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Characterisation of hepatitis B virus in Turkish blood donors, and the prevalence of the SP1 splice variant.

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3 1 **Characterisation of hepatitis B virus in Turkish blood donors, and the prevalence of the**
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6 2 **SP1 splice variant.**
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47 20 **Running title:** HBV characterisation in Turkish blood donors

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3 **23 Abstract**
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6 24 Hepatitis B is a disease of the liver that can manifest acutely, or persist chronically as a result of
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8 25 infection with the hepatitis B virus (HBV). Turkey has a moderate endemicity level of HBV
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10 26 infection, and all data published to date has shown this to be of genotype D, predominantly of
11
12 27 subgenotype D1. However the sequences of very few full genomes have been published. The
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15 28 aim of this study was to characterise the molecular profile of hepatitis B virus in asymptomatic,
16
17 29 first-time Turkish blood donors. These results confirm that genotype D, subgenotype D1 is the
18
19 30 most prevalent HBV strain in Turkey, accounting for 94% of cases. Subgenotypes D2 and D3
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21 31 were present as minority strains (4% and 2%, respectively). A singly-spliced HBV variant that is
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24 32 capable of forming defective HBV particles and has been associated with apoptosis and
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26 33 activation of T-cell responses was also detected in 52.5% of samples screened, co-circulating
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29 34 with wild type genomes.
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36 Introduction

37 Hepatitis B virus (HBV) carrier prevalence in Turkey is reported to be around 4%
38 [Degertekin and Gunes, 2008], which is in line with other Mediterranean and Middle Eastern
39 countries and meets the criteria for intermediate endemicity level. HBV belongs to the family
40 Hepadnaviridae, and has a 3.2kb genome that, when encapsidated within the virion, is a relaxed
41 circular DNA molecule that is only partially double-stranded. It was first reported in 1989 that
42 HBV could undergo splicing by the host's machinery in human hepatoma cells transfected with
43 full-length HBV [Su et al., 1989]. This 2kb variant is commonly termed SP1, and contains a 1223
44 nucleotide deletion beginning at the last codon of the core gene and spanning through to the
45 middle of the S gene. To date, this splice variant has been identified in chronic hepatitis B
46 patients infected with HBV genotypes A and D, and an additional 10 splice variants have been
47 identified in patients infected with genotypes A, B and D [Gunther et al., 1997].

48 HBV is divided into eight well-documented genotypes (A-H), some of which are further
49 divided into two or more sub-genotypes [Kay and Zoulim, 2007]. Recently, two putative new
50 genotypes, I and J, have been reported [Tatematsu et al., 2009; Tran et al., 2008]. Genotype D is
51 predominant in the Mediterranean basin, and is also frequently found in populations from
52 Europe, Africa and Asia. Of the HBV genotypes, this is the least defined geographically, which
53 may be a result of diverging earlier than the other genotypes [Norder et al., 2004]. The majority
54 of the current data on HBV in Turkey has come from patients with clinical Hepatitis B, with few
55 full genome sequences published. Sequencing of full genomes and the S gene from patients with
56 chronic HBV infection identified only genotype D in Turkish samples, and where subgenotyping
57 was documented, D1 was the most prevalent subgenotype, with a minority of subgenotype D2
58 strains present [Bozdayi et al., 2005; Sayiner et al., 2008; Serin et al., 2005; Sertoz et al., 2008].
59 The aim of this study was to characterise HBV strains obtained from HBsAg-positive plasma

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3 60 samples from asymptomatic, first-time, replacement blood donors collected in Ankara, Turkey,
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5 61 using serological and molecular techniques.
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9 63 **Materials & Methods**

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12 64 HBsAg-positive plasma samples were collected during 3 separate periods in 2004, 2005
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14 65 and 2006, with a total collection period of 13 months. Samples were heat-inactivated at 60°C for
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16 66 30 min prior to shipping on dry ice, and upon arrival in the UK were stored at -80°C until ready
17
18 67 to use. Viral DNA was extracted and quantified as described previously [Allain et al., 2003;
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20 68 Garmiri et al., 2009]. To generate sequence for the entire HBV genome from 50 samples, the
21
22 69 Expand High Fidelity PCR system (Roche) was used for two nested PCR assays. Firstly, a nearly
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24 70 full-length fragment of approximately 3000bp, minus a 50bp precore section was amplified as
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26 71 previously described [Zahn et al., 2008]. A second 300bp fragment including the basic core
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28 72 promoter/precore (BCP/PC) 50bp gap was produced to complete the full genome [Candotti et
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30 73 al., 2006]. PCR products were purified using the EZNA cycle pure kit (Omega; Crawley, UK).
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35 74 A semi-nested PCR was used to screen for the SP1 variant, using newly designed primers
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37 75 located in the core and S genes. SP1_core (5'-GTCGCAGAAGATCTCAATCTCGGG-3'; position
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39 76 2421–2444) was used as the sense primer in both first and second round PCR; SP1_R1 (5'-
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41 77 ATACAAAGGCATTAATGCAGGGTA-3'; position 1065–1042) and SP1_R2 (5'-
42
43 78 TGTGTAATGGAGCGGCAAAGCC-3'; position 1012–1034) were used as the anti-sense primers in
44
45 79 first and second round PCR, respectively. Using the Roche Expand High Fidelity system, a 50µL
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47 80 reaction mixture contained: 1X PCR buffer II (including MgCl₂; final concentration 2.5mM), 0.8
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49 81 mM dNTPs, 0.3 µM each primer, and 5.25 U HiFi. For samples with a viral load of x10⁸ IU ml⁻¹ or
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51 82 above, 3µl of viral DNA was used as first-round template; 5µl was used for samples with viral
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53 83 loads of x10⁴ – x10⁷ IU ml⁻¹ and 10µL for samples x10³ IU ml⁻¹ or below. Six microlitres of the
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3 84 first-round reaction was loaded into the second-round PCR. An identical touchdown cycling
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5 85 programme was used for both rounds, and consisted of: an initial denaturation step at 94°C for
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8 86 5 minutes, followed by 10 cycles of 94°C/40s; 65°C/45s (reduced by 1°C each cycle); 72°C/1
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10 87 min, and a following 30 cycles of 94°C/40s; 55°C/45s; 72°C/1 min. A final extension step was
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12 88 performed at 72°C for 10 minutes. In the presence of full-length HBV DNA (3.2kb) a 1.8kb
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14 89 product is amplified, in comparison to a 580bp product for the SP1 variant, visualised using
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16 90 agarose gel electrophoresis.

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19 91 Sequences with interesting deletions or insertions were examined further by fresh full
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21 92 genome or BCP/PC PCR reactions. Following addition of a 3' single adenosine overhang to the
22
23 93 PCR product, amplicons were ligated into either the pCR2.1 TOPO® TA vector (BCP/PC) or the
24
25 94 TOPO® XL vector (Invitrogen; Paisley, UK) as per the manufacturer's instructions. Following
26
27 95 transformation of chemically competent *E. coli* cells, plasmid DNA was isolated using a QIAprep
28
29 96 spin miniprep kit (Qiagen), and a minimum of 10 clones were sequenced.

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32 97 Sequencing and phylogenetics analysis was performed using Seqman, SeqBuilder
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34 98 (DNASTAR) and MacVector software. PAUP* version 4.0 beta 10 was used to calculate genomic
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36 99 distances. Microsoft Excel and Prism (version 4) were used for analysis of HBV DNA and HBsAg
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38 100 load.

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41 101 Antibodies against HBcAg (anti-HBc) and HBsAg (anti-HBs) were tested by ELISA with a
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43 102 Monolisa® anti-HBc PLUS kit (Bio-Rad; Hemel Hempstead, UK) and a Monolisa® anti-HBs PLUS kit
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45 103 (Bio-Rad), respectively.

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49 105 **Results**

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52 106 Over the collection period samples were taken from 20,960 first-time, replacement
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54 107 blood donors (18,898 male: 2,062 female). Routine screening identified 200 samples as positive
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3 108 for HBsAg (187 male: 13 female), giving a prevalence of 1% for males and 0.6% for females.
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5 109 Plasma was obtained for 199 HBsAg positive samples, and quantification of HBV DNA load by Q-
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7 110 PCR was successful in 176 (88.4%) of these samples. The median viral load was 1.19×10^3 IU ml⁻¹
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9 111 (range 1.62×10^1 - 8.95×10^8 IU ml⁻¹). In the remaining 23 samples (11.6%), HBV DNA was
10
11 112 undetected by Q-PCR. However amplification of the BCP/PC region was achieved by nested-PCR
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13 113 in one of the 23 samples, meaning detection of HBV DNA was possible for a total of 177/199
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15 114 samples (89%).
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19 115 Complete genomic sequences were obtained for 50 samples (GenBank Accession
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21 116 numbers: JF754586-JF754635), from 57 randomly selected samples in which this was attempted.
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23 117 Phylogenetic analysis identified all strains as belonging to HBV genotype D; 47 clustered as
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25 118 subgenotype D1 (94%), 2 as subgenotype D2 (4%), and 1 as subgenotype D3 (2%). Sequence
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27 119 analysis of the S gene revealed the predominant serotype to be *ayw2* ($n=41$), 5 strains specified
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29 120 *ayw3* and 1 strain was *ayw4*. An additional strain belonged to serotype *adw*, but could not be
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31 121 classified further, and serotype determination was not possible in one strain due to a deletion in
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33 122 this region (Table I).
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39 124 *PreC/C*

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42 125 One sample contained a deletion of 37 nucleotides, resulting in a frameshift mutation
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44 126 and premature protein truncation. In another strain, insertion of two adenine nucleotides in the
45
46 127 precore region caused a frameshift mutation, which is predicted to result in the inclusion of 31
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48 128 amino acids in the precore protein, whilst leaving the core protein unaffected. Cloning of the
49
50 129 BCP/PC for this strain identified the double adenine insertion in all 10 clones sequenced. Serum
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52 130 testing of these two samples identified the presence of anti-HBc. Another sample contained a
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54 131 single adenine insertion within the core region, introducing a premature stop codon and protein
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3 132 truncation. Serum was not available for anti-HBc testing of this sample. Seven samples (14%)
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5 133 contained mutations that abolished the precore initiation codon. The G1896A mutation, which
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8 134 introduces a premature stop codon and abolishes precore protein and HBeAg synthesis, was
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10 135 present in 33 strains (66%).
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14 137 *Pre-S/S*

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17 138 Mutations disrupting the initiation codon of Pre-S2 were identified in 3 samples (6%).
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19 139 Five samples contained deletions in the Pre-S/S transcript, ranging in size from 6 to 51
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21 140 nucleotides. These were present throughout the three ORFs. Three samples contained nonsense
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23 141 mutations that are predicted to result in premature truncation of the protein. One further
24
25 142 sample contained a 66 nucleotide deletion spanning the Pre-S1/Pre-S2 boundary, in addition to
26
27 143 a nonsense mutation leading to premature truncation of the S protein at amino acid residue 69.
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29 144 Testing for the presence of anti-HBs in the serum from which this strain was obtained was
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31 145 negative. Cloning of this sample identified the presence of wild-type species, in addition to a
32
33 146 much smaller product with a 1223 nucleotide deletion (positions 2449-489), predicted to result
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35 147 from splicing of viral mRNA. This deletion started at the final codon of the core gene and
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37 148 extended into the middle of the S gene.
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42 149 The mean overall amino acid divergence within the Pre-S/S region was 2.7% (range 0-
43
44 150 6.9%). The major hydrophilic region (positions 100-169) was more variable, with a mean amino
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46 151 acid substitution rate of 5.3% (range 0-20%). No substitutions associated with vaccine-escape
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48 152 mutants were identified in this cohort.
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53 154 *X gene*
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3 155 No deletions were present within the X transcript in any samples. However, one strain
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5 156 contained a 6 nucleotide insertion, leading to the inclusion of two additional amino acid residues
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8 157 (glycine and methionine) in the X protein, inserted between wild-type residues 79 and 80. The
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10 158 basal core promoter (BCP) variants, A1762T and G1764A were present as a dual mutation in 10
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12 159 strains, with an additional 6 samples carrying G1764A as a single change.
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16 161 *Pol*

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19 162 Six strains carrying deletions in the polymerase gene ranging between 6-66 nucleotides
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21 163 were identified; these were all located in the spacer domain of the protein. There were no
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23 164 disruptions to the highly conserved YMDD motif in any of the 50 strains sequenced. One further
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25 165 sample had a 6 nucleotide insertion within the RNase H domain of the protein.
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31 167 *SP1 screening*

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33 168 Screening for the presence of the SP1 variant was successful in 40/43 samples in which
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35 169 this was attempted (Figure 1). A full genome sequence was available for 27 of the 40
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37 170 successfully amplified samples. The SP1 variant was present in 10/10 (100%) samples with viral
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39 171 loads $\geq 10^6$, in 8/18 (44%) samples with viral loads of $\geq 10^4 - < 10^6$, and in 3/12 (25%) of samples
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41 172 with viral loads $< 10^4$.
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47 174 **Discussion**

48
49 175 Analysis of 50 full HBV genotypes extracted from asymptomatic Turkish blood donors
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51 176 selected at random supports previous findings that the most prevalent HBV strain in this country
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54 177 is subgenotype D1 and serotype *ayw2* [Bozdayi et al., 2005; Sertoz et al., 2008].
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3 178 In previously published complete HBV genomes from chronic Hepatitis B patients from
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5 179 Turkey, the G1896A variant was identified in 18% of samples, and these patients were HBeAg
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8 180 negative [Bozdayi et al., 2005]. In this cohort from healthy volunteer donors, the G1896A
9
10 181 precore stop variant was identified in 66% of samples. This is in keeping with data that shows a
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12 182 high prevalence of the G1896A mutation in samples from Mediterranean populations [Funk et
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14
15 183 al., 2002]. G1896A has previously been documented to be present in 15% of HBeAg-positive
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17 184 Turkish patients, compared to 85% of HBeAg-negative patients [Bozdayi et al., 1999; Bozdayi et
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19 185 al., 2005]. This mutation is of interest as it results in the prevention of HBeAg synthesis, and
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21 186 seroconversion of HBeAg to anti-HBe is correlated with a decline in viral load [Tedder et al.,
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23 187 2002]. The core promoter dual mutations (A1762T, G1764A) were identified previously in 3/11
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25 188 chronic-HBV infected patients, one of whom was HBeAg positive [Bozdayi et al., 2005], and was
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27 189 identified in 20% of the strains sequenced in this study. The double mutation has been
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29 190 implicated with a reduction in the generation of precore mRNA and HBeAg production, and is
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31 191 more prevalent in patients with advanced liver disease [Poustchi et al., 2008]. Given the
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33 192 relatively high frequency of these mutations in this study population, and their clinical
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35 193 implications, it may be of use to sequence the viral genome in subjects testing positive for HBV-
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37 194 infection in Turkey.

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42 195 This is the first time that screening of the SP1 variant has been conducted in
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44 196 asymptomatic blood donors. The data demonstrated that the SP1 variant was present in more
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46 197 than half of the samples screened (21/40), and was ubiquitous in samples with viral loads $\geq 10^6$.
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48 198 However, that SP1 is detected at a much lower frequency in samples with low viral loads (3/10
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50 199 VL < 10^4 IU/mL) may not be directly linked to viral load, but may be a matter of the ratio at
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52 200 which the transcripts are present and the ability of the PCR to detect very low levels of the
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54 201 spliced variant. Previous studies have examined the prevalence in symptomatic HBV-infected
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3 202 patients, and have correlated its presence with chronic infection and hepatocellular carcinoma
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5 203 [Lin et al., 2002]. The association between prevalence of the HBV splice variant and severity of
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8 204 liver disease is not yet elucidated; one study reported an association between a high proportion
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10 205 of spliced HBV: wild-type HBV in patients with more severe liver necroinflammation [Soussan et
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12 206 al., 2008], whereas a different study found no association between spliced HBV prevalence and
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15 207 disease status [Preiss et al., 2008]. Decreased levels of SP1 have been observed in patients with
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17 208 a lamivudine-resistant HBV strain, suggesting that antiviral therapy disrupts the balance
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19 209 between circulating HBV splice variants and full-length HBV genomes [Preiss et al., 2008].

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22 210 In addition, a novel 10.4kDa protein (HBV splice-generated protein; HBSP) arose from
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24 211 the fusion of a section of the polymerase and a new ORF created downstream of the splicing
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26 212 event [Soussan et al., 2000]. Detection by western blot analysis has revealed the presence of
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28 213 this protein in 46% of serum samples from chronic HBV carriers, as opposed to <1% of healthy
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30 214 HBV-negative controls [Soussan et al., 2003]. Functionally, this protein has been demonstrated
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32 215 *in vitro* to increase hepatocyte apoptosis [Soussan et al., 2000], and in humans and transgenic
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34 216 mice to induce a T-cell response [Mancini-Bourgine et al., 2007].
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50 223 and Transplant.
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3 311 **Legends:**
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8 313 **Table I: Molecular and serological characteristics of HBV strains from Turkish blood donors.**
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10 314 Summary of the features identified in the HBV strains sequenced, including deletions,
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12 315 truncations and insertions in the four ORFs, and viral load and SP1 distribution. (HBV: hepatitis B
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15 316 virus)
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20 318 **Figure 1: Representative gel of SP1 screening.** Amplification of full-length sequence results in a
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22 319 1802bp product, whereas spliced HBV DNA, which contains a 1223 nucleotide deletion,
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24 320 generates a 579bp product. Depending on the ratios present, both products may be amplified or
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26 321 alternatively only one amplicon is generated. Sample 1: TK89; Sample 2: TK88 (JF754601);
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28 322 Sample 3: TK129 (JF754618); Sample 4: TK32; Sample 5: TK28 (JF754624); Sample 6: TK74;
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30 323 Sample 7: TK171 (JF754623); Sample 8: TK178; Sample 9: TK63 (JF754608); Sample 10: TK38
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32 324 (JF754591); Sample 11: TK70). (Marker: Bioline, Hyperladder I; HBV: hepatitis B virus)
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325 Table I

Feature	Results				
	(No. of strains in which feature present/No. of strains screened)				
Genotype	D1 (47/50)	D2 (2/50)	D3 (1/50)		
Serotype	adw (1/50)	ayw2 (41/50)	ayw3 (5/50)	ayw4 (1/50)	Unknown (2/50)
X ORF	Deletions (0/50)	Insertions (1/50) (6bp)	Truncations (0/50)		
Pol ORF	Deletions (6/50) (6-66bp)	Insertions (1/50) (6bp)	Truncations (0/50)		
Pre-S/S ORF	Deletions (6/50) (6-66bp)	Insertions (0/50)	Truncations (4/50) (12-474bp)		
Pre-C/C ORF	Deletions (1/50) (37bp)	Insertions (2/50) (1-2bp)	Truncations (2/50) (369- 417bp)	G1896A (33/50)	
Viral load (VL) (IU/mL)	Undetectable (23/199)	<100 (26/199)	$1 \times 10^2 - 9 \times 10^3$ (120/199)	$1 \times 10^4 - 9 \times 10^6$ (22/199)	$>1 \times 10^7$ (8/199)
Splice variant (SP1) prevalence	$VL < \times 10^4$ (3/12)	$VL \geq \times 10^4 -$ $< \times 10^6$ (8/18)	$VL > \times 10^6$ (10/10)		

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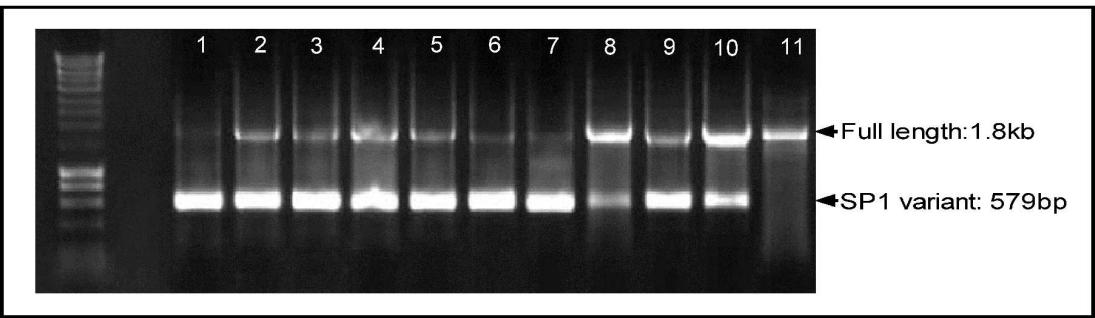
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330 **Figure 1**



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For Peer Review