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

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Introduction

- Hepatitis B, HBV, is a major health problem worldwide; 400 million people live with chronic HBV infection.
- Treatment for chronic HBV involves nucleoside/nucleotide analogues to suppress viral replication. However, resistance can develop while on long-term therapy (1) due to specific AVDR mutations in the HBV *pol* gene.
- Knowledge of the HBV genotype is also helpful in guiding clinical treatment and prognosis. The sequence of the HBV *pol* gene can determine the genotype of the virus (2).
- Reverse hybridization (INNO-LiPA) and Sanger sequencing are the most commonly used methods to determine the resistance mutations and viral genotype. However, neither method is quantitative. Furthermore, Sanger suffers from a lack of sensitivity, and INNO-LiPA may have hybridization failures.
- Next generation sequencing (NGS) has the potential to improve diagnosis by simultaneously sequencing thousands of individual viruses. Quantitative determination of mutation percentage can be calculated and HBV genotype determined.
- We describe our approach to validating a clinical diagnostic NGS assay for HBV genotyping and AVRD mutation analysis.

Methods & Materials

- An in-house developed assay for HBV genotype and resistance testing was studied using the GS Junior (454 Life Sciences). DNA was extracted using the MagNA Pure LC 2.0 (Roche Diagnostics). Codons 143 - 281 were amplified using a unique one-tube nested PCR of 15 µL of DNA, 0.07 µM of primers IL1 and IL2, 0.5 µM forward and reverse fusion primers, and Accuprime Taq DNA Polymerase HiFi kit (Life Technologies)

Primer	Sequence (5' → 3')
IL1	CGT GGT GGA CTT CTC TCA ATT TTC
IL2	AGA AAG GCC TTG TAA GTT GGC GA
Fusion Primer 1	CGTATCGCCTCCCTCGCGCCA-TCAG-10bpMID-GCTCAAGGAAMCTCTATGT
Fusion Primer 2	CTATGCGCCTTGCCAGCCCGC-TCAG-10bpMID-TGACANACTTTCCAATCAAT

- Where MIDs are the standard 454 MID sequences for amplicon sequencing
- A 3-step amplification strategy was employed. First annealing at 60C to allow the IL1/2 primers to amplify a 820bp target, then annealing at 52C to allow 454-fusion primers to amplify the 418bp product for sequencing and further amplification with annealing at 60C to complete the PCR.
- PCR products were purified by Agincourt AMPure XP beads, quantified, pooled, and sequenced on the GS Junior by bidirectional amplicon sequencing.
- Alignment of reads was performed by Amplicon Variant Analysis V2.7 (Roche), checked by Geneious software (Biomatters, NZ), and analyzed by DeepCheck (ABL-TherapyEdge, Luxembourg) to determine the *pol* gene mutations and HBV genotype.
- Interpretive criteria for antiviral resistance were based on the 2012 EASL Clinical Practice Guidelines (1)
- A positive control sample with a viral load of 2000 IU/mL and the ATCC plasmid were included with each NGS run for quality control purposes.
- Genotype (n=80) and resistance (n=80) were previously characterized by line-probe assay (INNO-LiPA HBV DR Assay, Version 2/3 and INNO-LiPA HBV Genotyping Assay; Innogenetics, Belgium). In addition, 24 proficiency samples from QCMD (UK) and the WHO std were sequenced.

- Sanger sequencing of the *pol* gene was performed with primers IL1 and IL2 using the 3730 DNA Analyzer (Applied Biosystems, Foster City) for discrepant results between the line-probe assay and NGS.

Results

- **Error rates** of less than 0.1% for the 9 AVDR codons were detected in the plasmid control.
- The error rate was dependent on the surrounding sequence motif. Homopolymer regions gave the highest error rate - a known limitation of 454 sequencing (3). However, no known antiviral drug mutations are located in these homopolymer regions.

- **Genotype Testing:** Concordance between INNO-LiPA and NGS was observed in 100/105 samples.

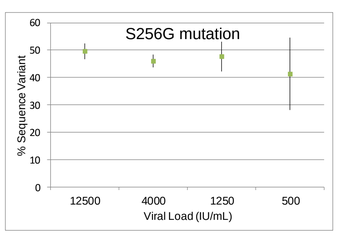
Table: Genotyping by next generation sequencing and INNO-LiPA.

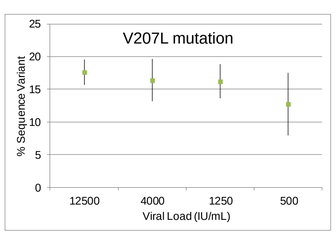
genotype	No. samples INNO-LiPA results	discrepancies
A	15	0
B	37	3
C	22	0
D	18	1
E	3	1
F	2	0
G	4	0
H	4	0

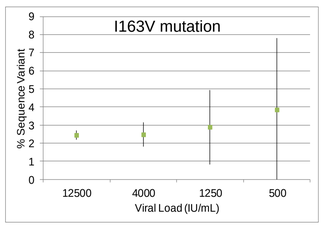
- Sanger sequencing confirmed the NGS genotype for all discrepant results.

- **Resistance testing** for 80 samples included mutations at the following codons: M204, L180, A181, N236, M250, V173, T184, S202, and I233.
- Concordance between INNO-LiPA and NGS for clinically relevant resistance loci was 85.0% (68/80).
- Six samples had a minor mutant subpopulation (<10% of the virus population with a base pair mutation at an AVDR codon) detected by NGS but not INNO-LiPA.
- Eight samples had *pol* codons that could not be detected by INNO-LiPA, probably due to sequence variations in the probe binding region that are not accounted for by the probes (hybridization failure). All samples had detectable 454 sequence at these codons.
- Two of these hybridization failure samples had a lamivudine mutation at the codon missed by INNO-LiPA (M204I).
- Three faint mutation bands seen by INNO-LiPA were missed by NGS; these samples had viral loads less than 1000 cp/mL. These discrepancies can be explained by sampling error.
- One sample failed to amplify properly due to mismatches in the fusion primer. A modified primer solved this problem.

- **Reproducibility** of mutation percentages was dependent on both mutation percent and sample viral load probably due to sampling error in the nested PCR, especially at low viral loads.







Discussion

- NGS yielded more accurate genotyping results than the INNO-LiPA method since the entire amplicon sequence is determined, whereas the INNO-LiPA method relies on interrogating only a few positions in the sequence. Additional mutations near these positions can result in probes not binding and incorrect genotype calling (5).

- Detection of low-level mutations (<10%) in samples with lower viral loads may generate false-negative results or variable mutation loads. Variability is better at higher % mutation, but still significant below 1000 IU/ml

- Since an average of 3000 sequence reads were obtained per patient sample, mutations present at very low levels might be detected by NGS that are not detectable by other methods. These mutations might indicate early signs of treatment failure.

- The one-tube nested PCR is ideally suited for clinical diagnostics since contamination risks are minimized, while maintaining a good limit of detection and simplifying sample processing.

- The sequencing reaction only detects codons 143 to 281. This sequence may miss other important mutations in the genome such as preCore mutations and other mutations in the *pol* gene shown to affect drug resistance in previous studies.

- Support for 454 technology will be terminated in 2016. However, similar sequencing strategies may be deployed for other NGS technologies.

- Long read lengths on the 454 allow for haplotype analysis. Haplotyping is important for determination of resistance where >1 mutation is necessary in the *pol* gene (eg. entecavir resistance). Haplotyping also allows for complementation analysis and for detecting recombinant genomes.

- Currently we do not have the bioinformatic capability to analyze haplotypes, though this may be available in the future versions of the ABL software.

Conclusions

- Utilizing a one-tube nested PCR targeting the HBV *pol* gene, genotyping and resistance testing for the most significant EASL mutations (1) can be performed with a single NGS reaction. This method proved to be very sensitive and specific.

- NGS can potentially provide clinicians with earlier detection and detailed analysis of resistance profiles, as well as accurate detection of genotype.

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