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Asimina Fylaktou, Dimitrios C Papaventsis, Maria Daoudaki, Mathaios Moskophidis, Thomas Reiberger, Nikolaos Malisiovas

Journal of Medical Virology

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Journal:	*Journal of Medical Virology*
Manuscript ID:	JMV-10-1854.R2
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	08-Sep-2010
Complete List of Authors:	FYLAKTOU, ASIMINA; Aristotle University of Thessaloniki, First Department of Microbiology PAPAVENTSIS, DIMITRIOS; General Hospital Papageorgiou, Virology Laboratory DAOUDAKI, MARIA; Aristotle University of Thessaloniki, Department of Biological Chemistry MOSKOPHIDIS, MATHAIOS; General Hospital Papageorgiou, Virology Laboratory REIBERGER, THOMAS; Medical University of Vienna, Dept. of Internal Medicine III, Div. of Gastroenterology & Hepatology MALISIOVAS, NIKOLAOS; Aristotle University of Thessaloniki, First Department of Microbiology
Keywords:	Hepatitis B virus, Epidemiology, Greece, Genotypes, Mutations

Molecular epidemiology of chronic Hepatitis B virus infection in Greece.

Fylaktou Asimina[1,2], Papaventsis Dimitrios[1], Daoudaki Maria[2*], Moskophidis Mathaios[1], Thomas Reiberger[3], Malisiovas Nicolaos[4]

[1]Virology Laboratory, Papageorgiou Regional General Hospital,

[2]Department of Biological Chemistry, [4]First Department of Microbiology, School of Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece

[3]Department of Internal Medicine III, Division of Gastroenterology & Hepatology, Medical University of Vienna, Vienna, Austria

Running title:

HBV genotypes in Greece

Abstract word count: 246 words

Full text word count: 2418 words

* Correspondence:

Maria Daoudaki PhD, Department of Biological Chemistry, School of Medicine, Aristotle University of Thessaloniki, University Campus Thessaloniki, 54124 Greece. Phone: +302310999167 Fax: +302310999004

e-mail: daoudaki@med.auth.gr

ABSTRACT

Virological data on chronic hepatitis B virus (HBV) infection in Greece are limited. HBV genotypes, surface antigen (HBsAg) subtypes, and HBsAg "a" determinant mutations among patients infected chronically with HBV, were investigated. Serum samples from 135 HBsAg positive patients were tested. Serologic (HBsAg, anti-HBs, HBeAg, anti-HBe), virologic (HBV DNA quantitation) and biochemical markers (serum alanine aminotrasferase/ALT, aspartate aminotrasferase/AST) were analyzed. HBV genotypes and HBsAg subtypes were determined by partial sequencing of the S gene. Genotyping was performed by using the NCBI online Genotyping tool and phylogenetic analysis. Nucleotide sequences were aligned pair wise with ClustalW and phylogenetic trees were constructed by the neighbour-joining method. Sequences were also used to predict HBV HBsAg subtypes. In six patients (4%), simultaneous presence of HBsAg and anti-HBs was determined, whereas 47 patients (35%) were HBeAg positive, 84 (62.5%) were anti-HBe positive, and 4 patients (3%) were characterized by the simultaneous presence of HBeAg and anti-HBe. Mean ALT was 238 IU/L (SD=576.84), and HBV-DNA levels ranged from $1.02x10^5$ to $2.2\ x10^7$ IU/ml. Genotype D was predominant (98%), with viral groups D/ayw2 (73%) and D/ayw3 (27%). Group A/adw accounted for the 1% of cases. Genotypes B and C were found exclusively in the Chinese immigrants (1%). Single or multiple point mutations were found in 35 cases (26%). Some of the most common mutations occurred at amino acid positions 129, 133, 134, 144, 145, including the 'vaccine escape' mutation G145R. Mutations analysis revealed that amino acid substitutions did not affect detection by commercial immunoassays.

Keywords: Genotypes; Mutations; An antigenic determinant

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is an important public health problem, despite the availability of a vaccine. Worldwide, according to the World Health Organization (WHO), 360 million people suffer from chronic HBV infection [Shepard et al., 2006], with an estimation of 600,000 deaths yearly due to the acute or chronic consequences of HBV infection [Lok, 2002]. In Greece, HBV infection is considered a low to moderate endemic disease and HBsAg carrier prevalence ranges from 0.29% to 2.6% [Raptopoulou et al., 2008].

HBV is a partially double-stranded DNA virus of the family *Hepadnaviridae* with a genome of 3.2 kb containing four partially overlapping open reading frames encoding the polymerase (P), core (C), HBsAg and X proteins. Based on the antigenic determinants of the HBsAg, HBV was classified into nine serological types, adw2, adw4, adr, adrq-, ayw1, ayw2, ayw3, ayw4, and ayr, [Couroucé-Pauty et al., 1978]. HBsAg contains the 'a' determinant, (amino acid residues 124 and 147) which is common to all the HBV genotypes [Weber, 2004; Lada et al., 2006].

The heterogenicity of HBV serotype (based on single amino acid substitutions of the HBsAg), might have a positive selective value which can act as an immune evasion strategy [Simmonds, 2001]. Based on complete genome nucleotide sequence divergence of more than 8%, eight naturally occuring HBV genotypes (A-H) have been described [Norder et al., 2004; Kurbanov et al., 2010]. There is a correlation between genotypes and antigenic subtypes, with a genotype associated with two or more different subtypes. Furthermore, subgenotypes have been identified within specific

genotypes, based on more than 4% sequence divergence over the entire genome [Norder et al., 2004; Kurbanov et al., 2010].

The prevalence of genotypes varies geographically, with genotype D being more common in the Mediterranean region. Subgenotypes within each genotype show distinct geographic distribution, except for those within genotype D which are found throughout the world [Norder et al., 2004].

Information concerning the genotype distribution of HBV in Greece is very limited [Katsoulidou et al., 2009]. In order to provide data on current HBV molecular epidemiology, the distribution of HBV genotypes, HBsAg subtypes and the mutations in the "a" determinant region, a retrospective study was conducted (years 2000-2007) among patients with chronic HBV infection in Thessaloniki, the second largest city in Greece. HBV genotypes were studied by partial sequencing of the surface gene, containing the HBsAg "a" antigenic determinant.

MATERIALS AND METHODS

Study population - Source of HBV DNA

This was an 8-year retrospective analysis of samples of 135 chronic HBV carriers admitted to Papageorgiou Regional General Hospital of Thessaloniki during the period from December 2000 until January 2007 for routine HBV DNA detection and quantitation. All patients, all their parents/guardians in the cases of children signed an informed consent for the protocol study, which was approved by the Local Ethics committee. Socio-demographic characteristics (gender, place of birth, place of residence and age) and medical history were recorded. Patients with HCV or HIV co-infection were excluded from the study.

Serological assays

Routine ALT and AST determinations were performed (normal range 10-37U/l for both enzymes). The HBsAg, anti-HBc (total), anti-HBc IgM, HBeAg, and anti-HBe were determined by a commercial enzyme immunoassay method (Abbott Laboratories, Dallas, TX, USA).

HBV DNA quantitative analysis

The PCR-based assay COBAS-AMPLICOR (Roche Molecular Diagnostics, Indianapolis, IN, USA), was used for serum HBV DNA quantitation according to the protocol of the manufacturer [DiDomenico et al., 1996; Noborg et al., 1999]. The HBV-DNA quantitation ranges from 60 to 38,000 IU/ml (300 to 200,000 HBV DNA copies/ml).

PCR amplification of HBV DNA S Gene

DNA was extracted from 200 µl of each serum sample by using QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The HBV DNA sequence of 259 nucleotide pairs (329-587) from the HBsAg region, was amplified by PCR with primers a) HB1 (5'-CAA GGT ATG TTG CCC GTT TGT -3') and b) HB2 (5'- AAA GCC CTG CGA ACC ACT GAA -3'). The amplified region corresponds to the amino acid (aa) residues 101-186 of the S antigen chain which contains the a-determinant region (a.a 124-147). PCR products were separated by agarose gel electrophoresis. The clean-up of PCR products was performed according to the QIAquick PCR Purification Kit Protocol (Qiagen GmbH, Hilden, Germany) using a microcentrifuge and at least 20 ng/µl of viral DNA was present in the purified product.

Direct Sequencing of PCR products

The purified HBV DNA was sequenced directly by Qiagen Sequencing Services using an ABI 3700 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA.). All sequences were analyzed in both, forward and reverse directions. The nucleotide sequence data reported in this study has been submitted to the DDBJ/EMBL/GenBank under accession numbers FJ178438 to 178573.

Determination of HBsAg Subtypes

The nucleotide sequences were translated into amino acid sequences according to the ORF of the partial S gene and the HBsAg subtypes were

predicted from the amino acids at positions 122 (K for *d* and R for *y* determinants), 127 (P for *w1-2*, T for *w3* and L-I for *w4*) and 160 (K for *w* and R for *r*) [Magnius and Norder, 1995]. Discrimination between ayw1 and awy2 was based on positions 134 and 159 (F and A, for ayw1 and Y and G for ayw2, respectively) [Norder et al., 1992].

HBV Genotypes determination and Phylogenetic Analysis

HBV genotype was determined by sequence analysis of the 195 bp fragment from the HBV "a" determinant, using the genotyping tool available at the National Library of Medicine's National Center for Biotechnology Information (NCBI) [Rozanov et al., 2004]. In addition, HBV genotype of each isolate was deduced by comparison of the partial surface protein sequences with the sequences of the corresponding genomic regions of all HBV strains available in the GenBANK/EMBL/DDBJ database by using the BLAST program, version 2.2.8 [Altschul et al., 1990; Altschul et al., 1997]. A phylogenetic analysis was carried out by pairwise comparison of the partial S gene sequences of representative HBV strains for all genotypes from the GenBANK/EMBL/DDBJ database with the 135 sequences obtained from the sera of the patients with chronic HBV infection, by using ClustalW [Thompson et al., 1994]. Construction of the phylogenetic tree was carried out with ClustalX (version 1.83) by using the alignment file, obtained by analysis with ClustalW. By using this program the distances between all pairs of sequences were calculated, and this was followed by application of the neighbor-joining method to the distance matrix. Confidence values for the groups in the tree (bootstrap values on a scale from 1 to 1000) were also calculated by using ClustalX.

Dendrograms showing the phylogenetic relationships among the HBV isolates of the present study and prototype strains was plotted in the PHYLIP format output by using the TreeView software (version 3.0), which was obtained from the website of the University of Glasgow.

Statistics

Statistical analysis of data was performed using the Statistical Program for social sciences (SPSS version 16.0). Results were presented as mean values +/-standard deviation (SD). Comparisons between patients groups were performed using the students t-test or nonparametric Mann-Whitney U test. A p value <0.05 was considered significant.

RESULTS

HBV serology and DNA viral load

A total of 135 HBV carriers (93 males, 42 females) with ages ranging from 9 to 92 years (mean value 45.5 years), were enrolled in the study (Table 1). Of the 135 patients, one hundred twenty-four patients were ethnic Greek residents of Thessaloniki (Northern Greece) while the rest were immigrants from Albania (five patients), China (two patients), Turkey (two patients), and Georgia (two patients).

All patients with chronic HBV infection had elevated aminotransferase levels. Mean ALT and AST levels were 238 IU/L (SD= 576.84) and 152 IU/L (SD= 282.65), respectively. All patients were HBsAg positive, and the majority of them (62.5%; 84/135) were anti-HBe positive. Thirty-five per cent (47/135) of patients were HbeAg positive, while 3% (4/135) were both HbeAg positive and anti-HBe positive. Anti-HBc seropositivity was detected in 98% of the study population. In 4.4% (6/135) of patients, simultaneous presence of HBsAg and anti-HBs was detected. Serum HBV-DNA levels ranged from $1.02x10^5$ to $2.2x10^7$ IU/ml. Patients positive for both HBeAg and anti-HBe, had significantly higher DNA levels than HBeAg negative patients ($2.29x10^6$ vs. $1.3x10^6$; $p<0.01$).

Determination of HBV Genotypes

The results obtained by analysis of the nucleotide sequences from the 135 serum samples tested, are summarised in tables 1-3 and the phylogenetic trees in Figures 1 and 2. The sequences grouped within clusters

corresponding to genotypes A, B, C, and D of HBV. None of them grouped within genotypes E, F, G or H. All genotype clusters were supported by significant bootstrap values. Agreement between the plylogenetic analysis and the NCBI genotyping tool results was observed in all cases and an HBV genotype was assigned to all but one samples. The distribution of HBV genotypes found among the 135 serum samples was 98% for genotype D, which was almost exclusively prevalent, 1% for genotype A, and 0.5% for each genotype B and C, which were exclusively found in Asian immigrants. Recombination of the HBV genes from different genotypes was not observed. The sequences of the partial S gene from the 135 patients participated in the study are available at the GenBank/EMBL/DDBJ Database with accession numbers FJ17843–FJ 178573.

Determination of HBsAg Subtypes and association with HBV Genotypes

Partial sequencing of the S gene predicted the HBsAg subtypes in all but one case. Isolates from genotype D specified exclusively subtype *ayw2* (96/132, 73%) and subtype *ayw3* (36/132, 27%). Almost all these isolates grouped together in HBsAg subtypes in the phylogenetic tree (Fig.2). The only strain belonging to genotype A specified subtype *adw2*, was isolated from a chronic HBV native Greek carrier. In addition, the one strain belonging to genotype C was detected in a Chinese immigrant and belonged to subtype *adr*. The second strain detected from another immigrant from China belonged to genotype B, but the HBsAg subtype was not specified, as the HBsAg aminoacid sequence was only readable between positions 121 and 154. The

first part of the sequence seemed to specify subtype *ayw* (Thr^{126}), while the second part seemed to specify subtype *adw* (Thr^{143}).

No unusual genotype/HBsAg subtype combinations were found. The association of HBsAg subtypes with genotypes and seroconvertion status is shown in tables 2 and 3, respectively. The *ayw2* HBsAg subtype was predominant in both the HBeAg positive and the anti-HBe positive patients with 74% (32/43) and 71% (58/82), respectively. The *ayw3* subtype was less common with 26% (11/43) in the HBeAg positive patients and 29% (24/82) in the anti-HBe positive patients.

HBV genetic diversity

Single or multiple point mutations were found in 26% (35/135) of the cases as shown in Tables 1 and 3. Some of the most common mutations occurred at amino acid positions 129 and 134 (5 strains), 133 and 145 (4 strains, including the 'vaccine escape' mutant G145R), and 128, 144, 164, 174 (3 strains each). Most common mutations observed were M133T, Y134N, and S174N (3 isolates). In the anti-HBe positive group, the mutation rate was significantly higher in patients with the *ayw3* subtype compared to the *ayw2* group (42% vs. 15%; $p<0.01$). Such difference was not observed in the HBeAg positive group (27% vs 22%; $p>0.01$). Multiple point mutations were almost exclusively observed in the anti-HBe positive group and in two of the four patients belonging to the HBsAg positive /anti-HBs positive group (Fig.3). Most strains were specified as subtype *ayw2*. Interestingly, 5 and 4 point mutations were found in the two patients from the HBsAg positive / anti-HBs positive group (Fig.3). In the first case, an HBeAg positive leukemic patient

under chemotherapy and immunosupression, the point mutations were S136F, P142L, S143L, D144E, and the 'vaccine escape' mutation G145R. In the latter case, an anti-HBe positive chronic HBV carrier, the point mutations were T123N, M133L, G145R, and V177A.

DISCUSSION

In this 8-year retrospective study, important data were obtained on the HBV genotypes/subtypes and mutations in HBsAg in chronic HBV carriers in Thessaloniki, Greece. Two HBV genotypes A and D, were found among native Greeks, with genotype D being almost prevalent exclusively (D: 98% vs. A: 1%). Genotype D was also the only genotype found in Turkey [Bozdayi et al, 2005; Ozasln et al 2007] and the predominant genotype in Serbia [Lazarevic et al., 2007].

The remaining 1% derived from two HBV strains from Chinese immigrants, were specified as genotypes B and C. The one isolate belonging to genotype A in this study was grouped with a reference sequence for A2, suggesting a European origin. As expected for samples from the European population, this strain belong to subtype adw2.

The presence of both HBsAg and anti-HBs, was found in 4% of the study population. The selection of HBsAg variants is a possible mechanism underlying the presence of both HBsAg and anti-HBs. Residue changes in the "a" determinant of the S protein, the main target of anti-HBs antibodies, could lead to escape from recognition by the host immune system. However, HBV escape mutants may also occur naturally in chronic HBV carriers due to the exclusive pressure of the host immune system. The results of the present study are comparable to those reported by Yamamoto et al., [1994], Kohno et al., [1996] and Lada et al., [2006].

The direct sequencing of a relatively short fragment of the HBV genome was shown to be efficient for the determination of both viral genotype and HBsAg subtype, as well as mutations with potential impact on the HBsAg

antigenicity. Mutational studies on HBV S genes are important in understanding the failure of protection with current HBV vaccines and the conflicting results of HBsAg detection with different diagnostic kits. This can be due to the fact that the specificity of the antibodies used in diagnostic assays and vaccines are targeted to these regions, and especially the “a” determinant. The results from this study, confirmed the good performance of the tests used, even in the presence of multiple point mutations.

In conclusion, data from this study contribute to the investigation of HBV genotype and HBsAg subtype distribution in Greece. Partial S gene sequence analysis determined that the HBV genotype D and ayw2 HBsAg subtype, were predominant in patients with chronic HBV infection from northern Greece. The clustering of all DNA sequences into genotype groups corresponded to their respective HBsAg subtype. Analysis of the point mutations revealed that amino acid substitutions at immunodominant epitopes involved in B or/and T cell recognition did not affect detection by commercial immunoassays. Additional data will be required in order to determine the frequency of HBV genotypes in Greece.

ACKNOWLEDGEMENTS

The authors would like to thank all medical doctors and technicians working in the Virology Laboratory, Papageorgiou Regional General Hospital, Thessaloniki, Greece, that assisted in the study. The authors would also like to acknowledge Abbott Hellas for financing the S gene partial sequencing of the HBV strains.

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Table 1. Main demographic characteristics of the study group and information concerning genotypes and subtypes

	Number of patients	genotypes/Subtypes (No of patients)
Gender (male/female)	135 (93/42)	
Age (mean years) (range)	45.5 (9–92)	
Origin		
Greece (GR)	124	D/ayw3 (30) D/ayw2 (93) A2/ayw2 (1)
Albania(Al)	5	D/ayw3 (4) D/ayw2 (1)
China (Ch)	2	C/adr(1), B/Nd*
Georgia (GeO)	2	D/ayw3 (2)
Turkey (TU)	2	D/ayw3 (1), D/ayw2 (1)

*(Nd:not determined. Patients sequences are reported in DDBJ/EMBL/GenBank under accession numbers FJ178438 to 178573)

Table 2. HBsAg Subtypes association with HBV Genotypes

	Genotype							
HBsAg subtype	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**TOTAL**
ayw2	**-**	**-**	**-**	**96**	**-**	**-**	**-**	**96**
ayw3	**-**	**-**	**-**	**36**	**-**	**-**	**-**	**36**
adw2	**1**	**-**	**-**	**-**	**-**	**-**	**-**	**1**
Adr	**-**	**-**	**1**	**-**	**-**	**-**	**-**	**1**
No subtype	**-**	**1**	**-**	**-**	**-**	**-**	**-**	**1**
Total	**1**	**1**	**1**	**132**	**-**	**-**	**-**	**135**

Table 3: Correlation of the HBV serology profile with the different subtypes, mutations in the a determinant.

HBV serology profile	*Total no (%)*	*ayw2*	*ayw3*	*Mutations*	*HBV-DNA (IU/ml)*	*ALT IU/ml*
HBsAg+/HBeAb+	82 (61%)	58 (71%)	24 (29%)	ayw2 (15%), ayw3 (42%)		
			HBV829GR	A128L	3260000	2610
			HBV1010GR	A128V	7070000	88
			HBV548GR	A128V	206000	674
			HBV641GR	D144E	2430000	30
		HBV998GR		E164G, S174N, S167L	3760000	57
			HBV205GR	F158L	925000	58
			HBV275GR	M133T	2260000	41
			HBV400GR	M133T	580000	42
			HBV895GR	M133T	1870000	77
			HBV1059GR	P127A, Q129R	1750000	240
		HBV1387GR		P142T, D144V	943000	1147
		HBV868GR		Q129G	12200000	393
		HBV552GR		Q129H,G130R	7660000	108
		HBV1141GR		S174N	1870000	1095
		HBV204GR		T126S	436000	215
		HBV982GR		T131I	3420000	85
		HBV785GR		Y134H	8400000	132
		HBV863GR		Y134N	818000	560
			HBV1586GR	Y134N	912000	289

HBsAg+/HBeAg+*	43* (31.6%)	29* (67%)	11* (26%)	ayw2 (22%), ayw3 (27%)		
		HBV928GR HBV1002GR HBV1696GR HBV262GR HBV1642GR HBV742GR HBV1774GR	HBV1010GR HBV1090GR HBV690GR** HBV1586GR	A128V E164V E164V G145A I126T P127L Q129H Q129R S136F V177L Y134N	4550000 13000000 7330000 1370000 NT 1200000 2740000 7700000 NT 8900000 912000	
HBsAg+/HBeAg+/HBeAb+	3 (2.2%)	3	-			
		HBV595GR		S174H	11000000	107
HBsAg+/HBcAb-/HBeAg+	1 (0.7%)	1	-	-		
HBsAg+/HBsAb+	5(3.4%)	4 (80%)	1 (20%)			
		HBV804GR HBV420GR HBV347GR		T123N,M133L,G145R,V177A D144E,S143L,P142L,S136F,G145R S136F	NT 1260000 2120000	39 6 24
HBsAg+/HBsAb+/	1	1				

HBeAg+/HBeAb+	(0.7%)					
TOTAL	135*	96*	36*			

*In the HBsAg+/HBeAg+ group there are three patients with different subtypes than ayw2 or ayw3, one with adr (**) subtype with mutation I126T, the second has adw2 subtype and the third one has no detectable subtype

Fig.1

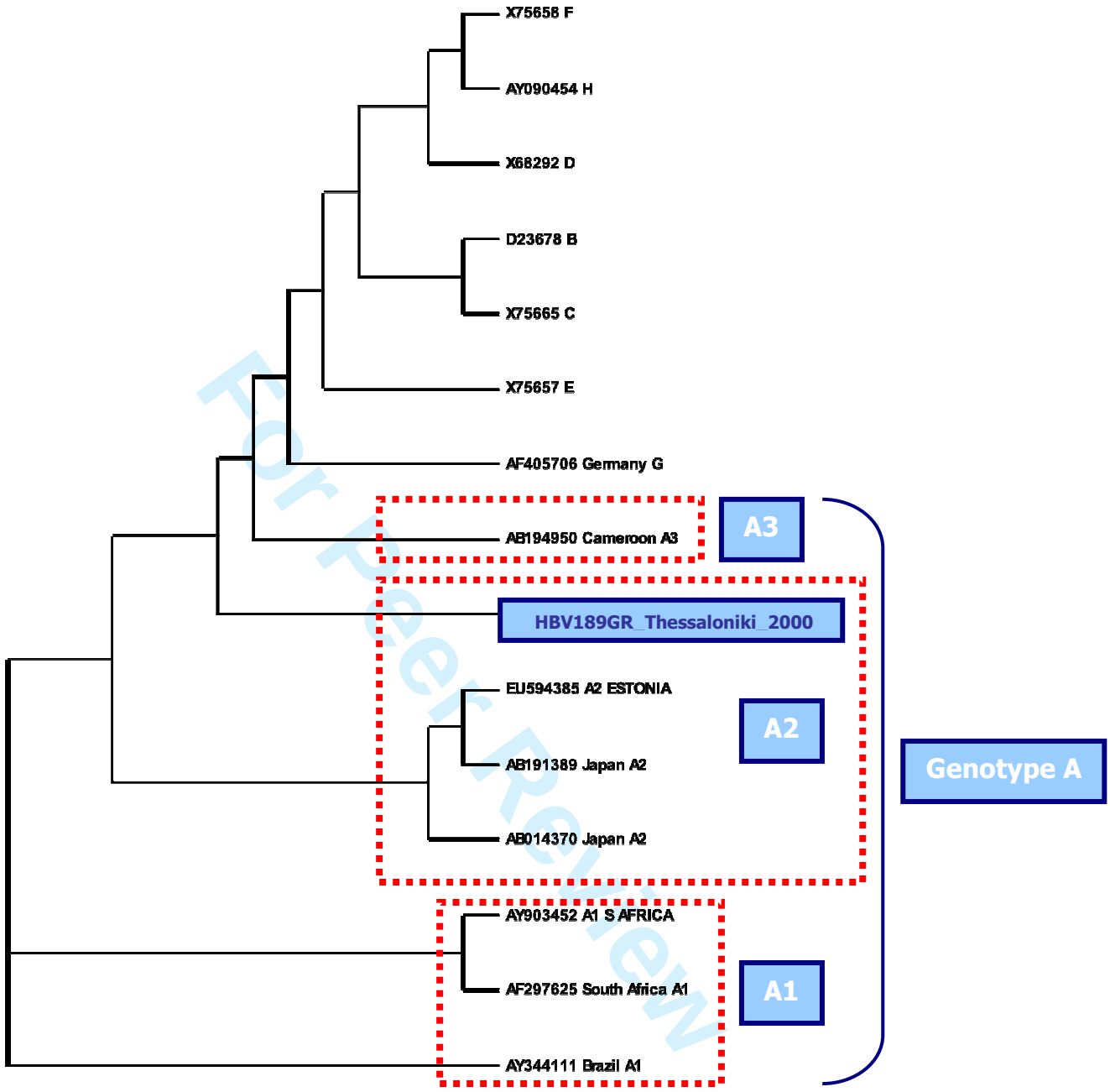

Fig. 2

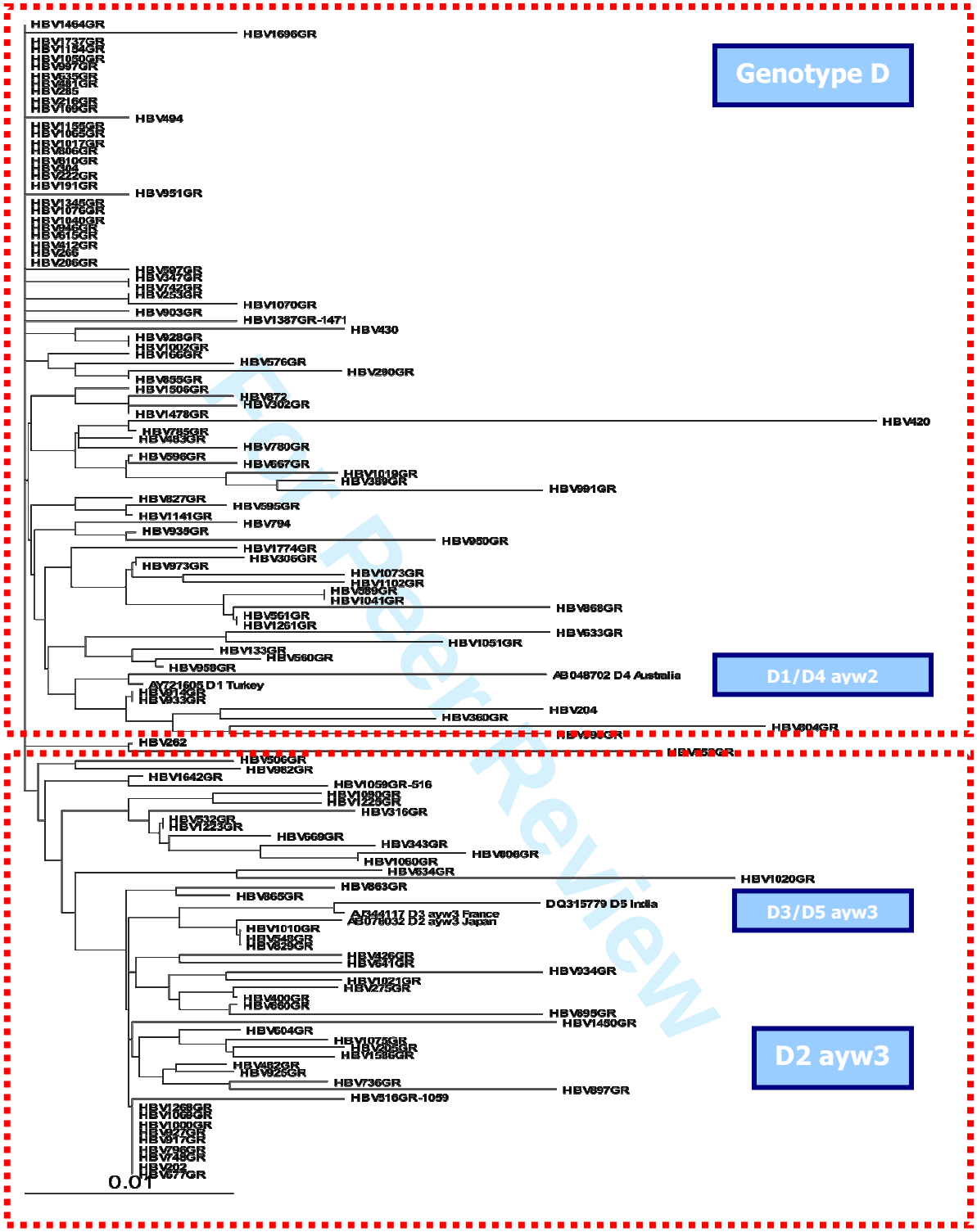

Fig. 3. Comparison with reference sequences for mutant isolates a) HBV804GR and b) HBV420GR

a)

AA-Position: 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142
Sequenced: AGA AAC TGC ACG ACT CCT GCT CAA GGA ACC TCT TTG TTT CCC TCC TGT TGC TGT ACA AAA CCT
Translation: R N C T T P A Q G T S L F P S C C C T K P
Reference: K T C T I P A Q G T S M F P S C C C T K P
Trace/Link: 782 783 784 785

AA-Position: 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163
Sequenced: TCG GAC AGA AAT TGC ACC TGT ATT CCC ATC CCA TCA TCC TGG GCT TTC GGA AAA TTC CTA TGG
Translation: S D R N C T C I P I P S S W A F G K F L W
Reference: S D G N C T C I P I P S S W A F A R F L W
Trace/Link: 786 787 788

AA-Position: 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182
Sequenced: GAG TGG GCC TCA GCC CGT TTC TYC TGG CTC AGT TTA CTA GCG CCA TTT GTT CAG TGG
Translation: E W A S A R F F/S W L S L L A P F V Q W
Reference: E W A S V R F S W L S L L V P F V Q W
Trace/Link: 789 790 791

b)

AA-Position: 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141
Sequenced: TGC AAA ACC TGC ACG ACT CCT GCT CAA GGA ACC TCT ATG CAT CCC TCC TGT TGC TGT ACC AAA
Translation: C K T C T T P A Q G T S M H P S C C C T K
Reference: C K T C T I P A Q G T S M F P S C C C T K
Trace/Link: 487 488

AA-Position: 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162
Sequenced: CTT TTG GAA AAA AAT TGC ACC TGT ATT CCC ATC CCA TCA TCC TGG GCT TTC GGA AAA TTC CTA

Translation: L L E K N C T C I P I P S S W A F G K F L
Reference: P S D G N C T C I P I P S S W A F A R F L
Trace/Link: 489 490 491 492 493 494

AA-Position: 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183
Sequenced: TGG GAA TGG GCC TCA GCC CGT TTC TCC TGG CTC AGT TTA CTA GTG CCA TTT GTT CAG TGG TTC
Translation: W E W A S A R F S W L S L L V P F V Q W F
Reference: W E W A S V R F S W L S L L V P F V Q W F
Trace/Link: 495