Journal of Infection (2011) 63, 66–75

BIAM British Infection Association

www.elsevierhealth.com/journals/jinf

Hepatitis B genotypes/subgenotypes and MHR variants among Moroccan chronic carriers

Bouchra Kitab [a], Abdellah Essaid El Feydi [b], Rajaa Afifi [b], Omar Derdabi [b], Younes Cherradi [b], Mustapha Benazzouz [b], Khadija Rebbani [a], Ikram Brahim [a], Hanane Salih Alj [c], Fabien Zoulim [d], Christian Trepo [d], Isabelle Chemin [d], Sayeh Ezzikouri [a], Soumaya Benjelloun [a,*]

[a] *Laboratoire des Hépatites Virales, Institut Pasteur du Maroc, Casablanca, Morocco*
[b] *Service Médecine C, CHU Ibn Sina, Rabat, Morocco*
[c] *Laboratoire de recherche sur les lipoprotéines et l'athérosclérose, Faculté des, Sciences Ben M'Sik, Université Hassan II, Morocco*
[d] *Unité 871, INSERM, Lyon, France*

Accepted 9 May 2011
Available online 17 May 2011

KEYWORDS
Hepatitis B virus;
Subgenotype D7;
ayw2;
MHR variant;
Morocco

Summary *Objectives:* The aim of this study was to determine the prevalence of Hepatitis B Virus (HBV) genotypes, subgenotypes, HBV surface antigen (HBsAg) subtypes and naturally occurring mutations in Major Hydrophilic region (MHR) of HBsAg among Moroccan patients with chronic HBV infection.
Methods: The study included 200 patients chronically infected with HBV. The HBV genotypes, subgenotypes, HBsAg subtypes and MHR variants were determined by direct sequencing of the HBV surface (S) gene and phylogenetic analysis.
Results: The S gene was successfully amplified in 134 patients. The mean age was 40.6 ± 12.2 years. Genotype D was predominant (90%, 120/134) and genotype A was less frequent (10%, 14/134). Genotype D strains belonged to subgenotypes D7 (70.8%, 85/120), D1 (25.8%, 31/120) and D2 (0.9%, 1/120). Three strains (2.5%) could not be classified in any subgenotype of genotype D. All genotype A strains belonged to subgenotype A2. HBsAg subtypes found were *ayw2* (82.1%, 110/134), *adw2* (10.4%, 14/134), *ayw3* (3%, 4/134) and *ayw4* (3%, 4/134). The global prevalence of MHR variants was 15% (20/134) with substitution P120T/S the most frequent (3.7%, 5/134). The occurrence of MHR variants was significantly associated with advancing age (>40 years) ($p = 0.003$) and independent of sex, HBeAg status, viral load, genotype, subgenotype and HBsAg subtype.

* Corresponding author. Laboratoire des Hépatites Virales, Institut Pasteur du Maroc 1, Place Louis Pasteur, 20360 Casablanca, Morocco. Tel.: +212 5 27016076/5 22 43 44 50; fax: +212 5 22 26 09 57.
E-mail address: soumaya.benjelloun@pasteur.ma (S. Benjelloun).

doi:10.1016/j.jinf.2011.05.007

Conclusions: This study provides the first description of predominance of HBV subgenotype D7/subtype *ayw2* among Moroccan HBV chronic carriers. It also showed a significant prevalence of naturally occurring MHR variants in Morocco.

Introduction

Hepatitis B virus (HBV) infection is a major public health problem. The World Health Organization (WHO) estimates that about 350 million persons are chronically infected with an increased risk of developing liver cirrhosis and hepatocellular carcinoma (HCC).[1] Morocco is considered as a country with intermediate endemicity of chronic HBV infection. To date, there are no published data describing the prevalence of HBV. However, some studies reported that the prevalence of hepatitis B surface antigen (HBsAg) was 2.5% among blood donors and 1% among health care workers.[2,3]

HBV, the prototype member of the Hepadnaviridae family, is a circular, partially double-stranded DNA virus with a genome of approximatively 3.2 kb in length, containing four overlapping open reading frames (ORFs: PreS/S, PreC/C, P and X).[4] HBV has considerable genetic variability related to high levels of virus production and absence of proofreading activity of the viral polymerase during the reverse transcription step of the replication cycle.[5] Initially, HBV isolates have been classified serologically into nine HBsAg subtypes (*ayw1*, *ayw2*, *ayw3*, *ayw4*, *ayr*, *adw2*, *adw4q−*, *adrq+*, and *adrq−*) based on the antigenic determinants of the HBsAg.[6] With the improvement of molecular biology techniques, HBV isolates has been classified into eight genotypes designated A to H, and defined by an intergroup divergence greater than 8% in the whole HBV genome or greater than 4% in the surface gene.[7] Recently, two new genotypes, I and J were proposed.[8,9] However, the designation of these additional genotypes has been questioned due to complex recombinations.[9,10] Except for genotypes E, G and H, subgenotypes have been identified in all other genotypes.[11,12] HBV genotypes and subgenotypes have a distinct geographical distribution and are associated with ethnicity. There is no clear correlation between HBsAg subtypes and HBV genotypes because several subtypes are found in more than one genotype.[13] Genotyping and HBsAg subtyping of HBV isolates are useful tools to understand the epidemiology of HBV infection and accumulating evidence suggests that HBV genotypes/subgenotypes influence the clinical outcome of HBV infection, including the risk of HCC.[14] Strong data indicated that genotype C was associated with faster liver damage and higher risk of HCC compared to genotype B.[15,16] Genotype D has been reported to be associated with severe liver disease compared to genotype A in some studies,[17,18] while the opposite was reported by others, with genotypes A and F more associated with HCC development than genotype D.[19] The influence of HBV genotypes on response to antiviral therapy has been also studied. Genotypes A and B have been associated with a better response to interferon-based therapies compared to genotypes C and D.[20]

A further correlate of the high genetic variability of HBV is the selection of point mutations in different viral genes under pressure of host immunity or antiviral treatment. HBsAg, the major envelope protein of HBV is composed of 226 amino acids (aa) and contains a central region called the Major Hydrophilic Region (MHR) from aa 100 to 169.[21] This region is highly immunogenic and is potentially under selective pressure of the immune system. Interestingly, the MHR contains a cluster of neutralizing B cell epitopes located between aa 124 and 147 and called "a" determinant.[21] Mutations within MHR have been involved in important medical and public health issues, such as vaccine escape, immunotherapy escape and lack of detection by commercials kits.[22] Such mutations might also arise naturally in chronic HBV carriers and were reported to be associated with viral persistence and severity of liver disease.[23,25]

In Morocco, little is known about the molecular characteristics of HBV strains. To date, two reports have been published and reported the predominance of genotype D/subgenotype D2.[26,27] In addition, in our previous study, phylogenetic analysis suggested that Moroccan HBV strains evolved on the Western margins of the genotype D distribution producing isolates subtly different from other D strains (D1−D4).[27] The aims of the present study were to determine (i) the distribution of HBV genotypes, subgenotypes and HBsAg subtypes in a significant number of Moroccan HBV chronic carriers and (ii) the prevalence and clinical significance of naturally occurring mutations in the MHR of HBsAg.

Materials and methods

Patients

The study population included 200 HBV chronic carriers (132 men and 68 women; age, 14−80 years) who were addressed to the Medical Center of Biology at the Pasteur Institute of Morocco and Ibn Sina hospital Rabat, during the period from November 2008 until May 2010 and defined by the persistence over $>$ 6 months of HBsAg. Patients originated from several regions of Morocco. All patients were negative for antibodies to hepatitis C virus (HCV), Hepatitis D virus (HDV) and human immunodeficiency virus (HIV). None of the patients were vaccinated for HBV or had received antiviral or immunoglobulin therapy. Blood and serum samples from all patients were collected at the time and stored at $-20\ ^{\circ}C$ until analysis. Informed consent was obtained from the patients.

Serological assays

All patients were tested for serological markers of HBV (HBsAg, anti-HBs, anti-HBc total, HBeAg, anti-HBe) and HCV (anti-HCV) using commercially, microparticle enzyme immunoassay kits (AxSYM, Abbott Laboratories), according to the manufacturers' instructions. Liver function tests including, alanine aminotransferase (ALT) and aspartate

aminotransferase (AST) were measured using commercially available autoanalyzers. The upper limit of normal (ULN) of ALT and AST was 40 IU/L and 31 IU/L, respectively.

HBV DNA quantitative assay

Serum HBV DNA was quantified by a fully automatic system (COBAS AmpliPrep/COBAS TaqMan, Roche Diagnostics) for HBV DNA extraction and real-time PCR quantification. The detection limit was 12 UI/mL.

Extraction of HBV DNA, amplification and direct sequencing of surface gene

HBV DNA was extracted from 200 μl of blood or serum sample by using QIAamp DNA Blood Mini kit (Qiagen), according to the manufacturer's instructions and eluted in 200 μl of sterile water. The HBV surface (S) gene was amplified by a nested PCR, using the outer primers HBV POL1 (nt 54 to 75; 5′-TTCCTGCTGGTGGCTCCAGTTC-3′) and POR4 (nt 826 to 810; 5′-TACCCAAAGACAAAAGAAAATTTC-3′)[28] for the first round and inner primers HBV123s (nt 123 to 141; 5′-TCGAGGATTGGGGACCCTG-3′) and HBV778r (nt 778 to 758; 5′-GAGGTATAAAGGGACTCAAG-3′) for the second round. The first-round PCR was performed for 40 cycles (95 °C for 20 s, 56 °C for 20 s and 72 °C for 2 min) and a final extension step at 72 °C for 4 min in a 25 μl reaction volume containing 50 ng of extracted DNA, 1X PCR buffer, 200 μM of each dNTPs, 1.5 mM $MgCl_2$, 25 pmol/μl of each outer primer and 1U Taq DNA polymerase (Invitrogen, France). One microlitre of the first-round PCR product was then subjected for the second-round PCR under the same conditions but with the inner primers. Appropriate negative and positive controls were included in each assay. After the second round, a 650 bp fragment was obtained and detected by electrophoresis in a 2% agarose gel. Positive PCR products were purified using the Exonuclease I/Shrimp Alkaline Phosphatase (GE Healthcare) and bidirectionally sequenced with inner primers, using BigDye Terminator version 3.1 kits and an ABI PRISM 3130 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence and phylogenetic analysis

Sequence analysis was performed with SeqScape® v2.5 software (Applied Biosystems). The HBV genotypes and subgenotypes were determined on the basis of phylogenetic relationship taking the first 600 nucleotides of the S gene with representative reference sequences from of all known HBV genotypes and different subgenotypes of genotype A (A1—A6) and genotype D (D1—D8). The sequences were aligned using the Clustal X V2.0 software.[29] Genetic distance was estimated using the Kimura two-parameter model and phylogenetic tree was constructed using the Neighbour-Joining (NJ) algorithm in the MEGA4 software.[30] The reliability of phylogenetic tree was tested by bootstrap analysis with 1000 replicates. The genome of the Woolly monkey HBV (GenBank accession number AF046996) was utilized as an out-group.

The S gene sequences generated in this work have been deposited in the GenBank database under accession numbers JF271686 to JF271746.

Prediction of HBsAg subtypes and analysis of S gene

The nucleotide sequences were translated into amino acid sequences according to the ORF of the S gene and the HBsAg subtype was predicted from the amino acids at positions 122, 127, 134, 159, and 160.[7,13,22] For identifying HBsAg variants, deduced amino acid sequences were aligned and compared with the consensus amino acid sequence of similar genotype in BioEdit software.[31] The corresponding substitutions in the polymerase were determined by translation of the sequences according to the polymerase ORF. To determine the consensus sequences, 180 HBV references sequences obtained from HBsAg carriers and belonging to different subgenotypes of genotypes A (A1 to A6) and D (D1 to D7) were imported from GenBank database, aligned with Clustal X V2.0 software and edited with BioEdit software. GenBank accession numbers of the HBV reference sequences used in the analysis are reported in the supplementary material.

Statistical analysis

Results were expressed as means ± standard deviation or percentage. One-way analysis of variance was conducted to compare mean quantitative values and the $\chi 2$ or Fischer's exact test for categorical variables. All *p*-values were two sided, and the difference was considered statistically significant for $p < 0.05$. All the statistical analyses were performed using Statistical Package for Social Sciences (SPSS) program (SPSS Inc., Chicago, IL, USA).

Results

Characteristics of patients

Two hundred patients with chronic HBV infection (HBsAg positive and anti-HBc IgG-positive) were studied. 66% were men and 34% were women. The mean age was 43 ± 12 years. HBV DNA of the S gene was successfully amplified from samples of 134 patients out of 200 patients analyzed (67%). The mean age of the 134 patients was 40.6 years (range, 14—80 years). 66.4% were men and 33.6% were women. The clinical characteristics of these patients are presented in Table 1.

Identification of HBV genotypes and subgenotypes

Phylogenetic analysis revealed that two HBV genotypes were circulating in Moroccan population with high bootstrap values: genotype D was predominant, accounting for 90% (120/134) and genotype A for 10% (14/134). All genotype A strains belonged to subgenotype A2. Among the genotype D strains, the majority (85/120, 70.8%) belonged to subgenotype D7, 25.8% (31/120) to subgenotype D1, and one isolate (1/120, 0.9%) to subgenotype D2. Three strains clustered as sister group to the D4/D7 clade and could not be assigned to any described subgenotype (2.5%) (Fig. 1).

Table 1 Baseline characteristics of patients.

Data	Patients (n = 134)
Age (years)[a]	40.6 ± 12.2
Sex ratio (Male/ Female)	89/45
HBeAg positive[b]	8 (6%)
anti-HBe positive[b]	126 (94%)
HBV DNA (IU/mL)[c]	5460 (108-10^8 UI/mL)
ALT (IU/L)[d,a]	59.12 ± 7.18
AST (IU/L)[e,a]	48.06 ± 6.88
Clinical status	
IC[b]	80 (59.7%)
CHB[b]	34 (25.4%)
LC[b]	12 (9%)
HCC[b]	8 (6%)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; IC, inactive HBsAg carrier; CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma.
[a] Mean ± SD.
[b] n (%).
[c] Median (range).
[d] Normal values ≤ 40 IU/L.
[e] Normal values ≤ 31 IU/L.

Prediction of HBsAg subtypes and association with HBV genotypes

The majority of Moroccan HBV strains belonged to HBsAg subtype *ayw2* (110/134, 82.1%) based on Arg122, Lys160, Pro127, Tyr134 and/or Gly159. 14 strains (10.4%) specified *adw2*, based on Lys 122, Lys160, Pro127, Phe134 and Ala159. Four strains belonged to *ayw3* (3%), based on Arg122, Lys160 and Thr127 and four strains belonged to *ayw4* (3%), based on Arg122, Lys160 and Ile127. In two *ayw* isolates (1.5%), the "*w*" sub-determinant could not be defined because of atypical substitution at Ser127. All strains coding for *ayw2* belonged to subgenotypes D1, D7 and the three genotype D strains of unknown subgenotype, *ayw3* was found in three strains of subgenotype D7 and the single strain of subgenotype D2, whereas all strains coding for *ayw4* belonged to subgenotype D7. All strains coding for *adw2* belonged to subgenotype A2.

Naturally occurring mutations in the major hydrophilic region of HBsAg

The comparison with consensus HBV sequence for the same genotype showed that twenty of 134 patients (15%) had amino acid substitutions in the MHR and eleven (8.2%) had amino acid substitution in the "a" determinant. Of these 20 patients, 13 had a single mutation (65%), whereas, seven (35%) had a combination of 2—8 mutations, making a total of 41 mutations (Fig. 2). 21 of the 41 mutations (51%) were located within the "a" determinant, 66.7% (14/21) in the first loop (positions 124—137) and 33.3% (7/21) in the second loop (positions 139—147). The most frequent variation was at position 120 with a rate of 3.7% (5/134), including P120S detected in one patient with HCC and P120T detected in four patients with chronic hepatitis B (Table 2).

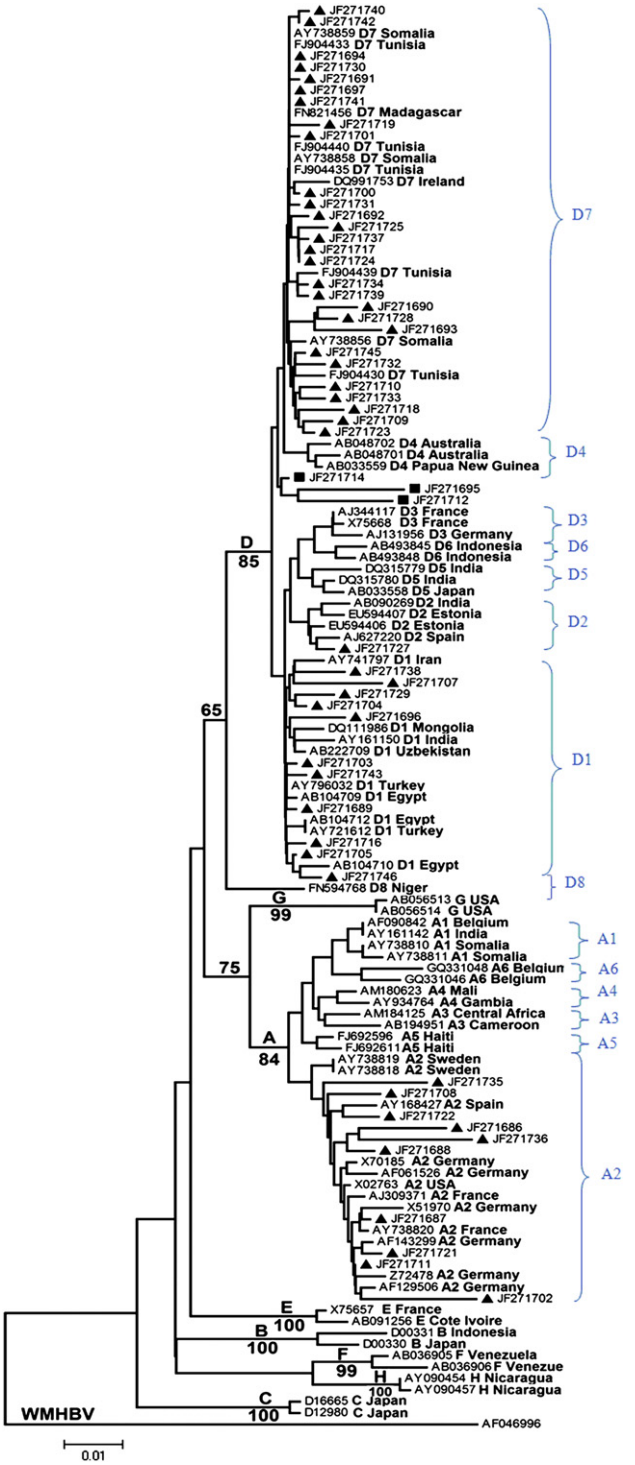

Figure 1 A phylogenetic tree constructed using the neighbour-joining (NJ) method, based on the first 600 nucleotides of S gene of HBV Moroccan isolates and the corresponding region of reference sequences retrieved from the GenBank database. Sequences from Morocco are marked by (▲). Moroccan sequences with unknown subgenotype are marked by (■). References sequences are identified by accession number and country of origin.

All MHR variants identified in our study have been previously described (Table 2). MHR variants with known functional effects are shown in Table 3. The variants

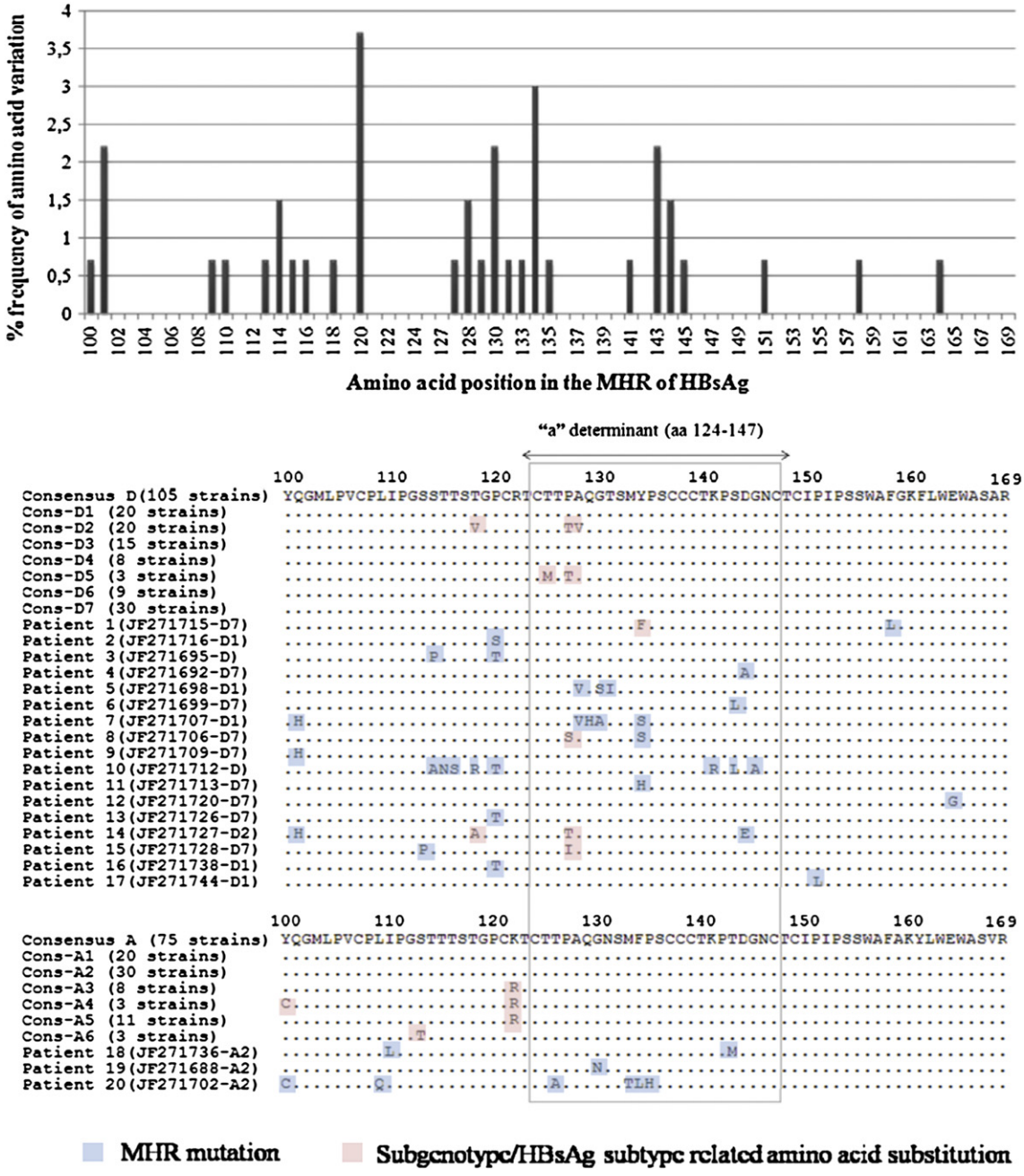

Figure 2 Frequency and distribution of amino acid substitutions in the MHR of HBsAg in 20 HBV carriers. A consensus amino acid sequence was built for genotype A and D by using 180 references sequences imported from GenBank database. The number of reference sequences for each HBV genotype/subgenotype was as follows: genotype A ($n = 75$) [A1 ($n = 20$), A2 ($n = 30$), A3 ($n = 8$), A4 ($n = 3$), A5 ($n = 11$), A6 ($n = 3$)]; genotype D ($n = 105$) [D1 ($n = 20$), D2 ($n = 20$), D3 ($n = 15$), D4 ($n = 8$), D5 ($n = 3$), D6 ($n = 9$), D7 ($n = 30$)]. Subgenotype/HBsAg subtype-related amino acid substitution: the amino acid substitution was associated with the subgenotype or HBsAg subtype and not regarded as MHR mutation.

involved in failure of HBsAg detection were found in samples of 13 patients (9.7%, 13/134). The variants involved in immunotherapy escape were detected in samples of eight patients (6%, 8/134) whereas variants associated with vaccine escape were detected in samples of five patients (3.7%, 5/134). The amino acid substitution G145A, which is associated with vaccine escape, was identified in one isolate (0.7%, 1/134).

The comparison of clinical and virological characteristics between patients with and without MHR variants revealed that advancing age (>40 years) was significantly associated with occurrence of MHR variants ($p = 0.003$), whereas, there was no significant association between mutations in the MHR and other clinical factors, including sex ($p = 0.33$), HBeAg status ($p = 0.220$), genotype ($p = 0.708$), subgenotype ($p = 0.448$), HBsAg subtype

Table 2 Clinical and virological characteristics of the 20 patients with amino acid substitutions in the MHR of HBsAg.

Patient N°	Sex	Age (year)	ALT	HBeAg/Anti-HBe	HBV DNA (UI/mL)	Genotype/subtype	Clinical Status	MHR mutation	Polymerase mutation
1	M	61	ND	−/+	48,571	D7/*ayw2*	HCC	F158L	−
2	F	60	1.5XULN	−/+	4,75,000	D1/*ayw2*	HCC	P120S	rtT128I
3	F	65	5XULN	−/+	8700	D/*ayw2*	CHB	S114P, P120T	rtF122S, rtT128N
4	F	33	1XULN	−/+	10^7	D7/*ayw2*	LC	D144A	−
5	M	23	1XULN	−/+	9591	D1/*ayw2*	CHB	A128V, G130S, T131I	rtR138K, rtN139H
6	M	61	1XULN	−/+	786	D7/*ayw2*	IC	S143L	−
7	M	48	1XULN	−/+	1680	D1/*ayw2*	IC	Q101H, A128V,Q129H, G130A, Y134S	rtR138S, rtV142E
8	M	51	1.5XULN	−/+	73,000	D7/*ayw2*	CHB	Y134S	−
9	F	50	1XULN	−/+	6270	D7*ayw2*	CHB	Q101H	−
10	M	45	1XULN	−/+	526	D/*ayw2*	IC	S114A, T115N, T116S, T118R, P120T, K141R S143L, G145A	rtF122C, rtN123K, rtH124L, rtH126Q, rtT128N
11	M	55	1XULN	−/+	145	D7/*ayw2*	IC	Y134H	rtV142A
12	M	51	1.5XULN	−/+	1,28,4737	D7/*ayw2*	CHB	E164G	−
13	F	49	3XN	−/+	10^8	D7/*ayw2*	CHB	P120T	rtT128N
14	M	27	1XN	−/+	1030	D2/*ayw3*	IC	Q101H, D144E	−
15	F	39	1XULN	−/+	529	D7/*ayw4*	IC	S113P	rtI121T
16	M	38	ND	−/+	ND	D1/*ayw2*	ND	P120T	rtT128N
17	F	52	1XULN	−/+	207	D1/*ayw2*	IC	P151L	−
18	F	25	2XN	−/+	2,34,977	A2/*adw2*	CHB	I110L, T143M	rtN118T
19	M	37	3XULN	−/+	10^7	A2/*adw2*	CHB	G130N	rtR138K
20	F	38	1XULN	−/+	993	A2/*adw2*	IC	Y100C, L109Q, T126A, M133T, F134L, P135H	rtD134G, rtV142T

IC, inactive HBsAg carrier; CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; ND, Not done; ULN, upper limit of normal; rt, reverse transcriptase.

Table 3 Amino acid substitutions in the MHR of HBsAg with known functional effects according to Avellon and Echevarria.[23]

Substitution in MHR	Isolate	Description		
		Vaccine escape	Immunotherapy escape	Failure of HBsAg detection
T116S	JF271712-D	−	−	+
T118R	JF271712-D	−	+	−
P120S	JF271716-D1	+	−	+
P120T	JF271712-D	−	+	+
	JF271726-D7			
	JF271738-D1			
	JF271695-D			
T126A	JF271702-A2	+	+	−
Q129H	JF271707-D1	+	+	−
T131I	JF271698-D1	−	−	+
M133T	JF271702-A2	−	−	+
F134L	JF271702-A2	−	−	+
S143L	JF271712-D	−	−	+
	JF271699-D7			
D144A	JF271692-D7	+	+	+
D144E	JF271727-D2	−	−	+
G145A	JF271712-D	+	−	−

($p = 0.418$) and HBV DNA levels ($p = 1.00$) (Data not shown).

Analysis of overlapping polymerase region

As the S gene overlaps completely with the polymerase (P) gene, the deduced amino acid sequence of the corresponding fragment of the polymerase was analyzed. This fragment represents an important part of the reverse transcriptase (rt) including the catalytic domains B and C which contains the motif YMDD, essential for reverse transcriptase activity. Of the 41 amino acid substitutions in the MHR, only twenty produced amino acid changes in the polymerase (Table 2). All of these changes clustered outside the B and C domains. rtT128N, corresponding to substitution sP120T in the surface gene, was the most frequent. No change was observed in the YMDD motif of the reverse transcriptase and no mutation that may provide antiviral resistance was detected.

Discussion

HBV has a wide genetic diversity with several genotypes, subgenotypes and HBsAg subtypes. Throughout the world, HBV genotypes/subgenotypes have a distinct geographical distribution associated with the distribution of human populations among the different continents and may reflect the patterns of human migrations.[7,32] Therefore, it has been successfully used to correlate the human migrations with epidemiology and introduction of new HBV strains in a given region. Morocco occupies a geographical area where there are significant population movements, especially with several European countries. Therefore, the determination of HBV genetic diversity among Moroccan chronic carriers is of interest. In the present study, phylogenetic analysis showed presence of HBV genotypes A and D in Moroccan population, with genotype D being predominant (D: 90% vs A: 10%). These results are in accordance with previous studies that show that genotype D is the most predominant in Morocco[27] and in Mediterranean countries.[7] Most of genotype D strains belonged to subgenotype D7 (70.8%), followed by subgenotype D1 (25.8%), and subgenotype D2 (0.9%). This is the first study describing the presence and the predominance of subgenotype D7 and subgenotype D1 in Moroccan HBV carriers. Subgenotype D7 was recently described and found prevalent in Tunisia by Meldal et al[33] that suggest that this subgenotype was the most predominant in the Maghreb. This hypothesis is reinforced by the results of our study. Subgenotype D7 has also been reported in Somalia,[7] Madagascar,[34] Central African Republic[35] and Ireland.[36] The subgenotype D1 is second most dominant in our population. This was in accordance with others studies reporting subgenotype D1 prevalent in Mediterranean area.[7,33] Subgenotype D2 is rare and found only in one strain. This result was in contrast with those previously obtained by Ezzikouri et al.,[27] which reported the predominance of subgenotype D2 in Moroccan population. In that study, the determination of HBV subgenotype was performed by Restriction Fragment Length Polymorphism (RFLP) analysis after the amplification of the Pre-S region of HBV genome, while in our study, the analysis is more precise because it is based on direct sequencing of the surface gene of HBV and phylogenetic analysis. On the other hand, subgenotype D2 is most prevalent in East Europe including Russia and the Baltic States.[37]

All genotype A strains from Morocco belonged to subgenotype A2. This subgenotype is prevalent in Europe and North America[7] and its presence in Moroccan population suggest the important role of movement of population from Europe to Morocco in introducing this HBV strain in Morocco. Interestingly, our study was the first to describe the HBsAg subtypes prevalent in Morocco. Subtype *ayw2* was the most predominant (82.1%) and found in all strains belonged to subgenotype D1 and most of strains belonged to

subgenotype D7. This result is in agreement with others studies which reported the predominance of subtype *ayw2* in Mediterranean countries.[7,24] Subtypes *ayw3* and *ayw4* were found at low prevalence (3% in both). In general, strains specifying *ayw2* and *ayw3* are only found in genotype D, while strains specifying *ayw4* occur in genotype E and rarely in genotype D.[7] All strains of subgenotype A2 specifying *adw2*. This result is consistent with previous data reporting the relationship between subtype *adw2* and genotype A, although strains encoding *adw2* can be found in genotypes B, C and G.[7]

Understanding the prevalence and types of HBsAg variants is of high importance, because this will affect policy decisions relating to vaccine and diagnostic reagents design especially in endemic populations.[38] Avellon and Echevarria indicated that the prevalence of such variants among random chronic carriers could be as high as 6–12%.[22] In addition, De Maddalena et al[39] found that genotype D strains carry more mutations in the "a" determinant of HBsAg with potential escape mutants in non-vaccinated subjects. Therefore, they indicate the need for careful surveillance of these variants in areas in which genotype D predominates. Morocco has an intermediate prevalence of HBsAg carriage with predominance of genotype D. However, the epidemiological situation of HBsAg variants is unknown. In the present study, we focused on variations in the Major Hydrophilic Region (MHR), the highly antigenic segment of HBsAg. The results show a significant prevalence of naturally MHR variants (15%) among the studied population. Comparable prevalences was also reported in others areas of the world, 14.8% in Argentina[40] and 17.2% in Iran.[41] However, direct sequencing of the PCR products cannot reveal a variant present in the sample as a minor population, and the true prevalence of MHR variants may be higher.

Moreover, it is essential to note that 8.2% of the patients analyzed had mutations in the "a" determinant which spans amino acids 124–147 of the MHR. The majority of the "a" determinant mutations (66.7%) was located in the first loop, whereas, only 33.3% were located in the second loop. According to Ogura et al,[42] mutations within the "a" determinant during the natural course of infection are predominantly observed in the first loop whereas those induced under immune pressure due to active and/or passive immunization are more frequently observed within the second loop. P120T/S was the most frequent substitution with a rate of 3.7%. It was also seen most frequently among Iranian and Serbian HBV chronic carriers.[43,44]

A large number of MHR mutants have been reported to be in association with failure of HBsAg detection, vaccine and immunotherapy escape. In our population, variants known to be involved in diagnostic failures were found in 9.7% of carriers although none of the samples had failed to be detected by the routine commercial kit utilized. In addition, variants associated with immunotherapy and vaccine escape were found in 6% and 3.7% of carriers, respectively. Interestingly, the most common substitution, P120T/S, has been described to be the most important mutant outside the "a" determinant that may cause diagnostic failures and also vaccine and immunotherapy escape. The natural occurrence of such variants in not previously vaccinated and treated patients is possible as reported by several studies.[23,40] However, the great danger of vaccine escape mutants is their emergence in the general population including HBV vaccinated individuals. The most frequent and well-documented vaccine escape mutation is a substitution of glycine to arginine at amino acid position 145 of HBsAg (G145R). HBV isolates with G145R are known to be transmitted despite vaccination against HBV.[45,46] In our study, one patient displayed substitution G145A, which has also reported as a vaccine-escape mutation. The overall prevalence of this mutation is 0.7%. This frequency was less than the frequencies reported in Spain and Serbia, 1.5% and 1.8%, respectively.[23,44] However, the presence of immune-escape mutants in our population should be considered in the immunization program.

The role of MHR mutations in the outcome and persistence of HBV infection remain unclear. Two studies has been reported a tendency for a higher prevalence of MHR variants in patients at advancing stage of liver disease.[25,47] In our study, the comparison of clinical characteristics between patients with and without MHR variants indicates a significant association between the occurrence of MHR variants and advancing age of the patients (>40 years). This result was consistent with that obtained by others studies[44,47] and could be explained by the fact that older age is more associated with longer history of HBV infection, correlated also with the predominance of HBeAg-negative form of chronic hepatitis B in our population.

Because the surface gene overlaps completely with that of the viral polymerase, mutations within the former could affect the latter. Therefore, we analyzed the effect of MHR variants detected in our samples on the corresponding fragment of HBV polymerase. We observed that the polymerase is more conserved and not all variants MHR were accompanied by amino acid substitutions in the polymerase. As reported recently, HBV polymerase and surface proteins may evolve independently despite the overlapping of the S and P genes. For allowing this independent evolution, HBV employ a mechanism by which an adaptive non-synonymous nucleotide substitution in the first position of a P codon is likely to remain synonymous in S, whereas an adaptive non-synonymous nucleotide substitution in the second position of an S codon is likely to remain synonymous in P.[48] The most frequent mutation was rtT128N, induced by substitution P120T in the S gene. This mutation was found to partially restore the in vitro replicative capacity of Lamivudine-resistant HBV.[49]

In conclusion, our results revealed the predominance of HBV genotype D, subgenotype D7 and *ayw2* HBsAg subtype among Moroccan chronic HBV carriers. In addition, a significant prevalence of naturally occurring MHR variants was detected. Considering that chronic carriers are the major reservoir of HBV infection, the selection such variants naturally in these patients could increase the problem of transmission of these variants in the general population. Therefore, epidemiological monitoring of naturally occurring MHR variants is essential, especially for immunotherapy and vaccination efficacy. Their role in the natural course of HBV infection should also be clarified by further functional studies.

Acknowledgements

This study was financially supported by a research grant from Novartis Pharma Morocco.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2011.05.007.

References

1. World Health Organization. *Hepatitis B 2008*. Accessed 10.04.10, at, http://www.who.int/mediacentre/factsheets/fs204/en; 2008.
2. Mrani S, Sakhsokh Y, Gille Y, Baaj A. Épidémiologie de l'infection par les hépatites virales B et C. *Cah Med* 2002;**4**:87—8.
3. Djeriri K, Laurichesse H, Merle JL, Charof R, Abouyoub A, Fontana L, et al. Hepatitis B in Moroccan health care workers. *Occup Med (Lond)* 2008;**58**:419—24.
4. Ganem D, Prince AM. Hepatitis B virus infection—natural history and clinical consequences. *N Engl J Med* 2004;**350**:1118—29.
5. Seeger C, Mason WS. Hepatitis B virus biology. *Microbiol Mol Biol Rev* 2000;**64**:51—68.
6. Magnius LO, Norder H. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology* 1995;**38**:24—34.
7. Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* 2004;**47**:289—309.
8. Tran TT, Trinh TN, Abe K. New complex recombinant genotype of hepatitis B virus identified in Vietnam. *J Virol* 2008;**82**:5657—63.
9. Tatematsu K, Tanaka Y, Kurbanov F, Sugauchi F, Mano S, Maeshiro T, et al. 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* 2009;**83**:10538—47.
10. Schaefer S, Magnius L, Norder H. Under construction: classification of hepatitis B virus genotypes and subgenotypes. *Intervirology* 2009;**52**:323—5.
11. Kramvis A, Kew M, Francois G. Hepatitis B virus genotypes. *Vaccine* 2005;**23**:2409—23. doi:10.1016/j.vaccine.2004.10.045.
12. Cao GW. Clinical relevance and public health significance of hepatitis B virus genomic variations. *World J Gastroenterol* 2009;**15**:5761—9.
13. Norder H, Courouce AM, Magnius LO. Molecular basis of hepatitis B virus serotype variations within the four major subtypes. *J Gen Virol* 1992;**73**:3141—5.
14. McMahon BJ. The influence of hepatitis B virus genotype and subgenotype on the natural history of chronic hepatitis B. *Hepatol Int* 2009;**3**:334—42.
15. Yu MW, Yeh SH, Chen PJ, Liaw YF, Lin CL, Liu CJ, et al. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: a prospective study in men. *J Natl Cancer Inst* 2005;**97**:265—72.
16. Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 2000;**118**:554—9.
17. Toan NL, Song le H, Kremsner PG, Duy DN, Binh VQ, Koeberlein B, et al. Impact of the hepatitis B virus genotype and genotype mixtures on the course of liver disease in Vietnam. *Hepatology* 2006;**43**:1375—84.
18. Thakur V, Guptan RC, Kazim SN, Malhotra V, Sarin SK. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. *J Gastroenterol Hepatol* 2002;**17**:165—70.
19. Livingston SE, Simonetti JP, McMahon BJ, Bulkow LR, Hurlburt KJ, Homan CE, et al. Hepatitis B virus genotypes in Alaska native people with hepatocellular carcinoma: preponderance of genotype F. *J Infect Dis* 2007;**195**:5—11.
20. Raimondi S, Maisonneuve P, Bruno S, Mondelli MU. Is response to antiviral treatment influenced by hepatitis B virus genotype? *J Hepatol* 2010;**52**:441—9.
21. Carman WF. The clinical significance of surface antigen variants of hepatitis B virus. *J Viral Hepat* 1997;**4**:11—20.
22. Echevarria JM, Avellon A. Hepatitis B virus genetic diversity. *J Med Virol* 2006;**78**:S36—42.
23. Avellon A, Echevarria JM. Frequency of hepatitis B virus 'a' determinant variants in unselected Spanish chronic carriers. *J Med Virol* 2006;**78**:24—36.
24. Sayiner AA, Ozcan A, Sengonul A. Naturally occurring MHR variants in Turkish patients infected with hepatitis B virus. *J Med Virol* 2008;**80**:405—10.
25. Song BC, Kim SH, Kim H, Ying YH, Kim HJ, Kim YJ, et al. Prevalence of naturally occurring surface antigen variants of hepatitis B virus in Korean patients infected chronically. *J Med Virol* 2005;**76**:194—202.
26. Sbai A, Bennani A, Benjouad A, Hassar M. HBV genotypes in Morocco. *J Clin Virol* 2007;**38**:184—5. doi:10.1016/j.jcv.2006.09.011.
27. Ezzikouri S, Chemin I, Chafik A, Wakrim L, Nourlil J, Malki AE, et al. Genotype determination in Moroccan hepatitis B chronic carriers. *Infect Genet Evol* 2008;**8**:306—12. doi:10.1016/j.meegid.2008.01.010.
28. Chemin I, Zoulim F, Merle P, Arkhis A, Chevallier M, Kay A, et al. High incidence of hepatitis B infections among chronic hepatitis cases of unknown aetiology. *J Hepatol* 2001;**34**:447—54.
29. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997;**25**:4876—82.
30. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular Evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007;**24**:1596—9.
31. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp* 1999;**41**:95—8.
32. Robertson BH, Margolis HS. Primate hepatitis B viruses-genetic diversity, geography and evolution. *Rev Med Virol* 2002;**12**:133—41.
33. Meldal BH, Moula NM, Barnes IH, Boukef K, Allain JP. A novel hepatitis B virus subgenotype, D7, in Tunisian blood donors. *J Gen Virol* 2009;**90**:1622—8.
34. Dupinay T, Restorp K, Leutscher P, Rousset D, Chemin I, Migliani R, et al. High prevalence of hepatitis B virus genotype E in Northern Madagascar indicates a West-African lineage. *J Med Virol* 2010;**82**:1515—26.
35. Bekondi C, Olinger CM, Boua N, Talarmin A, Venard V, Muller CP, et al. Characterization of hepatitis B virus strains from the Central African Republic: preliminary results. *Pathol Biol (Paris)* 2008;**56**:310—3. doi:10.1016/j.patbio.2007.12.007.
36. Laoi BN, Crowley B. Molecular characterization of hepatitis B virus (HBV) isolates, including identification of a novel recombinant, in patients with acute HBV infection attending an Irish hospital. *J Med Virol* 2008;**80**:1554—64.
37. Tallo T, Tefanova V, Priimagi L, Schmidt J, Katargina O, Michailov M, et al. D2: major subgenotype of hepatitis B virus in Russia and the Baltic region. *J Gen Virol* 2008;**89**:1829—39.
38. Gerlich WH. Diagnostic problems caused by HBsAg mutants-a consensus report of an expert meeting. *Intervirology* 2004;**47**:310—3.
39. De Maddalena C, Giambelli C, Tanzi E, Colzani D, Schiavini M, Milazzo L, et al. High level of genetic heterogeneity in S and P genes of genotype D hepatitis B virus. *Virology* 2007;**365**:113—24.

40. Pineiro YLFG, Pezzano SC, Torres C, Rodriguez CE, Eugenia Garay M, Fainboim HA, et al. Hepatitis B virus genetic diversity in Argentina: dissimilar genotype distribution in two different geographical regions; description of hepatitis B surface antigen variants. *J Clin Virol* 2008;**42**:381—8.
41. Mohebbi SR, Amini-Bavil-Olyaee S, Zali N, Noorinayer B, Derakhshan F, Chiani M, et al. Molecular epidemiology of hepatitis B virus in Iran. *Clin Microbiol Infect* 2008;**14**:858—66.
42. Ogura Y, Kurosaki M, Asahina Y, Enomoto N, Marumo F, Sato C. Prevalence and significance of naturally occurring mutations in the surface and polymerase genes of hepatitis B virus. *J Infect Dis* 1999;**180**:1444—51.
43. Bahramali G, Sadeghizadeh M, Amini-Bavil-Olyaee S, Alavian S, Behzad-Behbahani A, Adeli A, et al. Clinical, virologic and phylogenetic features of hepatitis B infection in Iranian patients. *World J Gastroenterol* 2008;**14**:5448—53.
44. Lazarevic I, Cupic M, Delic D, Svirtlih NS, Simonovic J, Jovanovic T. Prevalence of hepatitis B virus MHR mutations and their correlation with genotypes and antiviral therapy in chronically infected patients in Serbia. *J Med Virol* 2010;**82**:1160—7.
45. Stramer SL, Wend U, Candotti D, Foster GA, Hollinger FB, Dodd RY, et al. Nucleic acid testing to detect HBV infection in blood donors. *N Engl J Med* 2011;**364**:236—47.
46. Oon CJ, Lim GK, Ye Z, Goh KT, Tan KL, Yo SL, et al. Molecular epidemiology of hepatitis B virus vaccine variants in Singapore. *Vaccine* 1995;**13**:699—702. doi:10.1016/0264-410X (94)00080-7.
47. Roque-Afonso AM, Ferey MP, Ly TD, Graube A, Costa-Faria L, Samuel D, et al. Viral and clinical factors associated with surface gene variants among hepatitis B virus carriers. *Antivir Ther* 2007;**12**:1255—63.
48. Zaaijer HL, van Hemert FJ, Koppelman MH, Lukashov VV. Independent evolution of overlapping polymerase and surface protein genes of hepatitis B virus. *J Gen Virol* 2007;**88**:2137—43.
49. Torresi J. The virological and clinical significance of mutations in the overlapping envelope and polymerase genes of hepatitis B virus. *J Clin Virol* 2002;**25**:97—106.