *et al.* (2012). This result agree with Lhomme *et al*. (2012) fom France who reported that elevated levels of ALT, AST and ALP in HEV patients. RNA extraction efficiency was good, and high levels of RNA concentration were measured by Nanodrop. This is the first investigation about the occurrence of hepatitis E genotypes in Al-Muthanna province patients in Iraq. A total of 46 samples out of 72 samples have high RNA concentration were detected for genotypes I, III & IV. In this study HEV type I and III were detected in 23 samples out of 46 samples by using Applied Biosystems Real time PCR 7500 machine. Results are shown in Figures (2 and 3). The results showed that 10 samples (43.48%) were positive out of 23 samples and 13 samples (56.52%) were negative (Table 3), (Figure 4). There are significant difference ($P \leq 0.05$).The HEV type IV was detected in 23 samples out of 46 samples. These 23 samples include 13 samples were negative for genotype I & III and only 2 samples were positive for genotype IV and 11 samples were negative for genotype IV.

The others 10 samples only 3 samples were positive for genotype IV and (7) samples were negative for genotype IV. In totally, 5 samples showed positive results for HEV type IV (21.74%) (Table 4). 18 samples (78.26%) were negative results for HEV type IV, (Figure 5). There are highly significant differences ($P \leq 0.01$).

This results suggest that the genotypes I & III are the main causative agents of sporadic HEV infection in Al-Muthanna province/ Iraq. The reasons for the high HEV prevalence in this population are uncertain but may be due to, at least partially, to the contaminated water with virus and to the culinary culture of the local community. Thorough cooking of meat would help minimize the risk for HEV infection and could form part of a public health initiative in this area. This may imply that HEV can transmit from one person to another nearby through a certain route, such as faecal contamination and people movement; this may be exacerbated by non-viral factors, such as sanitary conditions, hosts, and facilities. Increased migration, tourism, and international trade, particularly of food products, may all contribute to the spread of HEV into new regions. Genotyping diversity as possible be attributed to the wars suffered by the country where delegations of soldiers of different nationalities and asset to this country.

The rate and mechanisms of human-to-human transmission and zoonotic transmission to humans vary geographically, thus contributing to the complexity of HEV molecular evolution (Purdy and Khudyakov, 2011).

Table.1 Distribution of samples study according to age groups

Age groups (years)	Samples no.	Percentage (%)
4-14	11	15.28
15-24	30	41.67
25-34	20	27.77
35-44	8	11.11
45-54	1	1.39
55-64	1	1.39
65-74	1	1.39
Chi-square (χ^2)	---	10.271 **
** ($P \leq 0.01$).		

Table.2 Comparison between HEV patients and healthy control in measuring TSB, GOT, GPT and ALP tests

Group	**No.**	**Mean ± SE**			
		TSB Mg/dl	GOT U/l	GPT U/l	ALP U/l
Patients	72	23.41 ± 12.38	887.17 ± 9.87	859.91 ± 13.28	206.56 ± 5.04
Healthy	10	0.884 ± 0.05	37.27 ± 4.58	34.14 ± 7.63	48.09 ± 15.42
LSD value	---	4.706 *	28.661 *	25.782 *	64.935 *
* ($P \leq 0.05$)					

Table.3 Distribution of HEV genotype I & III in the samples from Al-Muthanna province patients

Result	**Samples no.**	**Percentage (%)**
Positive	10	43.48
Negative	13	56.52
Total	23	100 %
Chi-square (χ^2)	---	4.981 *
* ($P \leq 0.05$).		

Table.4 Distribution of HEV genotype IV in samples study from Al-Muthanna province patients

Result	**Samples no.**	**Percentage (%)**
Positive	5	21.74
Negative	18	78.26
Total	23	100 %
Chi-square (χ^2)	---	12.059 **
** ($P \leq 0.01$).		

Figure.1 Distribution of samples study according to gender

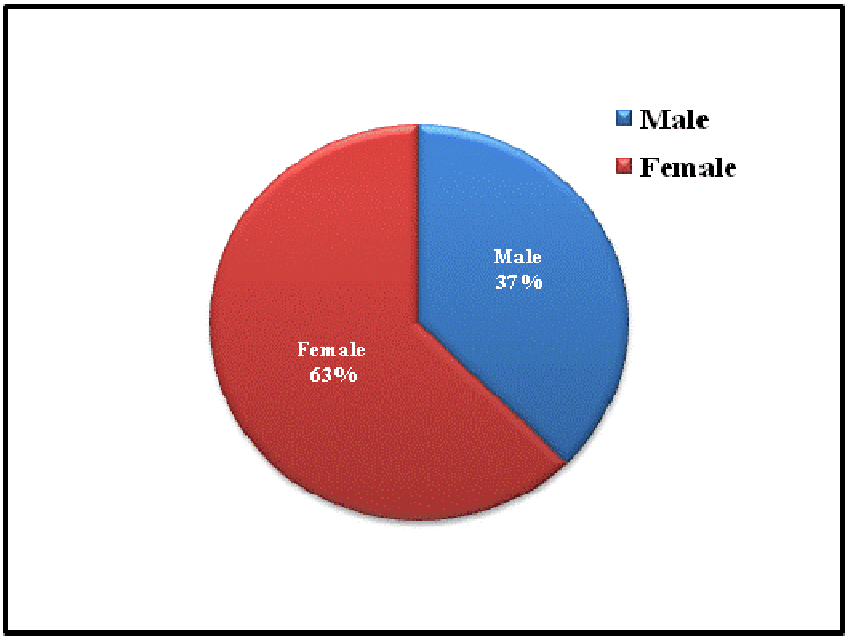


Figure.2 Real Time RT-PCR of HEV positive samples

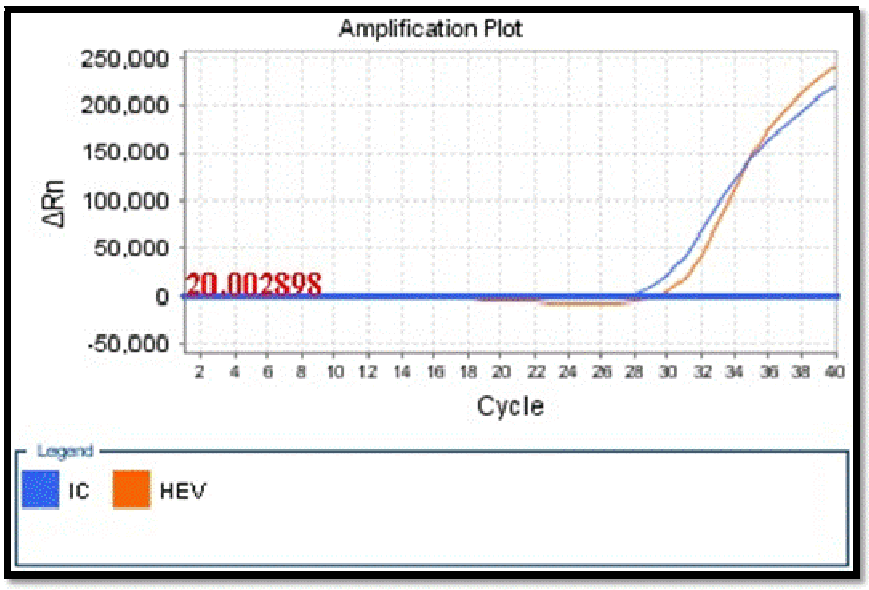


Figure.3 Real Time RT-PCR of HEV negative samples

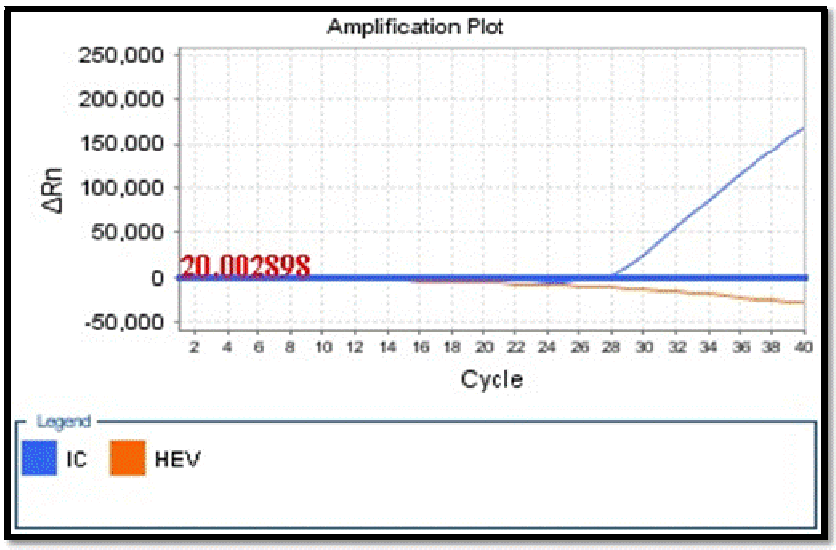


Figure.4 Distribution of HEV genotype I & III in the samples study

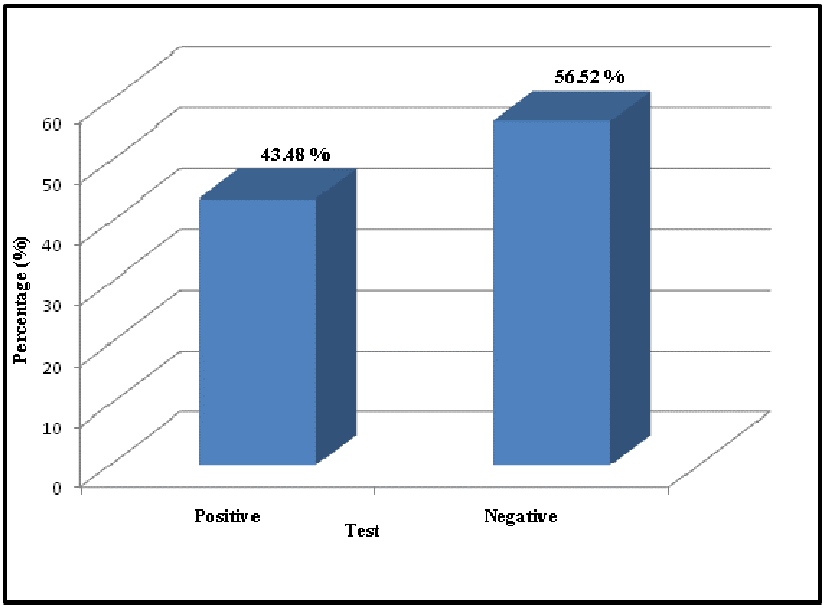
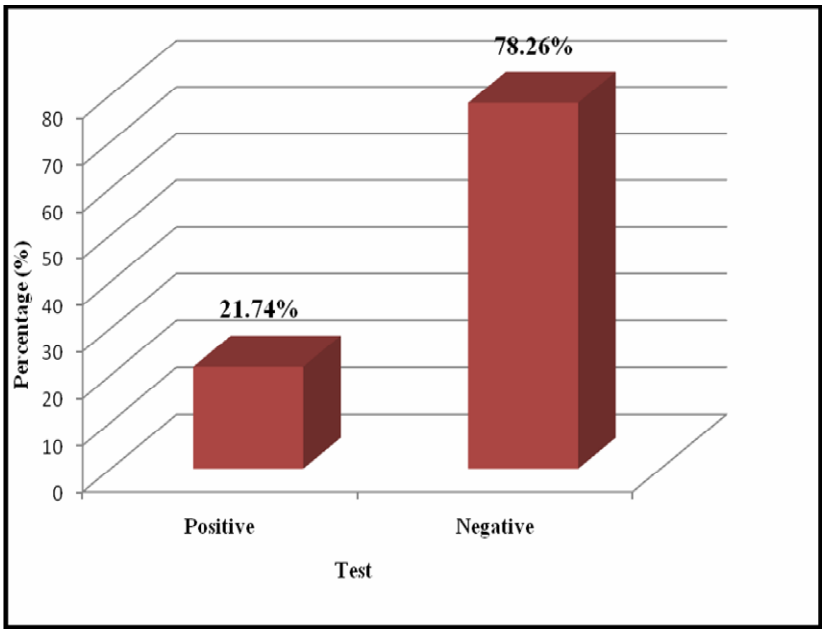


Figure.5 Distribution of HEV genotype IV in the samples study



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