Fulminant Hepatitis Failure in Adults and Children from a Public Hospital in Rio de Janeiro, Brazil

Damião Carlos Moraes dos Santos[1], José Manoel da Silva Gomes Martinho[2], Lucio Filgueiras Pacheco-Moreira[2], Cristina Carvalho Viana de Araújo[2], Barbara Cristina Euzebio Pereira Dias de Oliveira[2], Barbara Vieira Lago[3], Marcelo Alves Pinto[1] and Vanessa Salete de Paula[1]

[1]Laboratório de Desenvolvimento Tecnológico em Virologia, IOC, FIOCRUZ; [2]Hospital Geral de Bonsucesso; [3]Laboratório de Virologia Molecular, IOC, FIOCRUZ; Rio de Janeiro, RJ, Brazil

Fulminant hepatic failure (FHF) is characterized by massive hepatocellular injury, whose physiopathology is still unclear. Hepatitis B (HBV) is probably the most common viral cause of FHF, while hepatitis A (HAV) virus seem occurs less frequently. However, the host and viral factors that determine the outcome of these infections are poorly understood. In the present study, viral load and genotyping determining regions of HAV and HBV genomes were sequenced. Eight FHF patients and one patient with severe acute hepatitis (SAH) were included. Liver and blood samples were collected during liver transplantation or necropsy procedures. HAV-RNA and HBV-DNA were extracted from serum, biopsy and paraffin liver. Nucleotide sequencing of HAV-RNA was performed from VP1/2A and HBV-DNA from PreS/S region. The amplified samples were quantified by Real-Time PCR. The cases of HAV infection were due to subgenotype IA. The cases of HBV infection were due to genotype A2 and D4. The case of HAV/HBV co-infection was infected by genotype IA and D3. Hepatitis A and B infection were associated with genotypes most prevalent in Brazil. In hepatitis A infection the mean of period evolution was 13 days. In hepatitis B, FHF patients infected by genotype D have a shorter period of evolution than FHF patients infected by genotype A (mean 15 v. 53 days). There was no association with genotype-determining region with the severity of hepatitis, however nucleotide differences and high viral load could be observed among FHF.
Key-Words: Fulminant hepatitis failure, hepatitis A virus (HAV), hepatitis B virus (HBV) and genotype and viral load.

Fulminant hepatic failure (FHF) is a dramatic clinical syndrome resulting from the massive death of liver cells not adequately balanced by regenerative activity. It is usually characterized by the abrupt appearance of encephalopathy and coagulation disturbances in individuals with previously normal hepatic function. In these cases, liver transplantation is the only definitive therapeutic alternative. Several etiologies have been described to induce FHF. Among them, hepatitis A virus (HAV) and hepatitis B virus (HBV), and although rare, FHF presents a mortality rate of up to 80% without liver transplantation [1]. HBV infection is the most important cause of FHF, occurring in approximately 1% of the cases, while HAV occurs less frequently, from 0.5% to 1% of the cases. However, the majority of the patients present a self-limited course of hepatitis A and 5% to 10% of patients infected by HBV presents chronic course. In both cases, the clinical outcome may depend on several factors such as age and individual genetic characteristics. Nevertheless, the association between genotypes, clinical course, and excessive host response associated to marked reduction in viral load has been under discussion [2-4]. HAV is classified in the genus Hepatovirus within the *Picornaviridae* family. The genome is a positive strand RNA with approximately 7,500 nucleotides consisting of a 5' non-translated region, both structural and non-structural protein regions and a 3' non-translated region [5]. HAV strains correspond to a single serotype and have been classified into six genotypes [6,7].

HBV is a member of the Hepadnaviridae family, with a partially double stranded DNA genome that presents a very high genetic variability. Based on the nucleotide sequences, HBV has been classified into eight genotypes designed A through H which differ by more than 8% [8,9]. Due to its genetic diversity, a number of subgenotypes were described [9]. HBV genotypes and the majority of the subgenotypes show a distinct geographic distribution and early studies have been correlated to the severity of liver disease with the presence of some HBV genotypes [10].

Despite the advances in the understanding of the HAV and HBV, there is no data concerning the association between genotypes and fulminant hepatitis in Brazil. To explore if there are genotype differences in HAV and HBV that affect disease severity, the nucleotide sequences of genotype regions of the viruses from patients with extensive liver lesion were analyzed.

Material and Methods

Between January of 2003 and December of 2006, 33 patients with fulminate hepatic failure (FHF) were seen at the Liver Clinic/ Hospital Geral de Bonsucesso in Rio de Janeiro, Brazil. Among them 27% (9/33) were caused by viral hepatitis, eight with fulminant hepatic failure (FHF) and one patient with severe acute hepatitis (SAH). The clinical diagnostic of the patients were according to O'Grady of King's College [1]. Thus, the cases were classified as fulminant hepatitis by development encephalopathy between 7 days and 12 weeks from the onset

of jaundice. The case of SAH, however, presented coagulopathy without encephalopathy. Three cases of FHF were induced by HAV and five of them were induced by HBV. The case of SAH was induced by co-infection HAV/ HBV. Clinical and serological date from FHF patients such as prothrombin time activity (PTA), alanine aminotransferase (ALT), total bilirrubin and encephalopathy score were obtained from medical records during the intensive care period. The protocol of procedures was submitted to the Ethic Committee (Governmental Human Research) and reviewed at the Institutional Reviewer Board (CEP-FIOCRUZ.n°22/03).

Liver and Blood Samples

Liver and blood samples were obtained during liver transplantation procedures, except for patients that did not undergo liver transplantation, in these cases, only blood samples were obtained to study. Also, blood samples of three patients were not available, because they were retrospective cases; however their liver sections had been fixed in 10% buffered formalin and embedded in Paraplast, dehydrated in a graded series of ethanol and embedded in paraffin. The same above procedure was conduced for liver sample obtained by necropsy. In the case of fresh liver samples, fragments of hepatic explant were obtained and immediately after liver collection were snap frozen in liquid nitrogen-cooled (-196º C).

Serological Markers and Biological Test

All the patients were tested for viral hepatitis: anti-HAV IgM (Abbot, USA), HBsAg (Dia Sorin, Italian), anti-HBc IgM (BioKit, Spain), anti-HCV (Dia Sorin, Italian) and anti-HEV (BioKit, Spain).

Treatment of Paraffin-Embedded Liver Samples to Viral Genome Extraction

The treatment of paraffin-embedded liver samples for viral genome extraction was performed according to previously described methods [11]. Briefly, two sections of 7µm were deparaffinized overnight using xylene at 65ºC, followed by washes in absolute ethanol.

HAV RNA Extraction and PCR Amplification

RNA extraction and reverse transcription were realized by using a commercial assay QIAamp Viral RNA (QIAGEN, Valencia, CA, USA). Viral RNA was extracted from 140 µL of concentrated water samples and 60 µL of RNA were eluted. The cDNA was prepared at 50ºC over the course of 1h using 10 mL of RNA, random primers (Life Technologies, Gaithersburg, MD, USA) and the Super Script III reverse transcriptase (Life Technologies). After reverse transcription, the VP1/2A junction region of the HAV genome was amplified using nested PCR as described elsewhere [12].The PCR products were separated on 1.5% agarose gels and stained with ethidium bromide.

Quantification of HAV RNA

HAV RNA was quantified by real time PCR (TaqMan technology), using a standard curves previously described [13]. Amplification assays were performed in a final volume of 25 µL of TaqMan universal Mastermix (Applied Biosystems), containing 5 µL of extracted cDNA, 1.25 µL of assay containing forward primer (50-CTGCAGGTTCAGGGTTCTTAAATC-30, nt 86 to 109) reverse primer (50-GAGAGCCCTGGAAGAAAGAAGA-30, nt 219–240) and HAV-probe (FAM 50-ACTCATTTTTCACGCTTTCTG- 30, nt 198–218). The detection limit of the assay was 14 copies/reaction. The thermal cycling conditions consisted of denaturation for 10min at 94ºC followed by 40 cycles of 15-s at 94ºC and a final 1-min cycle at 60ºC.

HBV DNA Extraction and PCR

Viral DNA was extracted from serum or liver by using the-phenol-chloroform method after treatment with proteinase K, as described previously [14]. The HBV pre-S/S genomic region was amplified by using sense primer PS1 (5'-CCATATTCTTGGGAACAAGA-3', nt 2826-2845) and a mix of antisense primers S2 (5'-GGGTTTAAATGTATACCCAAAGA-3', NT 841-819) and (5'GTATTTAAATGGATACCCACACAGA-3', nt 841-819), able to amplify all HBV genotypes. PCR assays were performed under the following conditions: 35 cycles of 94ºC, 30s; 52ºC, 1 min; 72º C, 2 min; followed by a final elongation temperature at 72ºC for 7 min. Amplification products (50µL) were loaded on a 1.5% agarose gel, electrophoresed, stained with ethidium bromide and visualized under UV light.

Quantification of HBV DNA

HBV DNA was quantified by real time PCR (TaqMan technology), using a panel of reference sera containing given numbers of HBV DNA molecules, under the conditions described previously [15], with some modifications. Amplification assays were performed at a final volume of 25µL of TaqMan universal Mastermix (Applied Biosystems), containing 2 µL of extracted DNA, 1 µL of sense primer (5'-GGACCCCTGCTCGTGTTACA-3', nt position 184 to 203) and antisense (5'-AGAGAAGTCCACCMCGAGTCTAGA-3', nt position 273 to 249) primers designed in the S region and 0.3 µM of probe (5'-FAM TGTTGACAARAATCCTCACAAATACCRCAGA-TAMRA-3', nt position 218-247). After initial incubation steps of 2 min at 50ºC and 10 min at 95ºC, PCR assays consisted of 50 step cycles of 15s at 95ºC and 60s at 60ºC. Reactions were performed in a 7700 sequence detection system (Applied Biosystems). The detection limit of the assay was 10 copies/mL.

Direct Sequencing of HAV and HBV

Amplicons of expected size of HAV (247 bp) and HBV (1200 bp) were purified using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions. Direct nucleotide sequencing was performed in both directions using dye terminator reaction with dRhodamine terminator

reagents. Sense and anti-sense primers were used in the second round of PCR. Results were analyzed in an ABI Prism 3370 machine (Applied Biosystems, Foster City, CA, USA). Sequences reported in this paper have been deposited in the GenBank sequence database under the accession numbers EU395777-EU395785.

Sequence Analysis

The DNA star package was used for nucleotide alignment and for establishing amino acid sequences. Multiple alignments were initially performed with the Clustal X program [16]. Further adjustments were performed manually using visual correction based on sequence comparison generated with the Bioedit program. Phylogenetic trees were created using the neighbor-joining method and the Kimura two-parameter model by Felsenstein PHYLIP phylogenetic Inference Package, version 3.5. with MEGA 2.1 software [17]. Phylogenetic tree reliability was assessed by bootstrap re-sampling (1,000 pseudoreplicates).

Results

Serological Markers of Hepatitis A and Hepatitis B

Among the patients involved in this study, three had serological evidence of acute Hepatitis (anti-HAV IgM positive), five had evidence of acute hepatitis B (HBsAg and anti-HBc IgM positive) and one had serological evidence of acute co-infection HAV/HBV (anti-HAV IgM and HBsAg). All patients were negative for anti-HCV and anti-HEV IgM and none of them, had nor related use of hepatotoxic drugs such as acetaminophen, neither autoimmunity antibodies nor metabolical diseases.

Clinical and Biochemical Data from Fulminant Hepatic Failure Patients Induced by HAV Infection

The clinical characteristics of patients analyzed for HAV genotype are summarized in the Table 1. Among the patients with HAV infection, the time of evolution (period passed between the first signals and development of encephalopathy) ranged between 10 and 20 days; the mean was 13 days. Two patients died (HAV-3-03 and HAV-8-06); the patient HAV-3-03 who died after liver transplantation was a 3 year old child; the analyzed sample was paraffin liver. The patient HAV-7-05 recovered after liver transplantation and the analyzed samples were fresh liver and serum. Among the two patients who underwent liver transplantation (HAV-3-03 and HAV-7-05) the encephalopathy grade was IV.

HAV Mutations and Viral Load

In this study, the viral RNA was extracted from paraffin liver, serum and liver explants. Serum was investigated in two samples of hepatitis A (HAV-8-06, HAV-7-05). In the first one the viral load was 2.36×10^5 copies/mL and the second patient who recovered after liver transplantation

had 3.07×10^4 copies/mL in peripheral blood, in this latter case, 1.65×10^6 copies/mL in fresh liver sample collected from the explant were detected. It was not possible to measure the viral load from patient whose the HAV RNA had been extracted from paraffin liver. All of the three patients with fulminant hepatitis A under study were infected with genotype IA (Figure 1). Nucleotide sequences (210 nt) of the VP1/2A junction region of the samples were 97.4-99.2% homologous, no specific substitutions between fulminant hepatitis cases were observed.

The samples were also sequenced in 5'NTR (400nt) and compared with samples from acute hepatitis of sporadic cases sequenced previously in Brazil and other countries. In 5'NTR less nucleotide substitutions were found in patients with fulminant hepatitis than in patients with acute hepatitis.

Biochemical and Serological Data from Fulminant Hepatic Failure Patients Induced by HBV Infection

The clinical characteristics of patients analyzed for HBV genotype are also summarized in the Table 1. The mean age among the patients with HBV infection was 45 years old, and the time of evolution ranged from 8 to 56 days, a mean of 32 days. Four patients died, two of them, after the liver transplantation. The encephalopathy score among fulminant hepatitis B ranged from II to IV and the ALT values were higher than 631 UI/L in all patients.

HBV Mutations and Viral Load

In this study, the viral loads among patients with fulminant hepatitis range from undetermined to 1.24×10^7 copies/mL (Table I), none of them received antiviral therapy. In three patients that had the viral load measured in serum samples, the number of copies per milliliters was 10^5. In four patients infected with HBV the DNA was extracted from liver tissue, in the patient HBV-2-03 the viral load from liver fixed with paraffin was 5.59×10^5 copies/mL and in the patient HBV-6-05 the viral load from fresh liver was 1.24×10^7 copies/mL.

Among the fulminant hepatitis B patients, the following HBV genotypes/subgenotypes distribution was found: A2 (n=2), D4 (n=3). The isolates from this study were divergent among them (Figure 2). In genotype/subgenotype A2 the sequence variation was 0.2%. In homology between genotype/subgenotype D4 was 90.2-95.4%. Deduced amino acid sequence analysis of pre-S/S region showed that pre-S and S regions displayed several aminoacid variations, when compared to sequence consensus encountered in genotype A and D, available in GeneBank database.

Biochemical and Hepatitis Markers from the Severe Acute Hepatitis Patient Induced by Co-Infection HAV/HBV

The co-infected HAV/HBV patient, who developed severe acute hepatitis, presented ten days of evolution time (between the first symptoms and coagulation disturbances), did not

Table 1. Demographic, biochemical, clinical and serological data of fulminant hepatitis patients.

Patient number	Age	Gender	ALT (UI/L)	TB (mg/dL)	PTA %	HE Grade	Evolution (days)	Markers tested	Samples	VL (copies/mL)	G	Outcome
Hepatitis A												
HAV-3-03	3	F	1709	17.6	50.0	IV	10	Anti-HAV IgM	Paraffin liver	Undet.	IA	Death after LT
HAV-8-06	23	F	16	37.9	36.0	ND	10	Anti-HAV IgM	Serum	2.36×10^4	IA	Death
HAV-7-05	5	F	326	17.0	9.0	IV	20	Anti-HAV IgM	Serum / Liver explant	3.07×10^4 / 1.65×10^6	IA	LT
Hepatitis B												
HBV-5-04	49	F	775	21.4	15.3	IV	56	HBsAg	Serum / Liver explant	Undet. / Undet.	A2	Death after LT
HBV-4-04	24	M	964	45.0	25.0	II	50	HBsAg	Serum	7.94×10^5	A2	Death
HBV-1-02	26	M	631	22.6	30.7	III	30	HBsAg	Paraffin liver	4.12×10^5	D4	Death after LT
HBV-2-03	59	F	2855	20.7	9.0	II	15	HBsAg	Paraffin liver	5.59×10^5	D4	LT
HBV-6-05	67	M	1950	10.5	ND	IV	8	HBsAg	Serum / Necropsy	2.83×10^5 / 1.24×10^7	D4	Death
Co-infection												
HAVHBV-9-06	49	M	3240	18.4	ND	ND	10	Anti-HAV IgM / HBsAg	Serum	7.67×10^4 / 5.59×10^3	IA / D3	Recovery

ALT= alanine aminotransferase TB = T-bilirubin; PTA= Prothrombine Time Activity; HE= Hepatic Encephalophaty; ND= not determined; LT = liver transplantation; VL=viral load; G=genotype.

Figure 1. Phylogenetic tree based on 210-nt sequences of the VP1/2A region from Hepatitis A virus and depicting the genetic relatedness of isolates from this study (in bold) with other HAV strains previously characterized. The numbers at nodes indicate bootstrap percentages after 1,000 replications sampling. The horizontal bar provides a genetic distance scale.
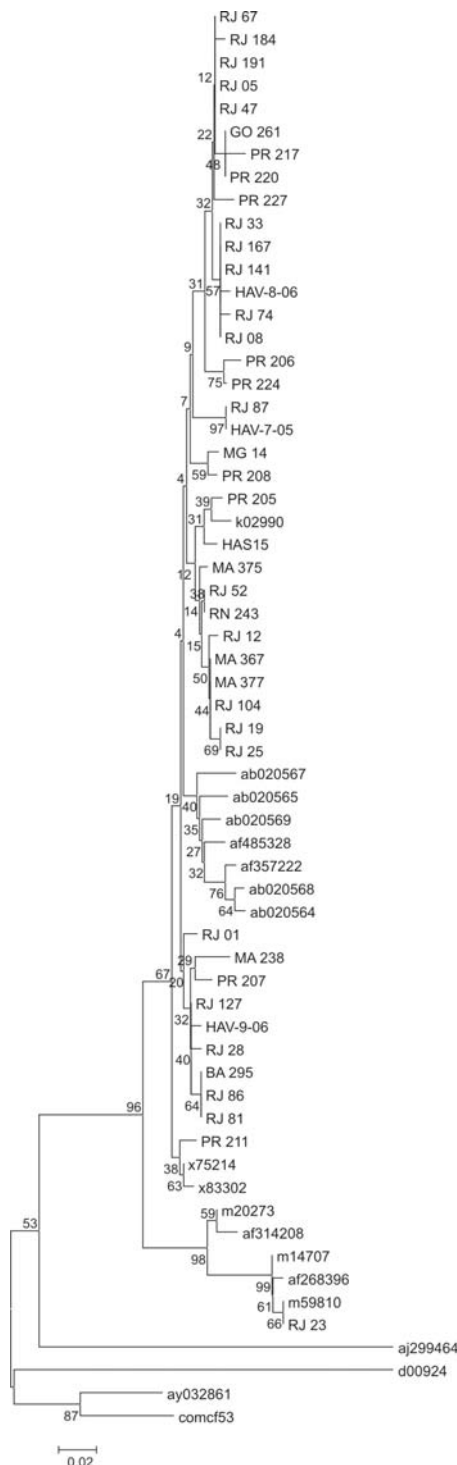
Figure 2. Phylogenetic tree based on 1200-nt sequences of the Pre S/S region from hepatitis B virus and depicting the genetic relatedness of isolates from this study (in bold) with other HBV strains previously characterized. The numbers at nodes indicate bootstrap percentages after 1,000 replications sampling. The horizontal bar provides a genetic distance scale.
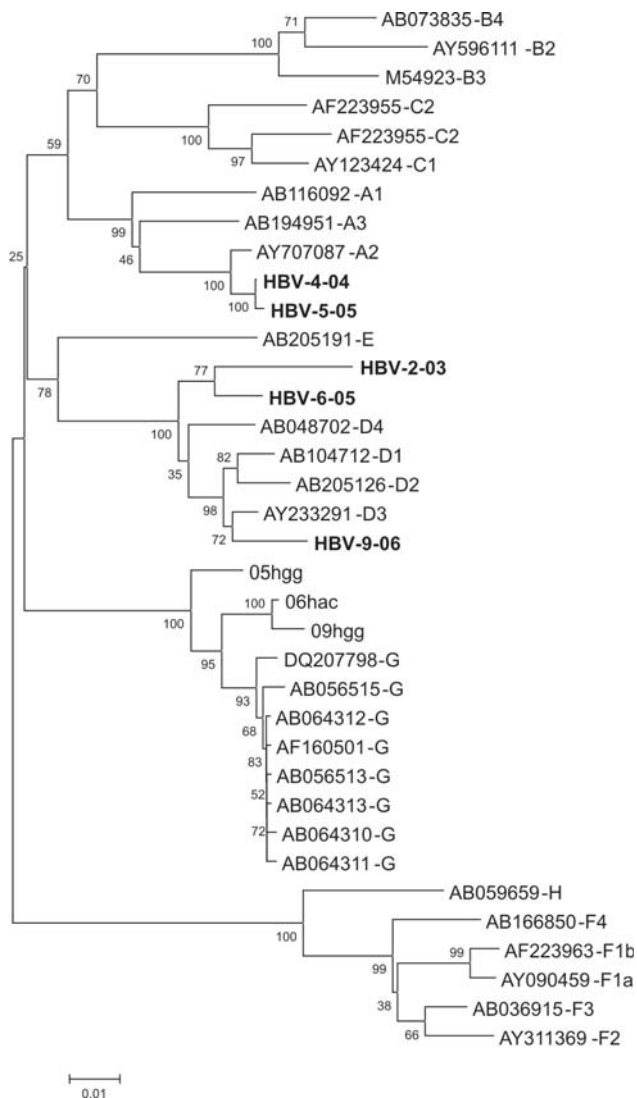
have early indicators of prognostic fulminant hepatic failure, classically did not develop encephalopathy and the PTA was not disposable in medical records. From this patient, only blood samples were analyzed.

HAV and HBV Mutations and Viral Load in the Co-Infected Patient

In the patient co-infected by HAV/HBV the viral load of HBV was 5.59×10^3 copies/mL and the genotype/subgenotype isolated was D3. The HAV viral load was 7.67×10^4 copies/mL

and the genotype isolated was also IA, as observed in other cases included in this study.

Discussion

The specific physiopathology of fulminant hepatic failure induced by viruses has not yet been well described, but some studies have suggested that the disease severity induced by these viruses may be related to genetic variability [2,18]. The clinical course severity of hepatitis A is associated with the age, being less severe in younger children, while that in older children and adults these diseases tend to be more symptomatic [19]. In this study 33% of FHF were caused by HAV, the mean age of fulminant hepatitis A was 13; two of the patients were less than five years old; previous studies have shown that hepatitis A is the most common detectable cause of fulminant hepatic failure in children [20-23]. A retrospective study showed that HAV is the most frequent aetiological agent of acute liver failure, being associated with 39% of cases eligible for liver transplantation [23].

All hepatitis A patients, including the patients co-infected with hepatitis B, were infected by genotype IA, the more prevalent in Brazil [24]; suggesting that in areas where the subgenotype IA is the most prevalent, FHF is associated with this genotype. There was no association with genotype-determining region with the severity of hepatitis A [3,4], however nucleotide differences can be observed among FHF [20].

The comparison of 5'NTR sequences showed less nucleotide substitutions in relation to samples from acute hepatitis A. However, Fujiwara and colleagues showed that patients can develop FHF independent of the number of nucleotide substitutions in 5' NTR of HAV and the number of substitutions encountered in the 5' central region does not appear to be the unique factor that defines the outcome of the clinical course. The variations in the 5' NTR region may influence replication of the virus and affect the virulence [2].

Few data are available concerning hepatitis A viral load and fulminant hepatitis failure. Rezende and colleagues, 2003, who showed that low viral load was the main factor associated with FH, suggested that HAV-related liver failure is due to an excessive host response associated with a marked reduction in viral load; in our study one sample had undetectable viral load, the recovery from paraffin liver of HAV RNA is difficult due to degradation. In fresh liver the viral load was 1.65×10^6 copies/mL; higher viral loads in patients with fulminant and severe hepatitis A may be associated with the pathogenesis of disease severity [25]. In this study, viral loads measured in serum samples were 10^4 copies/mL, these patients had the same viral loads previously reported for patients with fulminant hepatitis A [25].

Hepatitis B is endemic in the Brazilian Amazon Basin and several studies have revealed the association of HBV and fulminant hepatitis [26]. However, this is the first study, in Brazil, correlating HBV genotypes with FHF targeting to the

S-gene encoding the hepatitis B surface antigen. Hepatitis B is probably the most common viral cause of FHF and the incidence may be underestimated. As in other studies, hepatitis B virus prevails in patients with FHF and is responsible for the majority of cases [27-29].

In this study, genotypes A and D were associated with fulminant hepatitis; however these genotypes are common in patients with acute hepatitis B in Brazil. The FHF samples displayed several amino acid variations, found in sequence consensus encountered in genotype A and D. As observed in India [30] and in our study, the HBV genotype D was more prevalent than A among FHF. In east-Asian countries where genotypes B and C are found to be dominant, fulminant hepatitis genotype C is associated with the more aggressive clinical course related to chronic hepatitis with evolution to cirrhosis [31-34]. The genotype C has a short course of fulminant hepatitis in comparison to genotypes A, B and D [35]. However, the FHF patients infected by genotype D have a shorter period of evolution than FHF patients infected by genotype A (mean 15 *vs.* 53 days), suggesting that genotype D has a fast progression course.

In our study in four out of five patients infected with HBV the viral load measured was 10^5 copies/mL. A previous study showed that almost all of the patients who remained HBeAg-positive had HBV DNA levels that were persistently above 10^5 copies/mL in serum sample [23,36]. The samples extracted from paraffin liver had the same viral load as the serum samples, these results showed that it is possible to recovery DNA from paraffin liver to quantify and genotype HBV in fulminate hepatitis cases.

In patient HBV-6-05 who had serum and biopsy analyzed, the viral load in liver was observed to be two logs higher than in the serum sample, showing a massive replication in liver; the HBV DNA was classified into genotype D, the patient had a fast progression (8 days) to hepatic coma and death. The viral load of this patient was four logs higher than the case of SAH, which may suggests that the higher viral load may be related to poor clinical course and very fast progression to FHF.

In the patient co-infected with HAV and HBV the ALT values were the highest (3240 UI/L) and had a fast evolution, however the HBV viral load was the lowest, probably the immunologcial factors that cleared HAV infection contributed to the low replication of HBV and the recovery of patient.

In conclusion, hepatitis A and B infections are vaccine-preventable diseases and so a universal vaccination should be implemented to avoid fulminant hepatitis failure.

Acknowledgements

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