Multiplex Detection of 60 Hepatitis B Virus Variants by MALDI-TOF Mass Spectrometry

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BACKGROUND: Variations in the hepatitis B virus (HBV) genome may develop spontaneously or under selective pressure from antiviral therapy. Such variations may confer drug resistance or affect virus replication capacity, resulting in failure of antiviral therapy.

METHODS: A duplex PCR was used to amplify the region of the reverse transcriptase gene, the precore promoter, and the basal core promoter of the HBV genome. Four multiplex primer-extension reactions were used to interrogate 60 frequently observed HBV variants during antiviral therapy. Automated MALDI-TOF mass spectrometry (MS) was used for mutation detection. Capillary sequencing was used to confirm the MS results.

RESULTS: The limit of quantification was 1000 HBV copies/mL for multiplex detection of HBV variants. Fifty-three variants (88.3%) were analyzed successfully in at least 90% of the sera from 88 treatment-naive patients and 80 patients with virologic breakthrough. MS was able to detect twice as many minor variants as direct sequencing while achieving close to full automation. MS and direct sequencing showed only 0.1% discordance in variant calls.

CONCLUSIONS: This platform based on multiplex primer extension and MALDI-TOF MS was able to detect 60 HBV variants in 4 multiplex reactions with accuracy and low detection limits.

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Worldwide, 400 million people are estimated to be chronically infected with hepatitis B virus (HBV).[5] Chronic hepatitis B infection is the most common cause of liver cirrhosis and hepatocellular carcinoma, with an estimated 500 000–900 000 deaths per year. Continuing HBV replication increases the risk of progression to cirrhosis and hepatocellular carcinoma (1–4). Thus, antiviral therapy aimed at blocking virus replication is essential in managing chronic hepatitis B patients. A number of nucleoside or nucleotide analogs, such as lamivudine, adefovir dipivoxil, telbivudine, entecavir, and tenofovir, have been approved for antiviral treatment. Unfortunately, HBV variants frequently develop with long-term antiviral therapy (5). Some of these variants confer drug resistance, resulting in treatment failure that may lead to hepatitis reactivation and hepatic decompensation (6).

Periodic quantification of the HBV viral load is commonly used to monitor the response to antiviral treatment and for early detection of drug resistance (7, 8). Direct detection of important HBV variants is also effective in the early detection of drug resistance (9, 10). A number of viral variants have been well documented for their functional roles in drug resistance (5). Other variants have been detected in serum samples from patients undergoing antiviral therapy; however, the functions of these variants have not been carefully studied. The complexity of HBV variants may continue to increase with the use of new antiviral agents and new combinations of different agents.

A number of techniques are available for detecting HBV variation. Capillary sequencing can detect any sequence variation within a target sequence; however, it often fails to detect variants present at low frequencies because the signals of the mutant are dwarfed by the signals of the wild-type sequence. The commercialized line INNO-LiPA HBV DR assay (Innogenetics) uses reverse hybridization of amplified HBV DNA fragments together with specific nucleotide probes immo-

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[5] Nonstandard abbreviations: HBV, hepatitis B virus; MS, mass spectrometry; dNTP, deoxynucleoside triphosphate; SAP, shrimp alkaline phosphatase; SNP, single-nucleotide polymorphism.

bilized on nitrocellulose strips to detect drug-resistant variants at frequencies as low as 5% in mixed viral populations *(10)*; however, the assay is expensive, is difficult to adapt to incorporate newly identified variants, and has limited coverage with respect to the number of HBV variants. Recently, we and others adopted automated MALDI-TOF mass spectrometry (MS) for high-throughput detection of HBV variants *(9, 11)*. This assay has the capability to detect mutant strains down to a frequency of 5% in mixed viral populations. In this study, we designed and validated 4 highly multiplexed assays for the detection of 60 frequently observed HBV variants in a large number of patients undergoing different antiviral treatments.

Materials and Methods

PATIENT POPULATIONS AND SAMPLE COLLECTION
Serum samples were collected between August 2007 and June 2008 from 168 patients with HBV infection at Shenzhen Donghu Hospital, P.R. China, after informed consent was obtained. This study was approved by the Clinical Research Ethics Committee of The Chinese University of Hong Kong and Donghu Hospital, P.R. China. Of these 168 patients, 88 patients (71.6% males; age range, 18–62 years; median age, 32 years) had not received any antiviral therapy (treatment naive), and 80 patients (80% males; age range, 21–65 years; median age, 32 years) experienced a virologic breakthrough with an HBV DNA increase of >1 log from the nadir value and a concentration of >100 000 copies/mL. Peripheral blood was collected from each patient into red-top tubes containing no anticoagulant. After centrifugation within 1 h, 1.5 mL of serum was removed and stored at −80 °C. HBV DNA was extracted from serum with the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions [DNA Purification from Blood or Body Fluids (Spin Protocol)]. We used 800 μL of serum to extract DNA into a 50-μL elution volume. The viral load of each sample was determined with a fluorescence PCR kit (PG Biotech) *(12)*.

HBV VARIANT DETECTION
Four main steps were involved in the detection of HBV variants by MALDI-TOF MS. Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue8 lists the HBV variants selected, and Table 2 in the online Data Supplement provides the sequences and molecular weights of extension primers and extension products. All primers were purchased from Integrated DNA Technologies. All other reagents were purchased from Sequenom unless otherwise specified.

STEP 1: DUPLEX PCR TO AMPLIFY THE HBV REGIONS OF INTEREST
The reverse transcriptase and the basal core promoter/precore regions were selected because these 2 regions contain the functionally important variants as well as the variants frequently observed in patients undergoing antiviral therapy (see Table 2 in the online Data Supplement). We used 2 pairs of PCR primers (25IF-tag: 5′-gttggatgGACTCGTGGTGGACTTCTCTCA-3′ and 1004R-tag: 5′-ggatgCCCACAATTCKTTGACATACTT TCC-3′; 1593F-tag: 5′-acgttggatgACCTCTGCACGTY RCATGGA-3′ and 1950R-tag: 5′-ggatgGAGAGTAAC TCCACAGTAGCTCCAA-3′) to amplify these 2 regions in a duplex PCR that generates 2 amplicons of 754 bp and 358 bp, respectively. The lowercase letters in the primer sequences are sequence tags to increase the molecular weights of the PCR primers so that they do not interfere in the mass spectra. The 25-μL PCR reactions contained 5 μL HBV DNA, 200 nmol/L each of the primers 251F-tag and 1004R-tag, 100 nmol/L each of the primers 1593F-tag and 1950R-tag, 1× HotStar Taq buffer (with 1.5 mmol/L Mg^{2+}), 1.0 mmol/L additional Mg^{2+}, 200 μmol/L of each deoxynucleoside triphosphate (dNTP), and 0.5 U of HotStar *Taq* polymerase (Qiagen). PCR was initiated at 95 °C for 15 min, followed by 45 cycles of denaturation at 95 °C for 40 s, annealing at 57 °C for 30 s, and extension at 72 °C for 1.5 min. A final extension was performed at 72 °C for 3 min.

STEP 2: SHRIMP ALKALINE PHOSPHATASE TREATMENT
To remove the remaining dNTPs in the PCR reactions, we mixed 5 μL of PCR product with 2 μL of a shrimp alkaline phosphatase (SAP) solution containing 1.53 μL H_2O, 0.17 μL SAP 10× buffer, and 0.3 μL SAP enzyme (1.7 U/μL stock concentration). The reaction was performed at 37 °C for 40 min and stopped by inactivation at 85 °C for 5 min.

STEP 3: MULTIPLEX PRIMER-EXTENSION REACTIONS
The 60 selected HBV variants were analyzed in 4 separate primer-extension reactions (12-plex in assay 1, 17-plex in assay 2, 17-plex in assay 3, and 14-plex in assay 4). Sequences for the extension primers are listed in Table 2 in the online Data Supplement. The 9-μL extension reactions contained 7 μL of the PCR/SAP product, 0.2 μL of 10× iPLEX Buffer Plus, 0.2 μL iPLEX Termination Mix with modified dideoxy NTPs, 0.94 μL of the extension-primer mixture (Table 2 in the online Data Supplement lists the concentration of each primer), and 0.041 μL iPLEX Thermo Sequenase. Fig. 1 in the online Data Supplement shows a standard thermal profile for iPLEX reactions.

STEP 4: SAMPLE CONDITIONING AND MS ANALYSIS
We desalted extension products for MS analysis by adding 16 μL double-distilled H_2O and 6 mg

SpectroCLEAN resin. After centrifugation at 360*g* for 5 min, a Nanodispenser dispensed approximately 15 nL of reaction solution onto a SpectroCHIP in a 384-sample format. Data acquisition from the SpectroCHIP was performed by Bruker Compact MALDI-TOF MS (Bruker Daltonics), and data analyses were carried out with TyperAnalyzer Application, version 4.0 (Sequenom). Table 2 in the online Data Supplement lists the expected molecular weights for the extension primers and extension products.

CAPILLARY SEQUENCING

We randomly selected 70 samples for sequencing (35 from treatment-naive patients and 35 from patients with virologic breakthrough) to validate MS results. The same PCR products used for SAP treatment (step 1, above) were used for direct sequencing with the BigDye Terminator Cycle Sequencing Kit, Version 3.0 (Applied Biosystems) and the ABI 3100 Genetic Analyzer (Applied Biosystems). The sequencing primers were: 5′-gttggatgGACTCGTGGTGGACTTCTCTCA-3′ (PCR primer), 5′-ggatgGAGAGTAACTCCACAGTAGCTCCAA-3′ (PCR primer), 5′-GYGCCATTTGTTCAGTGGTTCG-3′, and 5′-AAACATAGAGGTTCCTTGAGCAGG-3′.

Results

Our main aim was to develop an assay for detecting HBV variants that (*a*) could analyze all functionally important mutations as well as other variants frequently observed during antiviral treatment, (*b*) was sufficiently flexible so that newly identified variants could be incorporated with ease, (*c*) could quantify low viral loads (e.g., 1000 copies/mL serum, (*d*) was universally applicable to all HBV genotypes, (*e*) was cost-effective, and (*f*) had a high throughput. An automated MALDI-TOF MS platform was chosen because it is has been widely used for multiplex detection of single-nucleotide polymorphisms (SNPs). Chemically, SNPs are indistinguishable from most HBV variants; however, multiplex detection of HBV variants (or variants of other viruses, such as HIV variants) is often much more difficult because viral variants occur at much higher densities on a viral genome, compared with human SNPs, and viruses may exist as quasi species, such that some viral variants may be present only in minor proportions, whereas heterozygous SNPs are present at a 1:1 ratio.

For these reasons, it is often not a trivial task to identify highly conserved genomic regions for PCR primer design, particularly if high sensitivity is required. Additionally, developing a highly multiplexed variant-detection assay requires that the extension primers used to interrogate the variants be carefully designed to avoid primer cross-hybridization.

HBV VARIANT SELECTION

We performed a literature search for all known HBV mutations directly associated with drug resistance (see Table 1 in the online Data Supplement). Mutations in the precore (G1896A and C1858T) and basal core promoter (A1762T and G1764A) were included. Variants that have frequently been observed during antiviral treatment but that have not been conclusively implicated in drug resistance were also included in the hope that when a large number of samples are analyzed in the future, statistical significance for their association with antiviral treatment may be observed for some of them.
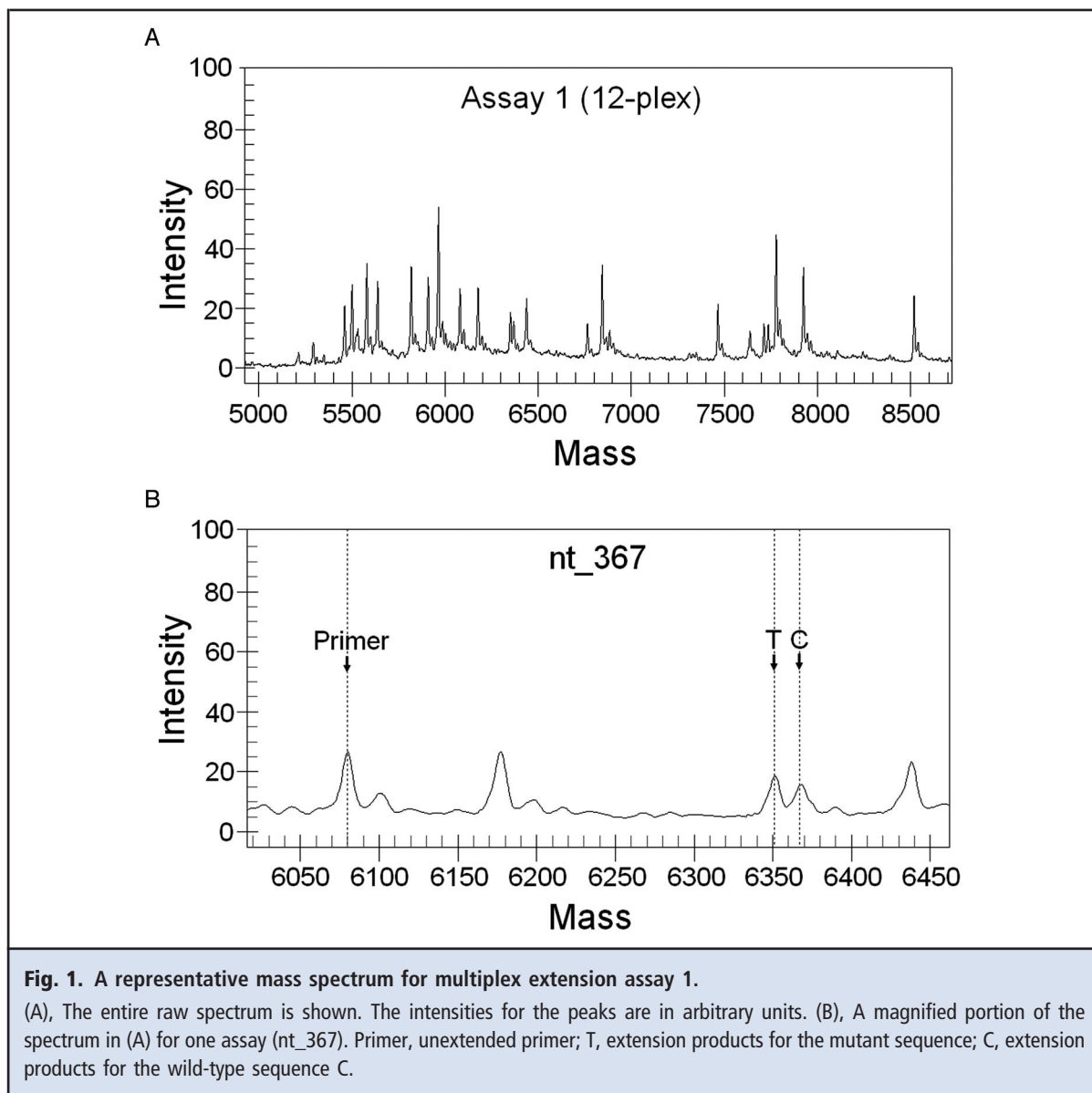
HBV DNA CONCENTRATIONS AND GENOTYPES

HBV DNA concentrations ranged from 1.2×10^3 to 6.3×10^8 copies/mL (median, 2.4×10^7 copies/mL) for the 88 treatment-naive patients and from 2.6×10^3 to 1.6×10^8 copies/mL (median, 2.5×10^5 copies/mL) for the 80 patients with virologic breakthrough. The viral load in treatment-naive samples was significantly higher than in samples from patients with virologic breakthrough ($P < 0.001$, Mann–Whitney U-test). Capillary sequencing of serum HBV DNA samples from 32 treatment-naive patients revealed that the HBV was genotype B in 23 patients (71.9%) and genotype C in 9 patients (28.1%). Sequencing of the HBV DNA from 33 samples from patients with virologic breakthrough showed that 23 (69.7%) of the patients had been infected with HBV genotype B and that 10 (30.3%) had been infected with HBV genotype C.

PCR AND EXTENSION PRIMER DESIGN

PCR primers were designed by aligning genomic sequences from different HBV genotypes (see Fig. 2 in the online Data Supplement). Because the selected variants were clustered in 2 genomic regions, we designed a duplex PCR assay that amplified all selected variants. With further PCR optimization, this duplex PCR assay consistently amplified HBV DNA when the input DNA copy number was >94 copies per PCR. This optimized assay allowed us to analyze all serum samples with viral loads of >1170 copies/mL serum.

For the primer-extension reactions, 60 extension primers are required for the 60 selected variants. Although MassARRAY Assay Design (Sequenom) can achieve a multiplex level as high as 36, we decided to design the assays at substantially lower levels of multiplexing so that new variants could easily be added in future updates. Therefore, we designed 4 multiplex primer-extension reactions (12-plex in assay 1, 17-plex

Fig. 1. A representative mass spectrum for multiplex extension assay 1.

(A), The entire raw spectrum is shown. The intensities for the peaks are in arbitrary units. (B), A magnified portion of the spectrum in (A) for one assay (nt_367). Primer, unextended primer; T, extension products for the mutant sequence; C, extension products for the wild-type sequence C.

in assay 2, 17-plex in assay 3, and 14-plex in assay 4; see Table 2 in the online Data Supplement).

The concentrations of the extension primers were further adjusted to compensate for differences in signal intensities in the mass spectra due to differences in sequence and molecular weight among the different extension primers. Adjustments were made on the basis of the real mass spectral data generated. Fig. 1 shows a representative mass spectrum for a multiplex assay after such adjustment.

DETECTION OF 60 HBV VARIANTS

After optimization of PCR and primer-extension reactions, we analyzed serum samples from 88 treatment-naive patients and 80 patients with virologic breakthrough. In this study, the call rate was defined in 2 contexts: the call rate per sample, which was the successful call percentage for each individual sample among all variants, and the call rate per variant, which was the successful call percentage for each variant among all samples. When not specified, call rate refers to the successful call percentage for all variants among all samples.

To evaluate the performance of the assays, we first looked at the call rate for each variant (defined as the percentage of successful calls in the selected samples tested for each variant). Thirty-two variants (53.3%) achieved a call rate of >98%. Fifty-three variants
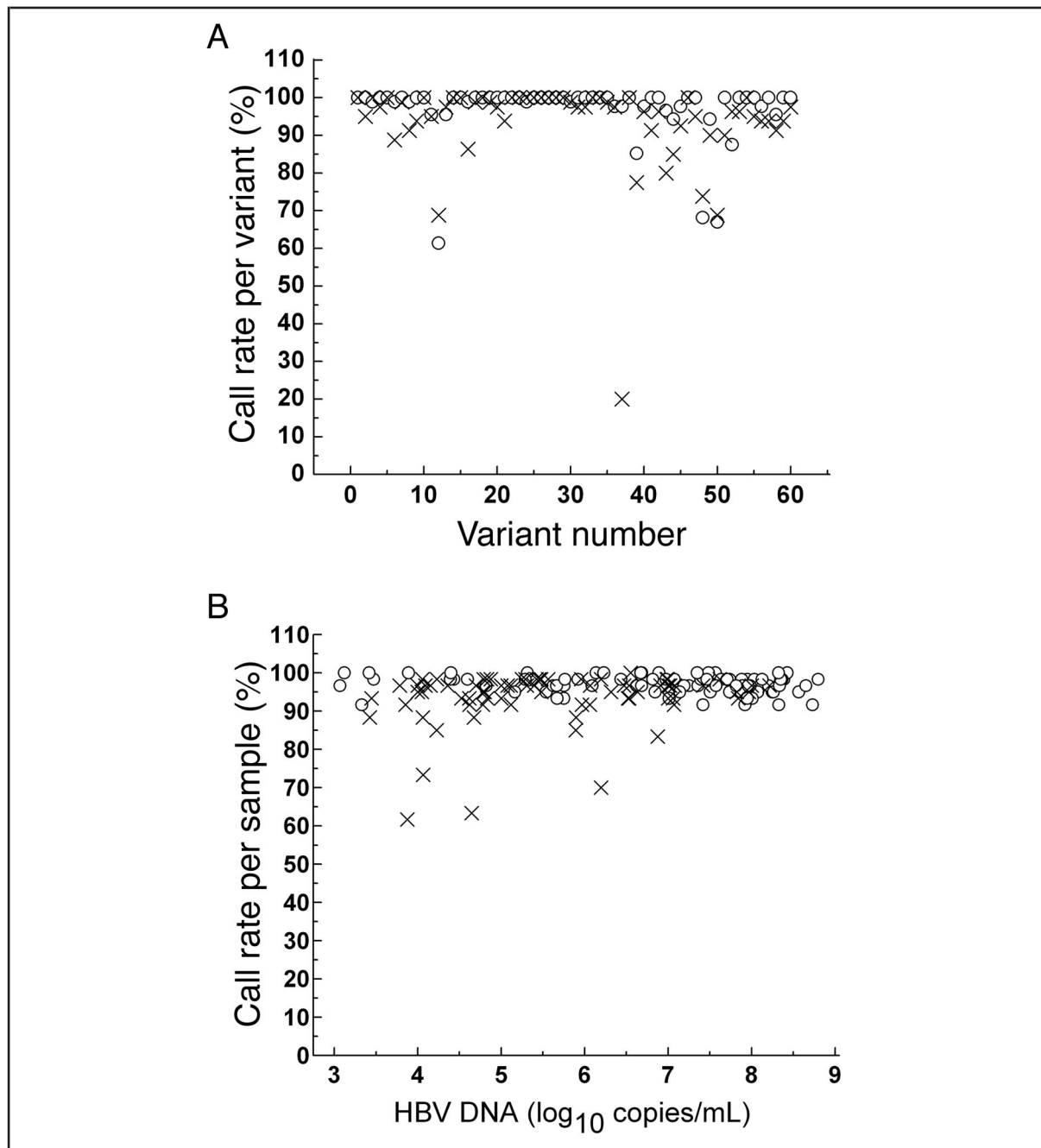
Fig. 2. Call rates for each variation and call rates for each sample.

(A), Call rates for each variant for each group (treatment-naive ○ and with virologic breakthrough ×). A majority of the variants had higher call rates (88.3% of the variants achieved a call rate >90%). (B), Call rates for each sample (shown by its viral load on the x axis). Samples from patients with virologic breakthrough were more likely to have lower call rates. All samples with call rates <90% were from patients with virologic breakthrough.

(88.3%) achieved a call rate of >90%. Fifty-six of the 60 variants (93.3%) achieved a call rate >80%. The overall call rate for all variants in all samples was 95.3%.

The call rates for each of the 60 variants in the 2 patient groups (treatment-naive and with virologic breakthrough) are shown in Fig. 2A. The overall call

rates for all variants in treatment-naive samples and in samples with virologic breakthrough were 97.0% and 93.4%, respectively. Additionally, the call rates of 14 variants in patients with virologic breakthrough were significantly lower ($P < 0.05$ for each of the 14 variants, Pearson chi-square test, degrees of freedom $= 1$) than those in treatment-naive patients. The higher call rates for the treatment-naive samples are at least partially due to higher viral loads in samples from treatment-naive patients (range, 1.2×10^3 to 6.3×10^8 copies/mL; median, 2.4×10^7 copies/mL) than in patients with virologic breakthrough (range, 2.6×10^3 to 1.6×10^8 copies/mL; median, 2.5×10^5 copies/mL).

Call rates per sample (defined as the percentage of successful calls for all 60 variants tested in each sample) were also analyzed. Fig. 2B shows call rates for each sample (HBV viral load shown on the *x* axis) in the 2 patient groups. Although the call rates per sample remained high (>90%) over a wide range of concentrations for the treatment-naive patients, a number of patients with virologic breakthrough had less than optimal call rates (7 with call rates of 80%–90% and 4 with call rates of <80%).

VALIDATION BY CAPILLARY SEQUENCING

We randomly chose 70 serum samples (35 from treatment-naive patients and 35 from patients with virologic breakthrough) for capillary sequencing to validate the MALDI-TOF MS results. The same PCR products used for MALDI-TOF MS analysis were used for sequencing. For the reverse-transcriptase region, 27 treatment-naive samples and 33 samples with virologic breakthrough were sequenced successfully. For the precore promoter and basal core promoter region, 29 treatment-naive samples and 33 samples with virologic breakthrough were sequenced successfully. The overall success rate of sequencing was 87%, although all of the samples had successfully been analyzed by MS.

Excluding some failed calls for certain variants in some samples, a total of 3380 variant calls were obtained for both MS and direct sequencing. Overall, MS and sequencing results were completely concordant for 3340 variant calls (98.8%), regardless of whether only the wild type or a mutant was present or whether both sequences were present. For 23 variant calls (0.7%), MS detected both a wild type and a mutant whereas sequencing detected only a wild type or a mutant. One representative example is shown in Fig. 3A in the online Data Supplement. For these calls, it is possible that sequencing missed the minor sequence (either wild type or mutant) because of a lower frequency of the minor variant (<20%). Some of these cases were validated by further cloning and sequencing (data not shown). For 12 variant calls (0.4%), sequencing detected both a wild type and a mutant

whereas MS detected only wild type or mutant. The majority of these MS calls were of lower signal quality, which is likely to cause an inability to detect a minor variant (see Fig. 3B in the online Data Supplement). For 0.1% of the calls (5 of 3380 calls), direct sequencing and MS were completely discordant. Further cloning and sequencing confirmed that direct sequencing was correct (data not shown).

Discussion

Viral variants can arise spontaneously or under selective pressures. They are frequently biologically and clinically important. Numerous methods are available for highly paralleled detection of viral mutations. For example, microarrays have been used to detect multiple human viruses (13), and multiplex PCR assays have been designed to detect HBV genotypes A–G with >93% accuracy (14). MALDI-TOF MS has been used to detect minor viral variants quantitatively (9, 11) and biased allelic expression (15).

In this study, we developed and validated a high-throughput platform for multiplex detection of 60 functionally known or frequently observed HBV variants, with a detection limit close to 1000 HBV copies/mL of serum. This platform is based on an automated MALDI-TOF MS capable of analyzing two 384-format chips (sufficient for 192 samples) in a single run. Because we have purposely designed the level of multiplexing to be well below the capability of iPLEX Gold, more HBV variants can be easily incorporated in the future. The cost for analyzing all 60 variants (excluding blood-collection and DNA-extraction costs) is <$10.

Direct sequencing and MS produced highly consistent data, with only 5 of 3380 variant calls being completely discordant. More frequently, MS detected the presence of both a wild-type sequence and a mutation, and direct sequencing did not. Of note is that virtually all heterozygous calls obtained by direct sequencing were found by careful manual inspection, whereas virtually all calls (homozygous or heterozygous) obtained by MS were done automatically by the software. Additionally, sequencing reactions failed for 13% of the amplicons that were successfully analyzed by MS.

The platform has some limitations. First, only HBV genotypes B and C were tested, because these 2 HBV genotypes are the most prevalent in the southern part of China (16). The performance of this test with other HBV genotypes will require further investigation. Second, 4 of the 60 variants had call rates of <80%, which was the main reason for the overall no-call rate of 4.7%. None of these 4 variants have known functions. Third, there was a performance bias against samples from patients with virologic breakthrough, because all 11 samples with call rates of <90% were from

those with virologic breakthrough. This bias may be caused by an overall lower viral load for the samples from patients with virologic breakthrough and by poor binding of some extension primers caused by spontaneous mutations in the HBV genome.

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