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6 HCV Genotyping 9G test for HCV 1a, 1b, 2, 3, 4 and 6 (6a, 6f, 6i and 6n) with high accuracy

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ABSTRACT

According to EASL guidelines and WHO recommendations, the accurate detection of HCV genotypes such as HCV 1a, HCV1b, HCV 2, HCV 3, HCV 4, and HCV 6 (6a, 6f, 6i, 6n) is crucial for the efficient treatment of hepatitis C. HCV Genotyping 9G test allows simultaneous genotyping of HCV 1a, 1b, 2, 3, 4, and 6 (6a, 6f, 6i, and 6n) in clinical samples in 30 min. The performance of the test was evaluated by comparison with sequence analysis. Serum samples (n = 152) from HCV-infected patients (n = 110) and healthy individuals (n = 42) were processed under blinded codes. The *k* coefficient (kappa) values indicated high agreement between the HCV Genotyping 9G test and sequencing. The sensitivity and specificity of the test were 99.1% and 99.7%, respectively. The results indicate that HCV Genotyping 9G test is rapid, reliable, sensitive, and accurate for screening and genotyping of HCV in the clinical specimens.

1. Introduction

Hepatitis C virus (HCV) infection is a leading cause of chronic hepatitis and related liver diseases including cirrhosis, hepatocellular carcinoma (HCC), and liver failure. 150 million people have chronic HCV infection around the globe and 700,000 people die each year from hepatitis C-related liver diseases (Lavanchy, 2011; WHO, 2016). The treatment of HCV infected patients has become intricate due to various genotypes and subtypes of HCV (Smith et al., 2014).

Globally, HCV1 is the most common (46.2%), followed by HCV 3 (30.1%), HCV 2 (9.1%), HCV 4 (8.3%), HCV 6 (5.4%), and HCV 5 (0.8%) (Messina et al., 2015). HCV 6 is dominant in South China, Myanmar, Laos, Vietnam, and Cambodia (Yamada et al., 2015). HCV 6 accounts for almost 20% of infections in Thailand (Wasitthankasem et al., 2015). The recent recommendations by European Association for the Study of the Liver (EASL) for the treatment of HCV infection validate that the treatment outcome significantly depends on the genotypes and sub-genotype (European Association for Study of, 2015). The 2016 WHO guidelines for correct hepatitis C treatment recommend HCV genotyping in clinical specimens (WHO, 2016).

Currently, treatment of HCV consist of individual or combination drugs such as pegylated interferon (PegIFN)-α, ribavirin, and the direct-acting antivirals (DAAs) such as sofosbuvir, simeprevir, ledipasvir, ombitasvir, dasabuvir. However, the choice of medicine or combination drugs and the duration of treatment varies depending on HCV genotypes 1a, 1b, 2, 3, 4, 5 and 6 (European Association for Study of, 2015; Mishra et al., 2015). Therefore, for optimal patient therapy, it is imperative to detect and discriminate the crucial HCV genotypes by using simple and accurate detection method.

Though the sequence analysis is a gold standard for HCV genotyping (Firdaus et al., 2015), it is time-consuming, expensive, and requires highly trained professional to process the samples. Thus, for the implementation of an accurate treatment regimen in the management of hepatitis C, there is a need to find an inexpensive genotyping test, which is rapid, simple, and precise. Nucleic acid tests such as real-time PCR (Beld et al., 2002; Hawkins et al., 1997) and line-probe assay (Cai et al., 2013) are comparatively rapid and cost-effective HCV genotyping methods. The Versant HCV 2.0 assay (LiPA 2.0), Abbott Realtime HCV Genotype II assay, and Trugene assay are the widely used assays for HCV genotyping. The LiPA 2.0 assay requires the use of interpretation

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charts to obtain the results. The Abbott Realtime HCV Genotype II assay uses four different primer sets and requires three separate reactions for HCV genotyping. Trugene assay is reported to be labor-intensive (Schutzbank et al., 2006). Comparison of Trugene assay and Versant HCV 2.0 with sequence analysis indicates that these tests can fail to differentiate HCV subtypes 1a and 1b, such results would lead to critical errors in the correct use of DAAs (Chueca et al., 2016). The Versant HCV 2.0 assay and the Abbott Realtime HCV Genotype II assay have limitations in identifying HCV genotype 6 (Yang et al., 2014). The limited accuracy of these three commercial assays is attributed to the low single-nucleotide polymorphism (SNP) discrimination ratio due to the high sequence similarity amongst the HCV genotypes.

In the present study, the performance of 6 HCV Genotyping 9G test was evaluated by using 152 patients' serum samples and comparing the results with a sequence analysis as reference. All samples were tested under blinded codes. The 6 HCV Genotyping 9G test is an application of the 9G technology (Song et al., 2013). In 9G technology, ssDNA oligonucleotide probes appended with the nine consecutive guanines are immobilized on the glass fiber membranes at specific positions. The immobilized probes hybridize with the complementary Cy5 labeled ssDNAs in the PCR product and show high SNP discrimination ratio resulting in accurate HCV genotyping. The HCV Genotyping 9G test allows screening and genotyping of HCV strains (HCV 1a, 1b, 2, 3, 4, and 6) in the clinical specimens. The HCV Genotyping 9G test provides the important information to physicians by accurate detection of six HCV genotypes in 30 min after RT-PCR at 25 °C.

2. Materials and methods

2.1. Clinical samples

This study tested 152 samples. The sample collection, treatment, and tests were carried out according to guidelines from the Ethical Clearance Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University. Clinical investigation has been conducted according to the principles expressed in the Declaration of Helsinki. Samples were collected during the period of June 2015-June 2016 in the Mahidol University, Bangkok. The samples were collected from males and females of 12–78 years with the average age of 50.37. Patients' locations included various provinces of Thailand, and other countries such as Vietnam, Malaysia, Cambodia, Laos, and Myanmar. Consent on the use of samples from patients was obtained from the ethical committee. The serum samples were from patients suspected of hepatitis C (n = 110) and from healthy people (n = 42) who did not have any history of HCV infection.

The HCV RNA extraction for all samples was performed on the NucliSENS easyMAG (bioMérieux, Boxtel, Netherlands), which automatically extracted nucleic acids from the clinical samples. A total of 200 µL of plasma was added to the lysis buffer and incubated for 10 min at room temperature. Magnetic silica particles were used for nucleic acid binding for 10 min at room temperature. Silica particles were washed with washing buffers A and B provided with the NucliSENS easyMAG kit. HCV RNA was eluted in 50 µL of Tris-elution buffer. The whole procedure was used with all of the 152 clinical samples. The eluted HCV RNA in the Tris-elution buffer was subsequently transcribed into cDNA by following the protocols of 6 HCV Genotyping 9G test. Results were blinded until all tests were completed.

2.2. 6 HCV Genotyping 9G test

The 6 HCV Genotyping 9G test includes viral RNA isolation, complementary DNA (cDNA) synthesis, PCR amplification, and detection of PCR amplicons. As shown in Fig. 1, primers in the 6 HCV Genotyping 9G test amplify the 5′ untranslated region (5′UTR) for genotyping of six HCV genotypes. The probes, which were selected according to the previous report (Nimse et al., 2011) allow the SNP discrimination for the accurate detection of HCV genotypes 1a, 1b, 2, 3, 4, 6a or 6f, and 6i or 6n at 25 °C in less than 30 min after PCR. The respective positions of the probes on the lateral flow strip membrane are depicted in Fig. 2.

The 6 HCV Genotyping 9G test is performed by adding 110 µL of hybridization solution (25% Formamide, 0.1% Triton X-100, 6x SSC) into the PCR tube containing 20 µL of PCR product. Then, 110 µL of this mixture was loaded into the sample port and allowed to stand for 20 min at 25 °C. After 20 min, 200 µL of washing solution (4x SSC) was loaded into the washing port and allowed to stand for 8 min at 25 °C. After 8 min, each 6 HCV Genotyping 9G test strips was scanned on the BMT Reader™ (Biometrix Technology Inc. Chuncheon, South Korea) and the results were automatically interpreted by the 9G Test™ Analyzer program.

In brief, the 9G Test™ Analyzer program uses the signal intensities for the respective probes for the interpretation of the results. As shown in Fig. 2, the HC probe serves as an internal standard for hybridization control. The HCV probe is used to detect the presence or absence of HCV in the sample. When 6 HCV Genotyping 9G test shows three signals corresponding to probes including the HC, HCV, and 1a or 2 or 3 or 4, a sample is designated as an HCV 1a or 2 or 3 or 4, respectively. The sample is identified as HCV 1b if the test shows four signals for probes including HC, HCV, 1a, and 1b, respectively. When the test shows three signals corresponding to the probes HC, HCV, and 6, the sample is genotyped as HCV 6a or 6f. Whereas, if the test shows four signals corresponding to the probes HC, HCV, 1a, and 6, the sample is genotyped as HCV 6i or 6n. The HCV probe indicates the presence or absence of HCV strain in the clinical samples. When, 6 HCV Genotyping 9G test shows two signals corresponding to the HC and HCV probes, the sample is identified to contain the HCV genotype other than the HCV 1a, 1b, 2, 3, 4, 6a, 6f, 6i, and 6n. However, for an HCV-negative sample, the test shows only one signal corresponding to the HC probe. Table 1 summarizes results of 6 HCV Genotyping 9G test for the screening and genotyping of HCV genotypes in the clinical samples.

2.3. Reference HCV genotyping

The sequences of amplified fragments (from clinical samples) were determined on Applied Biosystems (ABI) 3730XL DNA analyzer (Life Technologies Co., Carlsbad, CA, USA). The specific HCV genotypes were confirmed by comparing the obtained sequences with the reported sequences on the Basic Local Alignment Search Tool (BLAST) database of NCBI. The results of the sequencing analysis are in Table 1.

2.4. Statistical analysis

The k coefficient was used to assess consistency between 6 HCV Genotyping 9G test and sequencing results. The k values between 0.61 and 0.80 indicate good concordance between the two tests. The sensitivity, specificity, positive predictive (PPV) and negative predictive values (NPV) at 95% confidence interval (CI) were calculated. Statistical analysis were performed by using the statistical program Medcalc for Windows version 17.4.4 (Medcalc Software, Mariakerke, Belgium). A result was considered as true positive (TP) if the 6 HCV Genotyping 9G test and sequencing showed the same HCV genotype. A result was considered as true negative (TN) if the 6 HCV Genotyping 9G test and sequencing did not detect any HCV genotype in the sample. Results of 6 HCV Genotyping 9G test were considered as false positive (FP) if the number of a particular HCV genotype identified exceeds the number of that genotype detected by sequencing. Results of 6 HCV Genotyping 9G test were considered as false negative (FN) if the number of a particular HCV genotype detected is less than the number of that genotype detected by sequencing. Test results were classified as TP, TN, FP and FN. From these categories, sensitivity (TP/TP + FN), specificity (TN/TN + FP), PPV (TP/TP + FP) and NPV (TN/FN + TN)

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