African Journal of Microbiology Research Vol. 7(3), pp. 152-157, 15 January, 2013
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.241
ISSN 1996-0808 ©2013 Academic Journals

Full Length Research Paper

Study of genotypes and subgenotypes of Hepatitis B virus prevalent in big cities of Pakistan

Muhammad Hanif[1*], Perveen Zaidi[1], Salman Habib[1], Akhtar Ahmed[1], Abida Raza[2], Shoaib Ahmed[3] Shahnaz Murtaza[2] and Javaid Irfan[3]

[1]PCR and Molecular Biology Laboratory, Karachi Institute of Radiotherapy and Nuclear Medicine, Kiran, Karachi, Pakistan.
[2]Diagnostics Department, Nuclear medicines, Oncology and Radiotherapy Institute, Islamabad, Pakistan.
[3]Dow University of Health Sciences, Karachi, Pakistan.

Accepted 19 December, 2012

Hepatitis B virus (HBV) is a well known agent of acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Around 400 million people worldwide carry the virus including 4.5 million only in Pakistan. Very limited research work on HBV genotyping has been done so far in Pakistan and there is a high level of discrepancy and inconsistency among different group of researchers. The aim of present study was to determine the prevalent genotypes of HBV in three big cities Karachi, Rawalpindi and Islamabad, Pakistan. A total of 200 HBV DNA polymerase chain reaction (PCR) positive samples (135 male and 65 female) from three big cities of Pakistan were tested for genotyping by two step nested PCR. Genotype D was detected as the most prevalent (58.5%) genotype followed by mix genotype A and D (31.5%) and genotype A was only (10%). Phylogenetic tree of 19 reference sequences constructed by using Clustal X program shows that all belong to subgenotype D1.

Key words: Hepatitis B virus genotype, subgenotypes, HBV genotype D, Pakistan.

INTRODUCTION

Hepatitis B virus (HBV) infection is becoming a serious health threat in many developing countries like Pakistan and it has become the leading cause of acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. In Pakistan, HBV infection is not only a major health problem leading to significant morbidity and mortality but also catastrophic for its poor economy. In Pakistan, HBV infection rate is increasing day by day. The reason may be the lack of proper health facilities or poor economical status and less public awareness about the transmission of major communicable diseases like Hepatitis B virus, Hepatitis C virus and Human Immunodeficiency Virus (Hanif et al., 2009; Alam et al., 2007).

*Corresponding author. E-mail: hanifmuhammad@hotmail.com.

Genotyping of HBV is essential for characterization of patient groups and for epidemiological studies. The clinical significance of different HBV genotypes has become increasingly recognized in patients with acute and chronic infection. The course of HBV infection depends on several factors such as host genetic factors, age and genetic variability of the virus (Gunther et al., 1999; Hunt et al., 2000). In addition to the epidemiological importance, these genotypes may influence the disease pattern and response to treatment.

Ten (10) HBV genotypes (A to J) have been discovered so far (Zhang and Cao, 2011) and are known to have a distinct pattern of geographic distribution: genotype A is found in North America and Northern Europe, as well as in some parts of Africa (Bowyer et al., 1997) where as genotype B and C are common in Southeast Asia and genotype D is found universally (Okamoto et al., 1988). Genotype E has been reported from Western and Southeast

Table 1. Demographic, biochemical and HBV DNA PCR data among three cities of Pakistan.

Parameter	Karachi	Rawalpindi	Islamabad	Total (%)
Male	25	35	70	130 (65%)
Female	15	15	40	70 (35%)
Total	40	50	110	200
Median age (years)				35 (range 7-70)
Median ALT U/L	82	82	82	82 (10-488)
S Antigen positive	40	50	110	200 (100)
S Antigen Negative	0	0	0	0 (0)
HBV DNA positive	40	50	110	200 (100)
Genotype A	3	5	12	20 (10%)
Genotype D	27	30	60	117 (58.5%)
Mix Genotype A&D	10	15	38	63 (31.5%)

Africa (Bowyer et al., 1997). Genotype F has been detected in South and Central America (Bowyer et al., 1997) and genotype G has been reported in France and North America (Stuyver et al., 2000). In addition to these genotype I (Hannoun et al., 2000) and J (Tatematsu et al., 2009) have been reported as variants different from abovementioned from Vietnam and Japan, respectively. The data on distribution of HBV genotype is still emerging in Asia.

Very limited research work on HBV genotyping has been done so far in Pakistan and there is a high level of discrepancy and inconsistency among different group of researchers has been noted (Ahmed et al., 2009). For example, Idrees et al. (2004) reported that genotype C is the most pre-dominant, while genotype A was predominant in Sind Province, genotype C in North West Frontier Province (Idrees et al., 2004) and genotypes B and C in Punjab. In another study by Abbas et al. (2006) it was reported that genotype D is predominant genotype in Karachi, the largest city in Pakistan and also the capital of Sind Province (Abbas et al., 2006). None of the abovementioned studies have performed whole genome or partial genome sequencing of HBV (Ahmed et al., 2009).

In the present study, 200 HBV DNA polymerase chain reaction (PCR) positive patients from three big cities of Pakistan (Karachi, Rawalpindi and Islamabad) were selected for genotyping by two step PCR method described by Naito et al. (2001) with some modifications in cycling profile and PCR constituents. Phylogenetic tree of the reference sequences are constructed and discussed.

MATERIALS AND METHODS

Samples from different physicians and hepatologists of Karachi were sent to PCR and molecular biology lab, KIRAN and of Rawalpindi and Islamabad was sent to NORI Molecular Biology lab, Islamabad for HBV DNA detection by PCR methods. Genotyping of 200 HBV DNA PCR positive samples were carried out by two step PCR method as described by Naito et al. (2001). At the same time, data including date, age, sex and location were collected in KIRAN and NORI (Table 1). Further testing including liver function test (LFT) and enzyme-linked immunosorbent assay (ELISA) based detection of Hepatitis B surface antigen (HBsAg) was conducted according to manufacturer's protocols.

HBV DNA extraction

Sample preparation from ethylenediaminetetraacetic Acid (EDTA) plasma and serum was performed following the manufacturer's instructions using "INSTANT Virus DNA kit" (AJ Roboscreen, analytikajena Biosolutions, GmbH, Germany) where a synthetic internal control (IC) is included via extraction tubes to control DNA extraction and to indicate the inhibitory effects. DNA was eluted in 60 µl elution buffer.

Viral load quantitation

Viral load quantification was done by RoboGene® HBV Quantification kit (AJ Roboscreen, analytikajena Biosolutions, GmbH, Germany) at NORI. This allows amplifying all eight HBV genotypes (A-H) with equal efficiency by applying probes and primers specific for a subsequence of HBV-S gene encoding HBsAg. Upstream probes is labeled with 5'-end reporter dye (FAM for HBV DNA: Yakima Yellow for IC DNA) and downstream probe is labeled with quencher dye. A three step PCR protocol was followed: *Taq* activation step at 95°C for 4 min, then 50 cycles of synthesis at 57°C for 1 min, melting at 95°C for 30 s, stem formation and fluorescence detection 45°C for 30 s using Rotor-Gene™3000, Corbett Research, Australia. Data collected in the form of sigmoid growth curves was analyzed according to manufacturer's instructions keeping in view the parameters of slope correction and value, thresholds, CT value of IC DNA and R value, HBV DNA concentration is expressed in IU/ ml. Quantitation standards ranged from $5x10^2$ to $5x10^7$ IU/ml were provided along with the kit.

HBV genotyping

Genotyping was done following the two step PCR method described by Naito et al. (2001) with some modifications in cycling profile and PCR constituents. DNA material extracted for Real Time PCR quantification was used as template in regular PCR after 1:10 dilution. For regular PCR *GoTaq*® Green Master mix (Promega) 10 µl, universal primers (P1A and S1-2) (Table 2) 1 µl each, ddH_2O 2 µl, diluted template 6 µl. The cycling conditions were first incubation at 95°C for 10 min, then 30 cycles of 94°C for 20 s, 55°C for 20 s and extension at 72°C for 60 s. Multiplex nested PCR was performed

Table 2. Primer sequences used for HBV genotyping by nested PCR.

Primer	Sequence[a] (position, specificity, and polarity)
First PCR	
P1[b]	.....5'-TCA CCA TAT TCT TGG GAA CAA GA-39 (nt 2823±2845, universal, sense)
S1-2	.....5'-CGA ACC ACT GAA CAA ATG GC-39 (nt 685±704, universal, antisense)
Second PCR	
Mix A	
B2	.....5'-GGC TCM AGT TCM GGA ACA GT-39 (nt 67±86, types A to E specificity, sense)
BA1R	.....5'-CTC GCG GAG ATT GAC GAG ATG T-3' (nt 113±134, type A specificity antisense)
BB1R	.....5'-CAG GTT GGT GAG TGA CTG GAG A-3' (nt 324±345, type B specificity, antisense)
BC1R	.....5'-GGT CCT AGG AAT CCT GAT GTT G-39 (nt 165±186, type C specificity, antisense)
Mix B	
BD1	.....5'-GCC AAC AAG GTA GGA GCT-39 (nt 2979±2996, type D specificity, sense)
BE1	.....5'-CAC CAG AAA TCC AGA TTG GGA CCA-39 (nt 2955±2978, type E specificity, sense)
BF1	.....5'-GYT ACG GTC CAG GGT TAC CA-39 (nt 3032±3051, type F specificity, sense)
B2R	.....5'-GGA GGC GGA TYT GCT GGC AA-39 (nt 3078±3097, types D to F specificity, antisense)

[a]M , Nucleotide that could be either an A or a C; Y, nucleotide that could be either a C or a T; nt, nucleotide. [b]The sequence for primer P1 was determined by Lindh et al. (1998).

in two mixes. Mix 1 comprised of primers for genotype A, B and C while mix 2 contained primers for genotype D, E and F (Table 2). Each mix contained PCR DNA template from regular PCR product 2 µl, *GoTaq*® Green Master mix 08 µl, primers 1 µl each, ddH_2O 8 µl. The cycling conditions were first incubation at 95°C for 10 min, then 40 cycles of 94°C for 45 s, 63°C for 20 s and extension at 72°C for 60 s (ABI 9700). PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and evaluated under UV light (BioRad Gel Doc-XR, USA). The sizes of PCR products were estimated according to the migration pattern of a 50-bp DNA ladder (GeneRuler™ Fermentas).

Subgenotyping and sequencing

Subgenotyping of reference samples was done by comparing the sequences with already known HBV sequences from GeneBank after the phylogenetic analysis. After genotyping with nested PCR 19 samples were randomly selected for sequencing and were sent to Bio Basic Inc. Reference sequences of HBV Genotypes A-H and subgenotype of D (D1-D4) were downloaded from NCBI. Both reference and studied sequences were aligned and phylogenetic tree was constructed with Clustal X software, then viewed using Treeview program.

RESULTS

A total of 200 HBV DNA positive samples including 40 samples from KIRAN, Karachi and 160 samples from NORI, Islamabad (and Rawalpindi) were tested for genotyping. The demographic data shows that 130 samples were male and 70 were female patients, age range was 4 to 70 years with median age of 34.6 years (Table 1). The alanine amino transferase (ALT/SGPT) level of 98% sample was abnormally elevated with mean level of 82 U/L (normal range is 10 to 40 U/L). All samples were HbsAg positive and majority had viral load of $\geq X10^5$ IU/ml.

Genotyping

All samples were tested for HBV DNA by real time PCR and viral load quantification (data not shown) before genotyping by two step PCR method. Among 200 HBV DNA positive samples Genotype D was detected as the most prevalent genotype (117/200) including 27 samples from Karachi, 30 from Rawalpindi and 60 from Islamabad followed by mix genotype A and D (63/200) including 10 samples from Karachi, 15 from Rawalpindi and 38 from Islamabad and genotype A was only (20/200) including 3 samples from Karachi, 5 from Rawalpindi and 12 from Islamabad (Table 1).

Dominance of subgenotype D1

Genotype D was found to be the most prevalent (59%) amongst our patients moreover; Phylogenetic tree constructed using 19 reference sequences by using Clustal X program shows that all present sequences belong to subgenotype D1 (Figure 1).

DISCUSSION

The initial studies on HBV genotyping revealed that genotypes B and C are the most prevalent genotypes in Asian regions. It was because of the fact that all such studies were reported from Japan and China where genotype B and C are the most prevalent genotypes. Later on, it was found that all the seven HBV genotypes can be found in Asia (Toan et al., 2006). For instance, the predominant genotypes in India are genotype A and D (Thakur et al., 2002). The predominant HBV genotypes in Afghanistan were found to be genotype D (Amini-Bavil-

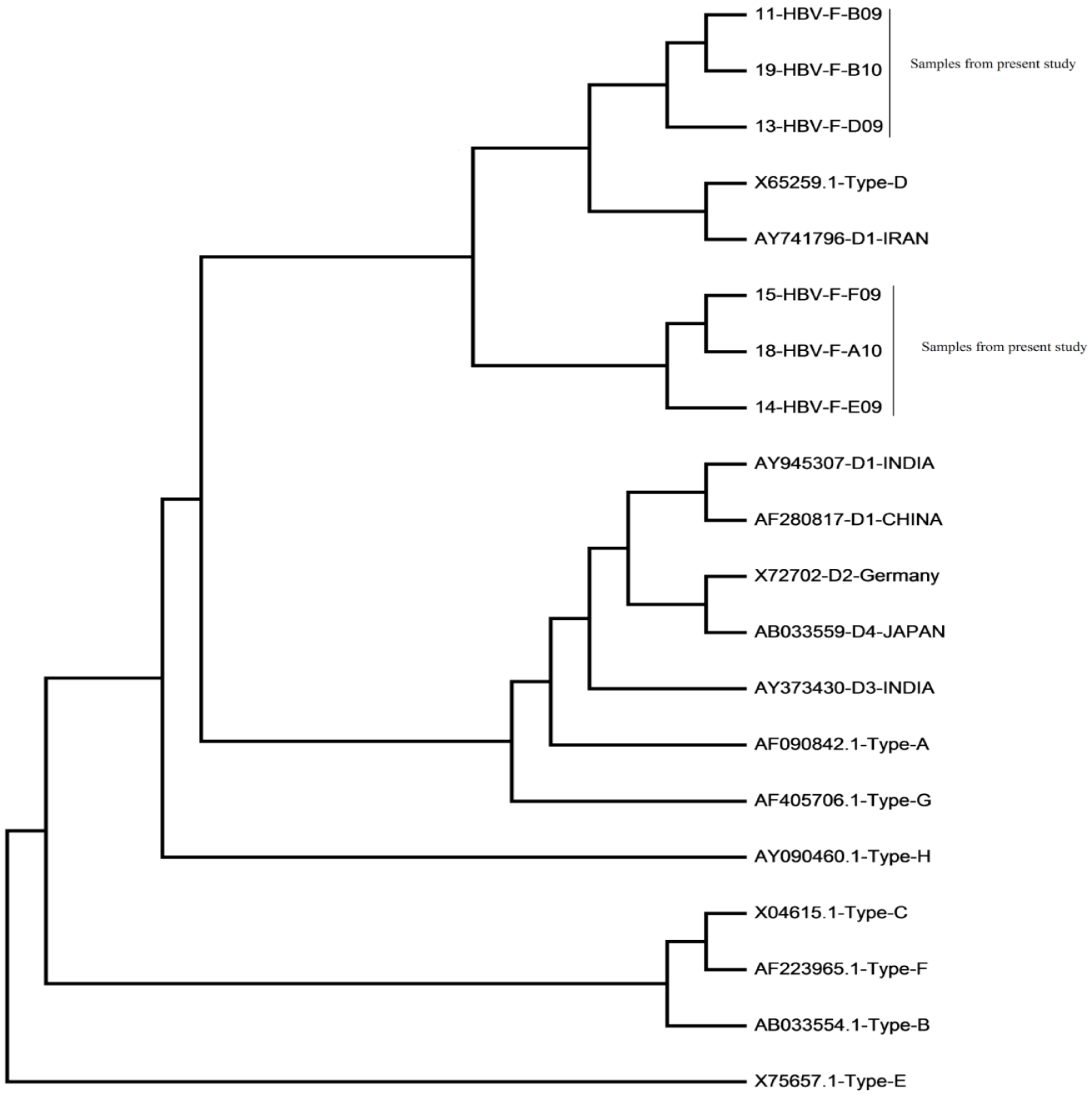

Figure 1. Phylogenetic tree was constructed using 19 sequences by using Clustal X program. Reference sequences are labeled with accession number followed by type. Vertical lines indicate the present study sequences. Genotype E was selected as out group. Phylogenetic analysis shows that all present sequences belong to HBV genotype D1.

Olyaee et al., 2006). Similarly, Zeng et al. (2004) reported 1.6% patients infected with multiple HBV genotypes. In Pakistan, studies from Karachi showing predominant gnotype D (Noorali et al., 2008; Baig et al., 2007; Alam et al., 2007), study from North West Frontier province also showing genotype D (Abbas et al., 2006) but study from Punjab is showing genotype C as predominant (Idrees et al., 2004). From Islamabad highest prevalence of genotype D followed by genotype B has been reported (Alam et al., 2007).

Majority of our patients had genotype D (58.5%) coinfected with type A (31.5%). This pattern is similar when compared to other studies in Pakistan (Noorali et al., 2008; Baig et al., 2007; Abbas et al., 2006; Idrees et al., 2004) except the study from Punjab, Lahore (Idrees et al., 2004). In south east Asia, genotypes B and C are more prevalent (Wong and Chan, 2003) but in India mixed pattern of genotypes A, C and D is observed (Gandhe et

al., 2003; Kumar et al., 2005). There are therapeutic implications of these finding as patients with genotype D have more severe disease (Kao et al., 2002) and are less responsive to interferon therapy as compared to genotype A and B (Sablon and Shapiro, 2004; Erhardt et al., 2000) and have higher HBV DNA levels (Westland et al., 2003).

Phylogenetic analysis of genotype D could distinguish four subgenotypes (D1-D5) (Baig et al., 2009). The only subtype in all 19 reference samples of present study was D1 which is almost similar to other studies from Pakistan where predominant subtype was D1 along with rare D2 and D3 (Ahmed et al., 2009; Baig et al., 2009). This subgenotype has mainly been shown in strains from Turkey, India, Iraq, Iran and Israel (Bläckberg et al., 2000). History of population migration from the Middle East to Pakistan is well known, which explains the predominance of HBV/D1 infection in this population. In neighboring India, genotype D is predominant with presence of subgenotypes D1, D2, D3 and D5 (Norder et al., 2004; Banerjee et al., 2006). Genotypes are important for designing effective treatments (Ding et al., 2003) and determining the genotype-specific mutations and antiviral drug resisance. The influence of genotypes on liver disease progression (Sablon and Shaprio, 2004; 2005) has already been recognized, therefore the determination of prevalent genotypes and subgenotypes will certainly help in the treatment of patients, studying the antiviral treatment response in the population and disease progression, and controlling and ultimately eradicating HBV from the Pakistani population.

REFERENCES

Abbas Z, Muzaffar R, Siddiqui A, Naqvi SA, Rizvi SA (2006). Genetic variability in the precore and core promoter regions of hepatitis B virus strains in Karachi. BMC Gastroenterol. 6:20.

Ahmed CS, Wang ZH, Zhou Bin, Chen JJ, Kamal M, Hou JL (2009). Hepatitis B virus genotypes, subgenotypes, precore and basal core promoter mutations in the two largest provinces of Pakistan. J. Gastroenterol. Hepatol. 24:569-573.

Alam MM, Zaidi SZ, Malik SA, Shaukat S, Naeem A, Sharif S, Angez M, Butt JA (2007). Molecular epidemiology of Hepatitis B virus genotypes in Pakistan. BMC Infect. Dis. 8(7):115.

Alam MM, Zaidi SZ, Shaukat S, Sharif S, Angez M, Naeem A, Saleha S, Butt JA, Malik SA (2007). Common genotypes of Hepatis B prevalent in injecting drug abusers (addicts) of North West Frontier Province of Pakistan. Virol. J. 28(4):63.

Amini- Bavil-Olyaee S, Alavian SM, Adeli A, Sarrami-Forooshani R, Sabahi F (2006). Hepatitis B virus genotyping, core promoter and precore/core mutations among afghan patients infected with hepatitis B: a preliminary report. J. Med. Virol. 78:358-364.

Baig S, Siddiqui AA, Ahmed W, Qureshi H, Arif A (2007). The association of complex liver disorders with HBV genotypes prevalent in Pakistan. Virol. J. 27(4):128

Baig S, Siddiqui AA, Chakravarty R, Moatter T (2009). Hepatitis B virus subgenotypes D1 and D3 are prevalent in Pakistan. BMC Res. Notes 2:1

Banerjee A, Kurvanob F, Datta S, Chandra PK, Tanaka Y, Mizokami M, Bhattacharya SK, Chakravarty R (2006). Phylogenetic relatedness and genetic diversity of HBV genotype strain isolated from Eastern India. J. Med. Virol. 78:1164-1174.

Bläckberg J, Braconier JH, Widell A, Kidd-Ljunggren K (2000). Long-term outcome of acute hepatitis B and C in an outbreak of hepatitis in 1969-72. Eur. J. Clin. Microbiol. Infect. Dis. 19:21-26.

Bowyer SM, van Staden L, Kew MC, Sim JG (1997). A unique segment of the hepatitis B virus group A genotype identified in isolates from S. Afr. J. Gen. Virol. 78(7):1719-1729.

Ding X, Gu H, Zhong ZH (2003). Molecular epidemiology of hepatitis viruses and genotypic distribution of hepatitis B and C viruses in Harbin, China. Jpn. J. Infect. Dis. 56:19-22.

Erhardt A, Reineke U, Blondin D, Gerlich WH, Adams O, Heintges T, Niederau C, Haussinger D (2000). Mutations of the core promoter and response to interferon treatment in chronic replicative hepatitis B. Hepatology 31:716-725.

Gandhe SS, Chadha MS, Arankalle VA (2003). Hepatitis B virus genotypes and serotypes in western India: lack of clinical significance. J. Med. Virol. 69:324-330.

Gunther S, Fischer L, Pult I, Sterneck M, Will H (1999). Naturally occurring variants of hepatitis B virus. Adv. Virus Res. 52:25-137.

Hanif M, Zaidi P, Kamal S, Hameed A (2009). 'Institution based cancer incidence in a local population in Pakistan: Nine year Data analysis'. Asian Pac. J. Cancer Prev. 10:225-228.

Hannoun C, Norder H, Lindh M (2000). An aberrant genotype revealed in recombinant hepatitis B virus strains from Vietnam. J. Gen. Virol. 81(9):2267-2272.

Hunt CM, McGill JM, Allen MI, Condreay LD (2000). Clinical relevance of hepatitis B viral mutations. Hepatology 31:1037-1044.

Idrees M, Khan S, Riazuddin S (2004). Common genotypes of hepatitis B virus. J. Coll. Physicians Surg. Pak. 14:344-347.

Kao JH, Liu CJ, Chen DS (2002). Hepatitis B viral genotypes and lamivudine resistance. J. Hepatol. 36:303-304.

Kumar A, Kumar SI, Pandey R, Naik S, Aggarwal R (2005). Hepatitis B virus genotype A is more often associated with severe liver disease in northern India than is genotype D. Indian J. Gastroenterol. 24:19-22.

Lindh M, Gonazalez JE, Norkrans G, Horal P (1998). Genotyping of Hepatitis B by restriction pattern analysis of a Pre-S amplicon. J. Virol. Methods 72:163-174.

Noorali S, Hakim ST, McLean D, Kazmi SU, Bagasra O (2008). Prevalence of Hepatitis B Virus genotype D in females in Karachi. Pak. J. Infect. Dev. Ctries. 2(5):373-378.

Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, Robertson BH, Locarnini S, Magnius LO (2004). Genetic diversity of hepatitis B virus strains derived worldwide: Genotypes, subgenotypes, and HBsAg subtypes. Intervirology 47:289-309.

Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y (1988). Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J. Gen. Virol. 69(Pt 10):2575-2583.

Sablon E, Shapiro F (2004). Hepatitis B and C genotyping: methodologies and implications for patient management. J. Gastroenterol. Hepatol. 19:S329-337.

Sablon E, Shapiro F (2005). Advances in molecular diagnosis of HBV infection and drug resistance. Int. J. Med. Sci. 2:8-16.

Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF (2000). A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J. Gen. Virol. 81(1):67-74.

Tatematsu K, Tanaka Y, Kurbanov F, Sugauchi F, Mano S, Maeshiro T (2009). A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. J. Virol. 83(20):10538-10547.

Thakur V, Guptan RC, Kazim SN, Malhotra V, Sarin SK (2002). Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. J. Gastroenterol. Hepatol. 17:165-170.

Toan NL, Song LH, Kremsner PG, Duy DN, Binh VQ, Koeberlein B, Kaiser S, Kandolf R, Torresi J, Bock CT (2006). Impact of Hepatitis B virus genotypes and genotype mixtures on the course of liver disease in Vietnam. Hepatology 25:1375-1384.

Westland C, Delaney W, Yang H, Chen SS, Marcellin P, Hadziyannis S, Gish R, Fry J, Brosgart C, Gibbs C, Miller M, Xiong S (2003). Hepatitis B virus genotypes and virologic response in 694 patients in phase III studies of adefovir dipivoxil. Gastroenterology 125:107-116.

Wong GL, Chan HL (2005). Molecular virology in chronic hepatitis B: genotypes. Hosp. Med. 66:13-16.
Zeng GB, Wen SJ, Wang ZH, Yan L, Sun J, Hou JL (2004). A novel hepatitis B virus genotyping system by using restriction fragment length polymorphism patterns of S gene amplicons. World J. Gastroenterol. 10:3132-3136.
Zhang Q, Cao G (2011). Genotypes, mutations, and viral load of hepatitis B virus and the risk of hepatocellular carcinoma. Hepat. Mon. 11(2):86-91.