

Infection with hepatitis G-virus and viral hepatitis in India

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Association of a new non-A-E hepatitis virus designated as hepatitis G virus (HGV) or GBV-C with acute and chronic hepatitis, particularly with fulminant hepatic failure is not clearly understood. In view of paucity of data on the prevalence of HGV in India where viral hepatitis is a major public health problem, we have examined the presence of HGV infection in patients with acute viral hepatitis (AVH), fulminant hepatic failure (FHF) and in normal healthy blood donors. HGV-RNA sequences were detected in patient's serum by reverse transcription plus nested polymerase chain reaction (RT-PCR) using primer sequences located in the conserved NS3 helicase region of the HGV genome. Serum samples collected from 36 acute viral hepatitis, 16 fulminant hepatic failure and 50 healthy voluntary blood donors who did not have symptoms of viral infection or liver disease were recruited for the study. HGV-RNA was detected in 6 (37.5%) of 16 patients with fulminant hepatic failure, in 7 (19.4%) of 36 acute viral hepatitis, and two (4%) in 50 control blood donors. Of the 6 HGV positive FHF patients, only one (1/6; 16.6%) was in non A-E category while 5 (20.0%) patients were HGV positive out of the 25 non A-E AVH cases. In both AVH and FHF, HGV was more frequently detected in (8/13; 61.5%) patients co-infected with other hepatotropic viruses and the most common co-infections were found to be HEV (6/8; 75%) and HBV (5/8; 62.5%). The frequency of hepatitis G virus is found to be certainly higher (37.5%) in fulminant hepatic failure than that in any other type of viral hepatitis in India. But since the virus is often detected in co-infection with either hepatitis B or E virus, which are known potential hepatitis agents, the role of HGV as an independent hepatitis agent is uncertain.

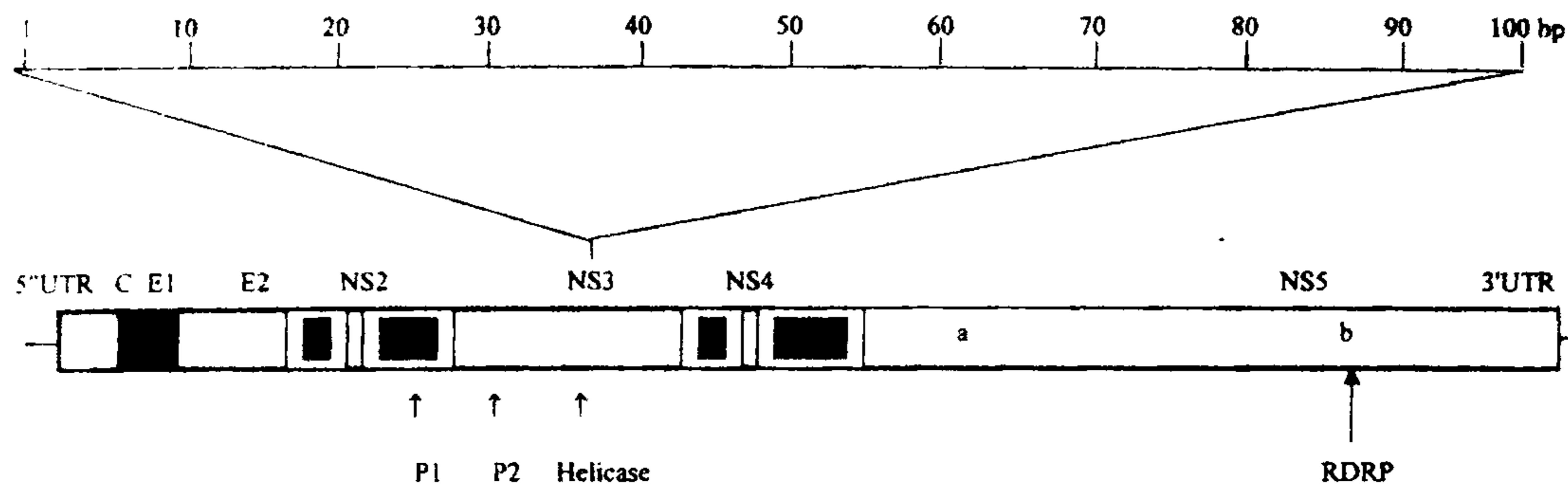
SEVERAL distinct hepatotropic viruses namely, hepatitis A, B, C, D and E viruses are known to cause viral hepatitis, each with a different severity of hepatic damage in humans but they certainly cannot account for all cases of hepatitis. Several epidemiological and experimental evidence indicated existence of additional hepatitis agents which may be transmitted parenterally^{1,2}. The virus can also be transmitted sexually³ and from

mother to child^{3,4}. Recent discovery of the hepatitis G virus (HGV) or the GB virus C (GBV-C)⁵⁻⁸, a single stranded RNA virus which belongs to the flavivirus family, has been considered to be a non-A-E hepatitis agent. HGV is distinct from hepatitis C virus but has a similar genomic organization with approximately 9,362 ribonucleotides. It contains a continuous open reading frame (ORF) which codes for a precursor protein of about 2,873 amino acids that is cleaved to structural and nonstructural proteins^{5,8}.

HGV-RNA sequences have been detected in sera from patients with non-A-E acute and chronic hepatitis and cirrhosis^{5,6,9,10}. HGV/GBV-C is often detected in patients who received multiple blood transfusion¹¹⁻¹⁴ or in hemodialysis patients¹⁵⁻¹⁸ and intravenous drug users¹⁹⁻²¹. The virus has a global distribution and is reported to be present in 1-3% in volunteer blood donors^{5,22,23}, a frequency higher than that observed for hepatitis C virus (HCV) or hepatitis B virus (HBV)^{19,22,24}. Recently, several authors²³⁻²⁵ have shown a high (50%) prevalence of hepatitis G virus, specifically in non A-E fulminant hepatitis and suggested that HGV may be involved in the aetiology of fulminant hepatic failure. This was supported by Heringlake *et al.*²² who found 11 (50%) of 22 German patients with fulminant hepatic failure were infected with HGV. They also observed that more than 50% of HGV-infected patients were co-infected with hepatitis B virus (HBV). In contrast, Sallie *et al.*²⁶ failed to detect HGV sequences in any of liver biopsy specimens from patients with fulminant hepatic failure. Recently, a very low (12.5%) prevalence of HGV has also been reported in Indian patients with fulminant hepatic failure²⁷. However, these authors failed to observe any co-infection along with HGV infection. In view of conflicting reports and paucity of information on the prevalence of HGV in India where viral hepatitis is a major public health problem, we have used reverse transcription and nested PCR to detect the presence of hepatitis G virus in patients with acute viral hepatitis, fulminant hepatic failure and in normal healthy blood donors.

The study included a total of 52 patients comprising 36 acute viral hepatitis, 16 fulminant hepatic failure and 50 healthy normal controls. This also included 10 non A-E cases (8 AVH and 2 FHF) diagnosed previously in our laboratory in order to enrich the non A-E cases. The blood samples were obtained from the patients who were not commercial blood donors, had not undergone intravenous drug injections, blood transfusions or hemodialysis and admitted in medical wards of Lok Nayak Hospital, New Delhi during the period between March 1996 and February 1997. Informed consent was obtained from all patients and control blood donors. The patients were evaluated on the basis of detailed history and physical examination. Acute viral hepatitis was defined as those which had acute self-limited disease and a

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P1 = Zn protease in NS2b
 P2 = Chymotrypsin-like serine protease
 RDRP = RNA dependent RNA polymerase

Figure 1. Genome organization of HGV showing structural (C, E1 and E2) and non-structural (NS2, NS3, NS4 and NS5) regions along with conserved motifs representing enzyme functions.

serum aspartate aminotransferase (AST) elevation of at least five fold or clinical jaundice or both. The diagnosis of fulminant hepatic failure was considered when after a typical acute onset, the patient became deeply jaundiced and went into hepatic encephalopathy within 4 weeks of onset of the disease with no past history of chronic liver disease. Acute hepatitis A was diagnosed by the detection of IgM antibody to hepatitis A virus (IgM anti-HAV) in the initial serum sample. Acute B virus hepatitis was diagnosed by the presence of either HBsAg or IgM anti HBc in the initial sample. Acute hepatitis C and E were diagnosed by the detection of anti-HCV and IgM anti-HEV virus respectively. Patients visiting medicine OPD for health check-ups or other complaints and volunteer blood donors who have no clinical and biochemical symptoms of hepatitis or viral infection were recruited as controls. Drawing of blood and separation of serum was completed under aseptic conditions within 4 h of collection and the serum samples were stored at -70°C until use.

Following initial clinical assessments of the patients, various biochemical parameters such as detailed haemogram, blood sugar, serum electrolytes, prothrombin time and liver function tests such as serum glutamate oxalacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase, serum bilirubin tests were done on every follow-up visit of patients who attended out-patient departments and also weekly on patients admitted in medicine ward of Lok Nayak Hospital, New Delhi. Special investigations such as ultrasonography of liver, gastro-intestinal endoscopy were performed whenever considered necessary.

Serological tests were performed in all the study subjects for all the known hepatitis viruses by using commercially available ELISA kits and their manufacturer's

manual. All the serum samples were analysed by serology for the detection of antibodies and antigens of different hepatotropic viruses. IgM antibodies against anti-HAV were detected by using HAV antibody EIA test kit (Abbott, USA), hepatitis B virus surface antigen (HBsAg) was detected by Eliscan elisa strips (Ranbaxy, England), IgM antibodies against anti-HBc using anticorase MB-96 (TMB kit, General Biological, Taiwan). Anti-HCV antibodies were detected by using INNOTEST HCV Ab III kit (Innogenetics NV, Ghent, Belgium) and IgM anti-HEV by using GLD HEV IgM ELISA kit (Genclabs, Singapore).

RNA was extracted from serum by acid-guanidinium-phenol-chloroform method of Chomczynski and Sacchi²⁸ with slight modifications^{29,30}. Briefly, the methods involved, 100 μl of patient's serum to which 500 μl of lysis buffer (4 M guanidinium thiocyanate (GITC), 0.7 M sodium citrate (pH 7.0), 2% sarcosyl and 0.1 M (final conc.) betamercaptoethanol were added. To this, 50 μl of 2 M sodium acetate (pH 5.0) was added and extracted once with phenol and chloroform-isoamyl alcohol (49:1) and kept on ice. After centrifugation at 12,000 rpm for 20 min at 4°C , the pellet was resuspended in 150 μl of lysis buffer (without β -ME) and precipitated in chilled isopropanol and kept at -70°C for 90 min, centrifuged and washed once with 70% ethanol. Finally, the pellet was dissolved in 25 μl of diethyl pyrocarbonate (DEPC) water and stored at -70°C until use. Reverse transcription for preparation of complementary DNA (cDNA) with the AMV-reverse transcriptase (Promega-Madison, WI, USA) and the PCR were performed in a single reaction tube using Cetus DNA Thermal Cycler (Perkin Elmer Cetus, Roche, New Jersey, USA). The outer and inner primer sequences³¹ for nested PCR were selected from NS3 helicase region of GBV-C genome^{5,8} (see

Figure 1) and the sequences (Gene Bank accession No. HGV: U44402 & U45966) were as follows:

Outer primers: Sense S1: 5'-GGC ACC TCG TGT TCT GCC A-3';

Antisense AS1-5'-AGG TCT CCG TCT TTG ATG AT-3'

Inner primers: Sense S2: 5'-CAT TC (A/C) AAG GCG GAG TGC GAG-3';

Antisense AS2-5'-(A/G) TC (T/C) TT GAT GAT GGA ACT GTC-3'.

Reverse transcription PCR was carried out as described earlier by us²⁹⁻³¹. For the first round of PCR, 5 μ l of extracted template RNA was added to 25 μ l of PCR master mix containing 10 mM Tris-HCl, pH 8.4; 50 mM KCl; 1.5 mM MgCl₂, 250 μ M dNTPs (Promega, Madison, USA), 20 pmoles each of HGV outer primers, (AS1, S1) and 0.5 units of Taq DNA polymerase (Promega, USA) and 1 μ l reverse transcriptase (10 units/ μ l, Promega, USA) were added and incubated at 42°C for one hour. Subsequently, PCR amplification was carried out after inactivating reverse transcriptase at 94°C for 5 min and 35 cycles of PCR with 30 s each of denaturation at 94°C, annealing at 50°C and extension at 72°C. Finally, in the last cycle the extension was prolonged for 8 min. The second round of PCR was performed by using 5 μ l of first PCR product as a template and amplified with the second PCR mix containing HGV inner primers (S2 and AS2). All compositions and temperature profiles were kept the same as that of the first PCR. A GBV-C positive control obtained as gift from Kendo Kiyosawa of Shinshu University, Japan and a negative control comprised of distilled water instead of RNA template were run parallelly along with the test samples. All safety precautions associated with the RT-PCR procedure were strictly followed. An expected second and the final PCR amplicon size of 101 bp was detected in an ethidium bromide-stained 3% Nusieve agarose gel (FMC Bioproducts, Rockland, ME, USA) and it matched the positive control used (see Figure 2).

Presence of HGV RNA in patient's serum as revealed by 101 bp RT-PCR product (Figure 2) was detected in 7 (19.4%) of the 36 acute viral hepatitis patients, 6 (37.5%) of the 16 patients with fulminant hepatic failure and in two (4%) out of the 50 normal control blood donors (Figure 3). Of the 52 patients in total, the male and female ratio was 39:13 = 3:1 and 50% of the patients belonged to the age group 21-30 years, the mean age being 27.78 \pm 9.83 years. No significant difference was observed between the age group for AVH and FHF cases as well as between the 13 HGV positive and the 39 HGV negative patients. Of 25 non A-E acute viral hepatitis cases which included additional 8 previously diagnosed non-A-E cases, 5 (20%) and of 6 non A-E fulminant hepatic failure 1 (16.6%) were positive for HGV-RNA

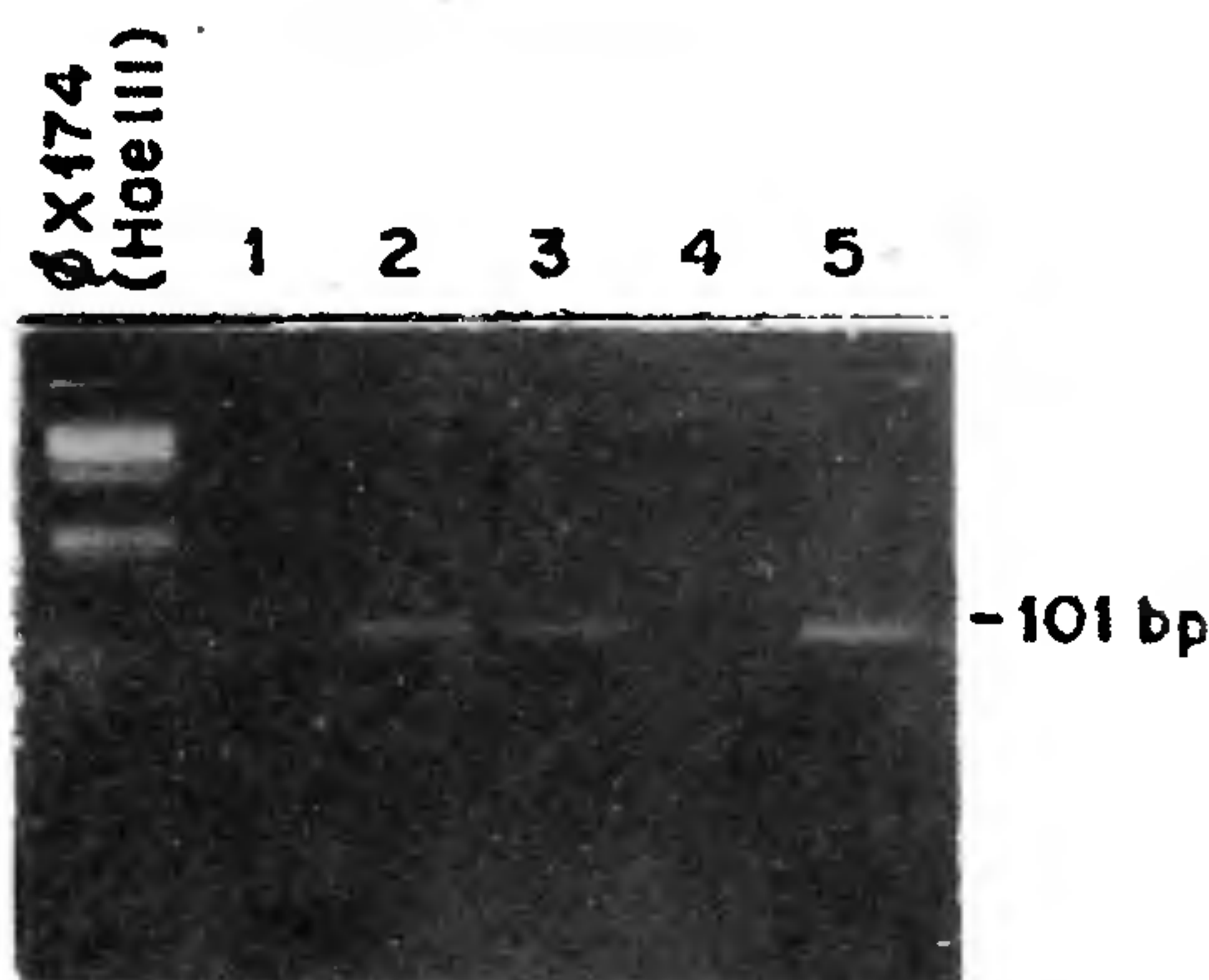


Figure 2. Ethidium bromide stained 3% Nusieve agarose gel showing 101 bp amplification product by reverse transcription and nested polymerase chain reaction using primer sequences from the conserved NS₃ helicase region of the HGV genome. Lanes 1 and 2 are negative and positive controls respectively and lanes 3, 4 and 5 are test samples showing HGV positivity in lanes 3 and 5. The extreme left lane shows HaeIII digested ϕ x 174 DNA molecular weight marker.

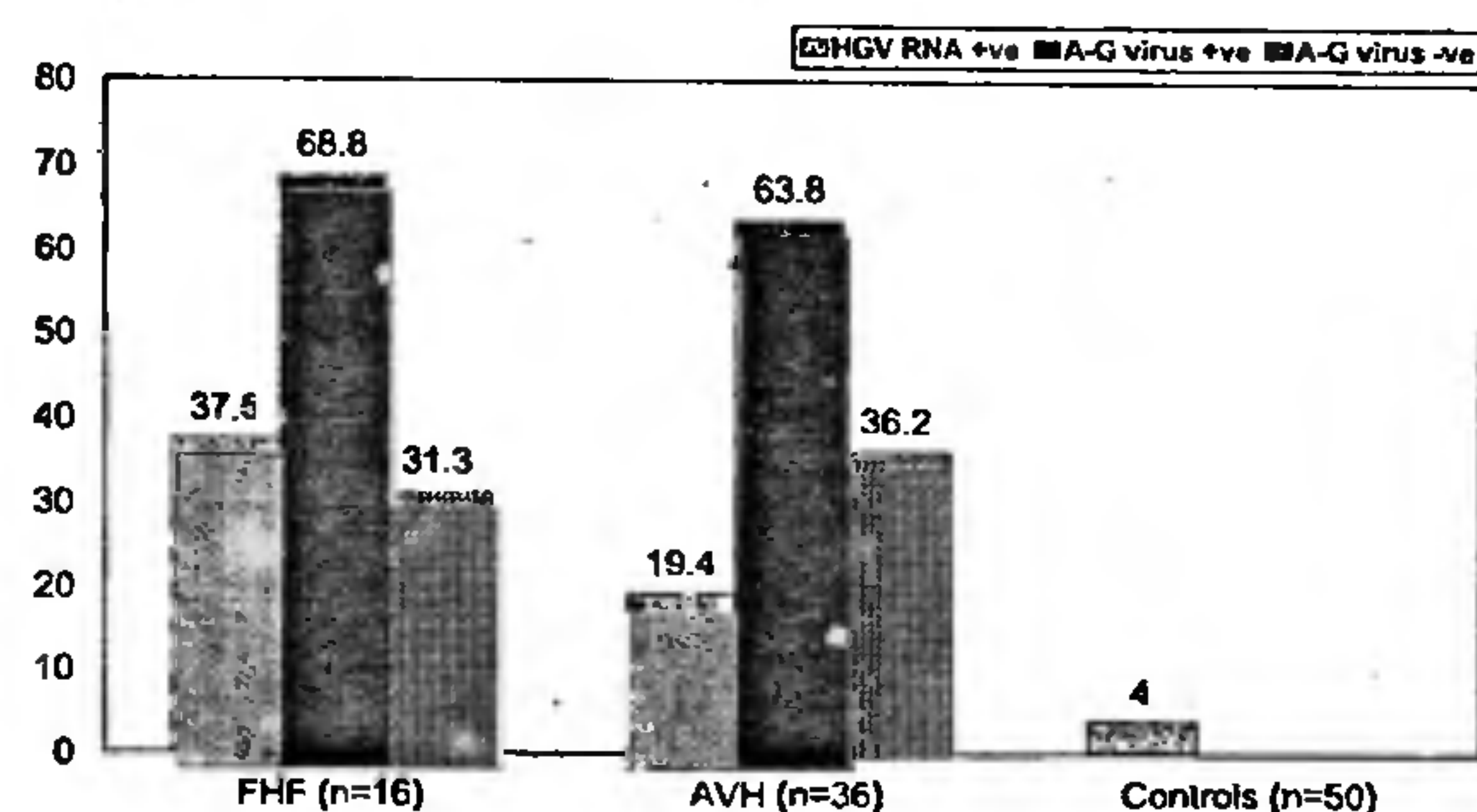


Figure 3. Prevalence of HGV RNA in FHF and AVH cases detected by RT-PCR.

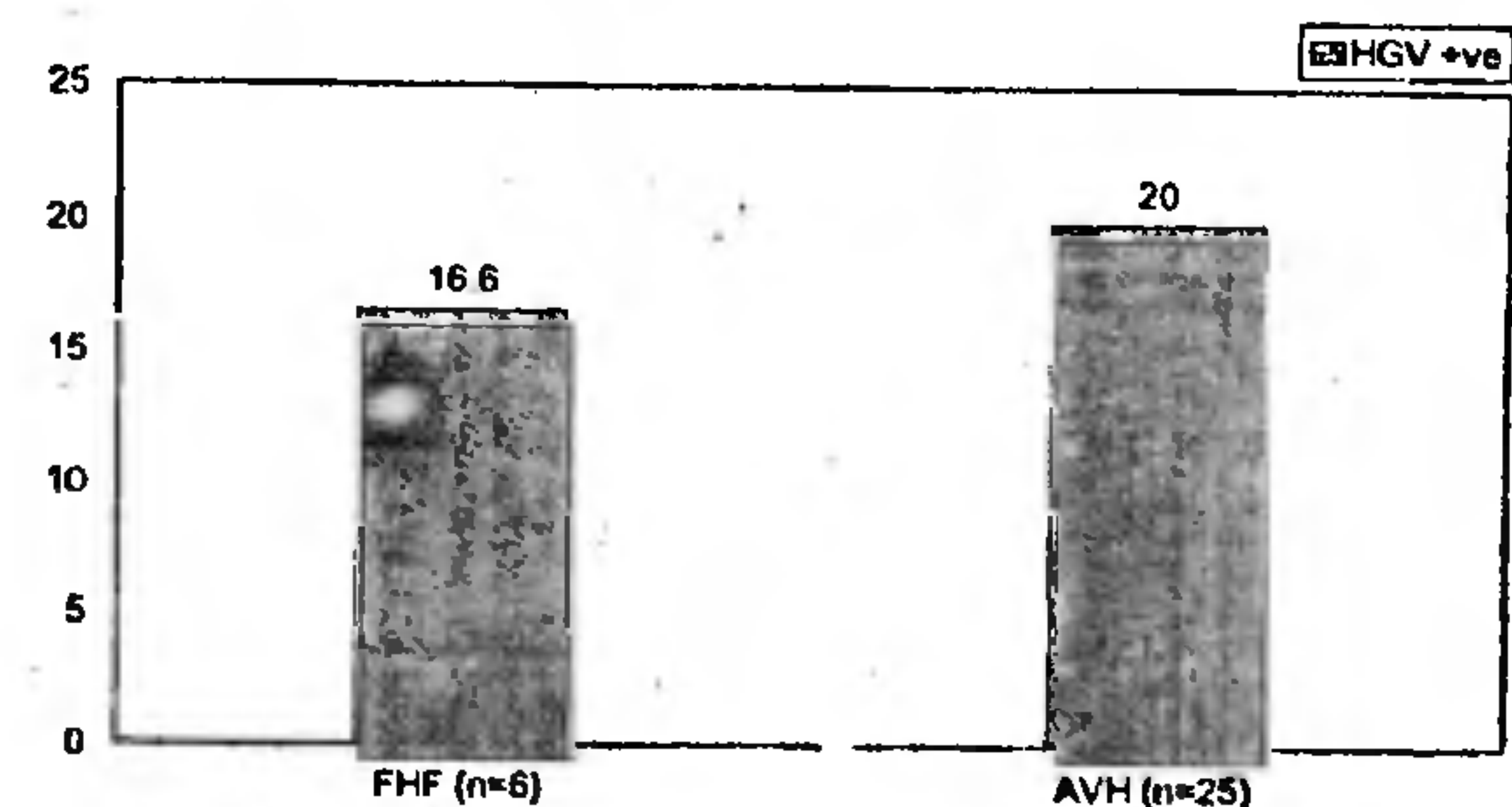


Figure 4. Prevalence of HGV RNA in non A to E cases of FHF and AVH as detected by RT-PCR.

(Figure 4). In 18 (34.6%) patients, 13 (36.2%) of AVH and 5 (31.3%) of FHF, no known viral agent could be

Table 1. Co-infection of other hepatotropic viruses with HGV in acute and fulminant hepatic failure patients

Type of hepatitis	Positive for HGV	HGV alone	HGV with co-infection of (n = 8)				Total cases with co-infection
			HAV	HBV	HCV	HEV	
FHF (n = 16)	6	1	-	3	-	4	5
AVH (n = 36)	7	4	-	2	1	2	3
Total	13	5 (38.5)	-	5 (62.5)	1 (12.5)	6 (75)	8 (61.5)

Figures in parentheses indicate percentage.

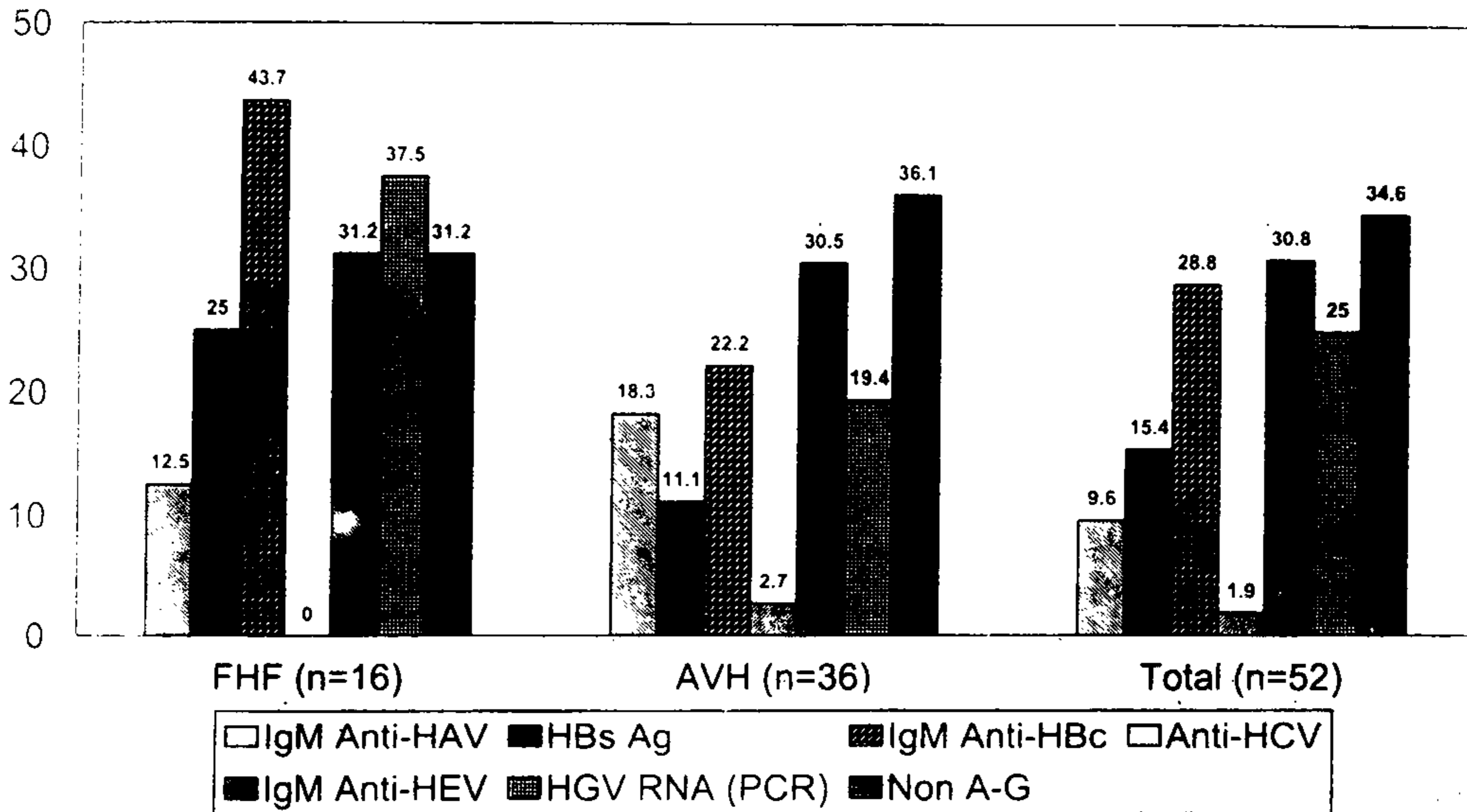


Figure 5. Frequency of different hepatotropic viruses in AVH and FHF cases as detected by specific viral markers and RT-PCR.

detected. Those samples which were found negative for HGV-RNA were checked for quality of RNA in their blood samples by amplifying β -globin gene in an RT-PCR. Interestingly, HGV infections were more frequently detected in patients co-infected with other hepatotropic viruses (8/13, 61.5%, Table 1). Figure 5 shows the frequency of different hepatitis virus types in AVH and FHF cases as detected by using specific viral markers and PCR. The most common co-infection in both AVH and FHF was found to be HEV (6/8; 75%) followed by HBV (5/8; 62.5%) (Table 1). No patient with HGV was found to have HCV infection except in one AVH patient who had also HEV and HGV infection along with HCV. Such triple infections involving HGV were found in 4 cases (6.6%); three cases with HBV, HEV, HGV and one with HCV, HEV and HGV. Clinically, no statistically significant difference was observed in general symptomology, physical sign and liver function between HGV positive and negative cases. Interest-

ingly, there were a total of 12 (23%) mortalities but none was infected with HGV alone.

Since quite a good number of the acute and chronic viral hepatitis cases fall in the category of non A-E hepatitis, it has been suggested that the newly identified hepatitis G virus may account for, at best, some of these cases. This was strengthened by the fact that several authors detected presence of HGV infection in about 50% of non-A-E cases only^{22,23,25}. They further showed that this new virus is most prevalent in fulminant hepatic failure. However, in the present study, out of 25 non A-E acute viral hepatitis patients, only 5 (20%) and 1 (16.6%) of 6 non A-E fulminant hepatic failure cases were found to be infected with HGV. This is in good agreement with the consensus reached in a recent meeting that HGV is responsible for no more than 20% of acute non A-E viral hepatitis³². The absence of any known viral agent in majority (25/31; 80.6%) of non A-E hepatitis indicates involvement of some yet unknown

hepatitis virus(es) or non-viral causes in these patients with unexplained hepatitis. The prevalence of HGV in normal population in India is not yet known. We have observed in the present study 4% (2 out of 50) of normal healthy blood donors positive for HGV RNA. Although similar results (4.7%) have been obtained for German population²², the frequency seems to be much lower (1–3%) in the US^{5,22}. It indicates that the prevalence of HGV in normal population may vary from country to country. Nevertheless, the infected individuals might be negative for HGV-RNA if the HGV-RNA is below the level of detectability. Therefore, the actual prevalence of HGV may be higher than that measured by current HGV-RNA tests. The frequency of HGV infection was however, reported to be much higher in commercial blood donors^{5,33,34}; it is about 3 times higher than what (1.3–1.7%) was observed for voluntary blood donors in the USA^{5,22,33}. This confirms that HGV has a global distribution and larger studies are needed to reconfirm the frequency of HGV in general population as well as in commercial blood donors in India.

In India, no information is available on the prevalence of HGV in acute viral hepatitis. This is the first report of a HGV-RT-PCR assay which shows the prevalence of hepatitis G virus infection to be 19.4% (7/36) in acute viral hepatitis (Figure 3). This is consistent with earlier reports^{5,23} on hepatitis G virus infection in acute viral hepatitis with rates varying from 0 to 34.0%. There have been several reports^{22,25,27,35} on the association of hepatitis G virus with the development of fulminant hepatic failure. Although Sallie and colleagues²⁶ could not detect HGV in FHF, several authors observed about 50% positivity of HGV in FHF^{22,25,35}. We have observed 37.5% (6/16) positivity of HGV in Indian patients with fulminant hepatic failure. In sharp contrast to our observations, many of these authors^{25,27} also failed to detect any co-infection along with HGV. In the present study, HGV infection was mostly seen with co-infection with other known hepatitis viruses. This is in good agreement with several reports from other regions^{4,22,36,37}. Infection with HGV alone could be detected in only 5 of the total of 13 HGV positive cases (5/13; 38.5%) while it is most frequent in cases with multiple infections (8/13; 61.5%). HEV and HBV have been found to be the most common co-infection both in AVH and in FHF. These results are in good agreement with the results of other authors^{22,25,26}. Interestingly, co-infection with HCV seems to be rarely associated with acute viral hepatitis and fulminant hepatic failure in India. Since only serological tests were performed and no PCR for these viruses, it is possible that their prevalence has been underestimated. Most intriguing is the observation of high prevalence of HEV-HGV co-infection since HAV and HEV are mainly enterically transmitted viruses which do not cause chronic hepatitis^{38,39}. Although several studies including the present one show that hepatitis

G virus is prevalent in patients who are at risk of developing parenterally acquired hepatitis, it is still not clear whether HGV alone could cause severe liver disease.

No specific clinical and biochemical marker could be associated with the patients positive for HGV RNA except past history of jaundice which was significantly higher (3/13, 23%) in the HGV positive than in negative cases. Although several studies including ours show that hepatitis G virus is prevalent in patients who are at risk of developing parenterally acquired hepatitis, it is still not clear whether HGV alone could cause severe liver disease or it acts as a co-factor or remains as a silent passenger⁴⁰. It could be possible that the virus is associated with non-liver diseases such as the immunosuppressed diseases or HIV-infected AIDS. Occurrence of high incidence of HGV infection in commercial blood donors, multiple transfused patients, intravenous drug users and haemodialysis patients which are also known high risk groups for HIV infections, indicates that HGV may play a role in AIDS. In fact, as high as 9% positivity of HGV-RNA has been detected in blood of HIV-1 infected patients⁴¹. Certainly additional studies are required to answer these questions.

A high prevalence of HGV in fulminant hepatic failure does not necessarily explain the aetiological role of the virus. Moreover, only a minority of cases of non-A-E hepatitis accounts for hepatitis G virus and there is no follow up study to show its progression to chronic hepatitis or cirrhosis. Further studies are therefore essential to determine the natural history, biological behaviour and clinical significance of this virus when infected alone or in combination with other hepatitis viruses.

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