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Hepatitis C Virus Genotyping Methods: Evaluation of AmpliSens® HCV-1/2/3-FRT Compared to Sequencing Method

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Background: Hepatitis C virus (HCV) genotyping is important for treatment and epidemiological purposes. The objective of this study was to evaluate the performance of AmpliSens® HCV-1/2/3-FRT kit in comparison to sequencing method for genotyping. *Methods:* A total of 17 samples collected from December 2009 to January 2011 were analyzed. Reverse transcriptase polymerase chain reaction (PCR) was performed, followed by sequencing technique. Results were analyzed based on sequence information in GenBank. A second genotyping method (AmpliSens® HCV-1/2/3-FRT) was done, which differentiates HCV genotypes by means of real-time hybridization-fluorescence detection. *Results:* From 17 samples, four were untypeable by AmpliSens® HCV-1/2/3-FRT. Eleven of 13 (84.6%) results showed concordant genotypes. A specimen that was determined as genotype 3a by sequencing was genotype 1 by the AmpliSens® HCV-1/2/3-FRT. Another specimen that was genotype 1 by sequencing was identified as genotype 3 by AmpliSens® HCV-1/2/3-FRT. *Conclusion:* HCV genotyping with AmpliSens® HCV-1/2/3-FRT using real-time PCR method provides a much simpler and more feasible workflow with shorter time compared to sequencing method. There was good concordance compared to sequencing method. However, more evaluation studies would be required to show statistical significance, and to troubleshoot discordant results. AmpliSens® HCV-1/2/3-FRT does differentiate between genotype but not until subtype level. J. Clin. Lab. Anal. 28:224–228, 2014. © 2014 Wiley Periodicals, Inc.

Key words: AmpliSens® HCV-1/2/3-FRT; HCV genotype; HCV subtype; sequencing methods; real time; RT-PCR

INTRODUCTION

Hepatitis C virus (HCV) was first discovered in 1989 as a predominant cause of transfusion-associated non-A-non-B hepatitis. At least six genotypes with more than 70 subtypes have been identified. Chronic HCV infection may lead to liver cirrhosis and hepatocellular carcinoma. Treatment with interferon and ribavirin has substantially reduced the risk of liver failure and malignancy. Response-guided therapy for chronic HCV is based on the genotype (1). Genotypes 1 and 4 respond poorly to treatment compared to genotypes 2 and 3. Hence, genotype determination is crucial before commencing treatment to achieve optimal outcome. Sequencing method is the gold standard for determining HCV genotype.

HCV is a spherical, enveloped virus that belongs to Flaviviridae family and Hepacivirus genus. It has a positive single-stranded RNA, approximately 9.7 kb in length, which contains a large open reading frame flanked by highly conserved untranslated regions, 5-UTR and 3-UTR. The open reading frame encodes a polyprotein that is processed into at least 10 proteins: core protein (C), envelope proteins (E1 and E2), NS2a, and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B).

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HCV has substantial nucleotide sequence diversity, with only 66–80% overall sequence similarity among strains belonging to different serotypes and genotypes. Analysis of different viral genomic region, for example, 5′NC (noncoding) region, E1 region, and NS5B region led to identification of six major genotypes, 1–6, and more than 70 subtypes within the genotypes. The main HCV types identified are numbered in the order of their discovery, that is, 1–6, whereas subtypes are identified by lowercase letters (2).

According to a study done by Tran et al., the most common genotypes in Malaysia are 1b, 1a, and 3 (3). However, a data from the Malaysian Liver Foundation revealed that genotype 3 was the most common type in Malaysia, followed by genotype 1 (4). The other genotypes including 4, 5, and 6 are rare in Malaysia and usually seen in immigrants (5).

HCV leads to hepatic inflammation, steatosis, and eventually to hepatic fibrosis and hepatocellular carcinoma. A number of factors associated with cirrhosis, including excessive alcohol ingestion, co-infection with HBV or HIV, human leucocyte antigen (HLA) B54, HCV genotype 1b, HCV quasispecies complexity, and higher levels of HCV viraemia. The clinical importance of genetic heterogeneity of hepatitis C lies in the fact that certain genotypes are associated with poor respond to treatment and more severe liver pathology. Thus, HCV genotyping is recommended before starting treatment. Several commercial assays are available to determine HCV genotypes using direct sequence analysis of the 5′NC region, which include Trugene 5′NC HCV Genotyping Kit (Siemens Healthcare Diagnostics Division, Tarrytown, NY), reverse hybridization analysis using genotype-specific oligonucleotide probes located in the 5′NC region, INNO-LiPa HCV II (Innogenetics, Ghent, Belgium), and Amplisens® HCV-1/2/3-FRT (InterLabService, Moscow, Russia). PCR kit variant AmpliSens® HCV-genotype-FRT was also in the market, which was intended to detect HCV genotypes 1a, 1b, 2, 3, and 4. However, the commercial production of this kit was discontinued by the manufacturer at the time of evaluation.

Most methods can identify the HCV in majority of cases, but distinction among certain genotypes is not possible, especially in the Southeast Asia (6). Therefore, nucleic acid sequencing and phylogenetic analysis of an appropriate subgenomic region remain the gold standard for determining HCV genotype (6).

The objective of this study was to evaluate the performance of AmpliSens® HCV-1/2/3-FRT kit in comparison to sequencing method. AmpliSens® HCV-1/2/3-FRT kit is an in vitro nucleic acid amplification test for qualitative detection and differentiation of HCV genotypes 1, 2, and 3 by means of real-time hybridization-fluorescence detection. It is hypothesized that sequencing method can perform better than AmpliSens® HCV-1/2/3-FRT.

MATERIAL AND METHODS

Samples

This was a cross-sectional study involving clinical samples that were sent to our molecular laboratory for quantitative hepatitis C polymerase chain reaction (PCR) and anti-HCV antibody tests from December 2009 to December 2010.

The sample size for this study was calculated based on the formula described by Lwanga and Lemeshow (7). With Z equals to 1.96, confidence level of 95%, anticipated population proportion (prevalence) of 0.54, absolute precision of 0.10, and 95% confidence interval, the minimum sample size was 67. However, after sequencing steps, only 17 samples were adequate for further test with AmpliSens® HCV-1/2/3-FRT.

Sequencing Method

HCV RNA amplification: HCV RNA was extracted from 140 μl plasma using QIAamp® Viral RNA Mini Kit and QiagenOneStep RT-PCR® (Qiagen GmbH, Germany) (cat no. 52904). To prepare for the amplification step, the extracted samples were mixed with QiagenOneStep RT-PCR®. The primers used targeted the NS5B region of HCV genome. The sequences of the forward primer: GCA GAA AGC GTC TAG CCA TGG CGT and reverse primer: CTC GCA AGC ACC CTA TCA GGC AGT. Then, RT-PCR was carried out in Automated Gene Amp PCR System 9700® (Applied Biosystems, Life Technologies, Grand Island, NY, USA).

Detection of PCR product

A 25 μl portion of PCR product was added to 8 μl of loading dye and subsequently electrophoresed on a 2% agarose gel (SIGMA-ALDRICH, Batch no. 069K1735) in TBE buffer (Promega Madison, Lot no. 24224502) at 100 V for 35 min. The gel was stained with GelRedTM (Lot 10G0125, Biotum, Inc). Hundred base pair (bp) DNA ladder (Promega Madison, G210A 21843705) and blue/orange 6× loading dye (Promega Madison, G190A 20319009) were used to identify the specified amplified fragment. The 100-bp-specific amplified bands were then visualized in ultraviolet transilluminator.

Sequencing

This was done with GenomeLab™ Dye Terminator Cycle Sequencing (Quick Start Kit) using forward primer.

Procedure involved manual preparation of DNA sequencing reaction (include master mix preparation and PCR) and ethanol precipitation. Sequence analysis was done using an automated sequence analyzer CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Inc, Indianapolis, IN, USA).

Genotype analysis

The nucleotide sequence data (base-call) produced by the automated sequence analyzer CEQ™ 8000 Genetic Analysis were analyzed with identical sequence information in the National Center for Biotechnology Information (NCBI) based GenBank (8).

Phylogenetic analysis

The phylogenetic analysis was done by MEGA 4.0 software. Before the phylogenetic tree was produced, the sequences were aligned together with outgroup using ClustalW.

AmpliSens® HCV-1/2/3-FRT

This PCR kit is an in vitro nucleic acid amplification test for qualitative detection and differentiation of HCV genotypes 1, 2, and 3 by means of real-time hybridization-fluorescence detection. Detection of HCV genotypes by PCR is based on the amplification of a pathogen genome specific region using specific primers. In real-time PCR, the amplified product is detected by using fluorescent dyes. These dyes are linked to oligonucleotide probes, which bind specifically to the amplified product. Monitoring of fluorescence intensities during the real-time PCR allows the detection of accumulating product without reopening the reaction tubes after the PCR run. AmpliSens® HCV-1/2/3-FRT PCR kit uses "hot-start," which greatly reduces the frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by separation of nucleotides and Taq-polymerase by using a chemically modified polymerase (TaqF), which is activated by heating at 95°C for 15 min.

HCV genotype detection was done according to the manufacturer instructions and the steps include:

(1) Total RNA extraction from blood plasma simultaneously with the recombinant internal control sample.
(2) Reverse transcription of cDNA on RNA template.
(3) Real-time PCR of HCV cDNA.

RESULTS

HCV genotypes of 17 clinical specimens were determined by AmpliSens® HCV-1/2/3-FRT and sequencing methods. AmpliSens® HCV-1/2/3-FRT was not able to genotype four samples, leaving 13 results for comparison. Eleven of 13 (84.6%) samples showed concordant genotype results. A specimen that was identified as genotype 3a by sequencing turned out as genotype 1 by the other method. In contrast, the other specimen that was genotype 1 by sequencing was identified as genotype 3 by AmpliSens® HCV-1/2/3-FRT. The genotyping results by method are shown in Table 1. As compared to sequencing, HCV subtype was not identifiable by AmpliSens® HCV-1/2/3-FRT. For this reason and for the small number of samples, statistical analysis was not feasible. HCV genotyping results are summarized in Table 2, by percentages.

TABLE 1. HCV Genotyping Results by Sequencing Method and AmpliSens® HCV-1/2/3-FRT

Sample ID number	Sequencing method	AmpliSens® HCV-1/2/3-FRT
1	3a	3
3	1a	Untypeable
5	3	3
7	3a	3
8	3a	3
9	3a	3
10	3a	Untypeable
13	3a	Untypeable
15	3a	1
16	1	3
17	1a	1
22	1a	1
23	3a	Untypeable
25	3a	3
29	1a	1
87	3a	3
91	3a	3
Total samples	17	13

TABLE 2. HCV Genotyping Results Summary

Genotyping results	Sequencing method $n = 17$ (%)	AmpliSens® HCV-1/2/3-FRT $n = 13$ (%)	Discordant results compared to sequencing method $n = 13$ (%)
Genotype 3 or 3a	12 (70.6)	8 (61.5)	1 (7.7)
Genotype 1 or 1a	5 (29.4)	3 (23.1)	1 (7.7)
Total	17 (100.0)	11 (84.6)	2 (15.4)

DISCUSSION

Eleven (84.6%) of 13 samples showed similar genotypes from both methods. Two samples showed discordant results; one subtype 3a by sequencing was genotype 1 by

AmpliSens® HCV-1/2/3-FRT, while one genotype 1 by sequencing was genotype 3 by AmpliSens® HCV-1/2/3-FRT. Four of 17 samples were untypeable, which could be due to sample degradation, storage conditions, and duration prior to testing with a second method as well as a repeat freeze-thaw.

HCV genotyping using real-time PCR method provides a much simpler and more feasible workflow compared to sequencing method, which requires more steps, longer time, and considerable technical expertise as well as laboratory setup. There was good concordance of 11 of 13 (84.6%) genotype results for AmpliSens® HCV-1/2/3-FRT compared to sequencing method. A larger evaluation study would allow statistical analysis for significant comparison.

The 5′UTR (5′NC) is a well conserved region, but has sufficient nucleotide sequence divergence to discriminate between most genotypes. It is the target region for most diagnostic HCV RNA PCR assays and genotyping based on the 5′UTR has a high concordance with genotype determined by sequencing of NS5B. However, it is not able to discriminate the HCV genotype 6c-1, which can be mistyped as HCV genotype 1/1b because of sequence homology. Syria et al. concluded that 5′NC regions genotyping methods give sufficient information for clinical purpose (in which the determination of the subtype is not essential) whereas NS5B genotyping methods are more reliable for subtype determination, which is required in epidemiological studies (9). The NS5B region has highly informative character and correlates well with HCV subtype definition. In contrast, the 5′UTR is highly conserved, hence does not provide a tool for differentiation between subtypes (10). In the Southeast Asian countries, where genotype 6 is frequently found, the NS5B genotyping method is recommended as the 5′UTR-based genotyping cannot distinguish certain isolates of genotype 6 from isolates of genotype 1 (11).

Many studies suggested that genotype determination by NS5B method is more reliable for subtype determination and can distinguish certain subtypes in genotype 6 from genotype 1 (9, 12). 5′NC region method (which is used by AmpliSens® HCV-1/2/3-FRT method) showed higher sensitivity but may lead to genotyping error (13). However, Syria et al. demonstrated that assays based on analysis of the NS5B region missed 23 (16.4%) of the 140 expected positive results, while 5′NC region based assays only missed 3 (3.1%) of the 98 expected positive results (9).

Most comparison studies only showed subtype differences in genotypes 1, 2, and 4, for example, versus 4c or differences between two genotypes, 1 and 6. Differences between genotypes 1 and 3 as portrayed in this study were rarely reported. Discrepancy of HCV genotypes as determined by analysis of partial NS5 (genotype 3a) and core sequences (genotype 1a) were reported in two Honduran HCV strains (14). Later in 2003, Notle et al. also found two distinct strains; genotype 2 (5′NC) and genotype 1 (NS5B) (15). Single region analysis may not represent the entire HCV genome. Analysis of two regions may give more accurate results.

Mixed-genotype infection could not be ruled out in the discrepant samples. It is difficult to determine the prevalence of mixed-genotype infections by currently available assays, including direct DNA sequencing, because they are designed to identify only the HCV genotype dominant in that particular population. Analysis of sequences is not accurate in identifying mixed infections. The prevalence rate of mixed-genotype HCV infection varies in different regions and for the same group of patients tested by different assays. Sequencing analysis of 5′NC region showed that 4.8% of 3,150 Indian patients were infected with mixed infection (16). Meanwhile, HCV genotype determination using a PCR-based genotyping kit in Iran showed 2% prevalence of mixed-genotype HCV infections (17). There are no published data on mixed-genotype HCV infection in Malaysia. Exclusion or confirmation of mixed genotype can be made by using methods other than sequence analysis.

The discordant results could also be an example of hybrid that was produced by separate intertype recombination events as reported in Sweden. A sequencing study done by Kalinina et al. found that 5% of HCV infections in St. Petersburg were caused by hybrid strains (18). In the future, perhaps repeat tests can be done from fresh samples and different method of genotyping should be used to confirm dissimilarities. Further sequence analysis and mapping of the crossover junction are required to prove hybrid viruses.

CONCLUSION

HCV genotyping with AmpliSens® HCV-1/2/3-FRT using real-time PCR method provides a much simpler and more feasible workflow with shorter time compared to sequencing method. There was good concordance compared to sequencing method. However, more evaluation studies would be required in order to allow statistical analysis for significant comparison, as well as to troubleshoot discordant results. AmpliSens® HCV-1/2/3-FRT does differentiate between genotype but not until subtype level.

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