Clinical evaluation of a newly developed automated massively parallel sequencing assay for hepatitis C virus genotyping and detection of resistance-association variants. Comparison with a line probe assay

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A B S T R A C T

Hepatitis C virus (HCV) infection is a leading cause of chronic liver disease, cirrhosis and hepatocellular carcinoma. Recently, HCV was classified into 6 major genotypes (GTs) and 67 subtypes (STs). Efficient genotyping has become an essential tool for prognosis and indicating suitable treatment, prior to starting therapy in all HCV-infected individuals. The widely used genotyping assays have limitation with regard to genotype accuracy. This study was a comparative evaluation of exact HCV genotyping in a newly developed automated-massively parallel sequencing (MPS) system, versus the established Line probe assay 2.0 (LiPA), substantiated by Sanger sequencing, using 120 previously identified-HCV RNA positive specimens. LiPA gave identical genotypes in the majority of samples tested with MPS. However, as much as 25% of LiPA did not identify subtypes, whereas MPS did, and 0.83% of results were incompatible. Interestingly, only MPS could identify mixed infections in the remaining cases (1.67%). In addition, MPS could detect Resistance-Associated Variants (RAVs) simultaneously in GT1 in 56.82% of the specimens, which were known to affect drug resistance in the HCV NS3/NS4A and NSSA genomic regions. MPS can thus be deemed beneficial for guiding decisions on HCV therapy and saving costs in the long term when compared to traditional methods.

1. Introduction

Hepatitis C virus (HCV) infection affects more than 185 million people worldwide, and causes 350,000 deaths yearly (Lee et al., 2014). Most people infected with acute hepatitis C could develop chronic infection, which can ultimately result in liver cirrhosis or hepatocellular carcinoma, because they are unaware of infection and a vaccine is currently unavailable. HCV is a 9.7 kb positive-sense single-stranded RNA genome of the family Flaviviridae, genus Hepacivirus, which contains the 5’ and 3’ untranslated regions (UTRs) that flank the ends of the single open reading frame, encoding 3 structural proteins (including the Core protein and two Envelope glycoproteins, E1 and E2) and 7 non-structural proteins (including P7, NS2, NS3, NS4A, NS4B, NSSA and NSSB) (Chevaliez and Pawlotsky, 2006; Ferenci and Reddy, 2011; Li et al., 2015; Morishi and Matsuda, 2012).

Assix confirmed HCV genotypes have a whole-genome nucleotide that differs by > 30% from the nucleotide sequence; a seventh genotype has been suggested, but not accepted universally yet. Each genotype can be subdivided into related multiple subtypes (67 confirmed STs), with a roughly estimated 15–30% difference from the nucleotide sequence among HCV subtypes (Cunningham et al., 2015; Messina et al., 2015; Quer et al., 2015; Smith et al., 2014). The distribution of HCV genotypes and subtypes vary substantially in different parts of the world; genotype 1 (GT1) to GT3 are distributed worldwide, GT4 and 5 are mainly found in Africa, and GT6 is endemic in Asia (Rossi et al., 2015). GT7 infection was reported in Canada from an infected central African immigrant (Messina et al., 2015). At present, treatment guidelines still require information of genotypes on patients infected with HCV before starting treatment, of which duration and recommend medicines of direct-acting agents (DAAs) must be managed with/without IFN and/or ribavirin, depending on the genotype. This also helps clinicians to predict a response to treatment (EASL, 2015; Panel, 2015).

Hence, current HCV treatment is still genotype-specific dependent, as many unknown strains are different and have impact on clinical treatment at the subtype level. This results from widely used methods in the past that identified subtypes poorly (Avo et al., 2013; Liu et al., 2015; Quer et al., 2015). The response rates vary between subtypes...
(Cunningham et al., 2015), as subtype identification seems to be important clinically for predicting responses and outcomes in, for example, subtype a and b in GT1. Furthermore, new information has been found that is useful in an era of increasingly used DAAs, which specifically target HCV proteins such as NS3/NS4A (protease inhibitors), NS5A (NS5A inhibitors) and NS5B (RNA-dependent RNA polymerase inhibitors). Therefore, Resistance-Associated Variants (RAVs) have been found increasingly too, particularly in infected naive patients with GT1 infection. GT1 infected patients are mostly found to have treatment failure with RAVs of first generation NS3/NS4A protease inhibitors (Ahmed and Felmlee, 2015). Several important pre-treatment mutations are known clearly to reduce treatment response to DAAs, for example, V36M/A and R155K/T in ST1a, and T54A/V/S and A156T/S/V in ST1b resist boceprevir and telaprevir (a first-wave NS3/NS4 inhibitor) (Kumthip and Maneekarn, 2015; Shepherd et al., 2015). In addition, the spontaneous occurrence of Q80K, which was found frequently in patients infected with HCV ST1a, reduced the efficacy of simeprevir (a second-wave NS3/NS4A protease inhibitor) (Izquierdo et al., 2014).

In addition, mixed HCV infections with two or more distinct HCV genotypes or subtypes are found commonly in high-risk groups; e.g. persons who inject drugs and homosexual men (Cunningham et al., 2015; Qiu et al., 2015). In the past, HCV genotype testing usually detected only the majority strain in patients. Therefore, the sensitivity of the methods used for HCV genotyping is important for detecting all HCV strains in patients. The minority strain differs from the majority strain will not be detected in infected patients by the method have low sensitivity. Therefore, genotype-specific treatment might only suppress treatment-sensitive detected strains, and result in persistence of a treatment-insensitive undetected strain (Cunningham et al., 2015).

A variety of technologies have been developed for HCV genotype and subtype determination. The Versant HCV Genotype 2.0 Assay (LiPA), as used widely in clinical diagnostic laboratories, is based on reverse hybridization with subtype-specific primers and probes targeting both the 5’UTR and core region (Bouchardeau et al., 2007; Verbeeck et al., 2008). However, these genes have limitation in accurate genotype results and they do not cover the regions that identify all subtypes inLiPA, but they are still used widely because of their technical simplicity and rapidity (Cabezas-Fernandez and Cabeza-Barrera, 2012).

However, determination of accurate HCV genotypes, subtypes, and mixed HCV infection is of major importance, because of the need to reach optimal treatment for HCV infection. This tool is necessary also for choosing optimal treatment prior to starting therapy in all HCV-infected individuals. In addition, it also supports physicians in predicting outcomes, and may indicate treatment tactics with new DAA-containing regimens in all HCV subtypes. The sequencing technique is applied to detect HCV strains, and thus improve the accuracy of genotype results as well as increase sensitivity for identifying all HCV subtypes. Nowadays, Sanger sequencing (first-generation sequencing) is still considered as a reference method for HCV genotyping by using the NS5B gene, but this technology has disadvantages in only performing one sample per run with only one gene. Thus, Sanger sequencing is inappropriate for use in a clinical laboratory. The newly developed technology for second-generation sequencing, also known as massively parallel sequencing or next-generation sequencing (NGS) technology, has high-sensitivity and appropriate for routine in clinical diagnostic laboratory related with turnaround time – that can perform with multiple genes in up to 15 samples per run. This novel development of massively parallel sequencing with an automatic system known as Sentosa SQ HCV Genotyping assay, is able to detect RAVs simultaneously with HCV genotyping. These RAVs are important for guiding treatment decisions in some infected patient subgroups.

This study evaluated the performance of newly developed automated massively parallel sequencing, which targets NS5B, NS3 and NS5A for determining accurate HCV genotypes and subtypes and detecting RAVs for optional identification. When comparing the Versant HCV Genotype 2.0 Assay with bi-directional Sanger sequencing as a reference method, the NS5B region is still considered as the gold standard for HCV genotyping, because it gives accurate identification of HCV subtypes and reliability in creating an epidemiological model of HCV strains (Enache and Enache, 2008; Gryadunov et al., 2010).

2. Materials and methods

2.1. Samples and genotyping samples

One hundred and twenty retrospective EDTA plasma samples from patients infected with HCV were used in this study. They were maintained at a temperature of ~ 80 °C at Ramathibodi Hospital, Thailand, and processed as recommended by the diagnostic virology laboratory. All of the samples were collected randomly without patient history-associated demographics or clinical information, and used to validate performance of the methodology in covering many subtypes, including HCV genotypes that were poorly identified previously by using the Versant Line Probe Assay (HCV genotyping LiPA2.0; Siemens Healthcare Diagnostics, Erlangen, Germany). Then, HCV genotyping was compared with the use of newly developed automated massively parallel sequencing (Vela Operations Singapore Pte Ltd, The Kendall, 50 Science Park Road, Singapore). Finally, the reference method was confirmed by bi-directional sequencing of the NS5B gene (Sanger sequencing; Applied Biosystems® 3500 Series Genetic Analyzer; Integrated DNA Technologies, Singapore). This study was approved by the Research Ethical Committee, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand.

2.2. HCV RNA extraction

LiPA 2.0 used the NucliSENS easy MAG (BioMérieux, Boxtel, Netherlands) to extract HCV RNA from 200 μL of plasma sample according to the manufacturer’s instructions. HCV RNA extraction was processed thoroughly by following the manufacturer’s instructions. HCV RNA was extracted from 530 μL of 15 clinical plasma samples and one system control, as performed by automatic RNA extraction of the MPS. Sanger sequencing used the Biosystems® 3500 Series Genetic Analyzer (Integrated DNA Technologies, Singapore) as part of the confirmation method to extract HCV RNA from 400 μL of plasma sample.

2.3. Target region for massively parallel sequencing

The Sentosa® SQ HCV Genotyping Assay (4 × 16) targets the 684-bp NS5B, 604-bp NSSA and 944-bp NS3 regions of the HCV genome amplified to produce DNA PCR product. More than 30 specific primers to the NS5B gene were used for massively parallel sequencing in order to cover > 98% of known HCV strains.

2.4. The Versant HCV Genotype 2.0 assay (LiPA)

The Versant HCV Genotype 2.0 assay (LiPA), utilize reverse hybridization, uses a biotinylated DNA PCR product specific to the 5’UTR and the core region of the HCV genome. This PCR product is produced using the Versant HCV amplification 2.0 kit. Next, hybridization step was performed using the Versant HCV Genotyping 2.0 kit on the Auto-LiPA 48 Genotyping Instrument. A biotinylated PCR product is
2.6. Sentosa

The consensus sequence was analyzed to identify the HCV genotypes and subtypes by using the Geno2pheno-web service (http://hcv.geno2pheno.org/index.php).

2.7. Reference sequences

All reference HCV (NS5B, NS5A, and NS3) genome sequences were obtained from the National Center for Biotechnology Information (NCBI) and Los Alamos National Laboratory (LANL) database. This study used the GenBank accession numbers NC004102 and EU256045 as references for the variant calling of subtype 1a and 1b, respectively.

3. Results

3.1. Identical HCV genotype levels

All 120 samples were identified as 15 genotype patterns by MPS method, there were 1a, 1b, 2a, 3a, 3b, 4d, 6b, 6c,6e, 6f, 6i, 6n 6n + 3b, 6q and 6 v. Comparison with LiPA 2.0 obtained 11 genotype patterns, there were 1a, 1b, 2a or 2c 3, 3a, 3b, 4a/4c/4d, 6a or 6b, 6(c-l) and 6(c-l) + 3 as follow details in Table 1. This study classified evaluation the results into four groups as shown in Fig. 1, the percentile of four HCV genotype result patterns was obtained from the comparison between LiPA and MPS methods. Of 87 identical HCV genotype and subtype results (72.5%), 35 were subtyped as 3a, 26 as 1b, 18 as 1a and 8 as 3b, by both the LiPA and MPS methods. The LiPA method could not identify subtypes in 30 (25%) samples, whereas the MPS method could, which classifies these samples as having similar results by the following the details in Table 2. All 30 samples of unequivocal subtypes were non-GT1, but 2 were found to be GT2, 5 GT3, 1 GT4 and 22 GT6.

3.2. Conflict of genotype and mixed-type infection results

Interestingly, one sample with patient code 40-3566 (0.83%) identified as GT1b by LiPA was found to be GT6v by MPS. Furthermore, in two remaining samples (1.67%), LiPA found one case of mixed-type GT6(c-l) and GT 3 infection in patient code 40-3664, while MPS found only GT6e infection. In another case with patient code 40-3651, LiPA found GT6(c-l), while MPS detected mixed-type GT6n and GT3b infection, as detailed in Table 3.

3.3. Confirmation of HCV genotyping using Sanger sequencing

Table 3 shows that the results of HCV typing obtained by each technique was confirmed by Sanger sequencing of 23 randomly selected samples from four patterns of HCV genotypes taken from 10 identical results, 10 similar results, all of a non-identical result (1 case) and all incompatible mixed HCV infection results (2 cases). The concordant result of HCV genotyping between Sanger sequencing and MPS was 95.65% (22/23). There maining 4.35% (1/23) of mixed infections were determined only by MPS, whereas, LiPA matched both genotypes and subtypes with Sanger sequencing in 43.48% (10/23). LiPA apparently was unable to identify HCV at the subtype level of 43.48% (10/23). In addition, incorrect identification of a genotype level of 4.35% (1/23) in patient code 40-3664 and 8.69% (2/23) was caused by false positive results of mixed infection (patient code 40-3566) and missed identification of mixed infection (patient code 40-3651).
Additional capabilities of detecting resistance-association variants

Of 44 infected patients with HCV GT1, 18 had GT1a and 26 GT1b. Since MPS can detect mutations simultaneously with frequency occurring in the GT1, pre-treated RAVs were found in 56.82% (25/44) of patients, which affected drug resistance in the NS3/NS4A and NS5A inhibitor. Sixty one point one percent (11/18) and 26.92% (7/26) of RAVs were divided into GT1a and GT1b, respectively by the following the details in Table 4. The Q80K mutation (NS3) was the most common RAV in GT1a at 55.56% (10/18), but it did not appear in GT1b. Another combination found Q30K and Y93H (NS5A) in GT1a at 5.56%, while Y93H was the most common RAV in GT1b at 19.23% (5/26). The T54S mutation also was found in GT1b patients at 3.85% (1/26) as well as an L31F mutation, but these were not found in patients infected with GT1a.

4. Discussion

According to the result shown in Fig. 1, LiPA differentiated the HCV genotype level accurately in the majority of cases. Therefore, LiPA is as effective as automated-MPS in testing HCV genotype levels. However, their ability to discriminate HCV subtype levels other than 1a and 1b accurately is very limited (Quer et al., 2015; Yang et al., 2014). Most GT6, some GT3 and all GT2 and GT4 infections clearly show that MPS is a more effective assay than LiPA, due to its high resolution for determining HCV subtyping levels, as shown in Table 2.

The confirmative results from the reference method (Table 3), clearly show that MPS identified the subtypes correctly in all 23 samples. In particular, it was able to identify different HCV genotypes in the case of mixed-type infections, while Sanger sequencing and LiPA were unable to do this. While MPS has the highest sensitivity for detecting low-frequency variants (< 20% of viral quasispecies), Sanger sequencing has lower or limited sensitivity for detecting mixed sequences

Table 1

The pattern of HCV genotype results obtained from the Sentosa HCV Genotyping-MPS versus the Versant-LiPA 2.0 assay for 120 samples.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sentosa-MPS</th>
<th>Versant-LiPA2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>1b</td>
<td>26</td>
<td>26</td>
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<tr>
<td>2a</td>
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</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>27</td>
</tr>
</tbody>
</table>

Gray box indicates to a group of similar results. Square indicates to an incompatibility of mixed HCV infection results. Underline indicates to non-identical genotype results.

Fig. 1. Percentage comparison of HCV genotype patterns between the VERSANT Line Probe Assay 2.0 (LiPA) and Sentosa® SQ HCV Genotyping Assay (automated-massively parallel sequencing). The 120 samples were separated into four groups: 1) those with identical results (when cases using LiPA had identical genotypes and subtypes to those using MPS); 2) those with similar results (when a subtype could not be identified in cases of LiPA); 3) those with non-identical results (when cases using the two methods gave conflicting genotypes); and 4) those with incompatible results of mixed HCV infection (when only one of these methods, either LiPA or MPS, found mixed-types of infection).
because the consensus sequence comprises > 20% of viral quasispecies. (Tsatis et al., 2010). Similarly, LiPA has limitation in accurately detecting low-level mixed viral variants (Bowden et al., 2005), and was not designed to identify mixed subtype infections confidently, which explains inability to detect mixed HCV infections of different subtypes in the same genotype. Therefore, LiPA is not a reliable assay for detecting true mixed HCV infection (Cunningham et al., 2015). Interestingly, despite patient ID 40-3488 having a low viral load of 219 IU/mL, MPS with bioinformatics analysis accurately determined the genotype as well as the reference method.

Therefore, these methods indicate that the only reliable assay for confirming the presence or absence of mixed HCV infection is MPS. In addition, the high sensitivity of MPS maybe essential in preventing a treatment failure and relapsed minority variant in the case of undetectable mixed-type infection after successful therapy. Furthermore, this study shows that MPS is a suitable assay for clinical use by identifying mixed HCV infection accurately. The limitation of this study was the small sample size of people at risk from mixed HCV infection, and MPS might be unable technically to detect a minor variant that contains < 1% of the total viral population (Cunningham et al., 2015). Furthermore, LiPA could not identify a subtype within non-GT1 samples (43.48%) in 23 confirmed cases (Table 3), and it misclassified 3 samples (13.04%). These results were similar to those in another study using Sanger sequencing that agreed with the majority of subtypes identified by deep sequencing in 100% and 88% of all HCV GT1 and HCV non-GT1 samples, respectively (Quer et al., 2015). While subtypes could not be determined in 3%-16% of HCV GT1, 20%-47% were indistinguishable or unidentified in non-HCV GT1 samples, and misclassifications were observed in 6%-14% by the Versant HCV Genotype 2.0 (Avo et al., 2013; Bouchardieu et al., 2007; Cai et al., 2013; Quer et al., 2015). Therefore, improvement to a reliable high-resolution assay for identifying the HCV subtype level should be considered. Additionally, increasing sensitivity for mixed-type HCV detection between different genotypes or subtypes would increase the potential for laboratory diagnosis.

Interestingly, more than half of the Thai patients infected with HCV GT1a had Q80K mutation. In several other studies, the Q80K polymorphism occurred naturally in detectable GT1a at approximately 19-48% (Kumphip and Maneekarn, 2015; Shepherd et al., 2015; Wyles and Gutierrez, 2014). Statistically significant known effects reduced the response rate to treatment with simeprevir (a second-wave NS3/NS4A protease inhibitor) when compared to GT1a infected patients that did not appear to have Q80K (Ahmed and Felmiie, 2015; Lenz et al., 2015; Sarrazin et al., 2015; Wyles, 2013). Thus, present concerns should involve avoiding the use of simeprevir in GT1a infected naive patients who carry Q80K. Similar to Y93H, frequent detection in GT1b has the effect of lower response to treatment with the N55A inhibitor. Furthermore, if the mutations are detected in more than one nucleotide position, they may support non-response to the treatment and lead to treatment failure. Therefore, RAV screening before starting treatment may be the way to increase the chances of reaching more appropriate treatment. It has been seen that mutation is common in GT1, but it may be the way to increase the chances of reaching more appropriate treatment. It has been seen that mutation is common in GT1, but it may be the way to increase the chances of reaching more appropriate treatment.
detected mixed HCV infection. It also has the optional performance of RAV detection simultaneously with HCV genotyping, which is performed on an automatic workflow. Therefore, MPS saves time, reduces manual steps and cuts costs in the long term when compared to traditional methods.

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