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Original Article

Molecular Detection of HGV RNA in Chronic Hepatitis Patients from Afzalipoor General Hospital in Kerman, Iran

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Abstract

Background and Aims: Viral hepatitis is a global health problem with a high mortality rate. Recently, a new Flavi-like virus, provisionally named hepatitis G virus (HGV), has been described. HGV does not induce an immune response that is consistently detectable by using recombinant proteins from prokaryotic expression, therefore studies have been conducted by using polymerase chain reaction (PCR) based system. HGV is a blood born virus that is parenterally transmitted, however incidence of severe hepatitis with HGV is rare, and most infections are subclinical or mild.

Methods: A total of 180 blood specimens from chronic hepatitis patients (80 were HBV and HCV positive and 100 were Non B-Non C hepatitis patients) were collected, plasma was separated and stored at -80° C. The specimens were examined by the method of RT-PCR.

Results: There were 36 male and 14 female patients, majorities (95%) were living in the city of Kerman, and average age was 35 years old. The rate of infection with hepatitis viruses were as follows: chronic liver disease, including 21 (52.5%) with chronic hepatitis B infection, 17 with chronic hepatitis C infection (42.5%) co-infected with HGV, respectively ($p = 0.03$). Of the 180 patients, 40 were HGV RNA positive (17.7%).

Conclusion: HGV co-infection is highly prevalent among Kerman blood donors who are infected with HBV or HCV. The results also reveal that population negative for HCV and HBV are a low risk group for HGV infection.

Keywords: Hepatitis G virus; RT-PCR; HBV; HCV

Introduction

Hepatic cirrhosis is an important public health problem; the major burden is caused by infection with primary hepatotropic viruses, Hepatitis B virus (HBV), hepatitis C virus (HCV) and hepatitis D virus (HDV). Many individuals who have clinical evidence of virus-induced chronic liver disease fall in the non-B, non-C category, suggesting the existence of one or more yet unidentified additional viral agent(s). HGV, a novel human Flavivirus is considered to be a putative hepatotropic agent. This virus is widely distributed throughout the world and its presence has been reported from different geographical areas (1). GB virus-C/hepatitis G virus (GBV-C/HGV) is an enveloped positive-stranded RNA virus with a genome of about

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9.4 kb, belonging to the Flaviviridae. It is distributed worldwide, and at least five major genotypes of this virus have been proposed based on the sequence analysis of the 5´ GBV-noncoding region 5´ (NCR) or E2 gene. HGV is transmitted through blood transfusion. A few cases of fulminant hepatitis due to HGV infection have been reported, but it remains unclear whether the hepatitis was actually caused by HGV (2). HGV does not induce an immune response that is consistently detectable by using recombinant proteins from prokaryotic expression; hence prevalence studies have been conducted by using polymerase chain reaction (PCR) based system. HGV-RNA has been detected in many human populations(3).Despite the availability of diagnostic tests for detection of hepatitis C virus (HCV) and hepatitis E virus (HEV), cases of acute and chronic hepatitis in which patients do not exhibit markers for any known hepatitis viruses still exist and are referred to as non-A–E hepatitis. A total of 4%–20% of acute hepatitis cases in Europe and the United States are associated with non-A–E hepatitis although the polymerase chain reaction (PCR) has significantly enhanced the ability to detect viral infections, 40% of fulminant hepatitis cases are of unknown etiology (4). Hepatitis A, B, C, D and E are well characterized with an unequivocal association with liver disease in human beings. However the role of HGV in hepatitis is controversial (4,5). The genome of HGV contains a 458 nucleotide long 5' non-coding region (NCR), a single open reading frame (ORF) encoding a polyprotein of about 2900 amino acids, and a 3' NCR of 315 nucleotides(6) . The detection of viral genome is carried out by RT-PCR for diagnosis of infection. We have attempted to determine the prevalence rate of HGV infection among healthy blood donors and blood donors with HBV or HCV infection in Kerman province, Southeast of Iran. HGV capsid protein is absent or defective and the capsid may be provided by another virus (12). Therefore, co- infection of HGV with other viruses is an interesting and important subject for investigation . The reported HGV infection rates in hepatitis C patients were approximately 20% (13,14) , but a quite lower co infection rate of HGV and HCV (5.6%) was also reported (12) . HGV RNA can be found in serum for 2-20 weeks after infection. Retrospective studies of stored sera show that HGV can cause a persistent infection up to7 to 16 years (15).

Contradictory data about the clinical importance of HGV infection and nucleotide sequence mutation of the viral isolates indicated that the pathogenesis and variability of the virus are not fully understood(16). In this study, HGV RNA in the 180 samples were detected by RT- PCR with two different sets of primers. The results of this study may contribute to the determination of HGV co infection frequency in HCV and HBV patients, choice of the methods with high sensitivity and the primers with high efficiency for HGV RNA detection (4).

Methods

Patients

A total of 80 consecutive patients with biopsy proven chronic hepatitis, who visited the out Patient Clinic of Afzalipoor Hospital, Kerman (southeast of Iran) between April 2006 and May 2007, were included in the study. Patients were diagnosed as having chronic hepatitis, using conventional clinical, biochemical and histological criteria and PCR for HBV and HCV. The mean duration of the disease was 10 ± 6 months. Anti-HCV or HBsAg positive patients with persistently elevated alanine amino transferase (ALT) levels (>50 IU/l), detectable HCV RNA or HBV DNA in serum, and a liver biopsy that indicates either portal or bridging fibrosis or at least moderate degree of inflammation and necrosis were included under HCV or HBV related chronic hepatitis categories. Patients with autoimmune liver disease, drug-induced hepatitis and alcoholic liver injury were excluded. A group of 100 patients (chronic non-B, non-C hepatitis) were seronegative for both HBV and HCV markers as revealed by ELISA and PCR studies. History of blood transfusion (HBT) was recorded in 6 patients in this group. Another group of 40 patients (chronic hepatitis B) was

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seropositive for HBsAg and HBV DNA and seronegative for anti-HCV and HCV RNA. Twelve patients had HBT in this group. A total of 40 patients (chronic hepatitis C) were seropositive for anti-HCV and HCV RNA and seronegative for HBsAg and HBV DNA. Fourteen patients in this group had HBV. The healthy controls (n=100) were drawn from healthy men and women voluntary blood donors with normal liver function test (LFT) profiles and were serologically negative for HBV and HCV. Twenty five individuals had HBT in this group.

Serology

Serum samples collected from all 100 chronic hepatitis patients and 100 healthy control subjects were included for serological tests for hepatitis B and C viruses. Enzyme Link immuno sorbent assay (ELSIA) for HBsAg was carried out by using commercial ELISA kit (RADIM, Italy). Third generation ELISA kits was used for detection of antibody to HCV (RADIM, Italy). The analysis was based on the 200 patients using SPSS 11.5 for Windows software (SPSS Inc.Chicago, Illinois, and USA). Prevalence rate and 95% confidence intervals (95% CI) were calculated.

Molecular test

Five ml of blood sample was collected from each patient in a sterile container with 100 µl EDTA 0.5 M and mixed. Plasma was separated and stored at –80°C till further testing. Plasma samples of HCV or HBV patients were obtained from the Afzalipoor hospital in Kerman, Iran. The 80 samples were confirmed to be HCV or HBV positive by PCR and RT-PCR Method (HBV &HCV RG kit, Qiagen, Germany). This test is a routine work in our laboratory for detection of HCV and HBV. Forty samples were HCV Positive and 40 samples were HBV positive, and four samples were both HCV and HBV Positive.

Viral RNA was extracted from 200µl plasma by using High pure viral nucleic acid kit (Roche, Germany) and resuspended in 50µl elution buffer, RNA stored at –80°C for further testing.

RT-PCR for HGV

All samples were tested for the presence of HGV RNA by Nested RT-PCR method. cDNA synthesis was subjected to PCR using specific primers for the 5'-UTR genome regions in all samples and this region is highly conserved in the HGV. Reverse transcription was performed at 42°C for 60 minutes in 20 ml reaction volume using avian myeloblastosis virus reverse transcriptase(AMV) (Bioneer, Korea) and external antisense primer (for the detection of genomic RNA), using a thermal cycler (MWG, Germany). Five µl of cDNA was added to 25 µl of reaction mixture containing 5 units Taq polymerase, 0.01% gelatin,0.6 µM of each primer, 200 µM of each deoxynucleotid triphosphate, 5 µl of reaction buffer (50 mM KC1, 10 mM tris-HCI, pH = 8.3) and 1.5 mM MgCl2.

Polymerase chain reaction was performed for forty cycles at 94° C for 60 s, 55° C for 70s and at 72° C for 1.5 min. The condition for the second amplification step was similar to the first one, 20 µl PCR reaction containing 1 µl of first step PCR product. For primers the inner was sense located at position 134, and anti sense primer located at position 467 were used. Samples containing 333 bp PCR products in agarose gel were considered as HGV RNA positive, stained with ethidium bromide, and visualized under UV light. Standard precautions were taken to avoid sample-to-sample contamination and PCR product carry-over (17).

In each step of PCR, sterile water, normal serum control and a negative serum (non reactive on multiple assays) were used as negative controls, and a positive UTR region of HGV cloned plasmid (kindly provided by Dr M N Al-Ahdal) was used as positive control.

Results

There were 36male and 14 female patients, majority (95%) were from Kerman, average age was 35 years old. The rest have chronic liver disease, including 21 (52.5%) with chronic hepatitis B infection, 17 with chronic hepatitis C infection (42.5%) both were co-infected with HGV, respectively (p = 0.03).Of the 180 patients, 40 were HGV RNA positive (17.7%). Among the chronic hepatitis B

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Table 1: Primer sequences for PCR and RT-PCR

Name	position	Sequence	location
G1	Sence	agg tgg atg ggt gat	108- 123
G2	Antisense	tgc cac ccg ccc tca ccc gaa	5 31- 510
G3	Sence	tgg tag gtc gta aat ccc ggt	134 – 155
G4	Antisense	ggr gct ggg tgg ccy cat gcw t	467 – 445

patients, alanine transferases (ALT) were between 52 and 79 U/l in the HGV RNA-positive and HGV negative patient groups, respectively (p = 0.4). Half of those (21/40) with positive marker of hepatitis G infection (HGV RNA) were cirrhotic, Whereas only 9.5 % of those without positive marker of hepatitis G infection were cirrhotic (N.S.). Similarly, among the patients with chronic hepatitis C, the mean ALT was 48 and 94 U/l in the HGV.

RNA-positive and HGV-negative patient groups, respectively (p= 0.05). Forty-two percent (17/40) of these patients with positive marker of hepatitis G infection were cirrhotic; in contrast to 15.6% of those without cirrhosis (N.S.).Twelve of the chronic hepatitis patients had background of end-stage renal disease.

Among the 100 samples which were positive for HBV or HCV, a couple of the samples were positive for all three viral markers (HBV, HCV, and HGV), and among 100 samples which were healthy blood donor (general population), only 2 of the samples were positive for HGV RNA. Age and sex distribution of HGV RNA, HBV and HCV positivity are shown in Table 3. There was male predominance and majority of cases belonged to age group of 20-39 years. There were no differences in age or sex between the HGV-positive and HGV-negative groups both for HCV and HBV infected patients. Overall the main risk factor for infection with hepatitis virus reported for all groups was a hospital stay with or without surgery, while a history of transfusion was reported by a minority of patients. The relationship between disease severity and the presence of HGV RNA in serum is presented in Table 2. No significant differences were found between the mean alanine aminotransferase levels in HGV infected and uninfected subjects .When histological disease severity was assessed, no significant differences were present in total HAI score, fibrosis interlobular necrosis, portal inflammation or degree of piecemeal necrosis (Table 4). The prevalence of HBeAg and anti-HBe was not statistically different in HGV-positive and HGV-negative chronic hepatitis B patients.

Discussion

Prevalence of HGV in healthy blood donors around the world is variable. The lowest prevalence rate was reported in one study from

Table 2: The prevalence of HGV infection in patients from Kerman, Iran.

Subject	n	HGV (%)
General population*	100	2(2)
Type-B hepatitis patients	40	21(52.5)
Type-C hepatitis patients	40	17(42.5)
Total	180	40(17.7)

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Table 3: Age and Sex wise distribution of patients for HGV positive.

Viral Markers *Positive /total*	Age group 20-29 M	20-29 F	30-39 M	30-39 F	40-49 M	40-49 F	Total
Type-B hepatitis patients	8/12	3/5	6/13	3/7	1/2	0/1	21/40
Type-C hepatitis patients	2/6	6/10	5/12	1/5	2/4	1/3	17/40
General population	0/18	0/7	0/48	0/15	2/8	0/4	2/100
Total	18/40	7/18	14/73	5/27	4/14	2/8	40/180

China (0.7%) compared to 5.7% in Vietnam. Overall, the prevalence of HGV-RNA among blood donors ranged from 1% to2% in most countries. However, the HCV and HCV-RNA positive blood donors have the highest prevalence rate of HCV-RNA. In patients with acute nonA-E hepatitis, the prevalence of HGV ranged from 2-14.6%, a range similar to that found among patients with chronic non-A-E hepatitis (4-17.9%). However, among patients with acute A, B or C hepatitis, the prevalence rate of HGV-RNA virus ranged from 9.5-41%. In patients with chronic HBV the prevalence of HGV-RNA ranged from 3.2-9.8% compared to 8.2-21% in patients with chronic HCV hepatitis. In patients with fulminant non-A-E hepatitis, only one study reported HGV-RNA. However rate of HGV infection in patients with hepatocellular carcinoma was about 6% and in patients with alcoholic or autoimmune hepatitis was close to 10% (4).

In this study a total of 180 patients with chronic hepatitis were tested for HGV RNA by RT-PCR. All the samples were also tested for

Table 4: Clinical and histopathological characteristics of 80 patients with hepatitis B and C

	Type B Hepatitis(40)		Type C hepatitis(40)	
	HGV Pos (21)	HGV Neg(19)	HGV Pos(17)	HGV Neg(23)
Mean Age±SD	35±6.5	32±7.5	28±4.3	37±6.6
Mean ALT±SD	65±14	55±13	71±23	46±18
Fibrosis Score±SD	1.5±1	2±0.6	2.5±0.7	2.4±0.2
Piecemeal necrosis's	1.6±2.5	1.9±3	3±2.1	2.9±2
Intralobular Necrosis±SD	2.5±1.2	2.1±1.2	2.3±0.9	2.4±1.1
Portal inflammation ±SD	3±0.3	3.1±0.2	3.4±0.3	3.6±0.2
HBe-Ag Positive	----	---------	10(58)	18(78)

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HBsAg and antibody to HCV. Fifty out of 180 (27.7%) were positive for HGV RNA. The result was confirmed twice by PCR. The prevalence rate of HGV infection observed was 27.7% in our study. In other countries prevalence rates range from 1- 14% (19,20). In a group of about 300 West Africans, 14% prevalence rate of HGV RNA was noted (21). Adhani *et al*, had found 1-2% positivity in blood donors in U.S.A (22). In a Korean study, HGV was detected in 1-2.5% of blood donors (23). In Western India Arankalle et al reported no positivity of HGV RNA in 51 voluntary blood donors (24). Whereas Jain et al in a study in Northern India found 24% of blood donors were positive for HGV RNA (25).The prevalence of 27.7% probably represents true prevalence rate of HGV infection our country. Though higher prevalence rate were observed among males and in age group of 20-39 years, the finding was not statistically significant. Co-infection of HGV with HBV and HCV suggests similar modes of transmission. HGV has been isolated in semen of spouses of infected persons (26).

Out of 40 donors positive for HBsAg, 21 were also positive for HGV RNA showing a co-infection of 52.5%. Similarly, out of 40 donors positive for antibody to HCV, 17 were positive for HGV RNA showing a co-infection rate of 42.5%. As both HBV and HCV are transmitted parenterally and by sexual route, it suggests that HGV also follows a similar transmission route. The co-infection rate of HGV with HCV is 42.5% depending on risk factors and with HBV is 52.5% .Prevalence of HGV RNA in healthy blood donors were 12% .In our study the prevalence of HGV infection did not show any significant difference among blood donors positive for HBsAg and with antibody to HCV. The co-infection rate is comparable to other studies mentioned.

In the absence of any reliable serological assay for the diagnosis of infection, HGV RNA detection by RT-PCR remains the only available diagnostic tool indicating an ongoing infection (27).

So far there is no conclusive evidence that HGV produces hepatitis. However presence of HGV in hepatitis cases casts a doubt on this finding .Although the disease activity of HGV is not yet fully elucidated viral RNA is detected more frequently in patients with liver disease than in control subjects. Conducting prevalence rate studies in blood donors may be helpful in future when the exact role of HGV in inducing hepatitis is known.

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