Subgenotypes and Mutations in the S and Polymerase Genes of Hepatitis B Virus Carriers in the West Bank, Palestine

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Abstract

The mutation rate and genetic variability of hepatitis B virus (HBV) are crucial factors for efficient treatment and successful vaccination against HBV. Until today, genetic properties of this virus among the Palestinian population remain unknown. Therefore, we performed genetic analysis of the overlapping S and polymerase genes of HBV, isolated from 40 Palestinian patients' sera. All patients were HBsAg positive and presented with a viral load above $10^5$ HBV genome copies/ml. The genotyping results of the S gene demonstrated that HBV D1 was detected in 90% of the samples representing the most prominent subgenotype among Palestinians carrying HBV. Various mutations existed within the S gene; in five patients four known escape mutations including the common G145R and D144E were found. Furthermore, a ratio of 4.25 of non-synonymous to synonymous mutations in the S gene indicated a strong selection pressure on the HBs antigen loops of HBV strains circulating in those Palestinian patients. Although all patients were treatment-naïve, with the exception of one, several mutations were found in the HBV polymerase gene, but none pointed to drug resistance. The study presented here is the first report to address subgenotypes and mutation analyses of HBV S and polymerase genes in Palestine.

Introduction

HBV infection remains a health problem worldwide with over two billion infected people and 600 000 deaths yearly [1]. Efficient treatment and vaccination
strategies are persisting challenges due to genetic heterogeneity of HBV DNA. The HBV DNA is only 3.2 kb long with four open reading frames encoding seven viral proteins, two of which are the viral polymerase and the small HBV surface (S) protein which is also named hepatitis B surface antigen (HBsAg). According to the overall nucleotide sequence variations of the entire genome, HBV is classified into nine genotypes (A-I) differing by at least 8% of the DNA sequence \([2, 3]\). These genotypes are furthermore divided into different subgenotypes that differ by at least 4% and are referred to with numbers \([4]\). Subgenotype distribution varies with geographic location; while subgenotype A2 is more common in northern Europe and the USA, A1 and other A subgenotypes are more prevalent in Africa. Genotypes B and C are prevalent in East and Southeast Asia, while D is described to be predominant in the Mediterranean, Near East and Oceania, beside its worldwide distribution \([2, 3]\).

Many studies on HBV subgenotypes analyze only the S gene, which is usually sufficient for accurate typing. At the antigen level, HBsAg is divided into nine major subtypes according to the combination of its common antigen determinant \(a\) with the subtype determinants \(d\) or \(y\), \(w1-4\) or \(r\), \(adw2\), \(adw3\), \(adw4\), \(adr\), \(ayw1\), \(ayw2\), \(ayw3\), \(ayw4\), and \(ayr\) \([5]\). The common antigenic \(a\) determinant in the S gene product is conserved in “normal” HBV strains and formed by conformational epitopes of the amino acids 124–147 \([6, 7, 8]\). Further heterogeneity is caused by point mutations, deletions and by genetic recombination with pre-S genes of different HBV strains \([9]\). HBV-infected recipients of hepatitis B vaccines or occult infected HBV carriers, who develop protective anti-HBs antibodies, may evoke HBV mutants encoding HBsAg with a more or less altered \(a\) determinant or untypical subtype determinants \([10, 11, 12]\). Such mutants can escape the host immune responses, and are therefore called “escape mutants”.

While the N-terminal domain of the viral polymerase forms the terminal protein (TP) linked to the viral DNA, its central domain forms the reverse transcriptase (RT), the coding region of which is largely overlapped by the S gene. The viral RT is an error prone-enzyme, as it lacks a proof reading function, producing HBV mixture of mutants and wild type. Therefore, mutations occur quite often and may be selected for during antiviral therapy \([13]\). The mutation rate of HBV is 10 times higher than that known for other DNA viruses, and is almost as high as that known for the retrovirus HIV \([14, 15]\). The HIV- and HBV-RT inhibiting drug, Lamivudine, is still widely used and is the only drug made available by the Palestinian Ministry of Health for antiviral treatment of HBV-infected patients. However, the highest resistance among licensed HBV antivirals has been attributed to Lamivudine with a yearly rate of 14-32%, reaching 70% after four years of treatment \([16]\). Primary mutations causing Lamivudine resistance are located within the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the viral pol/RT reading frame. An acute hepatitis B infection does not necessarily require therapy, as 90-95% of adults resolve the acute HBV infection and develop immunity \([17]\). However, children are at much higher risk for chronic infection after exposure to HBV \([18]\).
Using tools of molecular biology and bioinformatics, we analyzed the S and RT gene regions of HBV from 40 Palestinian HBV-patients. To the best of our knowledge, this study is the first analysis of mutations and variations within the S and RT genes of HBV conducted in Palestine.

**Methods**

**Ethics statement**

Patients’ data collection, using archived patients’ samples and performing this research were approved by Al-Makassed Islamic Charitable Hospital (MICH) and Al-Quds University ethics committees.

**Patients’ samples**

200 HBsAg positive serum samples from MICH were archived and subjected to viral load testing at the Virology Research Laboratory (VRL), Medical Research Center (MRC), Al-Quds University, Abu Dies, East Jerusalem, Palestine, where this research took place. HBsAg test was performed at MICH Central Laboratory using AxSYM kit HBsAg V2 on the Abbott AxSYM machine according to manufacturer instructions. MICH is the referral hospital for Palestine and the main teaching hospital associated with Al-Quds University Medical School. The archived sera were remnants of tested patients’ samples, which otherwise would have been discarded. Serum samples presented with a viral load above $10^5$ HBV genome copies/ml were subjected to genotyping. In all cases, patient’s names’ were substituted by “AQ-number” codes (AQ-1 to AQ-40). Age, sex and residency of patients were mainly retrieved from patient’s medical file based on MICH approval for archived samples. Once a child was tested positive for HBsAg, parents or guardians were questioned personally regarding the vaccination circumstances. Requests for parents’ serum samples were made orally.

**HBV DNA extraction and viral load**

HBV DNA was extracted from 200 µl serum using QIAamp DNA Mini kit (51304, Qiagen, Germany) according to manufacturer’s instructions. Real-time PCR (rt-PCR) was performed using an ABI Real Time PCR 7500 system (Applied Biosystems, USA). 2.5% of the 50 µl extracted DNA and TaqMan universal master mix (4304437, Applied Biosystems, USA) were used for amplification. The amplification targeted the x gene of the HBV using specific primer pair (XF: 5’-GAC GTC CTT TGT YTA CGT CCC GTC- 3’, XR: 5’- TGC AGA GGT GAA GCG AAG TGCACA- 3’) and probe (FAM 5’- ACG GGG CGC ACC TCT CTT TAC GCG G-3’–MGB [19]). Validated complete genome HBV-DNA (REF. 05960116, Clonit, Italy) at $10^6$, $10^5$, $10^4$, $10^3$, $10^2$ and $10^1$ copies/µl was utilized as standard in all rt-PCR assays. All standards, negative controls, and test samples were tested in duplicate manner. The amplification reaction started with 2 min at 50°C, followed by 10 min at 95°C and final 45 cycles as following: 95°C for 15 sec and 60°C for 1 min.
Amplification of the S and polymerase genes
Primer pair S6 sense (5′-TGG ATG TGT CTG CGG C-3′) and S6 antisense (5′-CKT TGA ACA DAC TTT CCA ATC AAT AG-3′) (validated primer sequences from the Institute of Medical Virology, National Reference Center for Hepatitis B and D Viruses, German Center for Infection Research (DZIF), University of Giessen, Germany) were used to amplify a 621 bp DNA fragment spanning the S and polymerase gene regions. Advantage 2 polymerase mix (639201, Clonetech, USA) was used for the amplification, which started with a single hot start step for 3 min at 95 °C followed by 40 cycles in the following order, 30 sec at 95 °C, 45 sec at 58 °C, and 1 min at 68 °C respectively. An additional extension step was performed for another 5 min at 68 °C. The PCR product was purified using Mini Elute PCR purification Kit (28004, Qiagen, Germany) according to manufacturer’s instructions. Sequencing was performed using Sanger chemistry (ABI PRISM 3130 Genetic Analyzer; Applied Biosystems, USA).

HBV Sequence analysis and typing
Sequences obtained with the forward and reverse primer were used for the sequence analysis of each PCR product belonging to each patient. Sequences were subjected to the NCBI BLAST search for initial identification of the HBV genotype (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nucleotides). For accurate sequence analysis, forward and reverse sequences were aligned with NCBI archived complete HBV genome sequences, representing the genotype identified in NCBI blast search, using the DNASTAR Lasergene MegAlign program (DNASTAR Inc., WI, USA). Lasergene MegAlign program marks nucleotides’ differences between the sequence set as reference and our sequences in red. Thereafter, the sequencing chromatograms of our sequences were inspected visually for these nucleotides and corrected if necessary. This procedure is essential to verify whether a detected nucleotide difference was due to real mutation or to a technical problem in the automatic chromatogram readings.

The corrected Palestinian sequences of the same subgenotype were entered in a new DNASTAR Lasergene MegAlign file, along with different NCBI archived sequences of the same subgenotype. S and polymerase genes for each Palestinian sequence were analyzed separately; synonymous and non-synonymous mutations for each sequence were recorded and searched for through scientific literature. The annotated 40 sequences (AQ-1 to AQ-40) were submitted to GenBank and assigned the accession numbers KC528610-KC528649.

Results
General patients’ data and demographics
Palestinian patients subjected to HBV subgenotype analysis were from all over the West Bank with 27.5% from southern Palestine (Hebron and Bethlehem districts), 45% from the middle (East Jerusalem and Ramallah districts) and 27.5% from
northern Palestine (Nablus, Jenin, Tulkarem and Qalqilya districts). The patients were between 8 months and 80 years old, distributed between 57.5% males to 42.5% females. Two samples from children were tested along with those of their mothers.

HBV infected children of the vaccination era
Of the 40 samples, five (12.5%) belonged to children at the age of 8 months, three years (two children), five and nine years. None of these children was delivered at MICH, which follows a strict TORCH test policy for pregnant women. All five children were infected with HBV subgenotype D1. With the exception of the eight-month-old child, the other four children were tested for HBsAg, as they were scheduled for surgery. Mothers of the eight-month and three-year-old children also had subgenotype D1 and both had sequences 99% identical with their children. The mother of the eight-month-old baby was not aware of her HBV infection; the child received the first two doses of hepatitis B vaccine (Engerix, GSK) but not HBIG and missed the third vaccine dose. The second mother was aware of her HBV infection and assured us the child did indeed receive the three doses of vaccine. Mother of the five-year-old child tested positive for HBsAg and presented with HBV viral load below 100 genome copies/ml. Guardians of the five, nine, and the other three-year-old children assured us that they received all three doses of active vaccination.

HBV genotypes and subgenotypes in the Palestinian patients
The analysis of the S gene from the 40 Palestinian samples revealed that 36 (90%) represented the D1 subgenotype. One (2.5%) sample belonged to the D3 subgenotype and three (7.5%) to the A2 subgenotype respectively.

Mutation analysis of the S gene in Palestinian HBV isolates
Analysis of the S gene in the 36 Palestinian D1 genotype isolates revealed 19 non-synonymous mutations, resulting in 17 different amino acid exchanges, six of which were located in the α determinant (Table 1). Most of these mutations (14) occurred in single cases, three occurred twice and two occurred three times, respectively. Simultaneously, seven synonymous mutations were also detected in the same 36 Palestinian D1 isolates (Table 2).

The only Palestinian D3 case showed six non-synonymous mutations, one of which was in the α determinant, and three synonymous mutations (Tables 3 and 4).

L209V was the only amino acid exchange detected in the S gene of the three A2 Palestinian genotypes, due to T779G point mutation. Two synonymous mutations were found in one of the three samples at position C406T (L84) and A436G (L94). The nucleotide position was defined based on A2 subgenotype GenBank archived sequence X51970.
Mutations occurring in the RT gene in Palestinian HBV isolates
Twenty-three non-synonymous RT gene mutations were recorded in the 36 D1 Palestinian HBV isolates (Table 5). Most mutations were caused by single nucleotide substitutions. However, the F122I mutation was caused by three different nucleotide substitutions at position 493. Three further mutations;

| Table 1. Non-synonymous mutations in the S region of Palestinian D1 subgenotypes. |
|----------------------------------------|-----------------|-----------------|-----------------|
| nt position | aa position | Occurrence | Reported function/detected in |
| 410:A/T    | I86F        | 1            | Unknown/Chronic HBV carriers with D1 subgenotype |
| 429:T/C    | I92T        | 1            | Unknown/Subgenotype C1 |
| 482:A/C    | I110L       | 1            | Unknown/solely anti-HBc-positive sera, genotype A |
| 484:T/G    | I110L       | 1            | Unknown/solely anti-HBc-positive sera, genotype A |
| 531: C/G   | T126S       | 1            | Escape mutation |
| 533: C/T   | P127S       | 1            | Escape mutation |
| 555:A/T    | Y134F       | 2            | Unknown/solely anti-HBc-positive sera, genotype D |
| 581:T/A    | S143T       | 1            | Unknown/solely anti-HBc-positive sera, genotype D |
| 586: C/A   | D144E       | 2            | Escape mutation |
| 587: G/A   | G145R       | 3            | Escape mutation |
| 720:C/T    | T189I       | 1            | Reduces HBsAg detection signal of genotype E |
| 753:A/C    | Y200F       | 1            | Unknown/naive patients |
| 765:G/A    | S204R       | 1            | Unknown/genotype E |
| 771:A/T    | Y206L       | 1            | Unknown |
| 772:G/T    | Y206L       | 1            | Unknown |
| 774:G/A    | S207N       | 2            | Unknown/solely anti-HBc-positive sera, genotype D |
| 784:T/A    | S210R       | 1            | Unknown/genotype A |
| 791:T/A    | L213I       | 3            | Unknown/genotypes D and C |
| 791:T/A    | L213F       | 1            | Unknown/solely anti-HBc-positive sera, genotype D |

Positions of the nucleotide (nt) mutation and the correlating amino acid (aa) are presented based on their location in the archived GenBank reference DQ315778. Occurrence reflects the number of samples (patients) in which the mutation was detected. Known escape mutations are in boldface. Exchanges marked with (*) are considered polymorphisms due to their prevalence in >10% of the 40 patients. Corresponding references and proposed functions are provided in the last two columns. Unreported mutations were considered novel.

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Mutations occurring in the RT gene in Palestinian HBV isolates
Twenty-three non-synonymous RT gene mutations were recorded in the 36 D1 Palestinian HBV isolates (Table 5). Most mutations were caused by single nucleotide substitutions. However, the F122I mutation was caused by three different nucleotide substitutions at position 493. Three further mutations;

| Table 2. Synonymous mutations in the S region of Palestinian D1 subgenotypes. |
|----------------------------------------|-----------------|-----------------|
| nt position | aa position |
| 457:A/G    | Q101Q        | 2            |
| 493:T/(A,C,G) | S113S    | 11*          |
| 499:T/(C,A) | T115T       | 7*           |
| 538:T/A    | A123A        | 2            |
| 562:C/A    | S136S        | 1            |
| 619:C/T    | S155S        | 4*           |
| 784:T/C    | S210S        | 1            |

Positions of the nucleotide (nt) mutation and the correlating amino acid (aa) are presented based on their location in the archived GenBank reference DQ315778. Occurrence reflects the number of samples (patients) in which the mutation was detected. Exchanges marked with (*) are considered polymorphisms due to their prevalence in >10% of the 40 patients.

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H124Y, S219P and I266K were caused by two different nucleotide substitutions at positions 499, 784 and 926 respectively (Table 5). Eleven synonymous gene mutations were recorded in the RT gene region of the 36 Palestinian D1 samples (Table 6). C619T (L169) was the most common synonymous mutation, occurring in four samples (Table 6). Other synonymous mutations occurred in 3, 2, or 1 sequence respectively.

Seven non-synonymous mutations and six synonymous mutations were found in the RT region of the only Palestinian D3 isolate (Tables 7 and 8). Six non-synonymous mutations and five synonymous mutations were detected in the RT gene region of the three Palestinian A2 samples (Tables 9 and 10). Non-synonymous mutations L217R and I253V and synonymous mutations Y252, G258, and K268 were present in all A2 samples.

Polymorphism and the ratio of non-synonymous to synonymous mutations

Seventeen non-synonymous and seven synonymous mutations were found in the S gene of the 36 Palestinian D1 subgenotypes (Tables 1 and 2). Three of the synonymous mutations were referred to as polymorphism due to their occurrence in more than 10% of the total 40 samples (Table 2). The ratio of non-synonymous (17) to synonymous non polymorphic mutation (4) in the S gene of the D1 subgenotypes was 4.25.

Table 3. Non-synonymous mutations in the S region of Palestinian D3 subgenotype.

| nt position | aa position | Reported function/detected in | Reference |
|-------------|-------------|-------------------------------|-----------|
| 528: C/T    | T125M       | Increases HBsAg reactivity in immunological diagnostic assays | [66]      |
| 753: A/T    | Y200F       | Unknown/antiviral therapy     | [64]      |
| 762: C/A    | P203Q       | Causes discrepant results in some HBsAg detection assays | [87]      |
| 766: T/A    | S204R       | Unknown, genotype E           | [34]      |
| 770:T/A, 771: A/C | Y206T | Unknown | Novel |
| 774: G/A    | S207N       | Unknown/solely anti-HBc-positive sera, genotype D | [34]      |

Positions of the nucleotide (nt) mutation and the correlating amino acid (aa) are presented based on their location in the archived GenBank reference JF754625. Corresponding references and proposed functions are provided in the last two columns. Unreported mutations were considered novel.

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Table 4. Synonymous mutations in the S region of Palestinian D3 subgenotype.

| nt position | aa position |
|-------------|-------------|
| 532:T/C     | T126T       |
| 562:C/A     | S136S       |
| 616:A/G     | S154S       |

Positions of the nucleotide (nt) mutation and the correlating amino acid (aa) are presented based on their location in the archived GenBank reference JF754625.

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Eleven synonymous mutations were found in the RT region of D1 subgenotypes (Table 6). One of these synonymous mutations and five of the 23 non-synonymous mutations were classified as polymorphisms, as they were prevalent in more than 10% of the 40 total samples (Tables 5 and 6). The ratio of non-synonymous (18) to synonymous (10) mutations without polymorphism was 1.8, which is significantly less than that for the S region.

**Discussion**

HBV genotype D was the most prominent among Palestinian patients. Only 7.5% of the samples had genotype A. The predominance of genotype D is consistent with regional reports from Egypt, Jordan, Israel, Syria, and Lebanon.
Genotype D has a worldwide distribution; but it is predominant in some regions, while a minor component in others. Regions of high genotype D prevalence occur in the Mediterranean and in large parts of Asia, except East and South East Asia [2]. The most prominent subgenotype among Palestinians was D1, detected in 90% of the samples. Subgenotype D1 is the most common subgenotype in Turkey, Greece, Iran, Pakistan, Egypt, Lebanon, Israel and others [2, 25, 26, 27, 28, 29, 30]. One single Palestinian sample belonged to D3 subgenotype. Subgenotype D3 is found prominently in Europe [2], but some regional studies report a low prevalence of D3 subgenotype [26, 27]. Surprisingly three Palestinian samples belonged to subgenotype A2. Subgenotype A2 is common in Northern and Central Europe and in the European offspring of Caucasians living in South Africa and USA [2]. Genotype A is barely reported in the region, with the exception of one report from Egypt, where a mix of genotypes

### Table 6. Synonymous mutations in the RT region of Palestinian D1 subgenotypes.

| nt position | aa position | Occurrence |
|-------------|-------------|------------|
| 555:A/T     | V142V       | 2          |
| 619:C/T     | L168L       | 4*         |
| 720:C/T     | H117H       | 1          |
| 774:G/A     | Q215Q       | 2          |
| 853:A/C     | R242R       | 1          |
| 888:C/A     | V253V       | 2          |
| 906:A/C     | S259S       | 1          |
| 907:T(A,C)  | L260L       | 2          |
| 909:G/A     | L260L       | 1          |
| 969:G/A     | R280R       | 1          |
| 987:C(G,T,A)| V286V       | 3          |

Positions of the nucleotide (nt) mutation and the correlating amino acid (aa) are presented based on their location in the archived GenBank reference DQ315778. Occurrence reflects the number of samples (patients) in which the mutation was detected. Exchanges marked with (*) are considered polymorphisms due to their prevalence in >10% of the 40 patients.

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### Table 7. Non-synonymous mutations in the RT region of Palestinian D3 subgenotypes.

| nt position | aa position | Reported Function/detected in | Reference |
|-------------|-------------|-------------------------------|-----------|
| 532:T/C     | Y135H       | Unknown/naive patients        | [69]      |
| 562:C/A     | L145M       | Unknown/naive patients        | [70]      |
| 616:A/G     | I163V       | Unknown                       | [71]      |
| 766:T/A     | S213T       | Candidate mutation associated with hepatocellular carcinoma | [74] |
| 770:T/A     | V214D       | Unknown/naive patient         | GenBank:FJ904404 |
| 895:T/A     | C256S       | Unknown                       | [71]      |
| 926:T/A     | I266K       | Unknown/patients treated with NRTIs | [68] |

Positions of the nucleotide (nt) mutation and the correlating amino acid (aa) are presented based on their location in the archived GenBank reference JF754625. Corresponding references and proposed functions are provided in the last two columns. NRTIs: nucleoside and/or nucleotide reverse-transcriptase inhibitors.

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D and A were detected in pediatric cancer patients [21]. Interestingly, one of the three patients with A2 genotype had received blood transfusion. We propose that this blood was not donated from local donor but rather from a foreign donor. However, we were unable to obtain further information regarding this or regarding the other two A2 samples.

The ratio of non-synonymous to synonymous non-polymorphic mutations is an indicator for the evolutionary relevance of a set of mutations [32]. Ratios below 1 imply that these mutations are genetically neutral as suggested by Gojobori et al. (1990) for HBV and other viruses [14]. A ratio of 4.25 for the S genes indicates a strong selection effect on HBV strains circulating in the Palestinian patients that were studied. Evidence for this selection may be the amino acid exchanges I110L and Y206L; each occurred twice in Palestinian patients and was caused each time by different nucleotide substitution events. The selection affected only the two last third of the S gene upstream of aa85, while the transmembrane helix I and the internal loop of HBsAg were completely conserved.

Six of the non-synonymous mutations in the D1 subgenotypes were found in the a determinant of the S gene, three were found upstream and eight downstream the a determinant respectively. Table 1 shows that the mutations found upstream–I86F, I92T, and I110L–and those found downstream–T189I, Y200F,
S204R, Y206L, S207N, S210R, L213I, and L213F–were recorded in different studies but were not associated with critical functions (Table 1).

The six mutations found in the a determinant displayed different characteristics. The amino acid exchange Y134F found in two patients correlated either with the HBsAg genotype A/adw2 [33] or genotype D/ayw2 [34]. S143 is typical for genotype A and T143 for genotype D [34]. The other four mutations found in the a determinant–T126S, P127S, D144E, and G145R–are known escape mutants [35, 36, 37, 38] and were detected in five Palestinian D1 samples accounting for 12.5% of the total 40 samples. Two of these samples presented with the escape mutations D144E and G145R simultaneously, while the other three samples presented with one single escape mutation each; G145R, T126S, or P127S.

Mutations in the a determinant of the S gene occur frequently in occult HBV infection, and are a potential risk to blood safety [39, 40]. This is the case, when HBsAg is seemingly absent in the presence of HBV DNA in serum, which was, however, not the case in our sample. Previous HBsAg assays often failed to detect HBsAg with mutations in the HBsAg loop but last generation assays can detect most HBsAg escape mutants if they are present in sufficient concentration [41, 42]. Different studies demonstrate that HBV viruses carrying antibody escape mutations have reduced binding affinity of anti-HBs antibodies to HBsAg, including vaccine generated antibodies, which may allow infection despite vaccination [10, 12, 35, 36, 43, 44]. All escape mutants detected in our study were found in patients, who were above 36 years of age, which means they were not subjected to vaccination. It is probably that an unrecognized antibody response of the patients against their own HBsAg had exerted some selective pressure in favor of classical escape mutants and mutations to an amino acid associated with another genotype like Y134F and S143T. According to earlier reports, S gene mutations accumulate in chronic HBV infection, particularly after development of immune pathogenesis or loss of HBeAg [45].

None of the six non-synonymous mutations found in the only Palestinian D3 sample was attributed to critical functional impact on viral activity (Table 3), in contrast, L209V, the only mutation found in the S gene of all three Palestinian A2 samples was reported in transplant recipients, who received HBIG [33] and in vaccinated individuals [46]. In the second report, authors propose that antibodies

| nt position | aa position | Occurrence |
|-------------|-------------|------------|
| 885:C/T     | Y252Y       | 3          |
| 903:A/G     | G258G       | 3          |
| 933:G/A     | K268K       | 3          |
| 987:A/C     | V286V       | 2          |
| 994:A/C     | R289R       | 2          |

Positions of the nucleotide (nt) mutation and the correlating amino acid (aa) are presented based on their location in the archived GenBank reference X51970.

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produced due to vaccination may not be effective in neutralizing HBV mutants including the L209V in genotype E [47].

Twenty-three, seven and six non-synonymous mutations were detected in the RT region of the Palestinian D1, D3, and A2 isolates respectively (Tables 5, 7, and 9). Although few of these mutations affect the viral polymerase activity, none was reported to confer drug resistance (Tables 5, 7, and 9).

Out of the 40 samples analyzed here, five belonged to children, who were vaccinated against HBV. These children were born at Palestinian public hospitals. Contrary to MICH policy and that of private clinics in Palestine, screening pregnant women for HBsAg and so administrating HBIG immediately after birth does not belong to the policy of the Palestinian Ministry of Health. Based on our data, we assume that all five children were suffering from breakthrough infections. Indeed, this is proven for the two cases with the children HBV sequences almost identical to their mothers. Breakthrough is strongly suggested in the five-year-old child as the mother tested positive for both HBsAg and HBV DNA. It had been shown earlier that the rate of perinatal transmission of HBV from infected mothers to offspring varies from 5 % to 31 % in HBsAg positive mothers to 85 % to over 90 % in HBeAg positive mothers or those presenting with high viral load [48, 49, 50, 51, 52, 53]. One factor favoring breakthrough in spite of immediate active vaccination at birth may be the fact that the vaccine has subgenotype A2 and HBsAg adw2, whereas the transmitted HBV in these cases were D1/ayw2. Vaccine-induced protection seems to be weaker against heterologous genotypes [18]. The fact that these children did not receive HBIG may have contributed to the breakthrough, even though the additional effect of HBIG to active vaccination has not been fully established [54]. It is widely accepted and recommended that the most efficient precaution for prevention of perinatal HBV transmission is screening pregnant women for HBsAg and the immediate administration of one infant dose of hepatitis B immune globulin (HBIG) along with the first hepatitis B vaccine dose within 12 hours after birth, followed by the second and the third vaccine dose at 1 and 6 month of age [55, 56, 57, 58, 59].

Screening mothers worldwide leads to significant reduction in the infection of their children [53, 60, 61, 62]. We hope that addressing this critical factor can advocate the introduction of screening pregnant women for HBsAg all over Palestine.

Finally, identifying specific genotypes and mutants of pathogens in different geographic areas is crucial for understanding clinical severity of the infection and developing control measures according to the predominant local strains, and can contribute to optimization of therapy, vaccines and diagnostic tools.

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Author Contributions
Conceived and designed the experiments: ZA MA. Performed the experiments: ZA NS. Analyzed the data: ZA MA. Contributed reagents/materials/analysis tools: MA DG SB. Wrote the paper: MA.

References

1. Ott JJ, Stevens GA, Groeger J, Wiersma ST (2012) Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. Vaccine 30: 2212–2219.

2. Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, et al. (2004) Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology 47: 289–309.

3. Kramvis A (2014) Genotypes and genetic variability of hepatitis B virus Intervirology 57: 141–150.

4. Ma Y, Ding Y, Juan F, Dou XG (2011) Genotyping the hepatitis B virus with a fragment of the HBV DNA polymerase gene in Shenyang, China. Virology journal 8: 315.

5. Courouce-Pauty AM, Soulier JP (1974) Further data on HBs antigen subtypes - geographical distribution. Vox sanguinis 27: 533–549.

6. Brown SE, Howard CR, Zuckerman AJ, Steward MW (1984) Affinity of antibody responses in man to hepatitis B vaccine determined with synthetic peptides. Lancet 2: 184–187.

7. Guerrero E, Swenson PD, Hu PS, Peterson DL (1990) The antigenic structure of HBsAg: study of the d/y subtype determinant by chemical modification and site directed mutagenesis. Molecular immunology 27: 435–441.

8. Howard CR, Stirk H, Brown SE, Steward MW (1988) Towards the development of synthetic hepatitis B vaccines. Viral Hepatitis and Liver Disease: 1094–1101.

9. Gunther S, Fischer L, Pult I, Sterneck M, Will H (1999) Naturally occurring variants of hepatitis B virus. Advances in virus research. 1999/06/29 ed: Academic Press. pp.25–137.

10. Carman WF, Zanetti AR, Karayiannis P, Waters J, Manzillo G, et al. (1990) Vaccine-induced escape mutant of hepatitis B virus. Lancet 336: 325–329.

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

12. Karthigesu BD, Allison LM, Fortuin M, Mendy M, Whittle HC, et al. (1994) A novel hepatitis B virus variant in the sera of immunized children. The Journal of general virology 75 (Pt2): 443–448.

13. Ghany M, Liang TJ (2007) Drug targets and molecular mechanisms of drug resistance in chronic hepatitis B. Gastroenterology 132: 1574–1585.

14. Gojobori T, Moriyama EN, Kimura M (1990) Molecular clock of viral evolution, and the neutral theory. Proceedings of the National Academy of Sciences of the United States of America 87: 10015–10018.

15. Locarinin S (2004) Molecular Virology of Hepatitis B Virus. Molecular Virology 24: 03–10.
16. Lai CL, Dienstag J, Schiff E, Leung NW, Atkins M, et al. (2003) Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 36: 687–696.

17. Yu JW, Sun LJ, Yan BZ, Kang P, Zhao YH (2011) Lamivudine treatment is associated with improved survival in fulminant hepatitis B. Liver international: official journal of the International Association for the Study of the Liver 31: 499–506.

18. Stramer SL, Wend U, Candotti D, Foster GA, Hollinger FB, et al. (2011) Nucleic acid testing to detect HBV infection in blood donors. The New England journal of medicine 364: 236–247.

19. Jursch CA, Gerlich WH, Glebe D, Schaefer S, Marie O, et al. (2002) Molecular approaches to validate disinfectants against human hepatitis B virus. Medical microbiology and immunology 190: 189–197.

20. Khaled IA, Mahmoud OM, Saleh AF, Bioumie EE (2011) Prevalence of HBV genotypes among Egyptian hepatitis patients. Molecular biology reports 38: 4353–4357.

21. Zekri AR, Hafez MM, Mohamed NI, Hassan ZK, El-Sayed MH, et al. (2007) Hepatitis B virus (HBV) genotypes in Egyptian pediatric cancer patients with acute and chronic active HBV infection. Virology journal 4: 74.

22. Masaadeh HA, Hayajneh WA, Alqudah EA (2008) Hepatitis B virus genotypes and lamivudine resistance mutations in Jordan. World journal of gastroenterology: WJG 14: 7231–7234.

23. Ben-Ari Z, Mor E, Bar-Nathan N, Shaharabani E, Shapira Z, et al. (2003) Combination hepatitis B immune globulin and lamivudine versus hepatitis B immune globulin monotherapy in preventing recurrent hepatitis B virus infection in liver transplant recipients. Transplantation proceedings 35: 609–611.

24. Antaki N, Haffar S, Ali Deeb S, Assaad F, Abou Harb R, et al. (2010) High prevalence of HBV genotype D in Syria and the clinical characteristics of hepatitis B e antigen-negative chronic hepatitis B. Epidemiology and infection 138: 40–44.

25. El Chaar M, El Jisr T, Allain JP (2012) Hepatitis B virus DNA splicing in Lebanese blood donors and genotype A to E strains: implications for hepatitis B virus DNA quantification and infectivity. Journal of clinical microbiology 50: 3159–3167.

26. Cox LE, Arslan O, Allain JP (2011) Characterization of hepatitis B virus in Turkish blood donors, and the prevalence of the SP1 splice variant. Journal of medical virology 83: 1321–1325.

27. Sayan M, Dogan C (2012) Genotype/subgenotype distribution of hepatitis B virus among hemodialysis patients with chronic hepatitis B. Annals of hepatology 11: 849–854.

28. Fylaktou A, Papaventsis D, Daoudaki M, Moskophidis M, Reiberger T, et al. (2011) Molecular epidemiology of chronic hepatitis B virus infection in Greece. Journal of medical virology 83: 245–252.

29. Garmiri P, Rezvan H, Abolghasemi H, Allain JP (2011) Full genome characterization of hepatitis B virus strains from blood donors in Iran. Journal of medical virology 83: 948–952.

30. Baig S, Siddiqui A, Chakravarty R, Moatther T (2009) Hepatitis B virus subgenotypes D1 and D3 are prevalent in Pakistan. BMC research notes 2: 1.

31. Ragheb M, Elkady A, Tanaka Y, Murakami S, Attia FM, et al. (2012) Multiple intra-familial transmission patterns of hepatitis B virus genotype D in north-easteren Egypt. Journal of medical virology 84: 587–595.

32. Hanada K, Shiu SH, Li WH (2007) The nonsynonymous/synonymous substitution rate ratio versus the radical/conservative replacement rate ratio in the evolution of mammalian genes. Molecular biology and evolution 24: 2235–2241.

33. Ghany MG, Ayola B, Villamil FG, Gish RG, Rojter S, et al. (1998) Hepatitis B virus S mutants in liver transplant recipients who were reinfected despite hepatitis B immune globulin prophylaxis. Hepatology 27: 213–222.

34. Weinberger KM, Bauer T, Bohm S, Jilg W (2000) High genetic variability of the group-specific a-determinant of hepatitis B virus surface antigen (HBsAg) and the corresponding fragment of the viral polymerase in chronic virus carriers lacking detectable HBsAg in serum. The Journal of general virology 81: 1165–1174.

35. Komatsu H, Inui A, Sogo T, Konishi Y, Tateno A, et al. (2012) Hepatitis B surface gene 145 mutant as a minor population in hepatitis B virus carriers. BMC research notes 5: 22.
36. Mele A, Tancredi F, Romano L, Giuseppone A, Colucci M, et al. (2001) Effectiveness of hepatitis B vaccination in babies born to hepatitis B surface antigen-positive mothers in Italy. The Journal of infectious diseases 184: 905–908.

37. Torresi J, Earnest-Silveira L, Deliyannis G, Edgerton K, Zhuang H, et al. (2002) Reduced antigenicity of the hepatitis B virus HBsAg protein arising as a consequence of sequence changes in the overlapping polymerase gene that are selected by lamivudine therapy. Virology 293: 305–313.

38. Zuckerman JN, Zuckerman AJ (2003) Mutations of the surface protein of hepatitis B virus. Antiviral research 60: 75–78.

39. Allain JP, Mihaljevic I, Gonzalez-Fraile MI, Gubbe K, Holm-Harristhoj L, et al. (2013) Infectivity of blood products from donors with occult hepatitis B virus infection. Transfusion 53: 1405–1415.

40. Gerlich WH, Bremer C, Saniewski M, Schuttler CG, Wend UC, et al. (2010) Occult hepatitis B virus infection: detection and significance. Digestive diseases 28: 116–125.

41. Lee JM, Ahn SH (2011) Quantification of HBsAg: basic virology for clinical practice. World journal of gastroenterology: WJG 17: 283–289.

42. Weber B (2005) Genetic variability of the S gene of hepatitis B virus: clinical and diagnostic impact. Journal of clinical virology: the official publication of the Pan American Society for Clinical Virology 32: 102–112.

43. Chang MH (2010) Breakthrough HBV infection in vaccinated children in Taiwan: surveillance for HBV mutants. Antiviral therapy 15: 463–469.

44. Locarnini SA, Yuen L (2010) Molecular genesis of drug-resistant and vaccine-escape HBV mutants. Antiviral therapy 15: 451–461.

45. Hannoun C, Horal P, Lindh M (2000) Long-term mutation rates in the hepatitis B virus genome. The Journal of general virology 81: 75–83.

46. Mathet VL, Cuestas ML, Ruiz V, Minassian ML, Rivero C, et al. (2006) Unusual naturally occurring humoral and cellular mutated epitopes of hepatitis B virus in a chronically infected argentine patient with anti-HBs antibodies. Journal of clinical microbiology 44: 2191–2198.

47. Mathet VL, Cuestas ML, Ruiz V, Minassian ML, Rivero C, et al. (2006) Detection of hepatitis B virus (HBV) genotype E carried–even in the presence of high titers of anti-HBs antibodies–by an Argentinian patient of African descent who had received vaccination against HBV. Journal of clinical microbiology 44: 3435–3439.

48. Stevens CE, Beasley RP, Tsui J, Lee WC (1975) Vertical transmission of hepatitis B antigen in Taiwan. The New England journal of medicine 292: 771–774.

49. Okada K, Kamiyama I, Inomata M, Imai M, Miyakawa Y (1976) e antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. The New England journal of medicine 294: 746–749.

50. Beasley RP, Trepo C, Stevens CE, Szmuness W (1977) The e antigen and vertical transmission of hepatitis B surface antigen. American journal of epidemiology 105: 94–98.

51. Beasley RP, Hwang LY, Lee GC, Lan CC, Roan CH, et al. (1983) Prevention of perinatally transmitted hepatitis B virus infections with hepatitis B virus infections with hepatitis B immune globulin and hepatitis B vaccine. Lancet 2: 1099–1102.

52. Burk RD, Hwang LY, Ho GY, Shafritz DA, Beasley RP (1994) Outcome of perinatal hepatitis B virus exposure is dependent on maternal virus load. The Journal of infectious diseases 170: 1418–1423.

53. Chang MH (2007) Hepatitis B virus infection. Seminars in fetal & neonatal medicine 12: 160–167.

54. Lee C, Gong Y, Brok J, Boxall EH, Gliudc C (2006) Effect of hepatitis B immunisation in newborn infants of mothers positive for hepatitis B surface antigen: systematic review and meta-analysis. BMJ 332: 328–336.

55. Piitt SS, Somily AM, Singh AE (2007) Outcomes from a Canadian public health prenatal screening program for hepatitis B: 1997–2004. Canadian journal of public health = Revue canadienne de sante publique 98: 194–197.

56. Spada E, Tosti ME, Zuccaro O, Stroffolini T, Mele A (2011) Evaluation of the compliance with the protocol for preventing perinatal hepatitis B infection in Italy. The Journal of infection 62: 165–171.
57. McMahon BJ, Bulkow LR, Singleton RJ, Williams J, Snowball M, et al. (2011) Elimination of hepatocellular carcinoma and acute hepatitis B in children 25 years after a hepatitis B newborn and catch-up immunization program. Hepatology 54: 801–807.

58. Harder KM, Cowan S, Eriksen MB, Krarup HB, Christensen PB (2011) Universal screening for hepatitis B among pregnant women led to 96% vaccination coverage among newborns of HBsAg positive mothers in Denmark. Vaccine 29: 9303–9307.

59. Hu Y, Zhang S, Luo C, Liu Q, Zhou YH (2012) Gaps in the prevention of perinatal transmission of hepatitis B virus between recommendations and routine practices in a highly endemic region: a provincial population-based study in China. BMC infectious diseases 12: 221.

60. Shepard CW, Simard EP, Finelli L, Fiore AE, Bell BP (2006) Hepatitis B virus infection: epidemiology and vaccination. Epidemiologic reviews 28: 112–125.

61. Ni YH, Huang LM, Chang MH, Yen CJ, Lu CY, et al. (2007) Two decades of universal hepatitis B vaccination in taiwan: impact and implication for future strategies. Gastroenterology 132: 1287–1293.

62. Willis BC, Wortley P, Wang SA, Jacques-Carroll L, Zhang F (2010) Gaps in hospital policies and practices to prevent perinatal transmission of hepatitis B virus. Pediatrics 125: 704–711.

63. Norouzi M, Ghorashi S, Abedi F, Nejatizadeh A, Ataei B, et al. (2012) Identification of Hepatitis B Virus Surface Antigen (HBsAg) Genotypes and Variations in Chronic Carriers from Isfahan Province, Iran. Iranian journal of public health 41: 104–111.

64. Kim JH, Jung YK, Joo MK, Yim HJ, Park JJ, et al. (2010) Hepatitis B viral surface mutations in patients with adefovir resistant chronic hepatitis B with A181T/V polymerase mutations. Journal of Korean medical science 25: 257–264.

65. Olinger CM, Weber B, Otegbayo JA, Ammerlaan W, van der Taelem-Brule N, et al. (2007) Hepatitis B virus genotype E surface antigen detection with different immunoassays and diagnostic impact of mutations in the preS/S gene. Medical microbiology and immunology 196: 247–252.

66. Araujo NM, Vianna CO, Moraes MT, Gomes SA (2009) Expression of Hepatitis B virus surface antigen (HBsAg) from genotypes A, D and F and influence of amino acid variations related or not to genotypes on HBsAg detection. The Brazilian journal of infectious diseases: an official publication of the Brazilian Society of Infectious Diseases 13: 266–271.

67. Geretti AM, Patel M, Sarfo FS, Chadwick D, Verheyen J, et al. (2010) Detection of highly prevalent hepatitis B virus coinfection among HIV-seropositive persons in Ghana. Journal of clinical microbiology 48: 3223–3230.

68. Margeridon-Thermet S, Shulman NS, Ahmed A, Shahriar R, Liu T, et al. (2009) Ultra-deep pyrosequencing of hepatitis B virus quasispecies from nucleoside and nucleotide reverse-transcriptase inhibitor (NRTI)-treated patients and NRTI-naive patients. The Journal of infectious diseases 199: 1275–1285.

69. Colonno RJ, Rose R, Baldick CJ, Levine S, Pokornowski K, et al. (2006) Entecavir resistance is rare in nucleoside naive patients with hepatitis B. Hepatology44: 1656–1665.

70. Katsoulidou A, Paraskevis D, Magiorkinis E, Moschidis Z, Haida C, et al. (2009) Molecular characterization of occult hepatitis B cases in Greek blood donors. Journal of medical virology 81: 815–825.

71. Geipel A (2011) Phänotypische Charakterisierung klinisch relevanter Hepatitis-B-Virus-Mutanten: university Gießen.

72. Costantini A, Marinelli K, Biagioni G, Monachetti A, Ferreri ML, et al. (2011) Molecular analysis of hepatitis B virus (HBV) in an HIV co-infected patient with reactivation of occult HBV infection following discontinuation of lamivudine-including antiretroviral therapy. BMC infectious diseases 11: 310.

73. Pollicino T, Isgro G, Di Stefano R, Ferraro D, Maimone S, et al. (2009) Variability of reverse transcriptase and overlapping S gene in hepatitis B virus isolates from untreated and lamivudine-resistant chronic hepatitis B patients. Antiviral therapy 14: 649–654.

74. Wu Y, Gan Y, Gao F, Zhao Z, Jin Y, et al. (2014) Novel natural mutations in the hepatitis B virus reverse transcriptase domain associated with hepatocellular carcinoma. PloS one 9: e94864.