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 I1 and IL2, 0.5 μM forward and reverse primer fusions, and Acumprime Taq DNA Polymerase HiFi kit (Life Technologies).

.. table:: Primer Sequence (5’ – 3’)
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<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
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<tbody>
<tr>
<td>IL1</td>
<td>CCGTGGGGGCTCCCTGACATG</td>
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<tr>
<td>IL2</td>
<td>TGGCTTGTGATGACAGTATGA</td>
</tr>
<tr>
<td>Fusion Primer 1</td>
<td>CGATATGTGCTTCGGGCTTTG</td>
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<tr>
<td>Fusion Primer 2</td>
<td>GATCTGTGCTCGCAGACCTTCATG</td>
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- Where MIDs are the standard 454 MID sequences for amplicon sequencing
- A 3-step amplification strategy was employed. First annealing at 60°C to the IL2/primers to amplify a 820bp target, then annealing at 52°C to allow 454-fusion primers to amplify the 418bp product for sequencing and further amplification with annealing at 60°C to complete the PCR.
- PCR products were purified by Agencourt 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 (Biornatters, 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 performed on primers I1 and IL2 using the 3730x DNA Analyzer (Applied Biosystems, Foster City) for discrepant results between the line-probe assay and NGS.

Results

- Sanger sequencing confirmed the NGS genotypes for all discrepant results.
- Resistance testing for 80 samples included mutations at the following codons: M204, L180, A181, N236, M205, V173, T184, S2020, and 3233.
- Concordance between INNO-LIPA and NGS for clinically relevant resistance locus was 85.0% (68/80).
- Six samples had a minor mutant subpopulation (<10% of the virus population with a base pair mutation at an AVRD 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 cpm/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 genotype 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 completion analysis and for detecting recombiant genotypes.
- 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.

Acknowledgements

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References


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