

Hepatitis G virus infection in India: prevalence and phylogenetic analysis based on 5' non-coding region

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Objectives: To determine the prevalence of hepatitis G virus (HGV) infection in western India and to carry out phylogenetic analysis of HGV isolates. **Methods:** Reverse transcription-polymerase chain reaction (RT-PCR) assay was used to detect HGV RNA in serum samples obtained from paid plasma donors, patients with hemophilia and voluntary blood donors. Nine Indian and one Kenyan HGV RNA-positive samples were sequenced in the 5' non-coding region (5'-NCR). Phylogenetic analysis based on the comparison of a 101 nucleotide fragment from a large number of HGV isolates from 22 countries (including Indian and Kenyan sequences obtained during the present study) was carried out. **Results:** HGV RNA positivity rates among paid plasma donors from a commercial plasmapheresis unit (7/43, 16.3%) and patients with hemophilia (5/44, 11.4%) were significantly higher than that in voluntary blood donors (0/51; $p=0.003$ and 0.019 , respectively). Among patients with acute non-A to E hepatitis and fulminant hepatic failure, 1 of 50 and 1 of 28 were HGV RNA-positive, whereas 6 of 49 (12%) patients with chronic liver disease had circulating HGV RNA. All Indian isolates belonged to genotype 2, whereas the Kenyan isolate formed a distinct branch within genotype 1 consisting of African isolates. **Conclusion:** Our results suggest existence of parenteral transmission of HGV in the Indian population. HGV was not an important cause of acute non-A to E hepatitis or fulminant hepatic failure among the patients investigated. Genotype 2 seems to be the most prevalent genotype in western India. [*Indian J Gastroenterol* 2001;20:13-17]

Key words: GBV-C/HGV, genotypes, viral hepatitis

Although the clinical significance of infection with the hepatitis G virus (HGV) is not yet clearly understood, the virus appears to be associated with both acute and chronic hepatitis. Association of HGV with fulminant hepatic failure (FHF) remains controversial.¹ There is evidence for the parenteral transmission of HGV.²

HGV has a positive-sense RNA genome, approximately 9.4 kilobases in length, that 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. Genotypic classification of HGV isolates identified worldwide is being attempted, mostly using the 5'-NCR region.³⁻⁸

We have attempted to determine the prevalence of HGV RNA among patients with hepatitis, certain high-risk individuals and voluntary blood donors from western India. We sequenced 9 Indian HGV isolates and one isolate from a Kenyan patient touring India (Kenyan isolate) in the 5' NCR and determined the phylogenetic status of these isolates in comparison with those sequenced earlier from different geographic areas.

Methods

Serum samples stored at -20°C were tested for HGV RNA. These were obtained from voluntary blood donors ($n=51$), hemophiliacs (44) and patients suffering from chronic liver diseases (49); these had been collected in Pune in 1997. Stored sera from paid plasma donors (43) with evidence of exposure to parenterally transmitted human immunodeficiency (HIV)⁹ and hepatitis C viruses (HCV)¹⁰ from a commercial plasmapheresis unit in 1989 were also tested. In addition, patients with non-A to E acute viral hepatitis (50) and fulminant hepatic failure (FHF; 28) from Pune, who constituted a subset of acute viral hepatitis cases investigated by us during the period 1994-97, were also investigated. In addition, serum from a Kenyan tourist visiting India for the first time, obtained 9 days after arrival for a liver illness (?subacute hepatic failure, ?reactivation of chronic liver disease), was also tested.

Polymerase chain reaction

All serum samples were tested for the presence of HGV RNA employing nested reverse transcription-polymerase chain reaction (RT-PCR). RNA was isolated using Trizol reagent (GIBCO BRL, Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. cDNA synthesis was carried out at 42°C for one hour using the external antisense primer. This was followed by 35 cycles at 94°C for 1 minute, 55°C for 2 min and 72°C for 3 min, for first-round PCR and 30 cycles for the second-round PCR. The PCR products were analyzed by 2% agarose gel electrophoresis and visualized using ethidium bromide. The primers used were:

External sense primer (89-106): 5' AGG TGG TGG ATG

Table 1: HGV isolates compared in this study

Country	No. of isolates	Accession Numbers
Australia	1	AF006975
China	1	U86154
Egypt	2	AB004505, AB004507
France	1	AF104403
Germany	1	AF006967
Greece	3	AF006966, AF006984, AF006985
Ghana	4	AB004508, AB004509, AB004511, AB004514
India	9	Present study*
Japan	5	AB012005, AB012010, AB012013, AB012022, AB004521
Kenya	1	Present study**
Korea	2	AB004522, AB004523
Myanmar	4	AB018651, AB018652, AB018655, AB018665
Nepal	2	AB004527, AB004528
Pakistan	2	AF003154, AF003162
Peru	2	AF006977, AF006978
Sweden	4	AF063855, AF063860, AF063861, AF063862
Spain	7	AF038789, AF038796, AF038800, AB004529, AF038782-84
Thailand	3	U91713, U91714, U917122
UK	5	AF003168, AF003171, AF003175- AF003177
USA	2	AB004530, AB004532
Vietnam	4	AB013189, AB013193, AB013203, AB013233
Zaire	2	AF003184, AF003186
Total	67	

*Accession Numbers: AF148543-AF148551

**Accession Number: AF148552

GGT GAT 3'

External antisense primer (512-492): 5' TGC CAC CCG CCC TCA CCC GAA 3'

Internal sense primer (114-135) 5' TGG TAG GTC GTA AAT CCC GGT 3'

Internal antisense primer (457-436): 5' GGA GCT GGG TGG CCT CAT GCTT 3'

Sequencing: Amplified PCR products (343 bases) were purified using mini column (Wizard; Promega, Madison, WI, USA) and sequenced in both directions using Taq dye terminator cycle sequencing kit (Perkin Elmer, NJ, USA) and an automatic sequencer (301 ABI; Perkin Elmer, NJ, USA).

Phylogenetic analysis

Phylogenetic analysis of HGV isolates from this study and previously described HGV DNA sequences obtained from the Genbank database was done using programs available in the software MEGA¹¹ and PHYLIP version 3.5c.¹² In MEGA, Jukes-Cantor (JC) algorithm was utilized employing the neighbor joining (NJ) method, and the reliability of different phylogenetic groupings was evaluated using the bootstrap test (1000 bootstrap replications). In PHYLIP, JC algorithm employing the NJ

method with or without mid-point rooted was used; the results obtained were evaluated using bootstrap analysis (SEQBOOT, 1000 bootstrap replications).

The phylogenetic analysis was carried out on the basis of comparisons of 340-nucleotide and 101-nucleotide fragments of the 5'-NCR of HGV⁴ representing all the available HGV sequences.

Statistical analysis

Fisher's exact test (one-tailed) was used to compare proportions.

Results

Prevalence of HGV RNA

Sera from none of the 51 voluntary blood donors tested positive for HGV RNA (Table 2); 7/43 (16.3%) paid plasma donors ($p=0.0031$ as compared to voluntary donors) and 5/44 (11.4%) hemophiliacs ($p=0.0187$) tested positive.

HGV RNA positivity rate in patients with non-A to E acute viral hepatitis (1/50) and FHF (1/28) were no different from that in voluntary blood donors, whereas that in patients with chronic liver disease (6/49; 12.2%) was higher ($p=0.05$). Serum sample from the Kenyan tourist tested positive.

Phylogenetic analysis

Ten of the 21 HGV RNA-positive samples (chronic liver disease 4, hemophiliacs 2, paid plasma donor 1, hemodialysis patient 1, non-A to E acute viral hepatitis 1, and the Kenyan tourist) were sequenced.

Analysis of a 101-nucleotide (-366 to -266) region, but not that of a 340-nucleotide 5'-NCR region revealed distinct efficient groups of various isolates (data not shown). Initial analysis was performed with 174 available sequences; for subsequent analysis, only representative sequences from various countries and groups (except those from the present study) were retained.

The 67 isolates analyzed fell into four major groups (genotypes) (Fig). A majority (43/64, 67.2%) belonged to group I (genotype 2); these included all the isolates from Australia (1), Egypt (2) France (1), Germany (1), Greece (3), India (9), Pakistan (2), Peru (2), Spain (7), Sweden (4) and USA (2), and 3 of 5 isolates from the UK, 1 of 2 isolates from Nepal, 1 of 2 isolates from Korea, 2 of 4 isolates from Vietnam and 2 of 4 isolates from Myanmar.

Table 2: Frequency of HGV RNA positivity

Category	No. positive / Tested (%; 95% CI)
Voluntary blood donors	0/51
Non-A-E acute viral hepatitis	1/50 (2%, 0% - 5.9%)
Chronic liver disease(s)	6/49 (12.24%, 3% - 21.4%)
FHF	1/28 (3.6%, 0% - 10.4%)
Hemophiliacs	5/44 (11.36%, 1.99% - 20.7%)
Paid plasma donors	7/43 (16.28%, 5.2% - 27.3%)

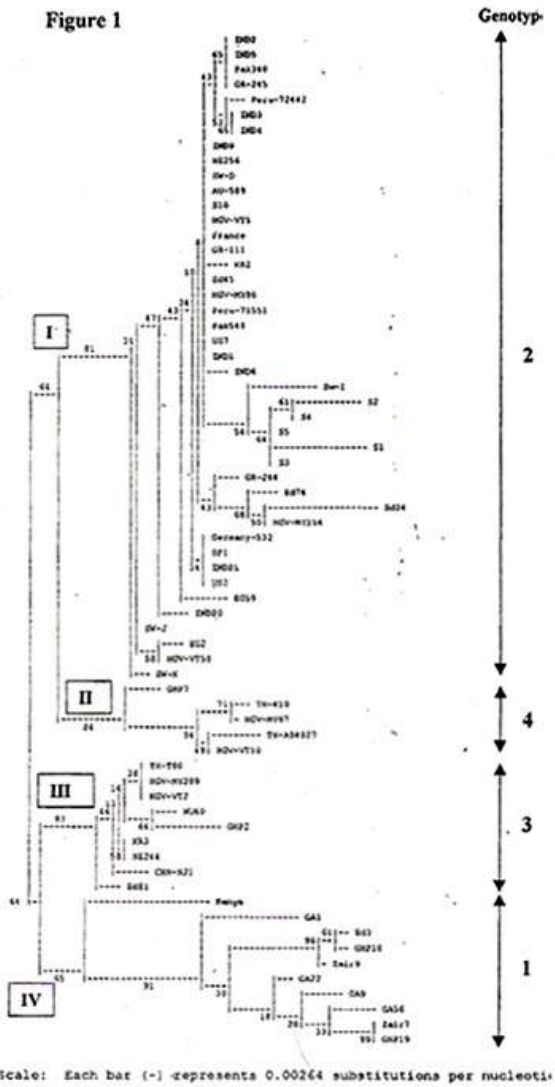


Fig: Phylogenetic analysis of the 5'-NCR of 93 HGV isolates employing MEGA software utilizing Jukes-Cantor distance and 1000 bootstrap analysis

Group II (genotype 4) consisted of 5 isolates (7.8%), including two from Thailand, one from Japan and one each from Myanmar and Vietnam.

Group III (genotype 3; 9 isolates, 14%) included one isolate each from China, Nepal, Thailand, Vietnam, the UK, Korea and Myanmar, and two isolates from Japan.

Group IV (genotype 1; 10 isolates, 15.6%) included mainly African isolates (Zaire, Ghana and Kenya), 1 of 5 isolates from the UK and 2 isolates from Japan.

Of the HGV isolates compared, the UK and Myanmar isolates segregated into three genotypes, i.e., 1, 3, 2 and 2, 4, 3, respectively, whereas the Nepalese, Korean and Thai isolates grouped into two genotypes. Though isolates from Zaire, Ghana and Kenya were of genotype 1, isolates from another African country, Egypt, clustered in genotype 2.

Discussion

A substantial proportion of patients with acute viral hepatitis and FHF from India test negative for known hepatitis viruses, i.e., A to E.^{13,14} HGV RNA testing of 50 patients with non-A to E acute viral hepatitis showed that HGV was not responsible for hepatitis in most of them, only one being HGV RNA positive. This is in contrast to reports from Argentina (30.6%)¹⁵ and Italy (39%)¹⁶ showing noticeably higher positivity rates. However, studies from the US,¹⁷ Japan^{18,19} and Brazil²⁰ did not show HGV as an important cause of acute viral hepatitis. HGV does not appear to be an important etiologic agent for FHF in the Indian patients investigated (1/28). A preliminary report from Delhi showed HGV RNA in 2 of 16 patients with non-A to E FHF.²¹

We found higher HGV RNA positivity rate (12.2%) among patients with chronic liver disease. A study from northern India²² also found 12% HGV RNA positivity among patients with cirrhosis; however, 24% of healthy blood donors in that study were HGV RNA-positive.

Significantly higher HGV RNA positivity among hemophiliacs and paid plasma donors with a high prevalence of antibodies to HIV⁹ and HCV¹⁰ confirms the importance of parenteral routes in the transmission of HGV. Absence of HGV RNA in 51 voluntary blood donors indicated that, as in Italy,²³ Germany²⁴ and the US,²⁵ HGV infection is infrequent in our population. South Africa (11.1%),²⁶ Vietnam (7.4%)²⁷ and Brazil (5.8%)²⁸ reported higher exposure of voluntary blood do-

nors to HGV. In China, a distinct difference between voluntary blood donors (0.8%) and paid donors (7.9%) was noted.²⁹

Phylogenetic analysis assesses evolutionary relationship between different viral isolates. Recent studies have shown that, unlike HCV, subgenomic analyses of different regions of the HGV genome do not lead to distinct differentiation of the various isolates.^{6,30} Based on the available 5' NCR sequences, Muerhoff *et al*³ proposed classification of the HGV isolates into three genotypes. In 1997, Smith *et al*⁴ and Linnen *et al*⁵ compared HGV 5' NCR sequences from a few additional countries, with comparable results. Based on restriction fragment length polymorphism analysis of the 5'-NCR too,⁷ HGV isolates were classified into 3 major groups.

The segment of HGV 5'-NCR used for phylogenetic analysis appears crucial for better segregation of HGV isolates into distinct genotypes/groups representing different geographic regions.⁴ Our data confirm this finding.

Use of a 148-nucleotide 5'-NCR segment led to the identification of a novel genotype of HGV isolates from Myanmar and Vietnam, the genotype 4; in our 101-nucleotide fragment analysis too, isolates formed a separate group, along with most Thai isolates and a Japanese isolate.

Overall, genotype 2 seems to be highly prevalent and is distributed worldwide. All our Indian isolates belonged to this genotype.

In conclusion, our study suggests that HGV infection has a low prevalence in western India. It confirms the usefulness of an 101-nucleotide fragment of the 5'-NCR in genotyping HGV, and places all Indian isolates in genotype 2. The Kenyan isolate emerged to be a distinct branch of genotype 1 represented mainly by African isolates.

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