Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 139–140
DOI: 10.14218/JCTH.2021.00135

Editorial

A Simple and Rapid Method for Quantitative Detection of Hepatitis B Virus Drug-resistant Mutations

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Received: 6 April 2021 | **Revised:** 10 April 2021 | **Accepted:** 12 April 2021 | **Published:** 16 April 2021

Citation of this article: Wang R, Xie Z. A simple and rapid method for quantitative detection of hepatitis B virus drug-resistant mutations. J Clin Transl Hepatol 2021;9(2):139–140. doi: 10.14218/JCTH.2021.00135.

Hepatitis B virus (HBV) is a small-enveloped virus enclosing a partially double-stranded DNA genome, belongs to the hepadnaviruses family.[1] To date, 10 genotypes (A–J) of HBV with distinct geographical distribution have been described, based on a divergence of at least 8% over the entire genomic sequence or >4% in the S gene sequence, with B and C being most prevalent and confined to Asia and Oceania.[2] HBV is a highly contagious pathogen that can lead to acute infection or chronic hepatitis B (CHB), cirrhosis and hepatocellular carcinoma (commonly known as HCC) in humans through immune anergy or upon immunosuppression.[3]

Although a global HBV vaccination program has been implemented in more than 200 countries and provided a significant decline in incidence of CHB, there are still approximately 292 million people worldwide suffering from CHB, with close to 1 million deaths occurring annually and maintaining the disease as a major global health problem.[4,5] Large-scale long-term prospective studies in the past decades have shown that antiviral nucleotide analogues (NAs) treatment of CHB patients may inhibit HBV replication without eliminating the virus, remitting HBV-related HCC in some patients and reducing associated morbidity and mortality but not completely.[6] Due to potent viral suppressive effects and good tolerance by patients taking the NAs for decades while experiencing limited side effects, these drugs have been widely used in the management of CHB treatment.[7]

Currently, NAs including lamivudine (LAM), entecavir (ETV), tenofovir disoproxil fumarate, telbivudine (LdT), adefovir dipivoxil, and tenofovir alafenamide are prescribed and available for CHB therapy in China.[8] All of these NAs represent the more preferred agents that work mainly by competitively inhibiting HBV DNA polymerase activity, with the incorporation of the natural endogenous intracellular nucleotides in assembled HBV DNA, causing DNA synthesis termination and suppressing viral replication.[9] In detail, LMT is often used in CHB patients with high HBV replication or decompensated liver disease; ETV and adefovir dipivoxil are not only suitable for patients in the acute phase of CHB, but also as alternative therapies for patients who have developed LAM resistance.

However, NA-associated resistance is a serious impediment to the treatment of CHB. For example, LAM was initially and extensively prescribed when resistance is impending. Resistance to LAM develops within 6 months of treatment, and emerges in ~20% after 1 year and at an accumulation rate of 70% following 5 years of treatment.[10] Moreover, resistance to LAM confers cross-resistance to ETV and LdT, which leads to lower antiviral efficacy and lower genetic barrier to the drugs.[11] Mutations targeting HBV polymerase/reverse transcriptase domains that are critical during viral replication, are responsible for conferring resistance to NAs.[12] High rates of HBV replication, combined with inadequately effective proofreading for HBV polymerase, is the basis for establishing mutations in the viral genome. Mutations that change the binding site between NAs and HBV represent the molecular mechanism underlying drug resistance. Mutation patterns such as M204V/I (primary resistance mutation) and L180M (secondary/compensatory mutation) were defined as joint resistance mutations across most genotypes of HBV and as involved in virological breakthrough or biochemical rebounds.[13,14] The M204V/I mutation in the C domain of polymerase represents one of the most common primary resistance mutations and directly results in high-level resistance to NAs, such as LAM, ETV, and LdT.[12,15] Thus, the monitoring and a high-speed feature detector of mutation for HBV drug resistance are necessary.

There are many types of laboratory tests that can be used to determine resistance mutations in the HBV reverse transcriptase region, with varying sensitivities. The most frequently used method for routine testing in clinical laboratories is PCR-based sequencing that is able to detect more than 20% of mutations among the total viral population.[11,16] Besides, PCR-restriction fragment length polymorphisms and reverse hybridization line-probe assays can consistently detect mutations present in 5% of the virus populations.[11] However, it should be pointed out that the emergence of HBV resistance mutations is silent and thus difficult to detect in a timely manner. Only until a certain

Abbreviations: CHB, chronic hepatitis B; ETV, entecavir; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LAM, lamivudine; LdT, telbivudine; NAs, nucleotide analogs.

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number of drug-resistant mutations have accumulated can they be detected by the commonly used sequencing methods. Thus, the key disadvantage of this method comes from the low sensitivity; that is, the variant strain can only be found when it exceeds 20% of the HBV sequence. Moreover, the detection approach by PCR combined with sequencing is time-consuming and high-cost, and the related data analysis is more complicated. Nevertheless, this method is generally used as the gold standard for the detection of resistance mutations.

To counter the disadvantages of the methods above, Liang *et al.*[17] developed a real-time PCR assay aimed at monitoring the reaction process and quantitatively detecting HBV resistance mutations. Compared with the above-mentioned commonly used methods, this method has a streamlined operation process and can detect resistance mutations with a rate of less than 10%, whereas its disadvantage is that it can only detect known mutation sites by using designed probes and primers. The investigators tentatively chose M204V/I mutations to be detected due to the widespread application of LAM, ETV, LdT, etc., and thus to be more readily available for blood samples from NA-resistant patients. Methodologically, the samples were collected from patients who were treated with NAs and were clinically confirmed to have viral breakthrough, primary nonresponse, or poor response. Then, they designed primers and probes for M204V/I that were screened and confirmed with M204V/I-positive sera, in order to verify the specificity and sensitivity rates. Finally, a linear standard curve for quantification was obtained by use of a 10-fold dilution of the plasmid, and the formula was used to calculate the viral load within extracted DNA.[17] Real-time PCR can quantify the mutated gene with relatively accurate measurement in units of copies/mL, while the current method uses ab approximate description via the percentage of mutation.

According to the results, Liang *et al.*[17] confirmed probes 204-2-VP2 and 204-2-IP2 with a positive rate of 100% and the false positive rate of 0%. Furthermore, the amplification curves showed a highly linear relationship between Ct values and the amount of serially diluted plasmid DNA for the primers and probes (R^2=0.996, slope=−3.723), indicating an appropriate quantitative detection of M204V/I. The limit of detection, sensitivity, and specificity were 10^3 copies/mL, 92.86%, and 100%, respectively. Meanwhile, the assay presented good reproducibility and accuracy.

In conclusion, a rapid and accurate assay is urgently needed to monitor HBV DNA polymerase/ reverse transcriptase gene mutations in patients undergoing NA treatment. The features of M204V/I detection for the pilot assay by real-time PCR provided high-efficiency, cost-effectiveness, and convenience, representing a high generalizability to quantitatively detect multiplex mutations in the target genes. This method is expected to present the association between specific mutations and the phenotypic resistance of an isolate with that mutation during the early stages of NA treatment.

Funding

RW was funded by the National Natural Science Foundation of China (82002130) and the Open Research Fund Program of the State Key Laboratory of Virology of China (2021IOV002); ZX was funded by the National Natural Science Foundation of China (82072266) and the National Major Science & Technology Project for Control and Prevention of Major Infectious Diseases in China (2017ZX10103004-004).

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Article conception and design (RW and ZX), drafting of the manuscript (RW), critical revision of the manuscript (ZX).

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