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Virological course of hepatitis A virus as determined by real time RT-PCR: Correlation with biochemical, immunological and genotypic profiles

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Abstract

AIM: To undertake analysis of hepatitis A viral load, alanine aminotransferase (ALT), and viral genotypes with duration of viremia, and to correlate these parameters with $CD4^+$/ $CD8^+$ lymphocyte populations that control cell-mediated immunity.

METHODS: Cell counts were carried out using fresh whole blood collected in EDTA vials using a fluorescence activated cell sorter. Hepatitis A virus (HAV) RNA was extracted from blood serum, reverse transcribed into cDNA and quantified by Real-Time polymerase chain reaction and was genotyped.

RESULTS: Among 11 patients, 10 could be analyzed completely. Of these, 3 had severe acute hepatitis (s-AH) and the remainder had a self-limited acute hepatitis A (AHA), with one patient with fulminant disease (encephalopathy Grade Ⅳ) dying on the 4th d. The ALT level was significantly higher both in AHA (1070.9 ± 894.3; P = 0.0014) and s-AH (1713.9 ± 886.3; P = 0.001) compared to normal controls (23.6 ± 7.2). The prothrombin time in s-AH patients (21.0 ± 2.0; P = 0.02) was significantly higher than in AHA (14.3 ± 1.1; P = 0.44). The $CD4^+/CD8^+$ ratio in AHA patients (1.17 ± 0.11; P = 0.22) and s-AH (0.83 ± 0.12; P = 0.0002) were lower than seen in normal healthy controls (1.52). Self-limited cases had peak viral load at the beginning of analysis while in s-AH patients this occurred at the 15th or 30th d. In acute and severe groups, one patient each belonged to genotype IA, with the remaining 8 cases belonging to genotype IIIA. The only fulminant hepatic failure case belonged to genotype IA. HAV viral load and ALT values collected during the entire course of the self-limited infection were directly correlated but this was not the case for s-AH patients.

CONCLUSION: Based on a small-scale study, the persistently higher viral load of s-AH might be due to diminished cellular immunity and hemolysis. The duration of viremia was dependent on the host, as the viral genotype had no apparent role in clinical outcome of AVH and s-AH cases.

Key words: Viral load; Real-time PCR; Immunological response; Severe acute hepatitis; Self-limited acute hepatitis

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INTRODUCTION

Hepatitis A virus (HAV) is a common cause of acute viral infections in humans[1]. It is a ubiquitous virus readily transmitted by the feco-oral route[2-5]. Nucleotide sequence analysis of HAV has classified the virus in seven different genotypes, which include human (Ⅰ, Ⅱ, Ⅲ, and Ⅶ) and simian (Ⅳ, Ⅴ, and Ⅵ) genotypes[6]. HAV infection follows a benign course; it is often asymptomatic in younger children, but can develop into a fatal fulminant form or severe acute hepatitis in older persons[7-9].

To date, however, there is limited knowledge of viral load, or the length of viral persistence both in the blood circulation and in fecal excretion. It has been reported that a relapse may occur 30-90 d after the initial onset of the disease[10-12] and virus has been detected in the stool of patients[13]. Recently, outbreaks of HAV have occurred among hemophiliacs receiving organic solvent and detergent-treated factor Ⅷ, a fact that stresses the potential usefulness of a reliable and widely applicable technique for quantifying viral load in blood samples[14-18]. The level and the length of HAV viremia involve the additional risk of the carrier becoming an infectious source of hepatitis A[19].

We undertook further examination of hepatitis A viremia during the course of infection to understand whether viral load was correlated with cell-mediated immunity. The pathogenetic mechanisms underlying hepatocellular injury in acute hepatitis are poorly understood[20]. There is general agreement that HAV infection does not evolve to chronic hepatitis in man[21], and immune mechanisms have been suspected of playing a major role in eliminating virus-infected liver cells[22-24]. The aim of this study was to undertake analysis of HAV viral load, alanine aminotransferase, and viral genotypes with the duration of viremia, and to correlate these parameters with populations of $CD4^+/CD8^+$ lymphocytes that controls cell-mediated immunity.

MATERIALS AND METHODS

Patients and blood samples

Patients attending the Medical Out Patient Department of Lok Nayak Hospital, New Delhi, with the characteristic symptoms of acute viral hepatitis such as jaundice, fever, general malaise, fatigue, nausea, vomiting, anorexia and right upper quadrant discomfort, were screened for the study. Ten mL of blood were collected by venipuncture from those patients, who gave consent for five different visits. The study was approved by the ethical committee of Maulana Azad Medical College, as per the Declaration of Helsinki (1995). Consecutive blood samples were collected from 10 acute hepatitis A patients on the 0^{th}, 7^{th}, 15^{th}, 30^{th} and 45^{th} d between July 2004 and June 2005. No sample was collected before the onset of symptoms. The '0^{th}' d was defined as the first day when the patient presented after the onset of jaundice. Ten healthy subjects who had no evidence of liver disease or dysfunction were taken as control.

Serological tests

Laboratory examination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), prothrombin time (PT), and total bilirubin levels were carried out by standard methods. IgM anti-HAV were detected by ELISA (HAVAB-MEIA); Abbott Laboratories, North Chicago, IL), IgM anti-HEV (Qiagen, Hilden, Germany), HBsAg (Qiagen, Hilden, Germany) and anti-HCV (Bio-Rad, San Francisco, CA, USA) were measured according to the manufacturer's protocol.

FACS analysis of T-lymphocyte profile

One milliliter whole blood was collected into a vial containing EDTA and was employed for $CD4^+$ (T helper) and $CD8^+$ (T suppressor) cell counts within 24 h of collection, using a Fluorescence Activated Cell Sorter (FACS) (Becton Dickenson Electronics Laboratory, Mountain View, California). This system quantifies $CD4^+$, $CD8^+$ and $CD3^+$ T lymphocytes as absolute numbers of lymphocytes per μL (mm^3) of blood, and the $CD4^+/CD8^+$ T lymphocyte ratio. Samples from healthy controls and patients were also run for cell counts using the manufacturer's protocol and reagents.

Primers, probe and standard for real-time amplification

HAV RNA was extracted using the QIAamp™ viral RNA extraction kit (Qiagen, Germany). Viral RNA was amplified using primers derived from the most constant region, the 5' non-coding region (5' NCR)[25,26]. The primers used were, forward primer HAV1 (22: 5'-TTT CCG GAG CCC CTC TTG-3'), as wild type (M14707) reverse primers HAV2 (85: 5'-AAA GGG AAA TTT AGC CTA TAG CC-3') and HAV3 (85: 5'-AAA GGG AAA ATT TAG CCT ATA GCC-3'), and HAV-probe (58: 5' -FAM-ACT TGA TAC CTC ACC GCC GTT TGC CT-TAMRA-3') and RNA standard representing the 5'NCR region was constructed according to Costa-Mattioli *et al*[27].

Fluorogenic quantitative Real-Time PCR and direct sequencing

RT-PCR was carried out with a HAV quantification kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. The total volume of the reaction mixture was 25 μL (15 μL of mastermix with 10 μL of the RNA template) in 0.2 mL tubes. The capillaries were sealed, centrifuged, and transferred to the Rotor Gene 3000 real-time PCR machine (Corbett Research, Sydney, Australia). Reverse-transcription was done for 15 min at 50℃ followed by 5 min denaturation at 95℃. The corresponding cDNA's were amplified by PCR (20 s at 95℃, 30 s at 50℃ acquiring FAM, and 20 s at 72℃) over 45 cycles, and an 87 bp fragment was obtained. The CT values from the clinical samples were plotted on the standard curve, and the number of copies was calculated automatically.

PCR amplification from part of the VP1/2A region of HAV genome was directly sequenced for genotyping[28-30]. Sequencing was done with an ABI Prism 310 Genetic Analyzer (ABI, Foster City, CA). Sequencing analysis was performed using ClustalW and the phylogenetic inference by version 1.81 of the PHYLIP software package (Professor J. Felsenstein, Department of Genetics, University of Washington, Washington, DC).

Statistical analysis

All data were analyzed by two tailed tests, and a P value less than 0.05 was considered significant. We used chi-square test and student's t-test as appropriate.

RESULTS

Comparison of clinical features between AVH and s-AH groups

The average age of all patients was 20.8 ± 15.5 years

Table 1 Clinical, biochemical and immunological characterization of different groups (AVH, *s*-AH, and normal control)

Characteristic	AVH[1]	*s*-AH[2]	Normal Control[3]
No. of cases	07	03	10
Sex (M/F)	3/4	3/0	7/3
Age (mean ± SD)	0.4 ± 17.9	27.7 ± 2.08	26.2 ± 3.6
ALT (mean ± SD; IU/L)	1070.9 ± 894.3	1713.9 ± 886.3	23.6 ± 7.2
AST (mean ± SD; IU/L)	621.3 ± 242.8	1614 ± 234.7	23.8 ± 5.8
T. Bilirubin (mean ± SD; mg/dL)	9.8 ± 3.8	24.5 ± 3.0	0.74 ± 0.43
Prothrombin Time (Seconds)	14.3 ± 1.1	21.0 ± 2.0	12.5 ± 0.5
Mean $CD4^+/CD8^+$	1.17 ± 0.11	0.83 ± 0.12	1.52 ± 0.11

ALT: AVH *vs* s-AH $P = 0.29$ (not significant); AVH *vs* NC $P = 0.0014$ (highly significant); s-AH *vs* NC $P = 0.001$ (highly significant); **AST:** AVH *vs* s-AH $P = 0.0034$ (highly significant); AVH *vs* NC $P = 0.00014$ (highly significant); s-AH *vs* NC $P = 0.00044$ (highly significant); **T. Bil:** AVH *vs* s-AH $P = 0.076$ (significant); AVH *vs* NC $P = 0.00017$ (highly significant); s-AH *vs* NC $P = 0.0023$ (highly significant); **PT:** AVH *vs* s-AH $P = 0.11$ (not significant); AVH *vs* NC $P = 0.44$ (not significant); s-AH *vs* NC $P = 0.02$ (significant); **Mean $CD4^+/CD8^+$:** AVH *vs* s-AH $P = 0.46$ (not significant); AVH *vs* NC $P = 0.22$ (not significant); s-AH *vs* NC $P = 0.0002$ (highly significant). **Note**: AVH[1] (Acute viral hepatitis); s-AH[2] (Severe acute hepatitis); NC[3] (Normal Control).

and ranged from 3 to 59 years. The average age and sex ratio of two groups of patients is shown in Table 1. 10 of 11 patients could be followed completely, of which 3 progressed to severe acute hepatitis (s-AH), which is defined on the basis of a prothrombin time (PT) < 40% of normal range. The remainder of patients had self-limited acute hepatitis A (AHA) and one died of fulminant hepatic failure (Grade Ⅳ encephalopathy) at the 4th d of follow up. There was no difference between the groups with respect to clinical symptoms, such as flu-like prodromes (including arthralgia, or headache), fever, nausea, vomiting, abdominal pain, pruritus, and diarrhea.

Comparison of biochemical features between AVH and s-AH groups

In Figure 1, the time course of viral load and serum transaminase ALT levels is presented for all patients. ALT values for both groups followed a decreasing trend towards normal from the initial to final day of follow up. The mean liver function profile of s-AH patients was higher compared to the AVH cases as shown in Table 1. The mean prothrombin time (PT) of s-AH patients was (21.0 ± 2.0; $P = 0.02$) significantly higher than that of acute cases (14.3 ± 1.1; $P = 0.44$).

Comparison of immunological profiles between AVH and s-AH groups

The mean immunological ($CD4^+/ CD8^+$) ratio in patients with acute viral hepatitis A was (1.17 ± 0.1) higher than that in the severe acute cases (0.83 ± 0.12). As shown in Table 1, the $CD4^+/CD8^+$ ratio in normal controls (NC) (1.52 ± 0.11) ratio was almost twice as high as in s-AH ($P = 0.0002$). There was no significant decrease in the immunological ratio of AVH cases compared to normal controls.

Comparison of viral load between AVH and s-AH groups

In the acute case, Ind-301, the viral load on the initial day was 4.5×10^5, and decreased further to $> 10^3$ copies/mL. As shown in Figure 1, Ind-303 and Ind-306 followed almost similar trends with viral loads of 2.6×10^5 and 1.2×10^5 respectively at 0th d while $< 10^2$ copies/mL still persisted at the end of follow up. The viral load of Ind-304 was 1.0×10^5 at start and complete elimination of the virus with zero copies/mL was seen at the end of follow up, as shown in Figure 1. Patients Ind-308 and Ind-310 displayed a peak viral load at 0th d like the cases described above, while Ind-309 peaked on the 15th d, but values then decreased to less than 100 copies/mL in all three. In the severe cases Ind-302 and Ind-305, the viral load on the initial day of follow up was 1.2×10^5 and 1.1×10^5 respectively, and reached a peak on the 30th d, quite different from the acute case shown in Figure 1. At the end of follow up, the viral load was significantly higher ($> 1.0 \times 10^5$) as compared to AVH. In-patient Ind-307, viral load reached the peak (4.6×10^5) at 15th d and decreased subsequently to 4.3×10^4 copies/mL at the end of the follow up.

Comparison of genotype (s) between AVH and s-AH groups

In Table 2, the maximal viral load was compared with genotyping and geographical distribution. As shown in phylogenetic tree (Figure 2), the patients Ind-301 (DQ179131) and Ind-302 (DQ179132) were categorized as genotype IA, while the remainder Ind-303 (DQ179133), Ind-304 (DQ179134), Ind-305 (DQ179135), Ind-306 (DQ179136), Ind-307 (DQ179134), Ind-308 (DQ182495), Ind-309 (DQ182496), and Ind-310 (DQ182497)) were classified as the ⅢA genotype. The only fulminant hepatic failure case, Ind-274 (DQ182500), belonged to genotype ⅠA.

DISCUSSION

Hepatitis A remains the most frequent form of viral hepatitis observed in a large number of countries[31,32]. Recent publications have demonstrated that the duration of the viremic phase is much longer than assumed[10,27]. A serum HAV viral load assay could therefore be helpful in the management of severe hepatitis A. Real-Time reverse transcription (Rotor Gene 3000, Corbett Research, Sydney, Australia), was used for the quantitative detection of the HAV genome in human sera in individuals who displayed varying disease courses[27,33,34]. The fluorescence signal due to the cleavage of the fluorogenic probe is generated only if the target sequence for the probe is amplified by the PCR. Therefore, no signals are generated by non-specific amplification.

The alanine aminotransferase level of AVH cases on the initial day was significantly higher ($\geqslant 10^3$) and the decreased upon subsequent follow up, which corresponds to earlier findings that demonstrate a direct correlation of viral load with serum ALT[27,35]. In our study, the mean prothrombin time in AVH was not higher while severe cases showed significant elevations compared to normal controls. This could be due to anemia (hemolysis) as this

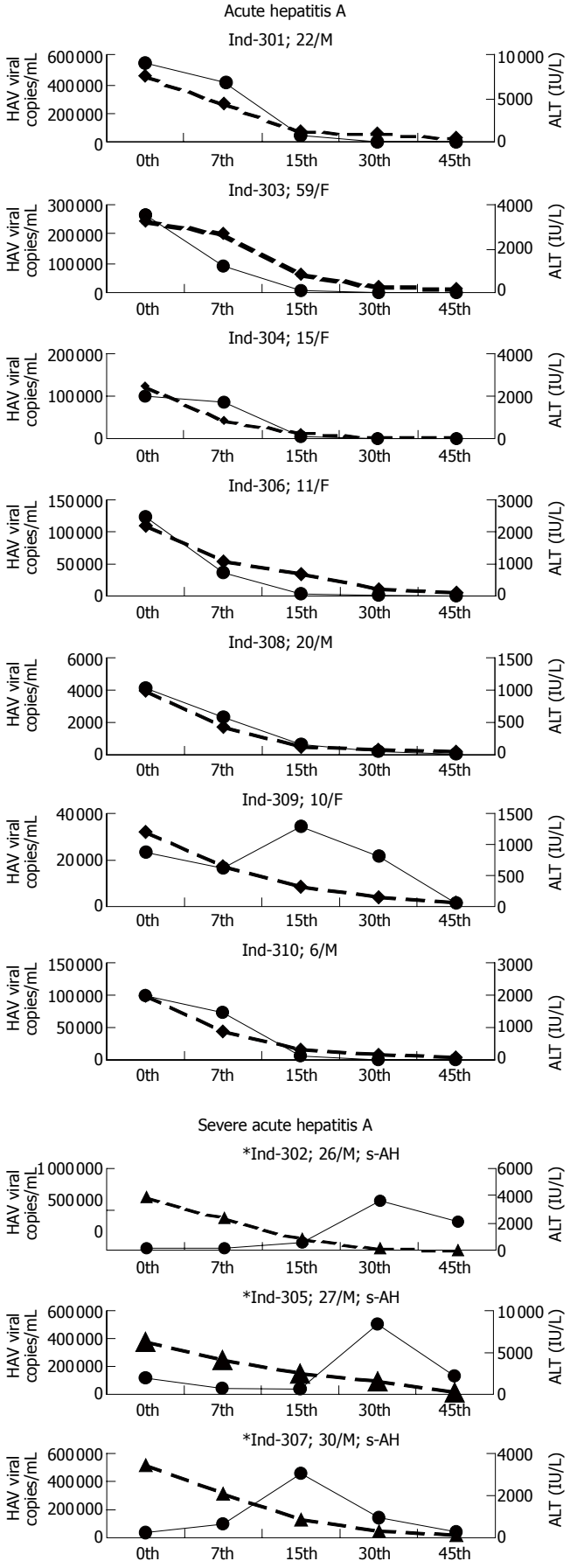

Figure 1 Relationship of hepatitis A virus (HAV) viral load (●) to levels of alanine aminotransferase (ALT) (▲) in serum at different day of follow up.

Table 2 Comparison of patient's genotypes to viral load and geographical distribution

Patients	Genotype	Maximal viral load Copies/mL	Geographical distribution
Ind-301	I A	4.5×10^5	New Delhi
[1]Ind-302	I A	6.0×10^5	New Delhi
Ind-303	III A	2.6×10^5	Uttar Pradesh
Ind-304	III A	1.0×10^5	Delhi
[1]Ind-305	III A	5.0×10^5	Delhi
Ind-306	III A	1.2×10^5	New Delhi
[1]Ind-307	III A	4.6×10^5	Uttar Pradesh
Ind-308	III A	4.1×10^3	New Delhi
Ind-309	III A	3.4×10^4	Haryana
Ind-310	III A	9.8×10^4	New Delhi

[1]Represents severe acute hepatitis.

is not uncommon during viral hepatitis[36]. We excluded glucose-6 phosphate dehydrogenase (G6PD) deficiency as a cause of hemolysis and anemia in our cases[36,37].

The immunological changes in acute cases was not significantly different from those in severe hepatitis A. The lymphocyte ratio (mean $CD4^+/CD8^+$) of the severe patients was, however, significantly lower, which could be due to diminished cellular immunity as compared to the normal controls. Previous studies have also shown that generation of $CD4^+$ T cells in the thymus is severely impeded as either a direct or indirect consequence of active viral replication[35,38]. We speculate that severe HAV infection may be triggered by diminished cellular immunity in susceptible patients, which may have increased the liver damage due to hepatitis A.

On the basis of our results, genome quantities measured on the first (0^{th}) d of clinical diagnosis in HAV infected humans reached peaked copies/mL in acute viral hepatitis and attained normality towards the end of follow up. The kinetics of peak viral load attainment in s-AH was quite different from that in the acute self-limited cases since at the end of follow up high copies/mL still persisted, as shown in Figure 1. The acute results confirm the levels recently estimated by Chudy *et al*[15]. The progression of severity due to diminished cellular immunity and hemolysis might be directly linked to high viral persistence throughout the follow up.

Most of the patients examined during acute self-limited illness belonged to genotype IIIA, other than Ind-301 who belonged to I A[6]. Among the severe cases, Ind-302 belonged to I A while other two belonged to IIIA, which means genotypic variations likely do not play a crucial role in determination of the viral load as described earlier by Normann[35]. The only FHF case, who died at the 4^{th} d of follow up, belonged to genotype I A. The question arises whether the duration of viremia is dependent on the genotype, or the immunological and/or biochemical profile. Our results showed that the duration of the viremia was dependent on the host, as the viral genotype had no role in acute self-limited illness and severe acute hepatitis A cases. This differs considerably from earlier

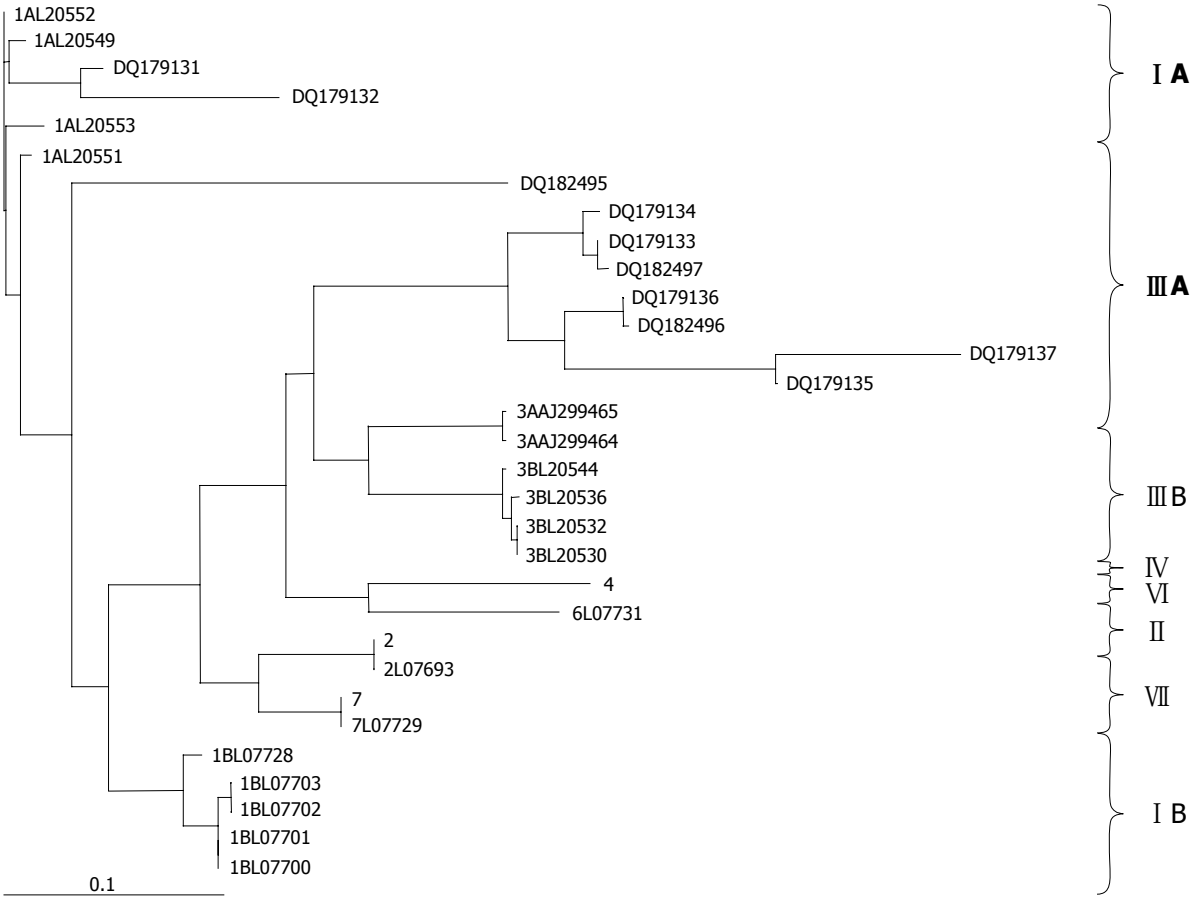

Figure 2 A neighbor-joining phylogenetic tree for isolates of hepatitis A virus based on the sequencing of the VP1-P2A region. Isolates DQ179131 (Ind-301), DQ179132 (Ind-302), DQ179133 (Ind-303), DQ179134 (Ind-304), DQ179135 (Ind-305), DQ179136 (Ind-306), DQ179137 (Ind-307), DQ182495 (Ind-308), DQ182496 (Ind-309), DQ182497 (Ind-310) were collected during the present study, in PCR Hepatitis Laboratory, MAM College and associated LNJ Hospital, New Delhi, India.

findings which showed that a long duration of viremia was found in patients infected with HAV genotype IA[35].

In conclusion, HAV viral load and alanine aminotransferase (ALT) values collected during the entire course of a self-limited acute infection were directly correlated, but this was not found in s-AH cases. The duration of viremia was dependent on the host (biochemical and immunological profiles), as the viral genotype had no role in the various groups studied. The mean prothrombin time in severe acute hepatitis was higher than seen in acute self-limited illness. The immunological ($CD4^+/CD8^+$) ratio of s-AH was quite low compared to the acute self-limited illness, and s-AH patients showed diminished cellular immunity and complications. There was no difference in the final clinical outcome and recovery of liver function was seen in all patients. The limitation of the study is that the number of patients examined was relatively small. Therefore, there is a need for further research on the duration and magnitude of HAV viremia in a large cohort of human patients to properly document complications and management.

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