Accurate Detection of Hepatitis B Virus G1896A Mutant by Developed Taqman-ARMS Followed a Strict Control System

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Background: Hepatitis B virus (HBV) G1896A mutation was associated with HBeAg seronegativity and hepatitis B related acute-on-chronic liver failure. In this study, we developed Taqman amplification refractory mutation system (Taqman-ARMS) and established a strict control system to detect HBV G1896A mutant. **Methods:** HBV viral DNA was isolated from 60 patient serum samples, and full-length HBV genome was cloned. Then, Taqman-ARMS was used to detect HBV G1896A mutant. **Results:** The assay has the sensi-

tivity of 1E+3 IU/ml G1896A template, and 0.1% weak population virus with G1896A could be found in mixtures. Total of all 60 clinical samples random collected were detected by Taqman-ARMS, the results were consistent with those by DNA sequencing. **Conclusion:** The proposed Taqman-ARMS real-time PCR method for the detection of G1896A mutation of HBV was rapid, simple, sensitive, specific, and applicable in the clinical setting. *J. Clin. Lab. Anal.* **30**:315–318, 2016. © 2015 Wiley Periodicals, Inc.

Key words: Taqman-ARMS; HBV; G1896A mutation

Unfortunately, more than 2 billion people over the world each year are infected (by World Health Organization report) with Hepatitis B virus (HBV) (1). HBV infection is one of the major serious risk factors for severe liver disease, liver cirrhosis, and hepatocellular carcinoma (HCC) (2). It was reported that HBV with G1896A and A1762T/G1764A mutations is associated with hepatitis B related acute-on-chronic liver failure (3–6). However, the G1896A mutation was also associated with HBV e-antigen (HBeAg) seronegativity (7). The G1896A mutation in particular creates a premature stop codon in the HBeAg open reading frame (ORF), thereby abolishing HBeAg production. Seroconversion to HBeAg-negative and e-antibody (HBeAb) positive status usually implies a decrease in viral replication, decrease in potential liver injury, improved prognosis, and decrease in HCC risk and is the major end point of therapy (8). The clinical course of HBV infection was monitored by biological markers in the serum, including liver enzymes and direct quantification of HBV DNA viral load. However, in many patients infection by G1896A mutant variants exhibited active liver disease with little or no HBeAg. So it is very important to accurately detect HBV infection with G1896A mutant.

Some assays have been developed to detect G1896A mutant. The gold standard method is direct

sequencing. However, DNA sequencing has the limitations in detecting low quantities. In this study, we developed Taqman amplification refractory mutation system (Taqman-ARMS) to real time detect G1896A mutant.

A total of 60 patient serum samples were randomly retrieved from the third people's Hospital of Nantong. HBV viral DNA was isolated from 200 µl patient serum samples using the QIAamp DNA blood mini kit (Qiagen Inc., Basel, Switzerland) and eluted into 50 µl buffer according to manufacturer's instructions.

Full-length HBV genome was cloned according to the method of Parekh et al. (9, 10). Briefly, clinical sample with chronic HBV infection were used. HBV DNA isolated by commercial kits was amplified by PCR in 50 µl of buffer containing 5 µl 10× LAMP buffer, 20 µM dNTP, 0.3 µM each primer, 2.5 mM Mg²⁺, and 5 U of LAMP DNA polymerase (Beijing Cowin Bioscience Co., Ltd.).

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PCR was carried on Bio-Rad Mycyle PCR under the following conditions: 94°C for 2 min, followed by 94°C for 30 sec, 60°C for 30 sec, and 72°C for 3.5 min for 35 cycles, with a final extension of 5 min. The PCR products were purified (Axygen Scientific, Inc.) and cloned into the pGEM-T Easy Vector Systems (Promega Corporation), and the plasmid was identified by sequencing. The clones that carried G1896 and A1896 were used as the assay standards and quantified by using NanoDrop 2000 (Thermal Fisher Scientific).

For identification of the double mutation at basal core promoter (BCP) region, HBV DNA was amplified and sequenced as follow: the p3 (5'-GCCACTTACATAAGAGGACTCTTGGACT-3') and p4 (5'-GAGAGTAACTCCACAGWAGCTCCAAATTCTTT-3') primers were used for amplification. The LAMP DNA polymerase was applied for amplification. The thermal program was 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, for a total of 40 cycles. The PCR products were separated by electrophoresis, purified using a Qiagen gel extraction kit, and sequencing was done with the BigDye terminator cycle sequencing reaction kit and Prism 3730 DNA analyzer (Applied Biosystems).

To avoid the base difference of HBV genotypes, reverse primer was designed to specifically detect G1896A. A strong A:G mismatch was added to the primer located at n-1 position of 3'-segment. Taqman probe was used as signal system. The sequences of primers and probes for detection of G1896A were as follows: ARMS-F: 5'-GGCTGTAGGCATAAATTGGT-3'; ARMS-R: 5'-GGGTCAATGTCCATGTCAT-3'; Taqman probe: 5'-FAM-CACCAGCACCATGCAACTTTT-TAMRA-3'. The Taqman-ARMS was performed in the 50 µl reaction mixture containing 0.15 µM primers and probe, 2.5 mM Mg²⁺ ions, 0.2 µM dNTP, 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT, 50 mM KCl, 50% glycerol, and 3 U Hotstart start Taq DNA polymerase (BioSci). The cycle condition was as follows: 95°C for 10 min, ten cycles of 95°C for 20 sec, 58°C for 25 sec, 72°C for 25 sec, and then follow 30 cycles of 95°C for 20 sec, 59°C for 45 sec, 72°C for 20 sec; the PCR was carried on 7,500 real-time PCR (Applied Biosystems).

Serial dilution of 1E+2 IU/ml ~ 1E+6 IU/ml G896A mutant plasmid was used to test the sensibility of Taqman-ARMS. After many repeats, the Taqman-ARMS has the limit of detecting 1E+3 IU/ml mutant plasmid (Fig. 1A).

1E+9 ~ 1E+6 IU/ml wild plasmid by ten-step dilution was applied to test the specificity of Taqman-ARMS; 1E+4 IU/ml and water was used as control. While detecting 1E+9 IU/ml and 1E+8 IU/ml wild template, the Ct value was 24.67 ± 0.39 and 27.42 ± 0.28 , respectively; and no curve was observed, when Taqman-ARMS was used to detect 1E+7 IU/ml wild template (Fig. 1A). So, we

established a Taqman-ARMS to detect G1896A, the effective detection range was 1E+3 IU/ml ~ 1E+7 IU/ml.

To evaluate distinguish ability of the assay, 1E+6 copies/ml mix population that contained 10%, 1%, 0.1%, and 0.01% G1896A mutant template were tested. In total, 0.1% (about 1E+3 IU/ml templates) mutant templates contained in mixtures could be found, and the Ct value was 28.75 ± 0.34 (Fig. 1B).

To establish the threshold Ct value, tens of repeats apply the Taq-ARMS to test the following samples: 1E+7 copies single mutation template, 1E+7 wild template, 1E+3 copies double mutation template, and 1E+7 copies mix plasmid containing 1E+3 copies double mutation. The threshold Ct value obtained was 30.

Considering detecting range of Taqman-ARMS effectively, we established a standard and controlled system to make sure the authenticity of the result of detecting clinical samples (Fig. 1D): First, when the HBV DNA level of the sample was higher than 1E+7 IU/ml, the sample was diluted before detection of G1896A mutant; while detection of G1896A mutant cannot be done when the HBV DNA level of the sample was lower than 1E+3 IU/ml. Second, when the DNA level control was checked, the Ct values were between 15 and 24, and the result was believable. Third, there was no curve observed in PCR negative control. Final, accorded with the condition above, and the S-curve observed, the result was effective.

A total of 60 clinical samples were collected from HBV infected patients and were detected by the Taqman-ARMS and sequenced double blindly. The results are summarized in Figure 1C. Twenty-eight samples (46.6%) carried G1896A mutant by Taqman-ARMS and sequencing. Two samples were determined with G1896A mutant by directly sequencing the concentration that was out the limit of Taqman-ARMS. Three samples were G1896A mutant by Taqman ARMS that could not be detected by sequencing due to low copy numbers contained in mix HBV population (Fig. 1B).

Detection and quantification of HBV G1896A mutant was reported previously using a TaqMan probe or SimpleProbe real-time PCR with additional melting analysis (8, 11). However, there are nucleotide polymorphisms next to G1896A mutant, the probe-based mutant detection method must be evaluated thoroughly (11). However it is easy to avoid this by using the SimpleProbe real-time PCR, but it is based on Nest-PCR, just a laboratory assay, and unable to avoid false-positive interference by using Sybrgreen as a signal system (11). Detection of HBV mutations has been greatly dependent on direct DNA sequencing, which has been considered to be the "gold standard." However, DNA sequencing only distinguished mutations more than 25%.

ARMS is a stable technology widely used in mutation (12–18). We developed ARMS to detect G1896 mutant

Fig. 1. (A) The sensibility and specificity of Taqman-ARMS for detecting G1896A mutant. (B) Taqman-ARMS was performed to detect weak population with G1896A contained in mixture. 1E+6 IU/ml mix population containing 10% ~ 0.01% G1896A mutant template was tested. (C) Comparison of HBV G1896A mutant detected by Taqman-ARMS and DNA directly sequencing. (D) Establishing rigorous control system to ensure accurate detection of HBV G1896A by Taqman-ARMS.

and used Taqman probe as signal system. Although it was stable and accurate, the effective detection limit of ARMS was 1E+3 IU/ml ~ 1E+7 IU/ml. To make sure the truth of result, we established serial strict controls to avoid false-positive and negative interference that have not been reported before. Within this context, Taqman-ARMS had sensitivity of detecting 1E+3 IU/ml HBV G1896A mutant, and 0.1% mutant could be found contained in mixture.

We had developed Taqman-ARMS assay to detect HBV G1896A mutant, and established a strict control to ensure the truth of the result detection by Taqman-ARMS. So, the Taqman-ARMS could be widely used in clinical analysis to detect HBV G1896A mutant.

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