HEPATITIS B VIRUS DRUG RESISTANCE AND SURFACE GENE ESCAPE MUTATION IN INDIA: ASSOCIATION WITH GENOTYPE AND VIRAL LOAD

Jagdish C. Kandpal[1*], Rishendra Kumar[1]
[1]Department of Biotechnology, Kumaun University Nainital, Uttarakhand, India
*Corresponding author email: jagdish.kandpal@yahoo.com

ABSTRACT
The Hepatitis B virus (HBV), reverse transcriptase (RT) region of the polymerase gene is largely overlapped by the surface gene (HBsAg). In-spite the high conservation of HBV- RT, infrequent mutations has been described. The aim of the study was to identify mutation for drug resistance within RT and HBsAg escape region, with respective of viral load and genotype in the Indian subcontinent. The study was done for 87 individual (mean age 33.44 ±15.04 years), between Apr 2017 to May 2018. HBV DNA of an infected individual was ranged, 608 to >7.0 ×10^7 IU/ ml (median log 6.41 log 10). Sub-genotyping and mutation determination was done in the amplified reverse transcriptase region of the HBV polymerase gene. Sub-genotype C1 (10.16%) and D5 (6.77%) was identified only in higher viral load cases. The frequency of HBV drug resistance mutation was 11.53% (3/26) for each sub-genotype D1, D2 and 9.52% (2/21) for sub-genotype A1. The drug resistance mutation was related to LAM (80V), ADV (236T, 236Y), ETV (250G, 250H), TDF (236T, 236Y), and LdT (80V). The frequency of HBsAg mutation was 96.15% (25/26) for sub-genotype D2, followed by 23.07% (6/26) for sub-genotype D1 and 4.76% (1/21) for sub-genotype A1. Most frequent escape mutation found was 128V, only on sub-genotype D2. This study was useful to determine the association of viral load, genotype and mutant identification for therapy selection. This finding made serious attention to the drug resistance and HBsAg escape mutation, if not diagnosed and treated.

KEYWORDS: Diagnosis, infection, polymerase gene, sequencing.

INTRODUCTION

HBV prevalence in India among south Asian countries has been grouped with an intermediate endemic region based on serological test (Puri and Srivastava, 2012). Polymerase chain reaction (PCR) test has been developed to detect and measure the amount of viral nucleic acid in clinical specimens, which are used to observe a person's infection status and to monitor treatment (Zoulim, 2006). Determinations of genotyping and sub-typing also have clinical as well as therapeutic importance in vaccine development, drug selection, and mutant identification (Shi *et al*., 2013). The advances in antiviral strategies, based on the development of new and more powerful nucleotide analogs (NA), have improved chronic HBV therapy management, including the prevention of allograft re-infection. HBV infection can occur even after patients are vaccinated either having occult infected carrier, or have developed protective anti-HBs antibody, by HBV mutants encoding HBsAg (Hino *et al*., 1995; Karthigesu *et al*., 1994). There is a lack of reliable studies on the mutated HBV. Mutations in the gene coding region of polymerase or reverse transcriptase can be associated with viral persistence or resistance to therapy by nucleotide analogs (Keeffe *et al*., 2006). Such mutants are able to escape the host immune responses. The N-terminal domain of the viral polymerase forms the terminal protein (TP) that linked to the viral DNA, and its central domain forms the coding region reverse transcriptase (RT), which is largely overlapped by the S gene. Irrespective the high conservation of HBV reverse transcriptase, infrequent mutations have been described (Blum *et al*., 1991). The main antiviral agents those are approved for management of chronic hepatitis B (CHB) infection includes two formulations of IFN- namely conventional, pegylated, and five nucleos(t)ide analogues (NAs), namely lamivudine (LMV), adefovir (ADV), telbivudine (LdT), entecavir (ETV) and tenofovir (TDF) (Scaglione and Lok, 2012; Yapali *et al*., 2014). Although IFN- has both antiviral and immune-modulatory activity only selected patients are treated with it because of side effects, on the other hand, the majority of chronic hepatitis B patients are treated with NAs only (Liaw and Chu, 2009). Nucleoside analogs can be easily applied orally but may often be required for an indefinite duration. About 25% of adults infected with HBV during childhood are reported to die from hepatocellular carcinoma (HCC) or liver cirrhosis (Park *et al*., 2012). Molecular biology tools can be used to identify and quantify viral genomes, sequence them, assign them to a genotype, and to find clinically relevant nucleotide or amino acid substitutions, such as those associated with resistance to antiviral drugs. Recent advances include Real-time target amplification methods for detecting and quantifying viral genomes and sequencing techniques, which can be utilized the application of HBV genotype in disease progression by Hepatitis B Virus infection. Hence, it is an urgent need to design process that would be appropriate for our society rather than following guidelines of European, South-East Asian countries or other continents.

MATERIALS & METHODS

Ethical statement

This study was supported by Kumaun University, Nainital and CORE Diagnostics, Gurugram and approved by its ethics committee. The informed consent form was obtained from all the cases or individual included.

Study subjects

A total of 87 infected cases with hepatitis B virus were enrolled in this study. The study cases were covering the patients of the major Indian sub-continent, North [n = 44 (Delhi = 42, Ghaziabad = 2)], East [n = 26 (Kolkata = 5, Patna =10, Cuttack = 2, Ling-raj = 1, the eastern adjacent country region; Dhaka = 7 and Kathmandu =1)], West [n= 10 (Nagpur =6, Dahisar =1, Ahmadabad =1, Baijaipur = 1, Jaipur =1), and South [n =7 (Mysore =3, Bangalore =2, Nampally =1, Hyderabad=1)]. The cases that recruited between April 2017 and May 2018 were part of our study. The mean age of the patients included in this study was 33.44 ±15.04 years. Blood was collected from the cases for HBV diagnosis and processed in the laboratory. The samples were tested by commercial Real-time PCR (QIAGEN, Germany) to determine the HBV DNA viral load. Consequently, samples were tested for HBV Genotyping or sub-genotyping and assigned for mutant identification.

Viral DNA extraction and HBV DNA amplification

Viral DNA was extracted from 500μl of plasma samples by QIAamp® DSP virus extraction procedure using silica column-based technology (QIAGEN, Germany). HBV DNA was detected and quantified by artus® HBV RG PCR kit (QIAGEN, Germany) according to the instruction of manufacture on the Real-time PCR system (Rotor-Gene Q, QIAGEN, Germany).

Genotyping with HBV RT- polymerase gene region

Primers used were; forward: 5'-TCGTGGTGG ACTTC TCTCAATT-3' and reverse; 5'-CGTTGA CAGAC TTT CCAATCAAT- 3' for the partial HBV polymerase gene region (Sayan and Dogan, 2012). The composition of 30μl reaction volumes of PCR master mix was contained 10X PCR buffer, 50 mM $MgCl_2$, 10 mM each of the four dNTPs, 10μM of each primer with a final concentration of 0.33μM and 5U of *Taq* DNA polymerase. The temperature parameters were; 95^0C for 15 minutes, followed to 45 PCR cycles at 95^0C for 45 seconds, 56^0C for 45 seconds, and 72^0C for 45seconds. Around 730 *bp* products recovered on agarose gel on amplification with primers. After that, clear and strong bands of PCR positive samples were selected for Sanger sequencing.

Sequencing reaction of the amplified product

The Reverse primer was used as a sequencing primer for all the samples. Amplified PCR products were directly sequenced in the ABI 3500xL Genetic analyzer (Applied Biosystem, USA) Instrument, using the Bigdye terminator (Version 3.1) cycle sequencing kit. For the sequencing reaction, thermal cycling, conditions used were 20 seconds on 95^0C, 25 seconds on 50^0C for 35 cycles, and 60^0C for 2 minutes. Data collection and assembly were done by 3500xL Genetic Analyzer data collection and sequencing analysis software (Version 1.0 and 5.4).

Sequence data analysis

Sequences were analyzed using KB^{TM} Basecalling (Version 1.4.1.8) sequence analysis software and saved as FASTA file format. Multiple sequence alignments and phylogenetic analysis were done using reference sequences available from Genbank.

Sub-genotyping determination

Obtained sequences were aligned with published sequences from the GenBank database with known HBV genotypes and sub-genotypes. Multiple sequence alignment was performed by using CLC Sequence Viewer, Version 6.1 (CLC, Denmark) software. HBV Genotype or sub-genotype was determined by phylogenetic analysis in CLC Sequence Viewer, Version 6.1 using the neighbor-joining method with a bootstrap analysis of 1000 replicates. This analysis is based on partial reverse transcriptase regions of HBV polymerase sequence. Genotype or sub-genotype of HBV was also determined by the Basic Local Alignment Search Tool (BLASTN) program, available in http://www.ncbi.nih.gov projects with reference to viral nucleotide sequences. That was done by BLAST of the query sequence with the known set of the sequence (Rozanov *et al.*, 2004).

HBV drug resistance and surface gene mutation analysis

HBV drug resistance mutation analysis was done by Geno2pheno [HBV] 2.0 analysis tools http://www. hbv. geno2pheno.org (Center of Advanced European Studies and Research, Bonn, Germany). This tool search for homology between the input sequence and its database sequence which are already stored. It identified HBV drug resistance mutation at amino acid positions 80, 169, 173, 180, 181, 184, 194, 202, 204, 215, 233, 236, and 250 in the reverse transcriptase domain of the polymerase gene segment. The overlapped surface gene was identified for mutations at amino acid position 119 to 145. The interpretation was done by both manually and Geno2pheno analysis software.

Statistical analysis

Statistics were analyzed using Microsoft Excel. The demographic variable was measured as mean ±SD. The significance of categorical variable was determined by x^2 test and *p*-value <0.05 considered statistically significant.

RESULTS

HBV DNA viral load said to be associated with the replication capacity of genotype or sub-genotype and the person immune status, hence were found the difference in viral load in the subjects.

Correlation of HBV sub-genotypes and viral DNA load

The association between viral load and sub-genotypes of the low viral load and high viral load, we found 28 samples of viral load below 2 x 10^4 IU/ml. From them sub-genotype A1 found in 7 subjects, sub-genotype D1 identified in 7 subjects, sub-genotype D2 in 12 subjects, and sub-genotype D3 in two subjects. The 59 samples of viral load above 2 x 10^4 IU/ml, we found 14 cases of sub-genotype A1, 6 cases of sub-genotype C1, 19 cases of sub-genotype D1, 14 cases of sub-genotype D2, two cases of sub-genotype D3 and four cases of sub-genotype D5. Here, sub-genotype C1 and sub-genotype D5 were found only on higher viral load samples (Table 1).

TABLE 1: Correlation of HBV sub-genotypes and viral load

Viral Load (IU/ml)	Sub-genotype	Cases count- 87	Frequency (%)
<20000		28	
	A1	7	25
	D1	7	25
	D2	12	42.85
	D3	2	7.14
>20000		59	
	A1	14	23.72
	C1	6	10.16
	D1	19	32.20
	D2	14	23.72
	D3	2	3.38
	D5	4	6.77

HBV drug resistance prediction

Approved drugs used to treat patients of HBV infection were Lamivudine (LAM), Adefovir (ADV), Entecavir (ETV), Tenofovir (TDF), and Telbivudine (LdT). To predict drug resistance, 5 mutations detected in the genotyping study were 236T, 236Y, 250G, 250H, and 80V. The frequency of these mutations in the genotyping studied group was 5.74 % (5/87). The prediction of drug resistance mutation was related to LAM (80V), ADV (236T, 236Y), ETV (250G, 250H), TDF (236T, 236Y), LdT (80V) (Table 2).

TABLE 2: Observed drug resistance mutation

Standard Drugs	Drug Resistance Mutations	Count	Sub-genotype _Region	Viral load (IU/ ml)	Age/ Gender
Lamivudine, Zeffix®	80V	1	A1_W (Ahmadabad)	$<2\times10^4$	32/ F
	none	86			
Adefovir, Hepsera®	236T	1	D1_W (Bijaipur)	$>2\times10^4$	30/ M
	236Y	1	D2_S (Bangalore)	$<2\times10^4$	44/ F
	none	85			
Entecavir, Baraclude®	250G	1	D1_W (Nagpur)	$>2\times10^4$	29/ M
	250H	1	D2_E (Dhaka)	$<2\times10^4$	33/ M
	none	85			
Tenofovir DF®	236T	1	D1_W (Bijaipur)	$>2\times10^4$	30/ M
	236Y	1	D2_S (Bangalore)	$<2\times10^4$	44/ F
	none	85			
Telbivudine, Tyzeka®, Sebivo®					32/ F
	80V	1	A1_W (Ahmadabad)	$<2\times10^4$	
	none	86			

E (East), W (West), S (South)

TABLE 3: Observed HBsAg escape mutation

Escape mutations SHB protein	Count	Sub-genotype_Region	Viral load (IU/ ml)	Age/ Gender
120A + 145A	1	D2_S (Hyderabad)	$>2\times10^4$	72/ M
120T	1	A1_W (Ahmadabad)	$<2\times10^4$	32/ F
126N + 128V	1	D2_N (Delhi)	$<2\times10^4$	22/ M
	6;	E (<2×10⁴, <2×10⁴, >2×10⁴, <2×10⁴, >2×10⁴, <2×10⁴); D2_E (Dhaka, Kolkata, Kolkata, Dhaka, Patna, Lingaraj);	E ($<2\times10^4$, $<2\times10^4$, $>2\times10^4$, $<2\times10^4$, $>2\times10^4$, $<2\times10^4$);	E (33/M, 20/M, 23/F, 28/M, 18/F, 60/M);
	9;	D2_N (Delhi, Delhi, Delhi, Delhi, Delhi, Ghaziabad, Delhi, Delhi, Delhi);	N ($<2\times10^4$, $>2\times10^4$, $<2\times10^4$, $>2\times10^4$, $>2\times10^4$, $>2\times10^4$, $>2\times10^4$, $>2\times10^4$, $>2\times10^4$); S ($>2\times10^4$, $<2\times10^4$, $<2\times10^4$, $>2\times10^4$)	N (60/M, 60/F, 21/F, 28/M, 23/F, 74/M, 26/M, 41/M, 50/M); S (33/M, 44/F, 55/M, 36/M)
	4	D2_S (Bangalore, Bangalore, Mysore, Mysore)		
128V	1;	D2_E (Dhaka);	$>2\times10^4$;	18/ F;
128V + 130R	1	D2_S (Mysore)	$<2\times10^4$	59/ M
128V+ 134H	1	D2 _E (Cuttack)	$<2\times10^4$	28/ M
128V + 145A	1	D2_E (Kolkata)	$>2\times10^4$	27/ M
129H	1	D1_N (Delhi)	$>2\times10^4$	25/ F
130R + 131N + 133L	1	D1_W (Bijaipur)	$>2\times10^4$	30/ M
131N + 133T	1	D1_W (Nagpur)	$>2\times10^4$	36/ M
134N	1	D1_N (Delhi)	$>2\times10^4$	31/ M
143L	1	D1_N (Delhi)	$>2\times10^4$	38/ M
144E	1	D1_W (Jaipur)	$>2\times10^4$	24/ F

E (East), N (North), W (West), S (South)

HBV escape mutation SHB protein

HBV polymerase gene segment also overlaps the envelope gene. Mutation in and around this domain can cause HBV reactivation due to escape mutation in immunized anti-HBs individual. To predict HBsAg escape mutation, 14 mutations detected in the genotyping study were 120A, 120T, 126N, 128V, 129H, 130R, 131N, 133L, 133T, 134H, 134N, 143L, 144E, and 145A. The prevalence of escape mutations in the genotyping studied group found was 16.09 % (14/87) (Table 3). Most frequent escape mutation found was 128V (Figure 1).
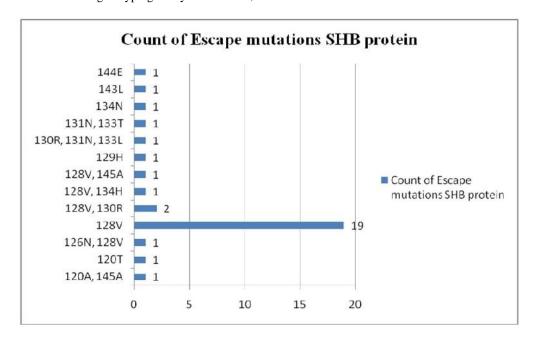

FIGURE 1: Count of HBsAg escape mutation**: In the SHB protein mutation group, 13 different patterns of HBsAg escape mutations were found: 144E, 143L, 134N, 131N + 133T, 130R + 131N + 133T, 129H, 128V +145A, 128V + 134H, 128V + 130R, 128V, 126N + 128V, 120T, 120A + 145A. Maximum number of escape mutation observed was of 128V.
In addition, there was no correlation observed for the presence of detected mutation with respect to patient age and gender.

DISCUSSION

In this study a wide range of HBV DNA viral load, 10.5 to $>2\times10^7$ IU/ml, was detected in plasma samples. HBV DNA viral load was categorized high viral load and low viral load. Measuring of HBV DNA levels in serum or plasma was useful for the clinical staging of chronic infection (Niitsuma et al., 1997), including assessment of infectivity and prognosis. High viral load of HBV DNA was known to be an independent risk factor for the development of fibrosis, cirrhosis, hepatocellular carcinoma, and drug resistance that can be accessed by measurement of HBV DNA levels was the most direct and effective way (Cai et al., 2008).

The sub-genotypes identified were A1 (24.13 %), C1 (6.89 %), D1 (29.88 %), D2 (29.88 %), D3 (4.59 %), and D5 (4.59 %). On the observation we found single sub-genotype C1. For genomic group A, only sub-genotype A1 occurred, which was more prevalent in India as well as in the world (Banerjee et al., 2006). However, in the case of genomic group D, multiple sub-genotypes D1, D2, D3, and D5 were found (Table 1), which was prevalent in India (Datta, 2008; Ghosh et al., 2013). Infection with HBV genotype A may lead to an increased risk of viral Transmission (Komatsu et al., 2011]. The high replication capacity of genotype C may be the reason for increased genotype-related severe hepatic damage (Kao, 2011). In India, sub-genotypes D1 and D3 were significantly associated with chronic and occult HBV infections, respectively (Chandra, et al., 2009). On analyzing the importance to determine sub-genotype and association with viral load, we have identified a higher viral load of sub-genotype C1 (10.16 %) and D5 (6.77 %). It could be due to high replication capacity of this genotype and also the person immune status that can lead to increase the incidence of vertical or horizontal transmission and apparent genotype associated liver damage (Komatsu et al., 2011; Kao, 2011). Therefore, the association of HBV genotype with viral load was important in estimating disease progression and provides to the clinician very important clues to overcome treatment response.

In our study, the frequency of drug resistance mutation was 11.53 % (3/26) for each sub-genotype D1 and D2 and 9.52% (2/21) for sub-genotype A1 on using standard approved drugs used to treat patients. A previous study from India showed that drug-resistant mutations in the polymerase gene are most frequent in genotype D in chronic hepatitis patients (Singla et al., 2013) and reported the presence of these mutations in treatment-naïve individuals. The probable dominance of genotype D in the Indian subcontinent and the frequent use of antiviral drugs, screening of such patients in the acute phase becomes extremely desirable for monitoring the possible infection of drug-resistant mutants. Therefore, HBV genotyping can predict the risk of adverse outcomes of fulminant disease, cirrhosis, HCC or can influence in managing decision of the HBV patients (Kao et al., 2000; Kato et al., 2005).

The drug resistance mutation either singly or in combination were related to LAM (80V), ADV (236T, 236Y), ETV (250G, 250H), TDF (236T, 236Y), LdT (80V) (Table 2). In the RT region of the HBV polymerase gene, L80V/I were reported in LMV-resistant HBV isolates which have shown disease exacerbation (Warner *et al*., 2007**).** The HIV and HBV RT inhibiting common drug, Lamivudine, was widely used. Lamivudine resistance can be caused primarily at location within the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the viral polymerase or RT reading frame. The adefovir resistant mutant was more common in patients than in treatment group patients. The major mutation of adefovir resistance has been associated with a mutation in the D domain at rtN236T (Pacheco *et al*., 2017; Pastor *et al*., 2009). Whereas, the addition of substitution rtM250 has associated with significant replication impairment, lies outside the HBsAg open reading frame and emerged during ETV combination treatment (Tenney *et al*., 2004**).** Disparate antiviral resistant mutations can result in reduced susceptibility to single or multiple antiviral agents. The emergence of these antiviral drug-resistant mutations contradicts the benefits of antiviral therapy. Moreover, the mutation can trigger hepatitis flares, liver failure, and even death because of uncontrolled HBV replication and cascade of liver inflammation.

Hepatitis B surface antigen (HBsAg) induces protection against HBV infection, as it is directly related to B-cell epitopes and, therefore considered the major target of neutralizing antibodies to act as an HBV vaccine (Kramvis *et al*., 2005). The frequency of HBsAg mutation was 96.15% (25/26) for sub-genotype D2, followed by 23.07% (6/26) for sub-genotype D1 and 4.76% (1/21) for sub-genotype A1 (p <0.000) (Table 3). Most frequent escape mutation found was 128V, only on sub-genotype D2 (Figure 1). Mutation in HBsAg region that cause amino acid substitutions (ELHadad *et al*., 2013), could affect the binding of anti-HBV antibodies and the detection of conventional serological assays (Torresi, 2002). A relationship in its support has been observed leading to HBV re-infection and, increased incidence of HCC in chronic HBV patients (Tian *et al*., 2007). Many investigations have shown the diagnostics challenges due to these mutations for HBsAg detection or for the consequences of vaccine and HBIg therapy (Carman, 1997; Coleman *et al*., 1999). It suggested the wide circulation of this mutant genotype was also possible in the Indian subcontinent. Hence, the strategy of viral monitoring was also important to determine the transmission of these variants and to stop accelerating public health threat.

Of the 32 identified cases of HBsAg escape mutations, sub-genotype D2 was associated with both low viral load-11/32 (34.37%) and high viral load- 14/32 (43.75%). Sub-genotype D1 was associated with only high viral load-18.75% (6/32). Whereas, one identified case of sub-genotype A1, was associated with low viral load (3.12%). The HBsAg escape mutations, either singly or in combination were clustered in 13 groups of the total 14 mutations identified (Table 3). The existence of low load of HBV DNA, absence of detectable viral factor HBsAg, in serum is a characterized feature of Occult hepatitis B infection, and considerable interest has been made on mutations within the surface gene and its regulatory regions. Thus, mutation clustering identified in immune-dominant regions of the surface protein is able to decrease the immune recognition of the virus. In addition, the deletions in the pre S1 region that impaired viral packaging and structural alteration in genomic regulatory regions leads to a strong reduction of HBsAg expression, as well as affecting posttranslational production of HBV proteins (Raimondo *et al*., 2008; El *et al*., 2010).

RT domain codons and SHB protein codons in this mutation analysis included were from amino acid 43 to 284 and 34 to 227 respectively. The extreme overlapping of the open reading frames of the HBV genome limits its possibility of fixation of mutations (Mizokami *et al*., 1997). These opposite aspects provide the substitution rate of HBV to an intermediate level between RNA and DNA viruses (Kidd-Ljunggren *et al*., 2002). The main factors for drug resistance mutation or escape mutation include viral dynamics, production and clearance of virus within the population. The absence of proofreading mechanism in the HBV genome and the frequent recombination creates this versatile mutation characteristic (Sayan *et al*., 2010; Shi *et al*., 2012). This study was limited by the fact that a suspected population or cases studied among those who attended the clinic or hospital in different regions and were likely to have liver complications. There was a lack of information about the patient's treatment strategy, or previously diagnosed with any other disease. People have been more traditional and didn't want to disclose their information.

CONCLUSION
In conclusion, our study focused on the findings of drug resistance and surface gene (HBsAg) escape mutation with their association with sub-genotype and viral load. This study is useful to determine the association of viral load, genotype and mutant identification for therapy selection. This finding made serious attention to the drug resistance and HBsAg escape mutation, if not diagnosed and treated. We have found HBV drug resistance and HBsAg escape mutation within the selected overlapping region. It is done by PCR followed DNA sequencing of the reverse transcriptase polymerase gene region. Therefore, this target region has useful application to identify sub-genotype, drug resistance and HBsAg escape mutation.

CONFLICTS OF INTEREST
No conflict of interest.

REFERENCES
Banerjee, A., Kurbanov, F., Datta, S., Chandra, P.K., Tanaka, Y., Mizokami, M., and Chakravarty, R. (2006). Phylogenetic relatedness and genetic diversity of hepatitis B virus isolates in Eastern India. Journal of Medical Virology, 78: 1164–1174.

Blum, H.E., Galun, E., Liang, T.J., Von-Weizsa-cker, F., and Wands, J.R. (1991). Naturally occurring missense mutation in the polymerase gene terminating hepatitis B virus replication. Journal of Virology, 65: 1836–1842.

Cai, T., Lou, G., Yang, J., Xu, D., and Meng, Z. (2008). Development and evaluation of real-time loop-mediated isothermal amplification for hepatitis B virus DNA quantification: a new tool for HBV management. Journal of Clinical Virology, 41: 270–276.

Carman, W.F. (1997). The clinical significance of surface antigen variants of hepatitis B virus. Journal of Viral Hepatology, 4: 11–20.

Chandra, P.K., Biswas, A., Datta, S., Banerjee, A., Panigrahi, R., Chakrabarti, S., De, B.K., and Chakravarty, R. (2009). Subgenotypes of hepatitis B virus genotype D (D1, D2, D3 and D5) in India: differential pattern of mutations, liver injury and occult HBV infection. Journal of Viral Hepatology, 16: 749–756.

Coleman, P.F., Chen, Y.C.J., and Mushahwar, I.K. (1999). Immunoassay detection of hepatitis B surface antigen mutants. Journal of Medical Virology, 59: 19–24.

Datta, S. (2008). An overview of molecular epidemiology of hepatitis B virus (HBV) in India. Virology Journal, 5: 156.

El, C.M., Candotti, D., Crowther, R.A., and Allain, J.P. (2010). Impact of hepatitis B virus surface protein mutations on the diagnosis of occult hepatitis B virus infection. Hepatology, 52: 1600–1610.

ELHadad, S.R., Alakilli, S.Y.A., Ramadan, H.A.L., and Baeshen, N.A. (2013). Novel mutations of hepatitis B virus surface antigen genotype D among chronic Egyptian patients. African Journal of Microbiology Research, 7: 814– 824.

Ghosh, S., Banerjee, P., Deny, P.Mondal, R.K., Nandi, M., Roychoudhury, A., Das, K., Banerjee, S., Santra, A., Zoulim, F., Chowdhury, A., and Datta, S. (2013). New HBV subgenotype D9, a novel D/C recombinant, identified in patients with chronic HBeAg-negative infection in Eastern India. Journal of Viral Hepatitis, 20 (3): 209–218.

Hino, K., Okuda, M., Hashimoto, O., Ishiko, H., Okazaki, M., Fujji, K., Hanada, R., and Okita, K. (1995): Glycine-to-arginine substitution at codon 145 of HBsAg in two infants born to hepatitis B e antigen-positive carrier. Digestive diseases and sciences, 40: 566–570.

Kao, J. H. (2011). Molecular epidemiology of hepatitis B virus. Korean Journal of Intern Medicine, 26: 255-261.

Kao, J., Chen, P., Lai, M., and Chen, D. (2000). Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology, 118: 554-559.

Karthigesu, V.D., Allison, L.M., Fortuin, M., Mendy, M., Whittle, H.C., and Howard C.R. (1994). A novel hepatitis

B virus variant in the sera of immunized children. The Journal of general virology, 75: 443-448.

Kato, H., Fujiwara, K., Gish, G., Sakugawa, H., Yoshizawa, H., Sugauchi, F., Ueda, R., Tanaka, Y., Kato, T., Miyakawa, Y., and Mizokami, M. (2005). Classifying genotype F of hepatitis B virus into F1 and F2 subtypes. World Journal of Gastroenterology, 11: 6295-6304.

Keeffe, E.B, Dieterich, D.T., Han, S.H., Jacobson, I.M., Martin, P., Schiff, E.R., Tobias, H., and Wright, A.T. (2006). Current Algorithm for the Treatment of Chronic Hepatitis B. Clinical.Gastroenterology, 4: 1-27.

Kidd-Ljunggren, K., Miyakawa, Y., and Kidd, A.H. (2002). Genetic variability in hepatitis B viruses. The Journal of general virology, 83 (6): 1267-1280.

Komatsu, H., Sugawara, H., Inui, A., Hiejima, F., Sogo, T., and Fujisawa, T. (2011). Does the spread of hepatitis B virus genotype A increase the risk of interfamilial transmission in Japan? Journal of Infection and Chemotherapy, 17: 272–277.

Kramvis, A., Kew, M., and François, G. (2005): Hepatitis B virus genotypes. Vaccine, 23: 2409-2423.

Liaw, Y.F. and Chu, C.M. (2009). Hepatitis B virus infection. Lancet, 373 (9663): 582–592.

Mizokami, M., Orito, E., Ohba, K., Ikeo, K., Lau, J.Y., and Gojobori, T. (1997). Constrained evolution with respect to gene overlap of hepatitis B virus. Journal of Molecular Evolution, 44: S83-S90.

Niitsuma, H., Ishii, M., Miura, M., Kobayashi, K., and. Toyota, T. (1997). Low-level hepatitis B viremia detected by polymerase chain reaction accompanies the absence of HBe antigenemia and hepatitis in hepatitis B virus carriers. American. Journal of Gastroenterology.92: 119-123.

Pacheco, S.R., Santos, M.M., Stocker, A., Zarife, M.S., Schinoni, M.I., Paraná, R., Reis, M.G., and Silva, L.K. (2017): Genotyping of HBV and tracking of resistance mutations in treatment-naïve patients with chronic hepatitis B. Infection and Drug Resistance, 10: 201-207.

Park. Y., Kim, B.S., Choi, K.H., Shin, D.H., Lee, M.J., Cho, Y., and Kim, H.S. (2012). A novel multiplex real-time PCR assay for the concurrent detection of hepatitis A, B and C viruses in patients with acute hepatitis. PLoS One, 7: e49106.

Pastor, R., Habersetzer, F., Fafi-Kremer, S., Doffoel, M., and Baumert, T.F. (2009). Hepatitis B virus mutations potentially conferring adefovir/tenofovir resistance in treatment-naive patients. World J Gastroenterology, 15: 753–755.

Puri, P. and Srivastava, S. (2012) Lower chronic hepatitis B in south Asia despite all odds: Bucking the trend of other infectious diseases. Tropical Gastroenterology, 33 (2): 89-94.

Raimondo, G., Allain, J.P., Brunetto, M.R., Buendia, M.A., Chen, D.S., Colombo, M., Craxi, A., Donato, F., Ferrari, C., Gaeta, G.B., Gerlich, W.H., Levrero, M., Locarnini, S., Michalak, T., Mondelli, M.U., Pawlotsky, J.M., Pollicino, T., Prati, D., Puoti, M., Samuel, D., Shouval, D., Smedile, A., Squadrito, G., Trepo, C., Villa,E., Will, H., Zanetti, A. R., and Zoulim, F. (2008). Statements from the Taormina expert meeting on occult hepatitis B virus infection. Journal of Hepatology, 49: 652–657.

Rozanov, M., Plikat, U., Chappey, C., Kochergin, A. and Tatusova, T. (2004). A web-based genotyping resource for viral sequences. Nucleic Acids Research, 32 (Web Server issue): W654–9.

Sayan, M., and Dogan, C. (2012). Genotype/subgenotype distribution of hepatitis B virus among hemodialysis patients with chronical hepatitis B. Annals of Hepatology, 11: 849-854.

Sayan, M., Senturk, O., Akhan, S.C., Hulagu, S., and Cekmen, M.B. (2010). Monitoring of hepatitis B virus surface antigen escape mutations and concomitantly nucleos(t)ide analogs resistance mutations in Turkish patients with chronic hepatitis B infection. International Journal of Infectious Disease, 14: 136-141.

Scaglione, S.J., and Lok, A.S. (2012). Effectiveness of hepatitis B treatment in clinical practice. Gastro-enterology, 142: 1360–1368.

Shi, W.F., Carr, M.J., Dunford, L.M., Zhu, C.D., Hall, W.W., and Higgins, D.G., (2012). Identification of novel inter-genotypic recombinants of human hepatitis B viruses by large-scale phylogenetic analysis. Virology, 427: 51–59.

Shi, W., Zhang, Z., Ling, C., Zheng, W., Zhu, C., Carr, M.J., and Higgins, D.G. (2013). Hepatitis B virus sub-genotyping: history, effects of recombination, misclassifications, and corrections. Infection Genetics and Evolutions, 16: 355–361.

Singla, B., Chakraborti, A., Sharma, B.K., Kapil, S., Chawla, Y.K., Arora, S.K., Das, A., Dhiman, R.K., and Duseja, A. (2013). Hepatitis B virus reverse transcriptase mutations in treatment Naïve chronic hepatitis B patients. Journal of Medical Virology, 85 (7): 1155–1162.

Tenney, D.J., Levine, S.M., Rose, R.E., Walsh, A.W., Weinheimer, S.P., Discotto, L., Plym, M., Pokornowski, K., Yu, C.F., Angus, P., Ayres, A., Bartholomeusz, A., Sievert, W., Thompson, G., Warner, N., Locarnini, R., and Colonno, R.J. (2004). Clinical Emergence of Entecavir-Resistant Hepatitis B Virus Requires Additional Substitutions in Virus Already Resistant to Lamivudine. Antimicrobial Agents and Chemotherapy, 48 (9): 3498–3507.

Tian, Y., Xu, Y., Zhang, Z., Meng, Z., Qin, L., Lu, M., and Yang, D. (2007). The amino acid residues at positions 120 to 123 are crucial for the antigenicity of hepatitis B surface antigen. Journal of Clinical Microbiology, 45: 2971–2978.

Torresi, J. (2002). The virological and clinical significance of mutations in the overlapping envelope and polymerase genes of hepatitis B virus. Journal of clinical virology, 25: 97–106.

Warner, N., Locarnini, S., Kuiper, M., Bartholomeusz, A., Ayres, A., Yuen, L. and Shaw, T. (2007). The L80I Substitution in the Reverse Transcriptase Domain of the Hepatitis B Virus Polymerase Is Associated with Lamivudine Resistance and Enhanced Viral Replication In Vitro. Antimicrobial Agents and Chemotherapy, 51 (7): 2285–2292.

Yapali, S., Talaat, N. and Lok, A.S. (2014). Management of hepatitis B: our practice and how it relates to the guidelines. Clinical Gastroenterology and Hepatology, 12: 16–26.

Zoulim, F. (2006). New nucleic acid diagnostic tests in viral hepatitis. Seminal Liver Disease, 26 (4): 309-317.