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Hepatitis C Virus RNA Real-Time Quantitative RT-PCR Method Based on a New Primer Design Strategy

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Viral nucleic acids are unstable when improperly collected, handled, and stored, resulting in decreased sensitivity of currently available commercial quantitative nucleic acid testing kits. Using known unstable hepatitis C virus RNA, we developed a quantitative RT-PCR method based on a new primer design strategy to reduce the impact of nucleic acid instability on nucleic acid testing. The performance of the method was evaluated for linearity, limit of detection, precision, specificity, and agreement with commercial hepatitis C virus assays. Its clinical application was compared to that of two commercial kits—Cobas AmpliPrep/Cobas TaqMan (CAP/CTM) and Kehua. The quantitative RT-PCR method delivered a good performance, with a linearity of $R^2 = 0.99$, a total limit of detection (genotypes *1* to *6*) of 42.6 IU/mL (95% CI, 32.84 to 67.76 IU/mL), a CV of 1.06% to 3.34%, a specificity of 100%, and a high concordance with the CAP/CTM assay ($R^2 = 0.97$), with a means ± SD value of −0.06 ± 1.96 log IU/mL (range, −0.38 to 0.25 log IU/mL). The method was superior to commercial assays in detecting unstable hepatitis C virus RNA ($P < 0.05$). This quantitative RT-PCR method can effectively eliminate the influence of RNA instability on nucleic acid testing. The principle of primer design strategy may be applied to the detection of other RNA or DNA viruses. *(J Mol Diagn 2016, 18: 84—91; http://dx.doi.org/10.1016/j.jmoldx.2015.07.009)*

Viral infections cause acute or chronic diseases, even cancer, in humans worldwide.[1] Viruses that commonly infect humans include both DNA and RNA viruses.[2–4] Current standard-of-care therapy for most DNA or RNA virus infections is antiviral treatment.[5] Quantitative nucleic acid testing has become a gold standard marker for clinical decisions regarding the use of antiviral therapy.[6] The most recently developed nucleic acid testing method is the real-time PCR-based assay.[7] However, commercially available assays require strict conditions for sample collection, transportation, storage, and processing because of the instability of viral nucleic acids,[8] in particular viral RNAs.[9]

The instability of viral nucleic acids is due to decay mechanisms. For example, the 5′-untranslated region (5′-UTR) of hepatitis C virus (HCV) is the most conserved region.[10] Thus, it is used for design targets in many commercial HCV RNA detection kits. However, the 5′-UTR has unique decay mechanisms due to its secondary structure. The 5′-UTR includes a region of the HCV internal ribosome entry site element,[11] which can be hybridized with the liver-specific microRNA (miR-122), to unlock and switch the 5′-UTR to an open structure,[12] and the RNA secondary structure can also melt with increasing temperatures to an open conformation.[13] The opened conformation induces the exposure of the 5′ end of the 5′-UTR to exonuclease-mediated degradation.[14] The HCV 5′-UTR strand can fold into several stem—loop secondary structures,[15] and the loop structures are more sensitive

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to cleavage by endonuclease than are the stem structures.[16] As described in the previous paragraph, the 5′-UTR of HCV RNA is prone to cleavage into short pieces of different lengths.[14,16]

Thus, when RNA is cleaved during sample collection, handling, and storage, decreased detection sensitivity or even a false-negative result may be obtained using the currently available commercial kits. For example, when serum samples from HCV-infected patients were stored at −70°C for up to 9 years, a significant loss of HCV RNA load was detected using Cobas Amplicor Monitor assay[17] (Roche Diagnostics Deutschland GmbH, Mannheim, Germany), and a 23% decrease in HCV RNA was observed in samples stored at −20°C for 6 months when measured using the branched DNA assay.[18]

To overcome the drawbacks of these commercial kits and to minimize the impact of the instability to nucleic acid testing, we developed a quantitative RT-PCR (RT-qPCR) method based on a new primer design strategy. We tested our method and new strategy with HCV RNA as a model.

Materials and Methods

Primer/Probe Design Strategy

Due to the decay mechanisms of HCV 5′-UTR described in the previous section, primers were designed according to the following rules: i) the 5′-UTRs of HCV genotypes *1* to *7* were aligned to identify conserved regions, which were selected as candidate regions for primer/probe design; ii) the 5′-UTR contains many stem−loop structures (Figure 1A), and we chose primer/probe sets from conserved sequences on stem structures, trying to avoid the loops structures; iii) because the base-pairing structures of the 5′-UTR might open at increasing temperatures or by miR-122 hybridization, the sequences near the 5′ end of the 5′-UTR are susceptible to cleavage by 5′-3′ exonucleases (Figure 1B), and therefore the probe and primers were designed close to the 3′ end of the 5′-UTR to prevent exonuclease degradation; iv) overlapping regions among the forward primer, probe, and reverse primer were avoided; and v) the covering loops were as few as possible for the target amplified fragment, which means that shorter amplified fragments were better.

Serum Sample Collection

We randomly collected 81 blood samples and their genotyping data from HCV-infected patients from the Beijing Ditan Hospital (Beijing, China) from May 2014 to October 2014. The blood samples were centrifuged immediately for the collection of serum. Each serum sample was divided into two aliquots. One aliquot was directly stored at −80°C as the HCV RNA nondegraded sample for baseline. The other aliquot was divided into two parts. For one part, 81 samples were incubated at room temperature for 6 hours and then stored at −80°C as the HCV RNA degraded sample. For the other part, 42 samples were randomly selected from the 81 collected samples and incubated

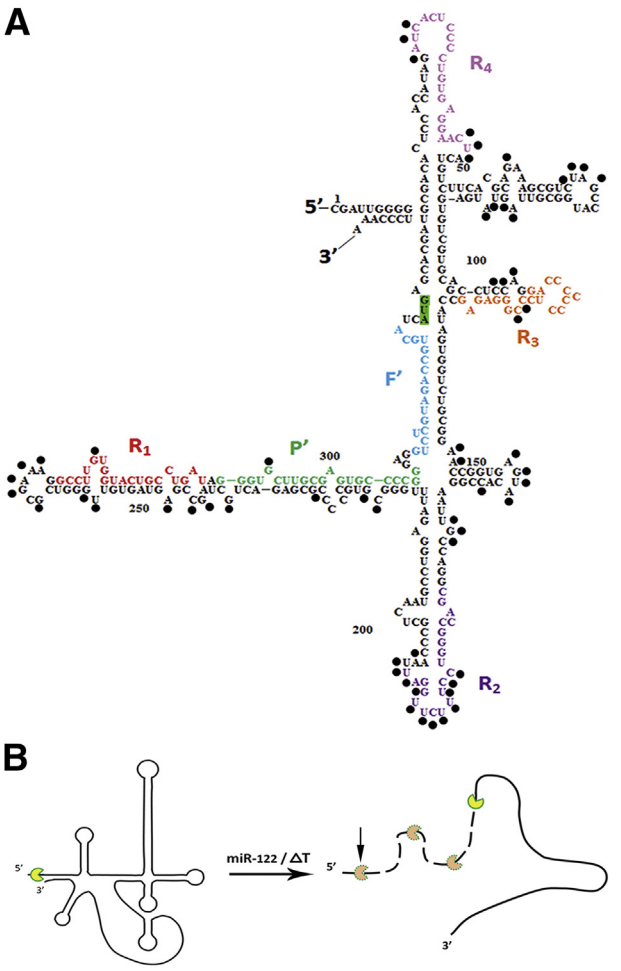

Figure 1 Primer/probe sets design strategy—based mechanisms. **A:** Nuclease cleavage site and primer/probe sets of 5′ untranslated region (UTR). Circles indicate endonuclease cleavage sites. Letters highlighted in green indicate the start codon for viral protein synthesis. **B:** Schematic illustration of secondary structure unlocked by miR-122 hybridizing or temperature increasing. **Down arrow** shows the first site where exonuclease-mediated degradation starts. Yellow symbol shows the 5′-3′ exonuclease at present place. Orange symbol show the 5′-3′ exonuclease at past place. ΔT, temperature increase; F′, complementary to forward primer F; miR-122, liver-specific mRNA; P′, complementary to probe P; R_1, R_2, R_3, and R_4, reverse primers for 62-, 157-, 222-, and 304-Bp targets, respectively.

at 37°C for 6 hours and then stored at −80°C as HCV RNA further-degraded samples. These treatment conditions simulated the maximum time delay between sample collection and detection observed in some Chinese hospitals in developing areas, at room temperature, and during hot summers. All aliquots were thawed at room temperature when ready for analysis. The study protocol was approved by the Ethics Committee of the National Center for Clinical Laboratories, and the study was conducted in accordance with the guidelines of the Declaration of Helsinki.

RNA Isolation

Two protocols were used for serum RNA purification. The QIAamp-based method was used for analysis of the HCV