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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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ABSTRACT

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 NS5A 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, NS5A and NS5B) (Chevaliez and Pawlotsky, 2006; Ferenci and Reddy, 2011; Li et al., 2015; Moriishi and Matsuura, 2012).

Assix confirmed HCV genotypes have a whole-genome nucleotide that differs by > 30% from the nucleartide 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 nucleartide 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

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(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). At present, the European Association for Study of the Liver (EASL) suggests avoidance Q80K in ST1a infected naive patients prior to starting treatment, as well as those re-starting treatment after previous treatment failure, in order to avoid a simeprevir containing-regimen (Au et al., 2015; EASL, 2015). Similar to M28T, Q30R/H, L31F/M and Y93C/H variants in ST1a, the Y93H variant found most frequently in ST1b (Lim and Gallay, 2014) offers reduced treatment response to daclatasvir and ledipasvir and other *NS5A* inhibitors (Wyles and Gutierrez, 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 NS5A and 944-bp NS3 regions of the HCV genome were 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

hybridized to the immobilized oligonucleotide probes. The probes, which are bound to a nitrocellulose strip, are specific for the 5′UTR and the core region of different HCV genotypes. Then alkaline phosphatase labeled streptavidin is bound to the biotinylated hybrid and follows by 5-bromo-4-chloro-3-indolylphosphate (BCIP)-*p*-nitroblue tetrazolium chromogen, which reacts to form a visible banding on the strip. HCV genotype and subtype determined from the band pattern using an interpretation chart.

2.5. Sanger sequencing

The DNA PCR product, generated by RT-PCR amplification of the NS5B region of HCV RNA by using the Veriti® Dx 96-Well Thermal Cycler, was sequenced by using Forward primer: AACTCCGTG TGGGAGGACTT and Reverse Primer: CTGGTCATAGCCTCCGTGAA. The consensus sequence was analyzed to identify the HCV genotypes and subtypes by using the Geno2pheno-web service (http://hcv.geno2pheno.org/index.php).

2.6. Sentosa® SQ HCV next generation sequencing-based workflow

Massively parallel sequencing was performed on the Ion Torrent PGM platform (Vela Operations Singapore Pte. Ltd, The Kendall, 50 Science Park Road, Singapore). The *Sentosa*® SQ HCV Genotyping Assay (4 × 16) is an automated next generation sequencing-based test. The nucleic acid extraction from samples and PCR set-up are performed on the *Sentosa*® SX101 instrument.After the nucleic acid extraction from samples and performing of RT-PCR set-upon the *Sentosa*® robotic SX101 instrument.Next, the RT-PCR reaction plate was taken out from the SX101 instrument and was seal with Pierceable foil heat seal by using PX1 PCR plate Sealer by setting the temperature at 170 °C for 3 min. The off-board RT-PCR amplification was performed on the Veriti® Dx 96-Well Thermal Cycler for 4 h 20 min. After completed off-board RT-PCR amplification, the reaction plate was transferred back to the workflow continued on the *Sentosa*® SX101for library preparation. After that, library preparation is restarted on the *Sentosa*® SX101, which generates 200 base-nucleotide fragments of a library, and pools DNA libraries that comprise 15 samples and one HCV System Control per run. Afterwards, the *Sentosa*® ST401 is used continuously for template preparation, followed by the *Sentosa*® SQ301 Sequencer. After completing the sequencing successfully, *Sentosa*® SQ Suite software performed primary analysis, such as signal processing and base-calling on raw sequencing data, generated by the *Sentosa*® SQ301 Server. Then, the data were transferred to the *Sentosa*® SQ Reporter for secondary analysis and generating a report that contained a full audit trail relating to the actions within the run, output files representing reads (uBAM), mapped reads (BAM), and variant call frequency (VCF).

The sequencing reaction was performed using the *Sentosa*® SQ Sequencing Kit on the *Sentosa*® SQ301 Sequencer, which measures hydrogen ions that are released due to nucleotide incorporation and conversion to an electrical signal. The initial traces on the *Sentosa*® SQ301 Server were processed and base-called. These calls were assembled into files representing the reads, which were all sequenced and then mapped using IonTorrent TMAP 4.0.5 to complete the HCV genome reference. Next, all mapped reads were down sampled to approximately 1000× coverage for assembly, with only NS5B (all reads) being assembled using the MIRA Assembler 3.4.1. After that, assembled NS5B contigs were aligned to all sequences in the NS5B database, using a chosen BLAST (Top Hit strain) if similarity was > = 80%. Subsequent hits were chosen if the alignment score was > = 0.98 of the top hit. Finally, a Phylogenetic Tree was used to interpret genotypes and subtype calling. For interpretation of mixed infection, reads from different HCV subtypes are separated prior to assembly and are assembled separately. Therefore if two or more contigs of different subtypes are generated, this is an obvious case of mixed infection. If two strains of the same subtype are present, this would still result in two separate contigs, but will require further analysis to ascertain the two contigs represents two separate strains.

In addition to variant calling during mapped data processing, subtypes 1a and 1b reads were handled separately in order to Downsampling and Assembly of the NS5A/NS3 step, which was similar to the NS5B Downsampling and Assembly step. Subtype 1a and 1b reads were mapped to 1a and 1b contigs and then aligned to the 1a and 1b reference, respectively. Finally, variants were called per codon and output termed amino acid change.

Moreover, all of nucleotide sequences from this method were submitted to Genbank and had been provided GenBank accession numbers (MF182660–MF182853).

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, 2, 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 by only 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).

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.

Genotype	Versant-LiPA2.0											
***Sentosa*-MPS**	1a	1b	2	2a/c	3	3a	3b	4a/c/d	6a/b	6(c-l)	6(c-l)+3	Total
1a	18											18
1b		26										26
2a			1	1								2
3a					3	35						38
3b					2		8					10
4d								1				1
6b									1			1
6c										1		1
6e										3	1	4
6f										3		3
6i										8		8
6n										5		5
6n+3b										1		1
6q										1		1
6v		1										1
Total	18	27	1	1	5	35	8	1	1	22	1	**120**

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.

3.4. 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 GT1aand 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

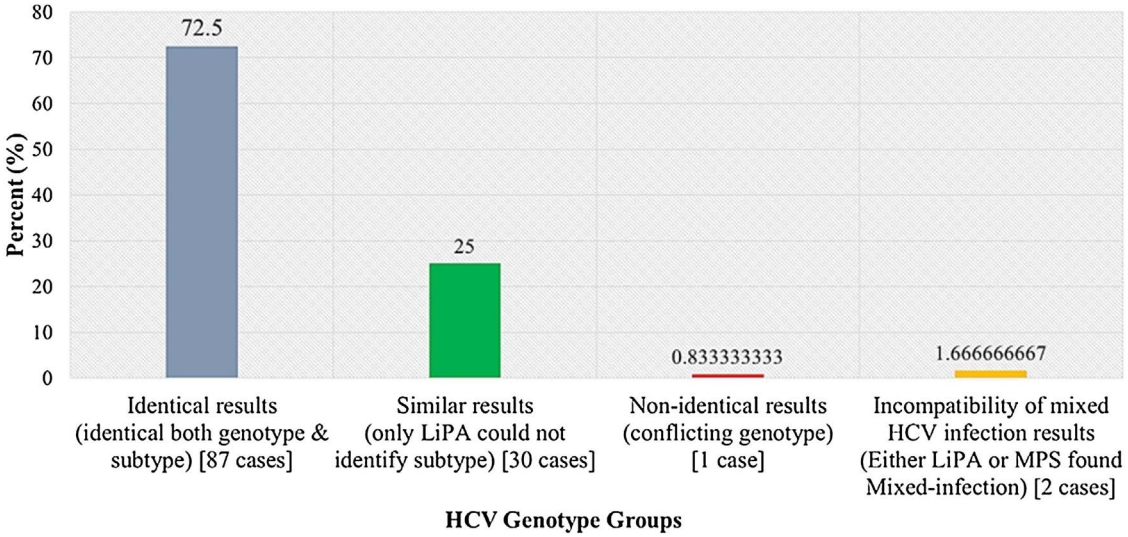

Fig. 1. Percentage comparison of HCV genotype patterns between the VERSANT Line Probe Assay 2.0 (LiPA) and *Sentosa*® SQ HCV Genotyping Assay (automated-massively parallel sequencing). The 120 samples were separated into four groups: 1) those with identical results (when cases using LiPA had identical 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).

Table 2
Comparison of HCV genotyping results between the MPS and LiPA method in 30 specimens with indeterminate subtypes (similar results).

No.	Patient code	Genotyping result	
		Versant HCV Genotype 2.0 LiPA	Sentosa SQ HCV Genotyping Assay (MPS)
1	40-3505	4a/4c/4d	4d
2	40-3506	2	2a
3	JHCG 1388	6(c-l)	6n
4	JHCG 1376	6a or 6b	6b
5	JHCG 1417	6(c-l)	6n
6	JHCG 1425	3	3b
7	40-3508	6(c-l)	6f
8	40-3511	6(c-l)	6q
9	JHCG 1469	6(c-l)	6i
10	JHCG 1480	6(c-l)	6i
11	JHCG 1482	6(c-l)	6i
12	JHCG 1491	6(c-l)	6i
13	40-3567	6(c-l)	6e
14	40-3568	6(c-l)	6n
15	38 − 0558	2a or 2c	2a
16	JHCG 1494	6(c-l)	6i
17	JHCG 1496	6(c-l)	6i
18	40-3645	6(c-l)	6n
19	40-3660	3	3a
20	JHCG 1509	3	3b
21	JHCG 1513	6(c-l)	6f
22	JHCG 1514	3	3a
23	JHCG 1515	6(c-l)	6i
24	JHCG 1519	3	3a
25	40-3674	6(c-l)	6c
26	40-3682	6(c-l)	6e
27	38-0582	6(c-l)	6f
28	40-3684	6(c-l)	6e
29	40-3692	6(c-l)	6n
30	JHCG 1562	6(c-l)	6i

because the consensus sequence comprises > 20% of viral quasispecies. (Tsiatis 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 explainsits 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 poor treatment outcome, treatment failure or viral recurrence, caused by hidden insensitive treatment 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; Bouchardeau 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.

Table 3
Comparison of HCV genotyping results obtained from 23 specimens by Sanger sequencing, LiPA and MPS methods, with 10 taken by identification, 10 by similarity, one was non-identifiable and two incompatible with mixed HCV infection.

No.	Patient code	Viral load (IU/mL)	Genotyping result		
			Directly NS5B Sanger sequencing	Versant HCV Genotype 2.0 LiPA	Sentosa SQ HCV Genotyping Assay (MPS)
1[a]	JHCG 1439	3,022,691	1a	1a	1a
2	40-3488	219	1b	1b	1b
3	40-3520	5,330,000	3a	3a	3a
4	40-3553	1,075,521	1b	1b	1b
5	JHCG 1492	2,793,458	1b	1b	1b
6	40-3646	156,973	3b	3b	3b
7	JHCG 1510	4,393,604	1a	1a	1a
8	JHCG 1518	11,051,463	3a	3a	3a
9	JHCG 1538	11,051	3a	3a	3a
10	JHCG 1543	1,826,305	3a	3a	3a
11[b]	40-3508	825,356	6f	6(c-l)	6f
12	JHCG 1469	2,502,365	6i	6(c-l)	6i
13	JHCG 1491	3,795,542	6i	6(c-l)	6i
14	40-3567	13,537	6e	6(c-l)	6e
15	40-3568	6,805,737	6n	6(c-l)	6n
16	JHCG 1494	655,832	6i	6(c-l)	6i
17	JHCG 1496	511,188	6i	6(c-l)	6i
18	JHCG 1513	278,112	6f	6(c-l)	6f
19	JHCG 1519	10,147	3a	3	3a
20	40-3692	80,517	6n	6(c-l)	6n
21[c]	40-3664	4,599,512	6v	**1b**	6v
22[d]	40-3566	6,998,899	6e	**6(c-l) + 3**	6e
23	40-3651	676,280	**3b**	**6(c-l)**	**6n + 3b**

[a] Identical genotype results (all cases in which LiPA gave identical genotypes and subtypes to MPS).
[b] Similarity of genotype results (all cases in which LiPA could not identify a subtype).
[c] Non-identifiable genotype results (all cases in which two methods gave conflicting genotype results).
[d] Incompatibility of mixed HCV infection results (when either LiPA or VeLa found mixed infection).

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% (Kumthip 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 Felmlee, 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 NS5A inhibitor. Furthermore, if the mutations are detected in more than one nucleartide 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 varies in subtype a and b, therefore, the correct assay for HCV genotyping and subtyping is good for surveillance or when detecting mutations in subgroups.

In conclusion, MPS or NGS is a technique for improving the reliability of subtype determination and increasing assay sensitivity for

Table 4
Mutation list obtained by the newly developed automated-MPS and predictions of phenotypic resistance to HCV direct-acting antiviral (DAAs) drugs.

Patient code	Genotyping result	Mutation result	
		RAVs	Resistance associated to
38 – 0538	1a	–	
JHCG 1390	1a	Q80K	2nd wave *NS3/NS4A* inhibitors
JHCG 1439	1a	Q30H, Y93H	*NS5A* inhibitors
JHCG 1458	1a	Q80K	2nd wave *NS3/NS4A* inhibitors
JHCG 1459	1a	Q80K	2nd wave *NS3/NS4A* inhibitors
40-3509	1a	Q80K	2nd wave *NS3/NS4A* inhibitors
40-3562	1a	–	
JHCG 1507	1a	–	
JHCG 1510	1a	Q80K	2nd wave *NS3/NS4A* inhibitors
JHCG 1526	1a	Q80K	2nd wave *NS3/NS4A* inhibitors
JHCG 1527	1a	Q80K	2nd wave *NS3/NS4A* inhibitors
40-3670	1a	–	
40-3690	1a	Q80K	2nd wave *NS3/NS4A* inhibitors
JHCG 1534	1a	Q80K	2nd wave *NS3/NS4A* inhibitors
JHCG 1537	1a	–	
JHCG 1541	1a	Q80K	2nd wave *NS3/NS4A* inhibitors
JHCG 1545	1a	–	
JHCG 1546	1a	–	
40-3476	1b	–	
JHCG 1399	1b	–	
JHCG 1416	1b	–	
JHCG 1423	1b	Y93H	*NS5A* inhibitors
JHCG 1426	1b	–	
JHCG 1436	1b	–	
JHCG 1464	1b	T54S	1st wave *NS3/NS4A* inhibitors
JHCG 1467	1b	–	
40-3488	1b	–	
40-3493	1b	–	
JHCG 1483	1b	–	
40-3553	1b	–	
40-3563	1b	–	
40-3570	1b	–	
40-3572	1b	L31F	*NS4A* inhibitors
38 – 0557	1b	–	
JHCG 1492	1b	Y93H	*NS5A* inhibitors
JHCG 1493	1b	–	
40-3656	1b	Y93H	*NS5A* inhibitors
38 – 0566	1b	Y93H	*NS5A* inhibitors
JHCG 1516	1b	–	
JHCG 1531	1b	Y93H	*NS5A* inhibitors
40-3685	1b	–	
38 – 0583	1b	–	
JHCG 1536	1b	–	
JHCG 1542	1b	–	

detecting 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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