Implementation of Next-Generation Sequencing for Hepatitis B Virus Resistance Testing and Genotyping in a Clinical Microbiology Laboratory

Christopher F. Lowe,^{a,b} Linda Merrick,^a P. Richard Harrigan,^c Tony Mazzulli,^{d,e} Christopher H. Sherlock,^{a,b} Gordon Ritchie^{a,b}

Division of Microbiology and Virology, Providence Health Care, Vancouver, British Columbia, Canada^a; Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada^b; BC Centre for Excellence in HIV/AIDS, Vancouver, British Columbia, Canada^c; Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada^d; Department of Microbiology, Mount Sinai Hospital/University Health Network, Toronto, Ontario, Canada^e

Sanger sequencing or DNA hybridization have been the primary modalities for hepatitis B (HBV) resistance testing and genotyping; however, there are limitations, such as low sensitivity and the inability to detect novel mutations. Next-generation sequencing (NGS) for HBV can overcome these limitations, but there is limited guidance for clinical microbiology laboratories to validate this novel technology. In this study, we describe an approach to implementing deep pyrosequencing for HBV resistance testing and genotyping in a clinical virology laboratory. A nested PCR targeting the *pol* region of HBV (codons 143 to 281) was developed, and the PCR product was sequenced by the 454 Junior (Roche). Interpretation was performed by ABL TherapyEdge based on European Association for the Study of the Liver (EASL) guidelines. Previously characterized HBV samples by INNO-LiPA (LiPA) were compared to NGS with discordant results arbitrated by Sanger sequencing. Genotyping of 105 distinct samples revealed a concordance of 95.2% (100/105), with Sanger sequencing confirming the NGS result. Resistance testing by NGS was concordant with LiPA in 85% (68/80) of previously characterized samples. Additional mutations were found in 8 samples, which related to the identification of low-level mutant subpopulations present at <10% (6/8). To balance the costs of testing for the validation study, reproducibility of the NGS was investigated through an analysis of sequence variants at loci not associated with resistance in a single patient sample. Our validation approach attempts to balance costs with efficient data acquisition.

As one of the leading causes of chronic hepatitis and hepatocellular carcinoma, hepatitis B virus (HBV) is a significant cause of morbidity and mortality worldwide. Knowledge of the HBV genotype and drug resistance mutations can assist in the management of chronic hepatitis B, preventing the development of complications and improving prognosis (1, 2).

Genotyping can be performed via multiple diagnostic methods, such as INNO-LiPA (LiPA), Sanger sequencing, restriction fragment polymorphism (RFLP), or PCR (2). LiPA has been commonly used for genotyping and resistance testing HBV. It involves PCR amplification of the target gene followed by hybridization of DNA products to probes bound to a membrane and detection by a chemiluminescent substrate. In Canada, the predominant HBV genotypes are B and C based on LiPA, with a variable prevalence of other genotypes depending on geography (3). LiPA has an analytical sensitivity of 100% at viral loads of >1,000 IU/ml, but sensitivity is reduced at lower viral loads (90% at 100 IU/ml). In general, LiPA had a success rate of 98%, but this is primarily based on genotypes A to C (4).

Treatment of chronic HBV usually requires long-term therapy, which predisposes the patient to the development of resistance and drug toxicity (5). Monitoring of resistance within clinical microbiology laboratories is primarily performed using PCR-based direct Sanger sequencing, restriction fragment length or mass polymorphism, and DNA hybridization. Each approach has its own limitations relating to sensitivity, specificity, target limitations, detection of novel mutations, and reporting of qualitative results (6). HBV in each infected individual is believed to exist as a collection of related but distinct variants (7). As a result, there are significant limitations with the most commonly utilized methods

for identifying HBV resistance: Sanger sequencing (relatively poor sensitivity and only detects consensus populations of >20%) and line-probe hybridization assays (unable to detect novel point mutations and susceptible to hybridization errors) (8). Ultradeep pyrosequencing (a method of next-generation sequencing [NGS]) can overcome these limitations with improved sensitivity to detect mutations, the ability to quantitate the presence of minor viral subpopulations, and the capability to identify novel resistance mutations (7–11).

NGS occurs in a massively parallel fashion. Depending on the technology, hundreds of thousands to millions of DNA molecules can be sequenced in one reaction. Despite the potential clinical utility of NGS, there are several barriers to utilizing NGS in clinical microbiology laboratories. These barriers include high cost, the complexity of the methods, the lack of molecular expertise, and bioinformatics/statistical capabilities (12). An additional barrier to adoption by clinical laboratories is the limited guidance for the clinical validation of this diagnostic modality. Clinical experience

Received 19 August 2015 Returned for modification 14 September 2015

Accepted 29 October 2015

Accepted manuscript posted online 4 November 2015

Citation Lowe CF, Merrick L, Harrigan PR, Mazzulli T, Sherlock CH, Ritchie G. 2016. Implementation of next-generation sequencing for hepatitis B virus resistance testing and genotyping in a clinical microbiology laboratory. *J Clin Microbiol* 54:127–133. doi:10.1128/JCM.02229-15.

Editor: A. M. Caliendo

Address correspondence to Christopher F. Lowe, clowe@providencehealth.bc.ca.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

TABLE 1 Sequences of the oligonucleotide primers used for PCR and NGS

Primer	Sequence (5' → 3') ^a
LiPA 1	CGT GGT GGA CTT CTC TCA ATT TTC
LiPA 2	AGA AAG GCC TTG TAA GTT GGC GA
MID primer 1 (HBVDR1)	CGTATCGCCTCCCTCGCGCCATCAG-10bpMID-GCTCAAGGAAMCTCTATGT
MID primer 2 (HBVDR2)	CTATGCGCCTTGCCAGCCCGCTCAG-10bpMID-TGACANACTTTCCAATCAAT

^a The underlined regions describe the fusion primer (including the key and MID) required for sequencing by the GS Junior sequencing system.

has been primarily derived from human genetics as is reflected in the guidance available for clinical laboratories through the CDC (12). There are recommendations for the validation of molecular diagnostic testing through the Clinical and Laboratory Standards Institute (CLSI), but these guidelines are difficult to translate for NGS due to issues such as the high costs of NGS and recommendations based on nonmicrobiology testing and quantitative PCR (12, 13). Our laboratory sought to introduce ultradeep pyrosequencing for HBV resistance testing and genotyping to provide more detailed and clinically relevant results. With several platforms available for NGS, our laboratory utilized the GS Junior system (454 Life Sciences, Branford, CT), as it provided a long read length. As the HBV *pol* amplicon to be sequenced is 418 bp, this capacity enabled the design of a single nested PCR to cover the desired region rather than multiple PCR and sequencing reactions. The following is a description of our approach to the clinical validation of the ultradeep pyrosequencing assay in a clinical microbiology laboratory.

MATERIALS AND METHODS

Patients and samples. NGS for genotyping was performed on plasma samples from 80 patients, which were previously characterized by LiPA. In addition, 24 samples from Quality Control for Molecular Diagnostics (QCMD, Glasgow, United Kingdom) proficiency panels were used. The 2nd World Health Organization (WHO) international standard for hepatitis B virus DNA was also included for testing. Resistance testing was studied on 80 clinical patient samples with a known mutant profile based on LiPA. Samples were selected for genotyping and resistance testing to ensure all genotypes and known clinically relevant mutations were included. ATCC 45020D plasmid DNA was utilized as a control sample for all runs.

A patient sample (genotype B; viral load of 1.25×10^6 IU/ml) with mutations at approximately 5%, 3%, 15%, 3%, 3%, and 45% at codons Y148F, I163V, V207L, V207M, N226T, and C256G, respectively, was diluted in Basematrix plasma (SeraCare, Milford, MA) to 12,500, 4,000, 1,250, 500 and 172 IU/ml. These dilutions were sequenced in replicates of 7 (at 12,500 and 500 IU/ml), 6 (at 4,000 IU/ml), and 10 (at 1,250 and 172 IU/ml) to determine the reproducibility of NGS at detecting mutations at various viral loads.

INNO-LiPA. The INNO-LiPA HBV DR assay (version 2/3) and the INNO-LiPA HBV genotyping assay (Innogenetics, Ghent, Belgium) were performed per the manufacturer's instruction.

PCR amplification and NGS. DNA was extracted from 500-μl plasma samples using the MagNA Pure LC DNA isolation kit on the MagNA Pure LC 2.0 (Roche Diagnostics, Mannheim, Germany) and was eluted in 50 μl. PCR amplified a 418-bp region of the HBV polymerase gene (codons 143 to 281). The PCR (50 μl) was performed on the Roche 480 PCR instrument, with the reaction mixture consisting of 15 μl of DNA, 0.075 μM primers LiPA 1 and LiPA 2 (Table 1), 0.5 μM forward and reverse fusion primers, and the AccuPrime Taq DNA polymerase high-fidelity kit (Life Technologies, Burlington, ON) with AccuPrime buffer 1.

The PCR was activated at 95°C for 30 s followed by a 3-step amplification. Step 1 consisted of 20 cycles of 95°C for 10 s, 65°C for 10 s, and 72°C for 5 s to preferentially amplify the LiPA 1 and LiPA 2 product. Step

2 included 5 cycles of 95°C for 10 s, 52°C for 10 s, 72°C for 5 s to initiate amplification with the fusion primers. Finally, step 3 consisted of 25 cycles of 95°C for 10 s, 70°C for 10 s, and 72°C for 5 s to complete the amplification. PCR products were purified using Agencourt AMPure XP (Beckman Coulter, Brea, CA), visualized on the Agilent 2100 high-sensitivity DNA analysis kit (Santa Clara, CA), and quantified using the QuantiFluor double-stranded DNA (dsDNA) system (Promega, Madison, WI). PCR products were diluted to 10^{-9} amplicons/ml, mixed in equimolar amounts to form the library, and purified a further 2 times by Agencourt beads. The library was sequenced on the GS Junior system (Titanium) following the manufacturer's protocols for sequencing in the forward and reverse directions using the Lib-A kit. The 95% limit of detection of the PCR amplicon was 125 IU/ml as determined by an endpoint dilution series of the WHO international standard.

Sequencing reads were sorted by sample according to their 10-bp multiplex ID (MID). Standard and extended MID sets were used for amplification as described in the GS Junior Amplicon Variant Analyzer (version 2.7 software; Roche). Performance of the GS Junior for each run is described in Table 2. All amplicons that passed the GS Run Browser (version 2.7) were included in the analysis. Sequences were then aligned to GenBank accession number [HE974363](#) (HBV genotype A1) by the 454 Amplicon Variant Analyzer software (version 2.7). FASTA files of the consensus sequences were sent to ABL TherapyEdge (Luxembourg) for genotyping and mutational analysis (DeepChek-HBV software, version 1.3; DeepChek-HBV Expert system, version 1.3; DeepChek-HBV Algorithms, version 6.0). For genotyping, homology testing was established after alignment based on a 20% consensus of the sample against the reference sequence. Drug resistance mutations and variant determinations were interpreted by ABL TherapyEdge using a proprietary pipeline for the correction and alignment of data, with preset data validators for homopolymers and insertions/deletions. Interpretation of resistance mutations was based on the 2012 clinical practice guidelines of the European Association for the Study of the Liver (EASL) (5).

Sequencing. Sanger sequencing of selected samples was performed on the ABI 3730 (Applied Biosystems, Burlington, ON) using primers LiPA 1 and LiPA 2 and a BigDye sequencing kit (Thermo Fisher Scientific Inc., Burlington, ON) as previously described (14).

TABLE 2 Read numbers per run on the GS Junior

Run no.	No. of samples	Total no. of reads	No of reads per sample
1	30	79,715	2,657
2	29	60,354	2,081
3	29	115,451	3,981
4	29	105,976	3,654
5	27	104,226	3,860
6	25	116,162	4,646
7	18	53,441	2,969
8	18	82,512	4,584
9	25	64,840	2,594
10	22	80,596	3,663
11	21	109,877	5,232
12	29	86,120	2,970
13	30	99,423	3,314

TABLE 3 Resolving discordant next-generation sequencing and INNO-LiPA genotyping results with Sanger sequencing results

INNO-LiPA	Next-generation sequencing	Sanger sequencing
B	C	C
B	C	C
B	D	D
D	C	C
E	B	B

RESULTS

Genotyping. A total of 105 unique samples were tested by NGS (by LiPA, the results were A = 15, B = 37, C = 22, D = 18, E = 3, F = 2, G = 4, H = 4). Concordance between LiPA and NGS was observed in 100/105 samples. Where samples were discordant between LiPA and NGS, Sanger sequencing was performed. In all five discrepant cases, the results from Sanger and NGS were concordant (Table 3).

Six samples were utilized to test inter-run and intrarun reproducibility (WHO 2nd international standard [A2], patient control [A], ATCC plasmid [A2], and 3 patient samples [2 Bs and 1 C]) over 8 runs on separate days by 2 different operators (total of 71 replicates tested). There was 100% concordance for reproducibility of HBV genotype calls.

Resistance testing. Eighty previously characterized HBV samples by LiPA were tested by NGS (A = 5, B = 25, C = 39, D = 5, E = 2, G = 4). Concordance between LiPA and NGS for clinically relevant resistance loci was 85.0% (68/80). Of the 12 samples that were nonconcordant, 8 were detected by NGS but not by LiPA, 3 were detected by LiPA but not by NGS, and 1 had discordant mutations by NGS compared to LiPA and vice versa (Table 4). Six of the eight samples detected by NGS and not by LiPA had low-level (<10%) mutant subpopulations detected by NGS. Specifically for non A to C genotypes, concordance was identified in 10/11 samples, with an S202S/G mutation identified by LiPA and not by NGS in a sample with a low viral load (Table 4, sample 3). In addition, LiPA produced indeterminate results in 8 samples (one or more codons without a result): four at M250V, two at N236T, two at S202I/G, two at V173L, and one at M204V/I. Except for two samples with mutations at M204I identified by NGS (Table 4, samples 7 and 12), drug resistance mutations were not identified at these indeterminate sites.

The initial PCR for sample 4 produced adequate copy numbers for ultradeep pyrosequencing but only 40 NGS reads. As a result of the low number of reads, sample 4 (Table 4) was repeatedly negative for S202G by next-generation sequencing. Further investigation of the PCR product on the Agilent gel revealed only a minor peak of the expected 530-bp product from the nested fusion primers (HVDR1 and HBVDR2). Over 90% of the product was 620 bp in size, which corresponded to a PCR product with the HBVDR2 primer and the LiPA 1 primer. The DNA was sequenced by Sanger sequencing, which identified 2-bp mutations in the primer binding site (target [mutations underlined]: TGG CAT ACT TTC CAA TCT AT). By utilizing modified primers for pyrosequencing, the sample was successfully tested and matched LiPA and Sanger sequencing results.

Reproducibility was evaluated based on the results of the patient positive control and the plasmid control. These controls were

included in every NGS run ($n = 13$), and the expected resistance patterns were detected in all repeats (plasmid—no detectable mutations; mean patient positive control—81.6% L180M and 99.3% M204I). In 2 of the 13 runs, the presence of V173L (2.2%) and M204V (4.3%, 2.4%) were also detected in the patient positive control. Analyzing the plasmid control for the resistance codons described in EASL (I169T, V173L, L180M, A181T/V, T184G, S202I/G, M204I/V, N236T, M250V), only 26 codon errors were identified out of a possible 365,148 codon reads (0.007% error rate for wild-type codons). None of these errors would have been identified by TherapyEdge since any mutations in one direction must be confirmed by reads in the opposite direction, and further, a threshold of 1% was established for the clinical report of a mutation.

To further assess the inter-run and intrarun variability of resistance testing, specifically the reproducibility of detecting low-level mutant subpopulations at low viral loads, a single patient sample fully susceptible to all HBV antiviral agents was evaluated over 2 different runs as described in the methods. Each sample underwent extraction, reverse transcriptase PCR (RT-PCR), and NGS. Detection of the 4 loci with sequence variants of <10% (Y148F, I163V, N226T, and V207M) at different viral loads was as follows: 28/28 at 12,500 IU/ml, 24/24 at 4,000 IU/ml, 40/40 at 1,250 IU/ml, 27/28 at 500 IU/ml (1 undetected variant at the L207M loci), and 27/40 at 172 IU/ml. Reproducibility of the exact percentage of sequence variants at various viral load dilutions is shown in Table 5, with increasing variability noted for variants of <10% at low viral loads.

DISCUSSION

Evaluation and implementation of an NGS assay for hepatitis B genotyping and resistance testing were conducted, with results indicating 100% accuracy for genotyping and 95% accuracy for resistance testing. Implementation of novel molecular diagnostics has been challenging in the absence of a highly sensitive standard for comparison. In this study, discrepant results were arbitrated by a 3rd test (Sanger sequencing) that is less sensitive, in which only the predominant subpopulation present at >20% can be detected (15). The decreased sensitivity was evident when evaluating discrepant patient samples with low viral loads and the rare mutant subpopulations shown in Table 3; however, resolving discordant genotype results was more reliable with Sanger sequencing than resistance testing, as genotyping is based on the predominant sequence of the S gene and is not dependent on the detection of rare mutations (2).

NGS assays are increasingly used in clinical laboratories due to their increased sensitivity and their ability to provide comprehensive and detailed information. Experience with NGS for clinical laboratories has been primarily from molecular genetics laboratories for rare diseases and malignancies, with recommendations available for approaches to the introduction of testing in clinical laboratories (12). Application of NGS to clinical microbiology laboratories is limited. Validation of this HBV NGS assay was based on recommendations for molecular testing in microbiology labs and NGS for genetics (12, 13, 16). A specific challenge to NGS clinical validation has been the difficulty in balancing the high costs with the need for data, as was the case for reproducibility (16). Assessing the reproducibility of NGS based on the recommendations for current molecular PCR assays was considered impractical given the vast number of permutations of HBV mutations identified with NGS. The use of a single-tube nested PCR amplifying a 418-bp region reduced cost and simplified sample

TABLE 4 Comparison of NGS, LiPA, and Sanger sequencing for mutations at clinically relevant loci

Sample	Genotype	Viral load (copies/ml)	LiPA	NGS	Sanger	NGS +/LiPA – ^a	NGS –/LiPA +	Comment
1	B	12,549	L180M M204V S202S/G	100% L180M 9% T184L 2% A194T	L180M T184L A194T	Y	Y	
2	C	4,999	L180L/M A181A/T A194A/T S202S/C/I M204/V	100% M204V 5% L180M 100% M204V	M204V M204V A181A/T	N	Y	Required 1/10 dilution for testing.
3	G	392	V173V/L L180L/M S202S/G M204M/V	15% V173L 18% L180M 18% M204V	NA ^b	N	Y	
4	C	2,435	L180M S202S/G M204V	100% L180M 100% M204V	L180M S202S/G M204V	N	Y	Mutation at the NGS primer binding site
5	C	1,800,000	L80L/V L180L/M M204M/I/V	3% V173L 20% L180M 45% M204I 17% M204V	NA	Y	N	
6	C	107,000	T184T/SCGA L180M M204V	96% T184A 100% L180M 2% S202G 100% M204V	NA	Y	N	
7	C	2,616,910	L80L/V	2% L180M 99% M204I	NA	Y	N	No band at M204I on LiPA
8	C	1,421,970	V173L L180M A181T	87% V173L 92% L180M 93% A181T 4% M204I	NA	Y	N	
9	C	93,000	L180L/M M204M/V N236N/T	1% V173L 13% L180M 13% M204V 81% N236T	NA	Y	N	
10	B2	2,917	L80I L180L/M M204I	85% L180M 97% M204I 3% M204V	NA	Y	N	
11	C	2,500	L80V L180L/M M204V/I	14% V173L 91% L180M 84% M204I 14% M204V	NA	Y	N	
12	C	16,000	No mutations	90% 204I	NA	Y	N	No bands at 173, 204, and 250 on LiPA

^a +, Positive; –, negative; Y, yes; N, no.^b NA, not available.

processing, as it required only amplification and sequencing of 1 amplicon to capture all clinically relevant mutations and genotypes. Previously, conventional two-tube nested PCR approaches have been used to produce the sequencing library for HBV mutational analysis (7, 17). However, in a diagnostic laboratory, conventional nested PCR poses an unacceptable contamination risk. Our single-tube nested PCR allowed for the increased sensitivity of nested PCR with reduced contamination risk since PCRs are not opened after the first round of amplification. More significantly, a patient sample with sequence variants at nonclinically significant loci that were present in rare (<10%) subpopulations was selected for reproducibility studies to avoid the need to test multiple samples repeatedly. In lieu of identifying and testing

multiple samples to ensure all mutations (at various percentages of subpopulation and viral load) were present, one patient sample (Table 5) was used as a pragmatic method to examine reproducibility and overcome the issue of validation cost.

The introduction of NGS revealed clinical challenges to reporting and to interpreting test results. Potentially clinically relevant mutations that are included in the EASL HBV guidelines were identified by NGS but not by LiPA in 11.3% (9/80) of patient samples. Eight samples also had indeterminate results by LiPA, including 2 at M204I (detected by NGS); this was likely the result of the hybridization failure of the probes due to sequence variation, a known limitation of LiPA (18). Although more low-level resistance mutations are detected, the reproducibility studies

TABLE 5 Variability in variant analysis with decreasing viral loads

Variant	Viral load (IU/ml)	Mean (%)	Coefficient of variation	95% Confidence interval
148F	12,500	5.1	19.3	4.4–5.9
	4,000	5.5	15.6	4.9–6.2
	1,250	5.1	59.8	2.8–7.3
	500	4.9	53.3	2.9–6.8
163V	12,500	2.4	10.2	2.3–2.6
	4,000	2.48	27.0	2.0–3.0
	1,250	3.0	56.7	1.8–4.3
	500	3.8	103.4	0.9–6.8
207L	12,500	17.6	11.0	16.2–19.1
	4,000	16.4	19.5	14.0–18.8
	1,250	16.2	16.2	14.3–18.2
	500	12.7	37.9	9.1–16.3
207M	12,500	3.5	19.5	3.0–4.0
	4,000	3.6	43.2	2.5–4.8
	1,250	3.8	71.7	1.8–5.8
	500	4.0	94.2	1.2–6.7
226T	12,500	2.0	23.7	1.7–2.4
	4,000	2.1	31.0	1.6–2.6
	1,250	2.7	72.0	1.3–4.2
	500	4.5	86.8	1.6–7.3
256G	12,500	49.6	5.8	47.5–51.7
	4,000	46.1	5.1	44.4–47.8
	1,250	46.9	10.5	43.2–50.5
	500	41.4	32.0	31.6–51.1

revealed high variation for the percentage of mutant subpopulations, especially at low viral loads near the limit of detection (Table 5) (19). In addition, implementing a reliable control for low-level mutants was a challenge, as the mutations were only detected in 15.4% (2/13) of the patient controls. These mutations, while only intermittently detected, represented real mutations and were present above the error rate of our assay. Low-level variants previously would not have been reliably detected by LiPA, and their clinical significance is unclear. The requirement for each run to detect low-level variants in the control would be impractical, as the vast majority of the runs would have to be rejected despite no issues with the plasmid control and other predominant mutations in the patient controls. However, with this potential uncertainty regarding the reliable detection of low-level mutations, we engaged local hepatologists regarding the limitations of these mutants and their guidance in reporting and interpreting these previously undetected low-level mutations. From that discussion, it was decided to report the presence of the mutation but not the specific percentage (i.e., all low-level mutations would be reported as <10%). Reporting based on a more sensitive assay can potentially have significant repercussions on pharmacy costs and clinical outcomes. The clinical relevance of detecting these variants in HBV is still unclear (20–22), but experience in resistance testing for HIV patients with low viral loads (and rare mutations) has suggested that they may have long-term clinical impacts (23). With increased adoption of NGS for clinical applications, further study is required to understand the potential clinical impact of the additional information identified by NGS, such as low-level mu-

tant subpopulations. Understanding the clinical relevance of these previously undetectable rare subpopulations can result in improved patient care, potentially utilizing NGS as a screening test before initiation of antiviral therapy (and selecting an alternative if these subpopulations are detected) or enabling an earlier change in antiviral therapy (22). On a practical level, in British Columbia, provincial coverage for the cost of lamivudine and tenofovir is based on resistance testing, and NGS may result in a change in practice as more patients may become eligible for tenofovir than those that are based on the LiPA (<http://www2.gov.bc.ca/gov/content/health/practitioner-professional-resources/pharmacare/prescribers/limited-coverage-drug-program/limited-coverage-drugs-tenofovir>).

For ongoing monitoring and quality control, 2 positive controls were tested on every run: a known patient positive control and a plasmid control. The plasmid control provided an estimate of error through the nested PCR, emulsion PCR, and ultradeep pyrosequencing reaction since only a single sequence should be present. Wild-type HBV samples would inherently have sequence variants, and the detection of rare reads could affect interpretation (normal variants versus the intrinsic error rate of NGS). Use of the plasmid control avoids this potential interpretation bias (8, 10). The error rate of NGS has been estimated to be <0.05%, with conservative cutoffs of 1% (8, 24), which has been within the observed results of this HBV NGS assay for resistance and genotyping (<0.1%). In addition, as a quality check before clinical reporting of an NGS run, a minimum threshold of 2,000 reads per sample was set for acceptance of the sequencing result. As a result, theoretically, detection of a 1% subpopulation can then be identified based on the presence of 20 copies above the expected error rate of the assay.

Clinical validation of NGS for microbiology laboratories has not been standardized and is a limitation of this study. In an attempt to introduce new technology while being mindful of costs, reproducibility experiments for low-level sequence variants were performed on nonclinically relevant sites to enable all testing to be done on 1 sample. However, the selected loci spanned the entire 418-bp polymerase product and would be representative of the reported resistance sites. The mutation L80I/V has been associated with lamivudine resistance but in conjunction with the presence of M204I/V (25). The coverage of this NGS assay does not extend to L80I/V, but in discussion with clinicians, the lack of L80I/V was acceptable, as this loci is not included in the EASL guidelines and lamivudine resistance would still be identified through M204I/V. Sequencing errors can also be a potential issue with NGS, particularly for 454 platforms at sequences with homopolymers (26). However, this did not represent a significant issue, as the amplified *pol* target did not contain homopolymer regions around the clinically relevant mutation sites. Error rates are also platform dependent. In addition, analysis of such vast amounts of sequencing data is a significant barrier for clinical laboratories without in-house bioinformatics support. A third party bioinformatics company was utilized for analysis, but detailed, customized interpretation of data, such as haplotyping and coinfections, was not conducted in this study. Haplotyping can provide details regarding viral subpopulation complexity. This may be useful for antiviral resistance requiring multiple mutations (e.g., Entecavir), though they may predominantly colocalize to the same viral genome (27). Further study is needed to determine whether there is a clinical effect of differentiating patients with mutations present

(that may or may not colocalize to the same viral genome) as tested by LiPA or Sanger sequencing compared to patients with mutations localized to a predominant haplotype (NGS). With respect to coinfections, the software utilized for bioinformatics did not determine coinfections at the time of the study. Updates for this software have since been implemented through a high-resolution subtyping method (28). Finally, the initial limit of detection for the amplification of the *pol* gene was insensitive (20,000 IU/ml) using NGS fusion primers in a single round of PCR. This was overcome by implementing a nested PCR as described above (120 IU/ml). The sensitivity for detecting rare sequence variants can be compromised in patients with low viral load, as few or none of the low-level variants may be present in the aliquot amplified. For practical purposes, a cutoff was established for testing at 1,000 copies/ml (or 172 IU/ml) for mutations identified at proportions of >10%. Due to the potential for false-negative results of low-level mutations, as 70% of these variants were not detected at 172 IU/ml, a comment will be appended to the report for viral loads of <500 IU/ml indicating this limitation. Discussion with end-users about the establishment of cutoffs is critical to ensure they understand the limitations of the test.

Molecular diagnostics have advanced rapidly in the past decade, and it is imperative for clinical microbiology and virology laboratories to integrate these methods to provide additional information to clinicians and improve patient care. Many barriers still need to be addressed with respect to cost, clinical interpretation, bioinformatics, proficiency testing, and quality control. With limited guidance for clinical microbiology laboratories, we have presented our approach to the development and validation of an NGS assay for HBV genotyping and resistance testing. As molecular assays advance to provide information that was previously undetected or unknown, it is critical to collaborate and engage relevant clinicians on reporting algorithms. Technology continues to advance rapidly with the impending discontinuation of the 454 platform and the introduction of a new generation of sequencers, but we believe our approach, including the pragmatic approach to the assessment of reproducibility, can translate to the validation of future platforms.

ACKNOWLEDGMENTS

We acknowledge Roche Diagnostics for technical support and assistance with the 454 Junior.

REFERENCES

- Zoulim F, Locarnini S. 2009. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology* 137:1593–1608. <http://dx.doi.org/10.1053/j.gastro.2009.08.063>.
- Guirgis BS, Abbas RO, Azzazy HM. 2010. Hepatitis B virus genotyping: current methods and clinical implications. *Int J Infect Dis* 14:e941–e953. <http://dx.doi.org/10.1016/j.ijid.2010.03.020>.
- Congly SE, Wong P, Al-Busafi SA, Doucette K, Fung SK, Ghali P, Fonseca K, Myers RP, Osiowy C, Coffin CS. 2013. Characterization of hepatitis B virus genotypes and quantitative hepatitis B surface antigen titres in North American tertiary referral liver centres. *Liver Int* 33:1363–1369. <http://dx.doi.org/10.1111/liv.12222>.
- Qutub MO, Germer JJ, Rebers SP, Mandrekar JN, Beld MG, Yao JD. 2006. Simplified PCR protocols for INNO-LiPA HBV genotyping and INNO-LiPA HBV PreCore assays. *J Clin Virol* 37:218–221. <http://dx.doi.org/10.1016/j.jcv.2006.08.006>.
- European Association for the Study of the Liver. 2012. EASL clinical practice guidelines: management of chronic hepatitis B virus infection. *J Hepatol* 57:167–185. <http://dx.doi.org/10.1016/j.jhep.2012.02.010>.
- Kim JH, Park YK, Park ES, Kim KH. 2014. Molecular diagnosis and treatment of drug-resistant hepatitis B virus. *World J Gastroenterol* 20:5708–5720. <http://dx.doi.org/10.3748/wjg.v20.i19.5708>.
- Rodriguez C, Chevaliez S, Bensadoun P, Pawlotsky JM. 2013. Characterization of the dynamics of hepatitis B virus resistance to adefovir by ultra-deep pyrosequencing. *Hepatology* 58:890–901. <http://dx.doi.org/10.1002/hep.26383>.
- Solmone M, Vincenti D, Prosperi MC, Bruselles A, Ippolito G, Capobianchi MR. 2009. Use of massively parallel ultradeep pyrosequencing to characterize the genetic diversity of hepatitis B virus in drug-resistant and drug-naïve patients and to detect minor variants in reverse transcriptase and hepatitis B S antigen. *J Virol* 83:1718–1726. <http://dx.doi.org/10.1128/JVI.02011-08>.
- Margeridon-Thermet S, Shulman NS, Ahmed A, Shahriar R, Liu T, Wang C, Holmes SP, Babrzadeh F, Gharizadeh B, Hanczaruk B, Simen BB, Egholm M, Shafer RW. 2009. Ultra-deep pyrosequencing of hepatitis B virus quasiespecies from nucleoside and nucleotide reverse-transcriptase inhibitor (NRTI)-treated patients and NRTI-naïve patients. *J Infect Dis* 199:1275–1285. <http://dx.doi.org/10.1086/597808>.
- Rodriguez-Frias F, Tabernero D, Quer J, Esteban JI, Ortega I, Domingo E, Cubero M, Camós S, Ferrer-Costa C, Sánchez A, Jardi R, Schaper M, Homs M, Garcia-Cehic D, Guardia J, Esteban R, Buti M. 2012. Ultra-deep pyrosequencing detects conserved genomic sites and quantifies linkage of drug-resistant amino acid changes in the hepatitis B virus genome. *PLoS One* 7:e37874. <http://dx.doi.org/10.1371/journal.pone.0037874>.
- Ciftci S, Keskin F, Cakiris A, Akyuz F, Pinarbasi B, Abaci N, Dincer E, Badur S, Kaymakoglu S, Ustek D. 2014. Analysis of potential antiviral resistance mutation profiles within the HBV reverse transcriptase in untreated chronic hepatitis B patients using an ultra-deep pyrosequencing method. *Diagn Microbiol Infect Dis* 79:25–30. <http://dx.doi.org/10.1016/j.diagmicrobio.2014.01.005>.
- Gargis AS, Kalman L, Berry MW, Bick DP, Dimmock DP, Hambuch T, Lu F, Lyon E, Voelkerding KV, Zehnbauser BA, Agarwala R, Bennett SF, Chen B, Chin EL, Compton JG, Das S, Farkas DH, Ferber MJ, Funke BH, Furtado MR, Ganova-Raeva LM, Geigenmüller U, Gunselman SJ, Hegde MR, Johnson PL, Kasarskis A, Kulkarni S, Lenk T, Liu CS, Manion M, Manolio TA, Mardis ER, Merker JD, Rajeevan MS, Reese MG, Rehm HL, Simen BB, Yeakley JM, Zook JM, Lubin IM. 2012. Assuring the quality of next-generation sequencing in clinical laboratory practice. *Nat Biotechnol* 30:1033–1036. <http://dx.doi.org/10.1038/nbt.2403>.
- Clinical and Laboratory Standards Institute. 2009. Molecular diagnostic methods for infectious diseases; approved guideline—2nd ed. CLSI Document MM3-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
- Law JK, Ali JA, Harrigan PR, Sherlock CH, Savage KJ, Yoshida EM. 2006. Fatal postlymphoma chemotherapy hepatitis B reactivation secondary to the emergence of a YMDD mutant strain with lamivudine resistance in a noncirrhotic patient. *Am J Hematol* 81:969–972. <http://dx.doi.org/10.1002/ajh.20737>.
- Lindstrom A, Odeberg J, Albert J. 2004. Pyrosequencing for detection of lamivudine-resistant hepatitis B virus. *J Clin Microbiol* 42:4788–4795. <http://dx.doi.org/10.1128/JCM.42.10.4788-4795.2004>.
- Burd EM. 2010. Validation of laboratory-developed molecular assays for infectious diseases. *Clin Microbiol Rev* 23:550–576. <http://dx.doi.org/10.1128/CMR.00074-09>.
- Homs M, Buti M, Tabernero D, Quer J, Sanchez A, Corral N, Esteban R, Rodriguez-Frias F. 2012. Quasispecies dynamics in main core epitopes of hepatitis B virus by ultra-deep-pyrosequencing. *World J Gastroenterol* 18:6096–6105. <http://dx.doi.org/10.3748/wjg.v18.i42.6096>.
- Niesters HG, Zoulim F, Pichoud C, Buti M, Shapiro F, D'Heuvaert N, Celis L, Dautreloigne J, Sablon E. 2010. Validation of the INNO-LiPA HBV DR assay (version 2) in monitoring hepatitis B virus-infected patients receiving nucleoside analog treatment. *Antimicrob Agents Chemother* 54:1283–1289. <http://dx.doi.org/10.1128/AAC.00970-09>.
- Knapp DJ, McGovern RA, Poon AF, Zhong X, Chan D, Swenson LC, Dong W, Harrigan PR. 2014. “Deep” sequencing accuracy and reproducibility using Roche/454 technology for inferring co-receptor usage in HIV-1. *PLoS One* 9:e99508. <http://dx.doi.org/10.1371/journal.pone.0099508>.
- Bayliss J, Nguyen T, Lesmana CR, Bowden S, Revill P. 2013. Advances in the molecular diagnosis of hepatitis B infection: providing insight into the next generation of disease. *Semin Liver Dis* 33:113–121. <http://dx.doi.org/10.1055/s-0033-1345714>.
- Chevaliez S, Rodriguez C, Pawlotsky JM. 2012. New virologic tools for management of chronic hepatitis B and C. *Gastroenterology* 142:1303–1313. <http://dx.doi.org/10.1053/j.gastro.2012.02.027>.
- Homs M, Caballero A, Gregori J, Tabernero D, Quer J, Nieto L, Esteban R, Buti M, Rodriguez-Frias F. 2014. Clinical application of estimating hepatitis B virus quasiespecies complexity by massive sequencing: correla-

- tion between natural evolution and on-treatment evolution. PLoS One 9:e112306. <http://dx.doi.org/10.1371/journal.pone.0112306>.
23. Gonzalez-Serna A, Min JE, Woods C, Chan D, Lima VD, Montaner JS, Harrigan PR, Swenson LC. 2014. Performance of HIV-1 drug resistance testing at low-level viremia and its ability to predict future virologic outcomes and viral evolution in treatment-naïve individuals. Clin Infect Dis 58:1165–1173. <http://dx.doi.org/10.1093/cid/ciu019>.
 24. Homs M, Buti M, Quer J, Jardí R, Schaper M, Tabernero D, Ortega I, Sanchez A, Esteban R, Rodriguez-Frias F. 2011. Ultra-deep pyrosequencing analysis of the hepatitis B virus preCore region and main catalytic motif of the viral polymerase in the same viral genome. Nucleic Acids Res 39:8457–8471. <http://dx.doi.org/10.1093/nar/gkr451>.
 25. Warner N, Locarnini S, Kuiper M, Bartholomeusz A, Ayres A, Yuen L, Shaw T. 2007. The L80I substitution in the reverse transcriptase domain of the hepatitis B virus polymerase is associated with lamivudine resistance and enhanced viral replication *in vitro*. Antimicrob Agents Chemother 51:2285–2292. <http://dx.doi.org/10.1128/AAC.01499-06>.
 26. Becker EA, Burns CM, León EJ, Rajabojan S, Friedman R, Friedrich TC, O'Connor SL, Hughes AL. 2012. Experimental analysis of sources of error in evolutionary studies based on Roche/454 pyrosequencing of viral genomes. Genome Biol Evol 4:457–465. <http://dx.doi.org/10.1093/gbe/evs029>.
 27. Ko SY, Oh HB, Park CW, Lee HC, Lee JE. 2012. Analysis of hepatitis B virus drug-resistant mutant haplotypes by ultra-deep pyrosequencing. Clin Microbiol Infect 18:E404–E411. <http://dx.doi.org/10.1111/j.1469-0691.2012.03951.x>.
 28. Struck D, Lawyer G, Ternes AM, Schmit JC, Bercoff DP. 2014. COMET: adaptive context-based modeling for ultrafast HIV-1 subtype identification. Nucleic Acids Res 42:e144. <http://dx.doi.org/10.1093/nar/gku739>.