Rev. Inst. Med. trop. S. Paulo
51(5):269-272, September-October, 2009
doi: 10.1590/S0036-46652009000500006

DISTRIBUTION OF HEPATITIS B VIRUS GENOTYPES AND VIRAL LOAD LEVELS IN BRAZILIAN CHRONICALLY INFECTED PATIENTS IN SÃO PAULO CITY

Rosana ALCALDE(1), Fernando Lucas MELO(3), Anna NISHIYA(4), Suzete Cleusa FERREIRA(6), Mario Dante LANGHI JÚNIOR(6), Simone Sena FERNANDES(1), Luis Augusto MARCONDES(5), Alberto José Silva DUARTE(1) & Jorge CASSEB(1,2)

SUMMARY

The objective of the present study was to evaluate the serum viral load in chronically infected Hepatitis B virus (HBV) patients and to investigate the distribution of HBV genotypes in São Paulo city. Quantitative HBV-DNA assays and HBV genotyping have gained importance for predicting HBV disease progression, have been employed for assessing infectivity, for treatment monitoring and for detecting the emergence of drug resistance. Twenty-nine Brazilian patients with suspected chronic hepatitis B were studied, using real time PCR for viral load determination and direct DNA sequencing for the genotyping. The serology revealed chronic HBV infection in 22 samples. The HBV-DNA was positive in 68% samples (15/22). The phylogenetic analysis disclosed that eleven patients were infected with HBV genotype A, two with genotype F and two with genotype D. Thus, the genotype A was the most prevalent in our study.

KEYWORDS: HBV genotype; Region precore HBV; Viral load; Brazil.

INTRODUCTION

Hepatitis B virus (HBV) infects 400 million people worldwide[8,9], with a particularly high prevalence in Asia and Africa. HBV is the most common cause of liver disease worldwide[14,22] being a global health problem[7]. Some patients may be affected only mildly[9,22], while other patients may evolve to cirrhosis, hepatocellular carcinoma[8], and death from chronic hepatitis B[19].

HBV molecular virology and immunology were found to be more complex than initially thought, whereas the selection of precore HBV mutants was showed to be largely determined by the HBV genotype[5]. Several studies found an association between variants of the virus and genetic variations found in the host. However, only recently the impact of natural variability of the virus on the clinical course of disease has become the focus of research[17].

In Latin America there are more than 140,000 acute cases of hepatitis B per year and about 6.6 to 12 million people are chronic HBV carriers. More than 65% of these cases live in South America, where about 100,000 cases of acute hepatitis B occur annually[2]. In Brazil, there are more than 75,000 acute cases per year and HBsAg prevalence increases from South to North: 1% in the Southern and Central Western regions; 2% in the Southeastern region; 2.5% in the Northeastern region, and 8% in the Northern region. However, moderate to elevated HBsAg prevalence has been detected in candidate blood donors from the Southern states Santa Catarina (4.3%) and Paraná (8.3%)[2].

HBV genotypes have a distinct geographical distribution[11,19]. There are eight major HBV genotypes (A to H) prevailing in different parts of the world. Genotyping A is pandemic[8,20], B and C are commonly found in east Asia, and the latter has been associated with an increased risk of hepatocellular carcinoma[19]. Genotype D has been found worldwide with highest prevalence in Mediterranean area, Middle East and South Asia, particularly in India. The genotyping E is found in sub-Saharan Africa, F in South and Central America, G in France and in USA[1,12]. Genotype H so far seems to be restricted to Northern Latin America[12]. Coinfection with several HBV genotypes seems to be rare, below 5%[19].

In Brazil, the genotype A is the most prevalent and the genotypes B, C, D, and F have already been reported. Their distribution varies among Brazilian regions suggesting an influence of the immigration pattern for each region[18].

The objective of the present study was to evaluate the serum viral load and the virus genotype in chronically infected Hepatitis B virus (HBV) patients. The phylogenetic analysis disclosed that eleven patients were infected with HBV genotype A, two with genotype F and two with genotype D. Therefore, the genotype A was the most prevalent in our study in São Paulo city.

(1) Laboratório de Dermatologia e Imunodeficiências, LIM-56, Departamento de Dermatologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil.
(2) Instituto de Medicina Tropical de São Paulo, USP, São Paulo, SP, Brasil.
(3) Instituto de Ciências Biomédicas II, São Paulo, SP, Brasil.
(4) Fundação Pró-Sangue, Hemocentro de São Paulo, São Paulo, SP, Brasil.
(5) Departamento de Medicina Preventiva, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil.
(6) Santa Casa de São Paulo, São Paulo, SP, Brasil.
Correspondence to: Jorge Casseb, IMTSP/USP, IMT II, 3º andar, Laboratório de Dermatologia e Imunodeficiências, LIM-56/FMUSP, Departamento de Dermatologia, Faculdade de Medicina, USP, Av. Dr. Enéas de Carvalho Aguiar 470, 05403-000 São Paulo, SP, Brasil. E-mail: jcasseb@usp.br and rosana_fmusp@yahoo.com.br

MATERIALS AND METHODS

Blood samples: A total of 29 blood samples of patients with suspected chronic hepatitis B were collected. The serum was collected before the beginning of the antiviral treatment and stored at -70 °C. Age, sex and risk factors were obtained for infection with HBV and the time of infection estimated for patients with risk factors for blood transfusion and vertical transmission. The study protocol was approved by the Ethical Committee of the Institute of Infectious Diseases "Emílio Ribas" and written informed consent was obtained from all patients.

DNA extraction, Viral load determination, Nested-PCR and Sequencing: To detect hepatitis B viral DNA in the serum, HBV-DNA was isolated from 200 µL of serum using extraction method QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturers' protocol. Conventional PCR was carried out to amplify a 239 bp of DNA fragment.

HBV-DNA was quantified by TaqMan technology based real time PCR (quantitative polymerase chain reaction (qPCR)) as described by GARSON *et al.*[4], including albumen as positive control. Reactions were analyzed by Bio-Rad iCycler IQ Real Time PCR (Hercules, CA, USA). To test the performance of our primers and probes in real time PCR, we used 10^6, 10^5, 10^4, 10^3, 10^2, and 10 copies of HBV-DNA. Our results showed that amplification performance was good for all reactions.

Positive samples were amplified by conventional PCR for posterior genotyping. Polymerase chain reaction (PCR) and nested-PCR have been described by KANEKO *et al.*[6]. The HBV PCR products were sequenced utilizing ABI Prism Big Dye Terminator Ready Reaction Kit version 3.0 (Applied Biosystems, Foster City, CA). Reactions were analyzed by ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were validated after visual inspection and analysis using the Sequencher program version 4.0.5 (Gene Codes).

Phylogenetic analysis: The fifteen sequences with 239 base pairs from precore region generated (Table 1) were manually aligned with 21 reference sequences from genotypes A to H. The GenBank accession numbers for the reference sequences were: AY128092, X51970, AB241114, D00329, AB010289, X04615, AY123041, AB014381, M32138, X85254, X75657, AB032431, X69798, AB036910, AF223965, AF160501, AB064310, AF405706, AY090454, AY090457, AY090460.

Phylogenetic analyses were performed using both maximum likelihood (ML) criterion and Bayesian inference. ML searches were performed using GARLI v0.951.OsX-GUI[21] under an assumed general time-reversible (GTR) model with rates estimated from the data. Three independently runs were performed. Each run gave identical topologies and similar likelihood scores. The Bayesian analysis was performed with MrBayes[16] using a GTR + invariant (ι) and gamma (Γ_4). The MCMC search was run for 2 x 10^6 generations with trees sampled every 100^{th} generation (with a burn-in of 10%). The final tree presented is the maximum clade credibility tree inferred using TreeAnnotator v1.4.7 (available from http://beast.bio.ed.ac.uk) from the 1800 trees estimated by MrBayes.

RESULTS

A total of twenty-nine Brazilian patients with suspected chronic hepatitis B were studied. The serology revealed that 22 had HBsAg detection after six months from the first positive sample. HBV-DNA was positive in 68% samples (15/22), eight women and seven men, with a mean age of 42 years (range 31-51) and the HBV genotype was determined in 15 of 15 samples which presented detectable viral load (Table 1). In seven cases, the HBV-DNA viral load was undetectable by quantitative PCR; the cause of no amplification was low DNA serum viral.

Table 1
HBV viral load, conventional PCR and serology of the HBV

Patient ID	Gender	Age (years)	HBV viral load	HBV subtype	Accession number	Serology HBsAg	Serology anti-HBs
01	M	NC	3370	A	FJ404688	>250,000 / 0.05	Neg
02	M	34	*>500000	F	FJ404679	>250,000 / 0.05	Neg
03	M	51	1950	A	FJ404690	>250,000 / 0.05	Neg
04	F	39	3130	A	FJ404686	>250,000 / 0.05	Neg
05	F	NC	184	F	FJ404680	>250,000 / 0.05	Neg
06	F	NC	11200	A	FJ404682	>250,000 / 0.05	Neg
07	F	45	30780	A	FJ404685	>250,000 / 0.05	Neg
08	F	39	6450	D	FJ404691	>250,000 / 0.05	Neg
09	F	NC	18800	A	FJ404687	>250,000 / 0.05	Neg
10	M	53	67200	D	FJ404693	>250,000 / 0.05	Neg
11	M	48	259	A	FJ404684	0.844 / 0.05	Neg
12	F	45	3390	A	FJ404683	>250,000 / 0.05	Neg
13	F	31	461	A	FJ404681	>250,000 / 0.05	Neg
14	M	40	*>500000	A	FJ404692	>250,000 / 0.05	Neg
15	M	51	282	A	FJ404689	>250,000 / 0.05	Neg

M: Male; F: Female; NC: not collected; Plasma DNA-HBV viral load: copies/mL; *> upper limit of the assay; Neg: Negative; Kit HBsAg Architect (Abbott, USA); Kit anti-HBs Murex (Dartford, England)

The phylogenetic analysis disclosed that eleven patients were infected with HBV genotype A (73.3%), two with genotype F (13.3%) and two with genotype D (13.3%), as shown on Fig. 1. Therefore, the genotype A was the most prevalent in our study in São Paulo city.

DISCUSSION

HBV genotyping among chronic carriers was studied in São Paulo city. This small sample had a mean age of 42 years, and was made up predominantly of females. In the city of Salvador, Brazil, the majority of the cases corresponded to males, with a similar distribution age (30 to 40 years)[15]. Viral load was detected in fifteen samples, and two patients had viral load that were above the upper limit of detection. To our knowledge, this is the first study to describe HBV viral load done with Brazilian subjects.

Clinical studies have shown a high rate of HBV-DNA in chronic cases[7]. Quantitative HBV-DNA assays and HBV genotyping have gained importance for predicting HBV disease progression, employed for assessing infectivity, for treatment monitoring and for detecting the emergence of drug resistance[4,19]. A study showed that viral load is the major determinant of the risk of liver cirrhosis, hepatocellular carcinoma and death in chronic hepatitis B patients[19].

To our knowledge, this is the first time that HBV genotypes from patients living in São Paulo were typed. The genotypes showed a distinct and well-defined geographic distribution. In our study, genotype A was the most prevalent, but other subtypes were also typed, such as F and D. This distribution was similar to that found in Rio de Janeiro and Salvador, for example[13,15]. The higher prevalence of HBV genotype A might reflect the ethnic background of the Brazilian population, with its strong miscigenation[15]. The white population of São Paulo is descendant of Portuguese and other Europeans, predominantly Italians. Other studies have also shown that genotype A is most commonly found in North America, Africa and Northwestern Europe[15] and is the most prevalent in Brazil followed by genotypes B, C, D and F[18] as already documented among most other groups of chronic carriers, excluding hemodialysis patients[3].

In the Amazonian region, only genotype F was found among the native Brazilian tribes who have not had contact with whites, while genotype A was found among tribes with frequent contacts[13]. In a study

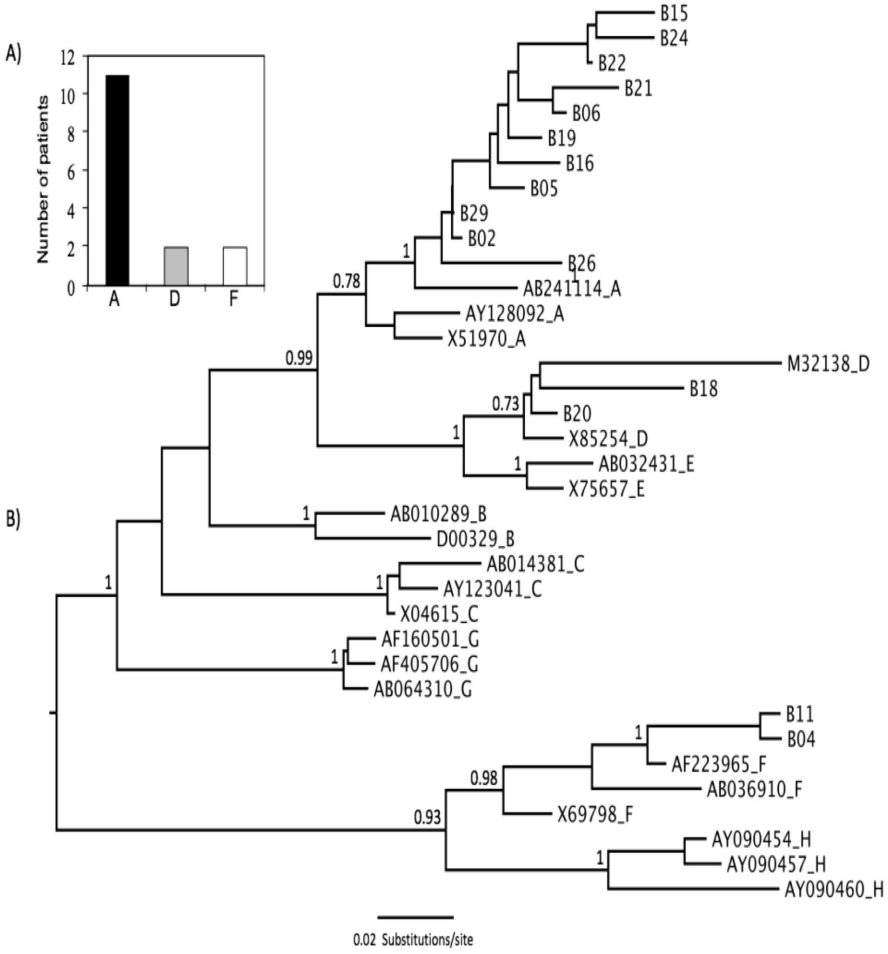

Fig. 1 - A) Frequency of genotypes found in 15 Brazilian HBV patients studied in São Paulo city. **B)** Maximum clade credibility tree based on 239 base pairs from pre-core region. The posterior probability values are shown for key nodes.

from MAYERAT *et al.*[10], it was suggested that the infection by HBV genotype A could be more frequently associated with chronic infection than genotype D. HBV genotype D has a universal distribution, however it seems to be more prevalent in Southern Europe, Middle East, Northern Africa and India[15].

Finally, for the first time the HBV genotype was performed in samples from patients living in São Paulo city. The HBV-DNA viral load was performed for clinical samples to follow up the patients. Viral load is a major marker of disease progression and for treatment guidelines. Summing up, quantitative real time PCR was a simple, sensitive, specific and reproducible assay for the measurement of HBV viral load in serum samples.

RESUMO

Distribuição dos genótipos do vírus da hepatite B e níveis de carga viral em pacientes brasileiros cronicamente infectados na cidade de São Paulo

O objetivo do presente estudo foi avaliar a carga viral no soro de pacientes cronicamente infectados pelo vírus da Hepatite B (HBV) e investigar a distribuição de genótipos HBV na cidade de São Paulo. PCR quantitativo do HBV e genotipagem ganharam importância para a previsão de progressão da doença, empregada para avaliar a infectividade, para tratamento e acompanhamento e para detectar o aparecimento de resistência aos anti-retrovirais. Vinte e nove pacientes brasileiros com suspeita de hepatite B crônica foram estudados, utilizando PCR em tempo real para a determinação da carga viral e seqüenciamento direto para determinação do genótipo. A sorologia revelou que 22 estavam, de fato, cronicamente infectados pelo HBV. O HBV-DNA foi positivo em 68% das amostras (15/22). Em sete casos, HBV-DNA foi indetectável por PCR quantitativo. A análise filogenética mostra que onze pacientes foram infectados com hepatite B genótipo A, dois com genótipo F e dois com genótipo D. Desta forma, o genótipo A foi o mais prevalente em nosso estudo.

ACKNOWLEDGMENTS

We thank all the patients who participated in this study and Abbott Laboratories for kindly providing the kits for serology.

REFERENCES

1. BARTHOLOMEUSZ, A. & SCHAEFER, S. - Hepatitis B virus genotypes: comparison of genotyping methods. **Rev. med. Virol., 14:** 3-16, 2004.

2. BERTOLINI, D.A.; PINHO, J.R.R.; SARACENI, C.P. *et al.* - Prevalence of serological markers of hepatitis B virus in pregnant women from Paraná State, Brazil. **Braz. J. med. biol. Res., 39:** 1083-1090, 2006.

3. FRANÇA, P.H.C.; COELHO, H.S.M.; BRANDÃO, C.E. *et al.* - The emergence of YMDD mutants precedes biochemical flare by 19 weeks in lamivudine-treated chronic hepatitis B patients: an opportunity for therapy reevaluation. **Braz. J. med. biol. Res., 40:** 1605-1614, 2007.

4. GARSON, J.A.; GRANT, P.R.; AYLIFFE, U.; FERNS, R.B. & TEDDER, R.S. - Real-time PCR quantitation of hepatitis B virus DNA using automated sample preparation and murine cytomegalovirus internal control. **J. virol. Meth., 126:** 207-213, 2005.

5. HADZIYANNIS, S.J. & VASSILOPOULOS, D. - Hepatitis B e antigen-negative chronic hepatitis B. **Hepatology, 34:** 617-624, 2001.

6. KANEKO, S.; FEINSTONE, S.M. & MILLER, R.H. - Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. **J. clin. Microbiol., 27:** 1930-1933, 1989.

7. KAO, J.H.; CHEN, P.J.; LAI, M.Y. & CHEN, D.S. - Genotypes and clinical phenotypes of hepatitis B virus in patients with chronic hepatitis B virus infection. **J. clin. Microbiol., 40:** 1207-1209, 2002.

8. LAI, C.L.; RATZIU, V.; YUEN, M.F. & POYNARD, T. - Viral hepatitis B. **Lancet, 362:** 2089-2094, 2003.

9. LOLE, K.S. & ARANKALLE, V.A. - Quantitation of hepatitis B virus DNA by real-time PCR using internal amplification control and dual TaqMan MGB probes. **J. virol. Meth., 135:** 83-90, 2006.

10. MAYERAT, C.; MANTEGANI, A. & FREI, P.C. - Does hepatitis B virus (HBV genotype influence the clinical outcome of HBV infection? **J. viral Hepatitis, 6:** 299-304, 1999.

11. MIYAKAWA, Y. & MIZOKAMI, M. - Classifying hepatitis B virus genotypes. **Intervirology, 46:** 329-338, 2003.

12. PALUMBO, E.; SCOTTO, G.; FALEO, G.; CIBELLI, D.C. & ANGARANO, G. - Prevalence of HBV genotypes in South American immigrants affected by HBV-related chronic active hepatitis. **Braz. J. infect. Dis., 11:** 311-313, 2007.

13. PINHO, J.R.R. - O vírus da hepatite B. In: SILVA, L.C. **Hepatites agudas e crônicas.** 3. ed. São Paulo, Sarvier, 2003. p. 15-25.

14. REHERMANN, B. & NASCIMBENI, M. - Immunology of hepatitis B virus and hepatitis C virus infection. **Nature Rev. Immunol., 5:** 215-229, 2005.

15. RIBEIRO, N.R.C.; CAMPOS, G.S.; ANGELO, A.L.D. *et al.* - Distribution of hepatitis B virus genotypes among patients with chronic infection. **Liver Int., 26:** 636-642, 2006.

16. RONQUIST, F. & HUELSENBECK, J.P. - MrBayes 3: Bayesian phylogenetic inference under mixed models. **Bioinformatics, 19:** 1572-1574, 2003.

17. SCHAEFER, S. - Hepatitis B virus: significance of genotypes. **J. viral Hepatitis, 12:** 111-124, 2005.

18. SITNIK, R.; SETTE Jr., H.; SANTANA, R.A.F. *et al.* - Hepatitis B virus genotype E detected in Brazil in an African patient who is a frequent traveler. **Braz. J. med. biol. Res., 40:** 1689-1692, 2007.

19. SORIANO, V.; PUOTI, M.; PETERS, M. *et al.* - Care of HIV patients with chronic hepatitis B: updated recommendations from the HIV-Hepatitis B Virus International Panel. **Aids, 22:** 1399-1410, 2008.

20. STUYVER, L.; DE GENDT, S.; VAN GEYT, C. *et al.* - A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. **J. gen. Virol., 81:** 67-74, 2000.

21. ZWICKL, D.J. - Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Austin, 2006. (Ph.D. Dissertation - The University of Texas at Austin).

22. SZOMOR, K.N.; DENCS, A.; TÓTH, G. *et al.* - Variability of the PreS1/PreS2/S regions of hepatitis B virus in Hungary. **Arch. Virol., 152:** 697-704, 2007.

Received: 19 November 2008
Accepted: 17 August 2009