Identification of Optimal Target Gene Regions for Hepatitis B Virus Genotyping by DNA Sequencing

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Key Words

Hepatitis B virus genotyping · DNA sequencing · Target gene region · Reference genotypes · NCBI genotyping tool · Referential genotyping guide

Abstract

Objectives: DNA sequencing is the gold standard for hepatitis B virus (HBV) genotyping. We investigated the intergenotypic discriminatory capabilities of various target gene regions over the entire HBV genome, introducing a novel data evaluation approach generally applicable in viral genotyping. **Methods:** Complete genome sequences of seventy HBV variants obtained from the sera of 50 Syrian patients were determined and assigned GenBank accession No. from JN257148 to JN257217. Nucleotide sequence contigs were analyzed together with the NCBI reference genome set of HBV genotypes. Nine target gene regions were analyzed by phylogenetic and scored BLAST analyses. Thirty-one overlapping 300-bp sequence segments over the entire genome were also analyzed using a scored BLAST analysis, and intergenotypic discriminatory capabilities were statistically estimated for each. **Results:** Intergenotype discrimination was extremely significant when targeting either the complete genome, the entire coding sequence of either P or S genes,

or any 300-bp sequence segment over the coding sequences of S protein or the polymerase N-terminal domain. Interestingly, intergenotypic discriminatory capability correlated negatively with intragenotype variation. **Conclusions:** The intragenotypic conservation of certain target gene regions determines the intergenotypic discriminatory capability and allows reliable genotyping with relatively short segments. Our referential genome-wide tabulated guide allows for selecting candidate target gene regions for sequencing-based HBV genotyping.

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Introduction

Hepatitis B virus (HBV) infection is highly prevalent worldwide, characterized by high infection and mortality rates, and hence considered one of the major public health issues. Chronically infected patients are at high risk of developing hepatic cirrhosis and hepatocellular carcinoma [1, 2]. HBV is a DNA virus with a circular partially double-stranded 3.2-kb genome [3]. At least eight HBV genotypes (A through H) have been identified based on genome-wide intergenotypic diversity [4]. Their occurrence is associated with ethnic origins and geographical

regions, and gives rise to various clinical outcomes, such as response to antiviral therapy, liver disease progression, mutant selection [5, 6] and vaccine effectiveness [7]. HBV genotyping, therefore, has become a valuable tool both epidemiologically towards tracing the infection routes and clinically in relation to patient management and therapeutic decisions [8, 9].

Among several molecular techniques described for HBV genotyping, DNA sequencing is the gold standard [1, 2, 10, 11] recommended to resolve result discordance [2] and detect recombinant genotypes [5]. To date, diverse target gene regions for HBV genotyping by DNA sequencing have been reported, such as the entire genome [6, 11], the S gene overlapping the reverse transcriptase region of the P gene [1, 4, 12] or the pre-S region [2, 13–15]. There is no consensus regarding the most suitable target region [2], and no comprehensive systematic research has been conducted to explore the optimum target gene regions over the entire viral genome. Our study aimed at investigating the intergenotypic discriminatory capabilities of various target gene regions over the entire genome of reference and clinical HBV sequences utilizing a novel data evaluation approach.

Materials and Methods

Specimens

Serum samples from 50 Syrian hepatitis B patients were drawn between August 2008 and April 2010. Only samples with HBV DNA concentrations ≥ 6 log copies/ml were included to increase the sensitivity of detecting various HBV variants in mixed virus populations. Written informed consent was obtained from all enrolled patients. Viral DNA was extracted from 200 μ l serum using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Germany), and 50- μ l-volume DNA extracts were kept at -80° for further testing.

Complete HBV Genome Sequencing

PCR and cycle sequencing reactions included up to 32-fold degeneracy primers (TIB MOLBIOL, Germany; table 1) matching all reference HBV genotypes as predefined by the NCBI Genotyping resource [16–18]. Three overlapping 1.2- to 1.7-kb-long PCR fragments were amplified each in a 50- μ l-volume reaction containing 1 μ M of each primer, 1.5 mM of $MgCl_2$ and 5 μ l of extracted DNA using GeneAmp High Fidelity PCR System (Applied Biosystems, USA). Thermal cycling was initiated at 94° for 2 min followed by 40 cycles of denaturation at 94° for 15 s, annealing at 55° for 30 s and extension at 72° for 3 min. An additional extension time of 5 s per cycle after cycle 10, and a final extension step at 72° for 7 min were added. PCR products were directly sequenced using the BigDye[®] Terminator v3.1 Cycle Sequencing Kit on the ABI PRISM[®] 3100-Avant[™] Genetic Analyzer (Applied Biosystems, USA). Sequence ambiguities due to quasispecies variation were resolved by DNA cloning using the TOPO TA Cloning Kit (Invitro-

gen, USA) and up to eight clones were analyzed. Nucleotide sequence contigs were assigned seventy GenBank accession No. from JN257148 to JN257217, and were further analyzed together with the NCBI reference genome set of HBV genotypes.

Phylogenetic Analysis

Ninety-four reference and clinical HBV genomes were aligned using ClustalW2 [19, 20]. The phylogenetic analysis was carried out by bayesian inference of phylogeny using the general time-reversible evolutionary model with a gamma-distributed rate variation across the sites and a proportion of invariable sites. Nine phylogenetic trees of various target gene regions including the complete genome and seven coding sequences (CDS) of the viral genes (P gene, polymerase CDS; S gene, large S, middle S and S protein CDS in addition to pre-S; X gene, X protein CDS; C gene, precore/core and core protein CDS) were separately constructed using MrBayes v3.1.2 [21, 22], and then displayed using TreeView [23].

Sequence Segment Analysis

Ninety-three reference and clinical HBV genomes were subtyped using the NCBI Genotyping tool [16]. Nine query sequences of various target gene regions each, including complete genome and seven viral CDS in addition to pre-S region, were compared to the predefined reference set based on scored BLAST pairwise alignments. Likewise, similarity scores of 31 overlapping 300-bp sequence segments with 100-bp incremental steps along the entire genome of each query sequence were imported into spread sheets for further analysis. The unpaired Student's t test was used to determine whether the intra- and intergenotypic similarity score means were significantly different in a dual genotype matrix; the difference was considered extremely significant when $p < 0.001$, very significant when $0.001 < p < 0.01$, and significant when $0.01 < p < 0.05$. The p value was adopted for ranking sequence segments in terms of intergenotypic discriminatory capability. In addition, the relative standard deviation (RSD) of intragenotypic similarity scores was used to estimate intragenotype variation. Pearson's correlation coefficients between Student's t test p values and RSDs were finally calculated.

Results

Phylogenetic Analysis

All nine phylogenetic trees exhibited common topological characteristics where genotype-specific reference sequences clustered in monophyletic clades discriminating all HBV genotypes, while all sequences from Syrian isolates clustered in the genotype D clade (fig. 1a). Exceptions were confined to phylogenetic trees of C and X gene CDS showing poor discrimination and/or misclassification of reference genotypes B and/or D, respectively. Figure 1b shows that the C gene CDS of reference genotypes E and D were not discriminated in distinct clades but rather clustered together in one clade, while those of reference genotype B appeared separately as sister groups

Table 1. Primers used for complete HBV genome sequencing

Primer ^a	5'–3' sequence	Degeneracy ^b	Annealing site ^c
<i>PCR and cycle sequencing primers</i>			
F1	GGG TCA CCW TAT WCY TGG GAA	8-fold	2814–2834
R1	CGT TGC CKR GCA ACS GGG TAA AGG	8-fold	1163–1140
F2	GTT TCT CYT GGC TCA GTT TA	2-fold	660–679
R2	AAA AAG TYR CAT GRT GMT GG	16-fold	1825–1806
F3	ACR THG CAT GGA GAC CAC CG	6-fold	1601–1620
R3	GAA CTG GAG CCA CCA GCA GG	1-fold	75–56
F4 ^d	TTT TTC MCC TCT GCC TAD TCA	6-fold	1821–1841
R4 ^d	CCC TAG AAA ATT GAG AGA AGT C	1-fold	283–262
<i>Cycle sequencing primers</i>			
F5	GTC TAG ACT CGT GGT GGA CTT CTC	1-fold	246–269
F6	YTG GCC WAA ATT CGC RRT C	16-fold	300–318
R5	GAA GAT GAG GCA TAG CAG CAG G	1-fold	433–412
R6	AAG CCA RAC AGT GGG GGA AAG C	2-fold	730–709
F7	CCC CAC TGT YTG GCT TTY AG	4-fold	715–734
R7_1	TTC KYT GMC ASA CYT TCC A	32-fold	997–979
R7_2	TAC KYT GMC ASA CYT TCC A	32-fold	997–979
F8	YTC GYC AAC BTA YAA GGC CTT T	24-fold	1098–1119
F9	GCT GAY GCA ACC CCC ACK GG	4-fold	1186–1205
F10	CTC TGC CGA TCC ATA CTG CGG AA	1-fold	1256–1278
R8	GAT TCA GCG CYG AYG GGA CGT A	4-fold	1447–1426
F11_1	TCA GRC AAG CHA TYY TCT GCT GG	24-fold	2064–2086
F11_2	TCA GRC AAG CHA TYY TTT GCT GG	24-fold	2064–2086
F11_3	TCA GRC AAG CHA TYY TGT GCT GG	24-fold	2064–2086
R9	GTK GAT ARG ATA GGG GCA TTT GRT GG	8-fold	2325–2300
R10	KSA GWT CTK CKG CGA CGC GGC	32-fold	2430–2410
F12_1	AAG GTG GGR AAY TTY ACS GK C	32-fold	2469–2490
F12_2	AAG GTG GGR AAY TTY ACT GK C	16-fold	2469–2490
F13	GCH TCA TTT TGY GGG TCA CCW TA	12-fold	2802–2824
R11	CAT GMY GTA GHT CTT GTT CC	12-fold	2850–2831
F14	YTG GGR TGG AGC CCT CAR KCW	32-fold	3070–3090
F15	CCT CCY GYY TCY RCC AAT CG	32-fold	3124–3143

^a Primers coded (F = forward primer; R = reverse primer).

^b Degeneracy fold equals the number of primers included in an equimolar mixture, computed by multiplying the numbers of bases represented by the degenerate base symbols, e.g., F1: GGG TCA CC[A/T] TAT [A/T]C[C/T] TGG GAA has $2 \times 2 \times 2 = 8$ -fold degeneracy.

^c Nucleotide number according to GenBank accession No. NC_003977.

^d An alternative PCR fragment was amplified when F3 and R3 failed to generate the third amplicon.

either to genotype C or to genotypes A, D and E. Moreover, one reference X gene CDS of genotype D was misplaced to the genotype A clade (fig. 1c).

Sequence Segment Analysis of Reference Genotypes

Average intra- and intergenotypic similarity scores between reference sequences were extremely significantly different among most genotype target regions. Exceptions were observed in C and X gene CDS regions of genotypes B and/or D, respectively, versus reference geno-

types A, C and/or E showing concurrently the highest intragenotypic variation in terms of RSD values (in %) of all genotypes (table 2).

Intergenotype discrimination was extremely significant over 13 of 31 (42%) genome-wide 300-bp sequence segments including segments 2–6 of S gene CDS and segments 24–31 of P gene sequences coding for the N-terminal domain. However, the remaining segment 1 of pre-S2/S sequences, segments 7–14 of P gene sequences coding for reverse transcriptase-like and C-terminal domains,

Fig. 1. a Unrooted phylogenetic tree of HBV complete genome, including both reference and clinical sequences, constructed by Bayesian inference of phylogeny of MrBayes v3.1.2 using the general time reversible evolutionary model with a gamma-distributed rate variation across the sites and a proportion of invariable sites. This phylogenetic tree (as well as polymerase, large S, middle S, and S CDS; data not shown) discriminates all reference HBV genotypes

in monophyletic clades. All 70 clinical HBV isolates from enrolled Syrian patients clustered in reference genotype D clade and are prefixed by 'WH', while names of the 24 HBV reference sequences are prefixed by the genotype followed by the GenInfo Identifier (GI) and a color-coded triangle. Posterior probabilities are shown for each split of genotype-specific monophyletic clades.

Fig. 1. b Unrooted phylogenetic tree of HBV core gene, including both reference and clinical sequences, constructed by Bayesian inference of phylogeny of MrBayes v3.1.2 using the general time reversible evolutionary model with a gamma-distributed rate variation across the sites and a proportion of invariable sites. This phylogenetic tree shows poor discrimination and misclassification of some reference genotypes. All 70 clinical HBV isolates from en-

rolled Syrian patients clustered in reference genotype D clade and are prefixed by 'WH', while names of the 24 HBV reference sequences are prefixed by the genotype followed by the GenInfo Identifier (GI) and a color-coded triangle. Posterior probabilities are shown for each split of genotype-specific monophyletic clades.

Fig. 1. c Unrooted phylogenetic tree of HBV X gene, including both reference and clinical sequences, constructed by Bayesian inference of phylogeny of MrBayes v3.1.2 using the general time reversible evolutionary model with a gamma-distributed rate variation across the sites and a proportion of invariable sites. This phylogenetic tree shows misclassification of reference genotype D. All

70 clinical HBV isolates from enrolled Syrian patients clustered in reference genotype D clade and are prefixed by 'WH', while names of the 24 HBV reference sequences are prefixed by the genotype followed by the GenInfo Identifier (GI) and a color-coded triangle. Posterior probabilities are shown for each split of genotype-specific monophyletic clades.

Table 2. Intergenotype discrimination and intragenotype variation of hepatitis B virus reference genotypes over various target gene regions^a

Target region	HBV genotype	Intergenotypic discrimination ^b								Intra-genotypic variation ^c
		A	B	C	D	E	F	G	H	
Complete genome	A	NA	ES	ES	ES	ES	ES	ES	ES	4%
	B	ES	NA	ES	ES	ES	ES	ES	ES	3%
	C	ES	ES	NA	ES	ES	ES	ES	ES	2%
	D	ES	ES	ES	NA	ES	ES	ES	ES	4%
	D (our study)	ES	ES	ES	NA	ES	ES	ES	ES	5%
	E	ES	ES	ES	ES	NA	ES	ES	ES	NA
	F	ES	ES	ES	ES	ES	NA	ES	ES	5%
	G	ES	ES	ES	ES	ES	ES	NA	ES	0%
	H	ES	ES	ES	ES	ES	ES	ES	NA	1%
Polymerase CDS	A	NA	ES	ES	ES	ES	ES	ES	ES	8%
	B	ES	NA	ES	ES	ES	ES	ES	ES	4%
	C	ES	ES	NA	ES	ES	ES	ES	ES	1%
	D	ES	ES	ES	NA	ES	ES	ES	ES	3%
	D (our study)	ES	ES	ES	NA	ES	ES	ES	ES	5%
	E	ES	ES	ES	ES	NA	ES	ES	ES	NA
	F	ES	ES	ES	ES	ES	NA	ES	ES	1%
	G	ES	ES	ES	ES	ES	ES	NA	ES	0%
	H	ES	ES	ES	ES	ES	ES	ES	NA	3%
Large S protein CDS	A	NA	ES	ES	ES	ES	ES	ES	ES	4%
	B	ES	NA	ES	ES	ES	ES	ES	ES	3%
	C	ES	ES	NA	ES	ES	ES	ES	ES	0%
	D	ES	ES	ES	NA	ES	ES	ES	ES	0%
	D (our study)	ES	ES	ES	NA	ES	ES	ES	ES	3%
	E	ES	ES	ES	ES	NA	ES	ES	ES	NA
	F	ES	ES	ES	ES	ES	NA	ES	ES	1%
	G	ES	ES	ES	ES	ES	ES	NA	ES	0%
	H	ES	ES	ES	ES	ES	ES	ES	NA	1%
Middle S protein CDS	A	NA	ES	ES	ES	ES	ES	ES	ES	2%
	B	ES	NA	ES	ES	ES	ES	ES	ES	2%
	C	ES	ES	NA	ES	ES	ES	ES	ES	3%
	D	ES	ES	ES	NA	ES	ES	ES	ES	1%
	D (our study)	ES	ES	ES	NA	ES	ES	ES	ES	2%
	E	ES	ES	ES	ES	NA	ES	ES	ES	NA
	F	ES	ES	ES	ES	ES	NA	ES	ES	1%
	G	ES	ES	ES	ES	ES	ES	NA	ES	0%
	H	ES	ES	ES	ES	ES	ES	ES	NA	1%
S protein CDS	A	NA	ES	ES	ES	ES	ES	ES	ES	1%
	B	ES	NA	ES	ES	ES	ES	ES	ES	1%
	C	ES	ES	NA	ES	ES	ES	ES	ES	1%
	D	ES	ES	ES	NA	ES	ES	ES	ES	1%
	D (our study)	ES	ES	ES	NA	ES	ES	ES	ES	2%
	E	ES	ES	ES	ES	NA	ES	ES	ES	NA
	F	ES	ES	ES	ES	ES	NA	ES	ES	2%
	G	ES	ES	ES	ES	ES	ES	NA	ES	0%
	H	ES	ES	ES	ES	ES	ES	ES	NA	1%
Pre-S sequence	A	NA	ES	ES	ES	ES	ES	ES	ES	9%
	B	ES	NA	ES	ES	ES	ES	ES	ES	7%
	C	ES	ES	NA	ES	ES	ES	ES	ES	8%
	D	ES	ES	ES	NA	ES	ES	ES	ES	3%
	D (our study)	ES	ES	ES	NA	ES	ES	ES	ES	11%
	E	VS	ES	ES	ES	NA	ES	ES	ES	NA
	F	ES	ES	ES	ES	ES	NA	ES	ES	3%
	G	ES	ES	ES	ES	ES	ES	NA	ES	0%
	H	ES	ES	ES	ES	ES	ES	ES	NA	2%
X protein CDS	A	NA	ES	ES	S	ES	ES	ES	ES	6%
	B	ES	NA	ES	ES	ES	ES	ES	ES	2%
	C	ES	ES	NA	ES	ES	ES	ES	ES	3%
	D	NS	S	NS	NA	NS	ES	ES	ES	9%
	D (our study)	ES	ES	ES	NA	NS	ES	ES	ES	11%
	E	ES	ES	ES	VS	NA	ES	ES	ES	NA
	F	ES	ES	ES	ES	ES	NA	ES	ES	0%
	G	ES	ES	ES	ES	ES	ES	NA	ES	1%
	H	ES	ES	ES	ES	ES	ES	ES	NA	6%
Precore/core protein CDS	A	NA	ES	ES	ES	ES	ES	ES	ES	5%
	B	NS	NA	NS	S	NS	ES	VS	ES	19%
	C	ES	S	NA	ES	ES	ES	ES	ES	2%
	D	ES	ES	ES	NA	S	ES	ES	ES	4%
	D (our study)	ES	ES	ES	NA	VS	ES	ES	ES	11%
	E	ES	ES	ES	ES	NA	ES	ES	ES	NA
	F	ES	ES	ES	ES	ES	NA	ES	ES	2%
	G	ES	ES	ES	ES	ES	ES	NA	ES	0%
	H	ES	ES	ES	ES	ES	ES	ES	NA	5%
Core protein CDS	A	NA	ES	ES	ES	ES	ES	ES	ES	5%
	B	S	NA	NS	VS	S	ES	S	ES	18%
	C	ES	VS	NA	ES	ES	ES	ES	ES	2%
	D	ES	ES	ES	NA	NS	ES	ES	ES	5%
	D (our study)	ES	ES	ES	NA	VS	ES	ES	ES	12%
	E	ES	ES	ES	ES	NA	ES	ES	ES	NA
	F	ES	ES	ES	ES	ES	NA	ES	ES	3%
	G	ES	ES	ES	ES	ES	ES	NA	ES	1%
	H	ES	ES	ES	ES	ES	ES	ES	NA	6%

Color version available online

^a Reference HBV genotypes as predefined by the NCBI Genotyping resource [17].

^b Difference between intragenotypic similarity scores of reference/clinical HBV genotypes (row headers) and intergenotypic similarity scores versus reference HBV genotypes (column headers) estimated in terms of unpaired Student's t test p value, and considered extremely significant (ES; $p < 0.001$),

very significant (VS; $0.001 < p < 0.01$), significant (S; $0.01 < p < 0.05$), or not significant (NS; $p > 0.05$). NA = Nonapplicable.

^c Variation of intragenotypic similarity scores of reference/clinical HBV genotypes (row headers) estimated in terms of RSD. Intragenotypic variation was not estimated for genotype E as only two reference sequences were predefined by NCBI.

Table 3. Intergenotype discrimination and intragenotype variation of hepatitis B virus reference genotypes over genome-wide 300-bp sequence segments^a

[illegible]

Table 3 (continued)

[illegible]

Color version available online

Fig. 2. Intergenotypic discriminatory capability of various 300-bp-long target gene regions over the entire sequence of HBV genome in general (a) and each HBV reference genotype (b). Target gene regions appear thinner when the sequence segment includes flanking sequences as well.

segments 15 and 16 of X gene CDS, segments 17 and 18 of X/C gene CDS, and segments 19–23 of C gene CDS showed either insignificant intergenotype discrimination of reference genotypes A, B, C and/or D, or different levels of intergenotype discrimination significance between various genotypes (table 3).

Intragenotypic similarity score RSD values of genome-wide 300-bp sequence segments varied in the ranges of 1–30, 1–34, 1–7, 1–18, 0–7, 0–1 and 0–12% for the reference genotypes A, B, C, D, F, G and H, respectively. Student's *t* test *p* values corresponding to intergenotypic discriminatory capability and RSD values indicating intragenotypic variation showed a medium to strong positive correlation (average Pearson's correlation coefficients ranged between 0.4 and 0.8).

Sequence Segment Analysis of Clinical Specimens

Genotype D was assigned to all 70 complete HBV genome sequences of this study for matching its reference sequences with the highest similarity scores in most genome-wide segments. However, reference genotype E matched the query sequence segments 17–20 of X and C gene CDS with either equal or higher similarity scores than those of reference genotype D in 65 of 70 query sequences.

Average intra- and intergenotypic similarity scores between query and reference sequences were extremely significantly different among most target regions except X gene CDS versus reference genotype E (table 2). Intergenotype discrimination was extremely significant over the 30 genome-wide 300-bp sequence segments except segment

11 of P gene sequence coding for the C-terminal domain and segments 19, 20 and 22 of C gene CDS versus reference genotype E, and segment 16 of X gene CDS versus reference genotype C (table 3). Intragenotypic variation was significantly higher in our study group than in the reference genotype D group for all studied target regions.

Integrity of the Analyzed Genomes

Among the 70 sequenced HBV genomes, 40 were 3,182-bp-long showing no deletions or insertions compared to the reference genotype D genomes. One- to 183-bp-long deletions in the pre-S, X and/or C regions and 2- to 9-bp-long insertions in X or C regions were found in 28 and 5 of 70 genomes, respectively. A premature stop codon in the precore region leading to an HBeAg-negative genotype was found in 34 of 70 genomes. Furthermore, 8 of 28 deletion-mutant genomes contained premature stop codons in the C and/or S CDS probably causing nonviability. In addition, the start codons of large or middle S were missing in 1 and 5 genomes, respectively.

Discussion

Sequence Analysis of Reference Genotypes

The NCBI Genotyping resource has collaboratively predefined a set of 23 complete reference genomes for all eight HBV genotypes, two to three sequences each, taking into account genetic polymorphisms and including both ancient and recent sequence data. A defined genotype is

assigned to a query sequence when at least one of its reference sequences is matched with the highest similarity scores using BLAST pairwise alignments [16]. Hence, the lower the similarity scores versus the other reference genotypes are, the more decisive is the genotyping analysis and the more discriminative is the target gene region. This is the rationale upon which our study was designed and carried out. Accordingly, these predefined HBV reference sequences were compared intra- and intergenotypically to find out the most discriminative target gene regions.

Our revisiting approach revealed that sequencing either the complete genome or any entire CDS of the viral P and S genes (polymerase, large S, middle S and S protein CDS as well as the entire pre-S region) can significantly differentiate all HBV genotypes. In contrast, genotypes B and/or D may be confounded with other genotypes by sequencing either C or X gene CDS, respectively. Similarly, phylogenetic analysis delivered consistent but less detailed findings.

Our study was extended to further analyze the intergenotype discriminative capability of 31 genome-wide 300-bp sequence segments using the scored BLAST-dependent NCBI Genotyping tool, showing that thirteen segments over the CDS of S protein and polymerase N-terminal domain were capable of discriminating all HBV genotypes significantly. Thus, any 300-bp sequence segments chosen over nearly half the length of the genome are very suitable target gene regions to reliably assign any genotype to a query sequence (fig. 2). The findings point to the fact that it is the reliability of the target gene region that counts for intergenotypic discriminatory capability regardless of the sequence length.

Taking the geographical distribution of HBV genotypes into account, it may be required to identify only the dominant genotypes in a given geographic area for clinical purposes. In this context, HBV genotyping assays may be specifically designed for differentiating between genotypes B and C which predominate in China and Japan [1, 2, 4, 24], using even suboptimal target gene regions such as pre-S2 and X gene if considered necessary. Likewise, differentiating the globally distributed genotype D from non-D may be of interest worldwide [2, 6, 10, 11], and for this aim it is wise to use the optimal target gene regions. Obviously, the optimal target gene regions for HBV genotyping are genotype dependent.

Interestingly, intergenotypic discriminatory capability of studied target gene regions correlated negatively with intragenotype variation. Additionally, poor discrimination between various HBV genotypes was obviously attributed to the genotypes showing more intragenotype

variation such as genotypes A, B and D and to a lower extent genotype C (table 3).

Sequence Analysis of Clinical Specimens from Syria

Genotype D was assigned to all tested clinical specimens based on both phylogenetic and scored BLAST analyses. However, equal or higher similarity scores in X and C genes versus reference genotype E by most query sequence contigs suggested an absence of significant differences in these gene regions between genotypes D and E. The lack of discrimination may be caused by the higher intragenomic variability in these genomic regions due to the high prevalence of HBeAg-negative variants in our cohort. These are associated with a tenfold higher mutation rate [25]. In fact, half of our patients had the precore stop mutant generating HBeAg negativity. Recombination with genotype E could be another possibility as reported elsewhere [26] but this is unlikely in our cohort because no genotype E infections were noted.

Poor discrimination from reference genotypes E and C appeared in miscellaneous sequence segments of polymerase C-terminal domain CDS as well as C and X gene regions. This finding was consistent with that of the reference genotype analysis above, though not on a par with it, which can be attributed to either (1) the extinguished effect of genetic variation due to managing a huge amount of data or (2) limited genetic variation of locally distributed genotype D variants that do not represent all subgenotypes of it. HBV subgenotypes were not explored in our study which could have explained such an observation. Moreover, our study may not represent all HBV strains circulating in the Syrian population since it was biased towards samples of high viral loads (>6 log copies/ml), while the viral loads of most samples are supposedly lower [27].

In conclusion, we introduce a novel data evaluation approach applicable for the genotyping of other viruses as well, and a referential genome-wide tabulated guide of optimal target gene regions for HBV genotyping applications. It appears advisable to identify any of those 300-bp target sequences over the viral S protein and polymerase N-terminal domain CDS which yield the best discrimination of all HBV genotypes. Further studies may reveal even shorter sequence segments of certain target gene regions capable of discriminating HBV genotypes significantly, but caution must be paid to potential recombination events that may occur genome-wide [28].

Disclosure Statement

The authors have no conflicts of interest to declare.

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