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

Zahid Hussain, Bhudev C Das, Syed A Husain, Sunil K Polipalli, Tanzeel Ahmed, Nargis Begum, Subhash Medhi, Alice Verghese, Mohammad Raish, Apiradee Theamboonlers, Yong Poovorawan, Premashis Kar

Zahid Hussain, Sunil K Polipalli, Tanzeel Ahmed, Nargis Begum, Subhash Medhi, Premashis Kar, PCR Hepatitis Laboratory, Department of Medicine, Maulana Azad Medical College, New Delhi 110002, India
Bhudev C Das, Division of Molecular Oncology, Institute of Cytology and Preventive Oncology, (ICMR), Noida, Sector 39, Uttar Pradesh, India
Zahid Hussain, Syed A Husain, Tanzeel Ahmed, Nargis Begum, Mohammad Raish, Human Genetics Laboratory, Department of Biosciences, Jamia Millia Islamia, New Delhi 110025, India
Alice Verghese, Advance Center for AIDS, National Institute of Communicable Diseases, New Delhi 110041, India
Apiradee Theamboonlers, Yong Poovorawan, Viral Hepatitis Research Unit, Department of Pediatric, Faculty of Medicine, Chulalongkorn University and Hospital, Rama IV Road, Bangkok 10330, Thailand
Correspondence to: Dr. Premashis Kar, Room No. 111, Department of Medicine, Maulana Azad Medical College, New Delhi 110002, India. hussainzahp@gmail.com
Telephone: +91-11-23230132 Fax: +91-11-23230132 Received: 2006-03-11 Accepted: 2006-04-21

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; \(P = 0.0002\)) 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

Hussain Z, Das BC, Husain SA, Polipalli SK, Ahmed T, Begum N, Medhi S, Verghese A, Raish M, Theamboonlers A, Poovorawan Y, Kar P. Virological course of hepatitis A virus as determined by real time RT-PCR: Correlation with biochemical, immunological and genotypic profiles. World J Gastroenterol 2006; 12(29): 4683-4688

http://www.wjgnet.com/1007-9327/12/4683.asp

INTRODUCTION

Hepatitis A virus (HAV) is a common cause of acute viral infections in humans\(^6\). 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\(^7-9\).

© 2006 The WJG Press. All rights reserved.
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.\textsuperscript{[10-12]} and virus has been detected in the stool of patients.\textsuperscript{[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.\textsuperscript{[14-18]} The level and the length of HAV viremia involve the additional risk of the carrier becoming an infectious source of hepatitis A.\textsuperscript{[19,20]}

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.\textsuperscript{[20]} There is general agreement that HAV infection does not evolve to chronic hepatitis in man.\textsuperscript{[21]} and immune mechanisms have been suspected of playing a major role in eliminating virus-infected liver cells.\textsuperscript{[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\textsuperscript{+}/CD8\textsuperscript{+} 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\textsuperscript{th}, 7\textsuperscript{th}, 15\textsuperscript{th}, 30\textsuperscript{th} and 45\textsuperscript{th} d between July 2004 and June 2005. No sample was collected before the onset of symptoms. The 0\textsuperscript{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\textsuperscript{+} (T helper) and CD8\textsuperscript{+} (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\textsuperscript{+}, CD8\textsuperscript{+} and CD3\textsuperscript{+} T lymphocytes as absolute numbers of lymphocytes per mL (mm\textsuperscript{3}) of blood, and the CD4\textsuperscript{+}/CD8\textsuperscript{+} T lymphocyte ratio. Samples from healthy controls and patients were also run for cell counts using the manufacturer’s protocol and reagents.

**Primer, probe and standard for real-time amplification**

HAV RNA was extracted using the QIAamp \textsuperscript{TM} 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).\textsuperscript{[25,26]} The primers used were, forward primer HAV1 (22: 5’-TTT CCG GAG CCC CTC TTG-3’), as wild type (Mi4707) reverse primers HAV2 (85: 5’-AAA GGG AAA ATT TAG CCT ATA GCC-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 TGCC-3’). Sequencing analysis of the 5’NCR region was constructed according to Costa-Mattioli et al.\textsuperscript{[27]}.\n
**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°C followed by 5 min denaturation at 95°C. The corresponding cDNA’s were amplified by PCR (20 s at 95°C, 30 s at 50°C acquiring FAM, and 20 s at 72°C) 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.\textsuperscript{[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
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 \( P = 0.0004 \) (highly significant); s-AH vs NC \( P = 0.0044 \) (highly significant); T. Bilirubin: AVH vs s-AH \( P = 0.076 \) (significant); AVH vs NC \( P = 0.0017 \) (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); \( \text{Mean CD4}^-/\text{CD8}^+ \): AVH vs s-AH \( P = 0.46 \) (not significant); AVH vs NC \( P = 0.02 \) (not significant); s-AH vs NC \( P = 0.0002 \) (highly significant). Note: AVH (Acute viral hepatitis); s-AH (Severe acute hepatitis); NC (Normal Control).

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 14.3 ± 1.1; 21.0 ± 2.0; 23.8 ± 5.8; T. Bilirubin: AVH vs s-AH was 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.

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.

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

| Characteristic | AVH\(^1\) | s-AH\(^1\) | Normal Control\(^2\) |
|---------------|-----------|-----------|---------------------|
| 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 \( P = 0.0004 \) (highly significant); s-AH vs NC \( P = 0.0044 \) (highly significant); T. Bilirubin: AVH vs s-AH \( P = 0.076 \) (significant); AVH vs NC \( P = 0.0017 \) (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 \( P = 0.02 \) (not significant); s-AH vs NC \( P = 0.0002 \) (highly significant). Note: AVH (Acute viral hepatitis); s-AH (Severe acute hepatitis); NC (Normal Control).

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 (\( \geq 10^4 \)) and the decreased upon subsequent follow up, which corresponds to earlier findings that demonstrate a direct correlation of viral load with serum ALT.[27,33] 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
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[35,36]. 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 (0th) day 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[35]. 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 IA[40]. Among the severe cases, Ind-302 belonged to IA 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[40]. The only FHF case, who died at the 4th day of follow up, belonged to genotype IA. 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

**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                |
| 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                    |
| Ind-305  | III A    | 5.0 × 10^5                  | Delhi                    |
| Ind-306  | III A    | 1.2 × 10^5                  | New Delhi                |
| Ind-307  | III A    | 4.6 × 10^5                  | Uttar Pradesh            |
| Ind-308  | III A    | 4.1 × 10^5                  | New Delhi                |
| Ind-309  | III A    | 3.4 × 10^5                  | Haryana                  |
| Ind-310  | III A    | 9.8 × 10^4                  | New Delhi                |

*Represents severe acute hepatitis.
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.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Usha K Baweja, Advance Center for AIDS, National Institute of Communicable Diseases, New Delhi, 110041 for evaluation and analysis of immunological markers by FACS, and Mr. Pradeep K Singhal, Professional Biotech Ltd. New Delhi, India for their excellent guidance and assistance in viral load determination.

REFERENCES

1 Centers for Disease Control and Prevention. Hepatitis surveillance report No.56. Atlanta GA: Centers for Disease control, 1995
2 Sjogren MH. Hepatitis A. In: Schiff ER, Sorrell MF, Maddery WC, editors. Schiff’s Diseases of the Liver. 8th ed. Philadelphia: Lippincott-Raven, 1999: 745-756
3 Hadler SC. Global impact of hepatitis A virus infection changing patterns. In: Hollinger FB, Lemon SM, Margolis HS, editors. Viral hepatitis and liver disease. Baltimore, MD: Williams & Wilkins, 1991: 14-20
4 Bölke E, Flehmig B. New epidemiological patterns of hepatitis A and B infections in Germany. Zentralbl Hyg Umweltmed 1995; 196: 511-514
5 Niu MT, Polish LB, Robertson BH, Khanna BK, Woodruff BA, Shapiro CN, Miller MA, Smith JD, Gedrose JK, Alter MJ. Multistate outbreak of hepatitis A associated with frozen strawberries. J Infect Dis 1992; 166: 518-524
6 Robertson BH, Jansen RW, Khanna B, Totsuka A, Nainan OV, Siegel G, Widell A, Margolis HS, Isomura S, Ito K. Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. J Gen Virol 1992; 73 (Pt 6): 1365-1377
7 Takahashi Y, Okuda K. Fulminant and subfulminant hepatitis in Japan—etiological considerations. Indian J Gastroenterol 1993;
NainanOV, Han X, Margolis HS. Duration of viremia in hepatitis A virus infection. J Infect Dis 2000; 182:12-17.

Yotsuyanagi H, Jino S, Koike K, Yasuda K, Hino K, Kurokawa K. Duration of viremia in human hepatitis A viral infection as determined by polymerase chain reaction. J Med Viral 1993; 40:35-38.

Fujisawa K, Yokosuka O, Ehata T, Imazeki F, Saisho H, Miki M, Omata M. Frequent detection of hepatitis A virus RNA in serum during the early convalescent phase of acute hepatitis A. Hepatology 1997; 26:1634-1639.

Sjoogren MH, Tanno H, Fay O, Sileo S, Cohen BD, Burke DS, Feighery RJ. Hepatitis A virus in stools during clinical relapse. Ann Intern Med 1987; 106:221-226.

Kedda MA, Kew MC, Cohn RJ, Field SP, Schwyzer R, Song E, Fernandes-Costa F. An outbreak of hepatitis A among South African patients with hemophilia: evidence implicating contaminated factor VIII concentrate as the source. Hepatology 1995; 22:1363-1367.

Chudy M, Budek I, Keller-Stanislawski B, McCaustland KA, Neidhold S, Robertson BH, Nübling CM, Seitz R, Löwer J. A new cluster of hepatitis A infection in hemophiliacs traced to a contaminated factor VIII preparation. J Med Virol 1999; 57:91-99.

Corey L, Holmes KK. Sexual transmission of hepatitis A in homosexual men: incidence and mechanism. N Engl J Med 1980; 302:435-438.

Mannucci PM. Outbreak of hepatitis A among Italian patients with haemophilia. Lancet 1992; 339:819.

Normann A, Graft J, Gerritzen A, Brackmann HH, Flehmig B. Detection of hepatitis A virus RNA in commercially available factor VIII preparation. Detection of hepatitis A virus RNA in commercially available factor VIII preparation. J Infect Dis 1992; 163:1175.

Inoue K, Yoshiba M, Yotsuyanagi H, Otsuka T, Sekiyama K, Fujita R. Chronic hepatitis A with persistent viral replication. J Med Virol 1996; 50:322-324.

Kurane I, Binn LN, Bancroft WH, Ennis FA. Human lymphocyte responses to hepatitis A virus-infected cells: interferon production and lysis of infected cells. J Immunol 1985; 135:2140-2144.

Vallbracht A, Gabriel P, Zahn J, Flehmig B. Hepatitis A virus infection and the interferon system. J Infect Dis 1985; 152:211-213.

Vallbracht A, Gabriel P, Maier K, Hartmann F, Steinhardt HJ, Müller C, Wolf A, Manncke KH, Flehmig B. Cell-mediated cytotoxicity in hepatitis A virus infection. Hepatology 1986; 6:1308-1314.

Fleischer B. Liver-derived cytotoxic T cells in hepatitis A virus infection. J Infect Dis 1989; 160:209-217.

Stapleton JT. Host immune response to hepatitis A virus. J Infect Dis 1995; 171 Suppl 1:59-14.

Cohen JJ, Tichovirtz JA, Purcell RH, Buckler-White A, Baroudy BM. Complete nucleotide sequence of wild-type hepatitis A virus: comparison with different strains of hepatitis A virus and other picornaviruses. J Virol 1987; 61:50-59.

Jansen RW, Newbold JE, Lemon SM. Complete nucleotide sequence of a cell culture-adapted variant of hepatitis A virus: comparison with wild-type virus with restricted capacity for in vitro replication. Virology 1988; 163:299-307.

Costa-Mattioli M, Monpoeho S, Nicand E, Aleman MH, Billaudel S, Ferre V. Quantification and duration of viraemia during hepatitis A infection as determined by real-time RT-PCR. J Viral Hepat 2002; 9:101-106.

Hussain Z, Das BC, Hussain SA, Asim M, Chattopadhyay S, Malik A, Poovorawan Y, Theamboonlers A, Kar P. Hepatitis A virus genotypes and clinical relevance: Clinical and molecular characterization of hepatitis A virus isolates from southern India. Hepatol Res 2005; 32:16-24.

Theamboonlers A, Jantaratadseem P, Chatchatee P, Chongsriraswat V, Mokum M, Poovorawan Y. Molecular characterization of hepatitis-A-virus infections, in the context of two outbreaks in southern Thailand. Ann Trop Med Parasitol 2002; 96:727-734.

Najarian R, Caput D, Gee W, Potter SJ, Renard A, Merryweather J, Van Nest G, Dina D. Primary structure and gene organization of human hepatitis A virus. Proc Natl Acad Sci USA 1985; 82:2627-2631.

Steffen R. Risk of hepatitis A in travellers. Vaccine 1992; 10 Suppl 1:569-572.

Flehmig B, Normann A, Bohnen D. Transmission of hepatitis A virus infection despite vaccination. N Engl J Med 2000; 343:301-302.

Monpoeho S, Dehéée A, Mignotte B, Schwartzbrod L, Marechal V, Nicolas JC, Billaudel S, Ferre V. Quantification of enzoevirus RNA in sludge samples using single tube real-time RT-PCR. Biotechniques 2000; 29:88-93.

Aranal C, Ferre-Aubineau V, Mignotte B, Imbert-Marcille BM, Billaudel S. Quantification of hepatitis A virus in shellfish by competitive reverse transcription-PCR with coextraction of standard RNA. Appl Environ Microbiol 1999; 65:322-326.

Normann A, Jung C, Vallbracht A, Flehmig B. Time course of hepatitis A viremia and viral load in the blood of human hepatitis A patients. J Med Virol 2004; 72:10-16.

Lyons DJ, Gilvary JM, Fielding JF. Severe haemolysis associated with hepatitis A and normal glucose-6-phosphate dehydrogenase status. Gut 1990; 31:838-839.

Raffensperger EC. Acute acquired hemolytic anemia in association with acute viral hepatitis. Ann Intern Med 1958; 48:1243-1253.

Adjukiewicz AB, Fox RA, Dudley FJ, Doniach D, Sherlock S. Immunological studies in an epidemic of infective, short-incubation hepatitis. Lancet 1972; 1:803-805.