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## Molecular epidemiology of chronic Hepatitis B virus infection in Greece

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3 **Molecular epidemiology of chronic Hepatitis B virus infection**  
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**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 aminotransferase/ALT, aspartate aminotransferase/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  $1.02 \times 10^5$  to  $2.2 \times 10^7$  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.

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**Keywords:** Genotypes; Mutations; An antigenic determinant

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## 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 occurring 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

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3 genotypes, based on more than 4% sequence divergence over the entire  
4 genome [Norder et al., 2004; Kurbanov et al., 2010].  
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8 The prevalence of genotypes varies geographically, with genotype D  
9 being more common in the Mediterranean region. Subgenotypes within each  
10 genotype show distinct geographic distribution, except for those within  
11 genotype D which are found throughout the world [Norder et al., 2004].  
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17 Information concerning the genotype distribution of HBV in Greece is  
18 very limited [Katsoulidou et al., 2009]. In order to provide data on current HBV  
19 molecular epidemiology, the distribution of HBV genotypes, HBsAg subtypes  
20 and the mutations in the “a” determinant region, a retrospective study was  
21 conducted (years 2000-2007) among patients with chronic HBV infection in  
22 Thessaloniki, the second largest city in Greece. HBV genotypes were studied  
23 by partial sequencing of the surface gene, containing the HBsAg “a” antigenic  
24 determinant.  
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## 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

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

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20 HBV genotype was determined by sequence analysis of the 195 bp fragment  
21 from the HBV “a” determinant, using the genotyping tool available at the  
22 National Library of Medicine’s National Center for Biotechnology Information  
23 (NCBI) [Rozanov et al., 2004]. In addition, HBV genotype of each isolate was  
24 deduced by comparison of the partial surface protein sequences with the  
25 sequences of the corresponding genomic regions of all HBV strains available  
26 in the GenBANK/EMBL/DDBJ database by using the BLAST program, version  
27 2.2.8 [Altschul et al., 1990; Altschul et al., 1997]. A phylogenetic analysis was  
28 carried out by pairwise comparison of the partial S gene sequences of  
29 representative HBV strains for all genotypes from the GenBANK/EMBL/DDBJ  
30 database with the 135 sequences obtained from the sera of the patients with  
31 chronic HBV infection, by using ClustalW [Thompson et al., 1994].  
32 Construction of the phylogenetic tree was carried out with ClustalX (version  
33 1.83) by using the alignment file, obtained by analysis with ClustalW. By using  
34 this program the distances between all pairs of sequences were calculated,  
35 and this was followed by application of the neighbor-joining method to the  
36 distance matrix. Confidence values for the groups in the tree (bootstrap values  
37 on a scale from 1 to 1000) were also calculated by using ClustalX.  
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3 Dendrograms showing the phylogenetic relationships among the HBV isolates  
4 of the present study and prototype strains was plotted in the PHYLIP format  
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8 output by using the TreeView software (version 3.0), which was obtained from  
9  
10 the website of the University of Glasgow.  
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### 12 13 14 15 **Statistics**

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17 Statistical analysis of data was performed using the Statistical Program for  
18 social sciences (SPSS version 16.0). Results were presented as mean values  
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21 +/-standard deviation (SD). Comparisons between patients groups were  
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24 performed using the students t-test or nonparametric Mann-Whitney U test. A  
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27 p value <0.05 was considered significant.  
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## 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  $1.02 \times 10^5$  to  $2.2 \times 10^7$  IU/ml. Patients positive for both HBeAg and anti-HBe, had significantly higher DNA levels than HBeAg negative patients ( $2.29 \times 10^6$  vs.  $1.3 \times 10^6$ ;  $p < 0.01$ ).

### 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

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

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36 Partial sequencing of the S gene predicted the HBsAg subtypes in all  
37 but one case. Isolates from genotype D specified exclusively subtype *ayw2*  
38 (96/132, 73%) and subtype *ayw3* (36/132, 27%). Almost all these isolates  
39 grouped together in HBsAg subtypes in the phylogenetic tree (Fig.2). The only  
40 strain belonging to genotype A specified subtype *adw2*, was isolated from a  
41 chronic HBV native Greek carrier. In addition, the one strain belonging to  
42 genotype C was detected in a Chinese immigrant and belonged to subtype  
43 *adr*. The second strain detected from another immigrant from China belonged  
44 to genotype B, but the HBsAg subtype was not specified, as the HBsAg  
45 aminoacid sequence was only readable between positions 121 and 154. The  
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3 first part of the sequence seemed to specify subtype *ayw* (Thr<sup>126</sup>), while the  
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5 second part seemed to specify subtype *adw* (Thr<sup>143</sup>).  
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8 No unusual genotype/HBsAg subtype combinations were found. The  
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10 association of HBsAg subtypes with genotypes and seroconversion status is  
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12 shown in tables 2 and 3, respectively. The *ayw2* HBsAg subtype was  
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14 predominant in both the HBeAg positive and the anti-HBe positive patients  
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16 with 74% (32/43) and 71% (58/82), respectively. The *ayw3* subtype was less  
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18 common with 26% (11/43) in the HBeAg positive patients and 29% (24/82) in  
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20 the anti-HBe positive patients.  
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### 27 **HBV genetic diversity**

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29 Single or multiple point mutations were found in 26% (35/135) of the  
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31 cases as shown in Tables 1 and 3. Some of the most common mutations  
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33 occurred at amino acid positions 129 and 134 (5 strains), 133 and 145 (4  
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35 strains, including the 'vaccine escape' mutant G145R), and 128, 144, 164,  
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37 174 (3 strains each). Most common mutations observed were M133T, Y134N,  
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39 and S174N (3 isolates). In the anti-HBe positive group, the mutation rate was  
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41 significantly higher in patients with the *ayw3* subtype compared to the *ayw2*  
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43 group (42% vs. 15%;  $p < 0.01$ ). Such difference was not observed in the  
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45 HBeAg positive group (27% vs 22%;  $p > 0.01$ ). Multiple point mutations were  
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47 almost exclusively observed in the anti-HBe positive group and in two of the  
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49 four patients belonging to the HBsAg positive /anti-HBs positive group (Fig.3).  
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51 Most strains were specified as subtype *ayw2*. Interestingly, 5 and 4 point  
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53 mutations were found in the two patients from the HBsAg positive / anti-HBs  
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55 positive group (Fig.3). In the first case, an HBeAg positive leukemic patient  
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3 under chemotherapy and immunosuppression, the point mutations were  
4 S136F, P142L, S143L, D144E, and the 'vaccine escape' mutation G145R. In  
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6 the latter case, an anti-HBe positive chronic HBV carrier, the point mutations  
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8 were T123N, M133L, G145R, and V177A.  
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## 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; Ozaslan 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



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3 antigenicity. Mutational studies on HBV S genes are important in  
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5 understanding the failure of protection with current HBV vaccines and the  
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7 conflicting results of HBsAg detection with different diagnostic kits. This can  
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9 be due to the fact that the specificity of the antibodies used in diagnostic  
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11 assays and vaccines are targeted to these regions, and especially the “a”  
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13 determinant. The results from this study, confirmed the good performance of  
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15 the tests used, even in the presence of multiple point mutations.  
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20 In conclusion, data from this study contribute to the investigation of  
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22 HBV genotype and HBsAg subtype distribution in Greece. Partial S gene  
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24 sequence analysis determined that the HBV genotype D and ayw2 HBsAg  
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26 subtype, were predominant in patients with chronic HBV infection from  
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28 northern Greece. The clustering of all DNA sequences into genotype groups  
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30 corresponded to their respective HBsAg subtype. Analysis of the point  
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32 mutations revealed that amino acid substitutions at immunodominant epitopes  
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34 involved in B or/and T cell recognition did not affect detection by commercial  
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36 immunoassays. Additional data will be required in order to determine the  
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38 frequency of HBV genotypes in Greece.  
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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(AI)	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

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



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

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

<b>HBsAg+/HBeAg+*</b>	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	
<b>HBsAg+/HBeAg+/HBeAb+</b>	3 (2.2%)	3	-			
		HBV595GR		S174H	11000000	107
<b>HBsAg+/HBcAb-/HBeAg+</b>	1 (0.7%)	1	-	-		
<b>HBsAg+/HBsAb+</b>	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
<b>HBsAg+/HBsAb+/-</b>	1	1				

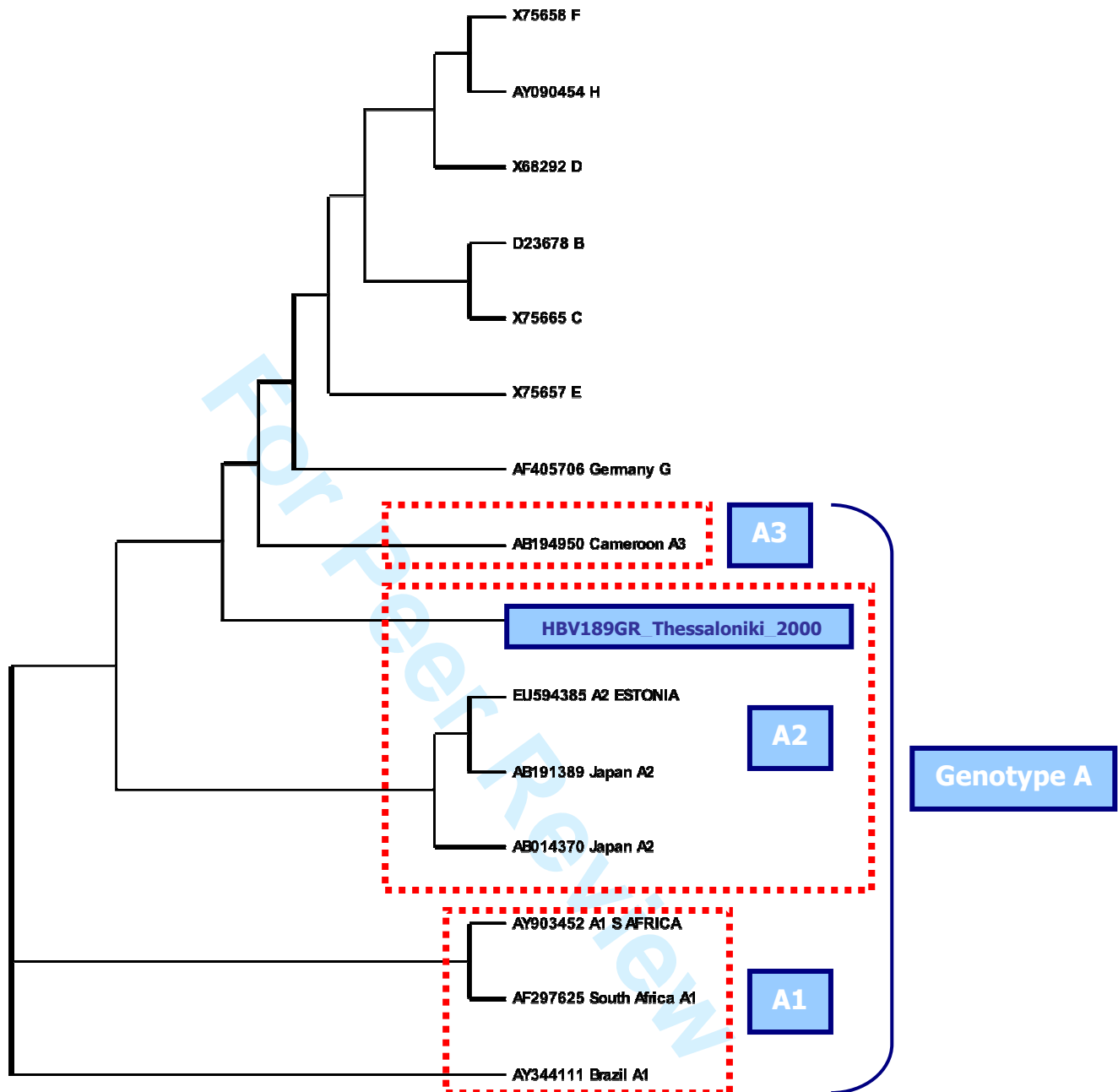
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<b>HBeAg+/HBeAb+</b>	(0.7%)					
<b>TOTAL</b>	135*	96*	36*			

\*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

For Peer Review

Fig.1



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**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

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7 Trace/Link: [489](#) [490](#) [491](#) [492](#) [493](#) [494](#)  
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10 AA-Position: 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183  
11 Sequenced: TGG GAA TGG GCC TCA GCC CGT TTC TCC TGG CTC AGT TTA CTA GTG CCA TTT GTT CAG TGG TTC  
12 Translation: W E W A S A R F S W L S L L V P F V Q W F  
13 Reference: W E W A S V R F S W L S L L V P F V Q W F  
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