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Sequencing Analysis of NS3/4A, NS5A, and NS5B Genes from Patients Infected with Hepatitis C Virus Genotypes 5 and 6

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Direct-acting antivirals (DAAs) with activity against multiple genotypes of the hepatitis C virus (HCV) were recently developed and approved for standard-of-care treatment. However, sequencing assays to support HCV genotype 5 and 6 analysis are not widely available. Here, we describe the development of a sequencing assay for the NS3/4A, NS5A, and NS5B genes from HCV genotype 5 and 6 patient isolates. Genotype- and subtype-specific primers were designed to target NS3/4A, NS5A, and NS5B for cDNA synthesis and nested PCR amplification. Amplification was successfully performed for a panel of 32 plasma samples from HCV-infected genotype 5 and 6 patients with sequencing data obtained for all attempted samples. LiPA 2.0 (Versant HCV genotype 2.0) is a reverse hybridization line probe assay that is commonly used for genotyping HCV-infected patients enrolled in clinical studies. Using NS3/4A, NS5A, and NS5B consensus sequences, HCV subtypes were determined that were not available for the initial LiPA 2.0 result for genotype 6 samples. Samples amplified here included the following HCV subtypes: 5a, 6a, 6e, 6f, 6j, 6i, 6l, 6n, 6o, and 6p. The sequencing data generated allowed for the determination of the presence of variants at amino acid positions previously characterized as associated with resistance to DAAs. The simple and robust sequencing assay for genotypes 5 and 6 presented here may lead to a better understanding of HCV genetic diversity and prevalence of resistance-associated variants.

Globally, 130 to 150 million people have chronic hepatitis C virus (HCV) infection. Without treatment, chronic hepatitis may lead to liver cirrhosis and hepatocellular carcinoma and the possible need for a liver transplantation (1–3). Hepatitis C virus has high genetic diversity and is classified into seven genotypes and at least 67 subtypes among the genotypes (4, 5). At the nucleotide level for the open reading frame, there is a 31 to 33% difference between genotypes and 20 to 25% difference between subtypes (6).

HCV genotype 1 is the most prevalent genotype and is widely distributed across the globe. Genotype 1 accounts for the majority of the HCV cases in North America, northern and western Europe, South America, Asia, and Australia (7, 8). Similarly, genotypes 2 and 3 have broad geographical distribution. In contrast, genotypes 4, 5, and 6 are common only in specific regions. Genotype 4 and genotype 5 are endemic to Egypt and South Africa, respectively. Genotype 6 has the highest prevalence in Southeast Asia and southern China. Although genotypes 5 and 6 are not widespread, there are an estimated 9.8 million and 1.4 million people infected with genotypes 5 and 6, respectively, worldwide (9–13). Better treatment options are needed for genotype 5- and 6-infected patients.

Due to the high prevalence and broad geographical distribution of genotype 1, early HCV research and development efforts were focused on this genotype. With the recent approval of HCV direct-acting antivirals (DAAs) active against multiple genotypes, such as Sovaldi (sofosbuvir), Harvoni (ledipasvir and sofosbuvir), and Daklinza (daclatasvir), there is an increased need to monitor the efficacy of pan-genotypic regimens and resistance-associated variants (RAVs) that may be present at baseline and posttreatment (14–24). However, compared to HCV genotype 1, genotypes 5 and 6 are not well-studied. A limited number of genotype 5 and 6 NS3/4A, NS5A, and NS5B sequences are deposited in public databases. Currently, there are few robust sequencing assays available for HCV genotype 5 and 6 patient isolates (25). Moreover, because many of the DAAs currently approved or being studied target either the protease (NS3), NS5A, or the polymerase (NS5B), an efficient strategy to amplify and sequence multiple genes is essential. With sequencing data, the detection and analysis of known gene-specific RAVs prior to treatment may guide selection of optimal HCV DAA combinations, allowing for improved sustained virologic response. Therefore, a robust sequencing assay is needed to allow for resistance testing and analysis. In this study, we developed a deep sequencing strategy of NS3/4A, NS5A, and NS5B for HCV genotype 5 and 6, allowing for the detection and analysis of RAVs and BLAST-based subtyping in HCV-infected genotype 5 and 6 patients.

MATERIALS AND METHODS

Clinical isolates. Plasma samples were collected from 7 HCV-infected genotype 5 and 25 HCV-infected genotype 6 treatment-naive patients enrolled in phase 2 sofosbuvir-containing treatment studies ($n = 22$) or commercial sources ($n = 10$) (ClinicalTrials.gov identifiers NCT01329978, NCT01641640, NCT01826981, and NCT01858766). The study protocols were approved by the institutional review board and ethics committee. All of the samples had patient consent for use. The samples originated from patients in the United States, South Africa, Thailand, and

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TABLE 1 HCV NS3/4A, NS5A, and NS5B primers for cDNA synthesis and PCR amplification

Name	Primer sequence (5′–3′)	Annealing temp (°C)
GT5a1_NS3/4A_PCR1_For	TGCTCCACCTTGGTAGGCTGACCGG	50
GT5a2_NS3/4A_PCR1_For	TGACCGGAACGTACATTTATGACC	50
GT5a1_NS3/4A_PCR1_Rev	GTGGAACTTTGTCAGTGGGATCCA	50
GT5a1_NS3/4A_PCR2_For	CCTATGGAGACGAAGGTCATCACG	55
GT5a1_NS3/4A_PCR2_Rev	GAGCAGTTCTGGGCAAAACACAT	55
GT6a1_NS3/4A_PCR1_For	GCCTTCTGAGGTTGGGCGCTTGGAC	55
GT6a1_NS3/4A_PCR1_Rev	CACATGTGGAACTTTGTCAGCGG	55
GT6a1_NS3/4A_PCR2_For	TGAGTGGCCTCCCGGTGTCAGC	60
GT6a1_NS3/4A_PCR2_Rev	GGGACTCCTCCAAGCGAGCGCTAAG	60
GT6e1_NS3/4A_PCR1_For	CCTGTTGTGTTCTCGCCTATGGAG	55
GT6e1_NS3/4A_PCR1_Rev	GACATCCTAGCAGGGTATGGAGC	55
GT6e1_NS3/4A_PCR2_For	GCAGACACGGCCGCGTGTGGCGAC	55
GT6e1_NS3/4A_PCR2_Rev	GAGGCATTCTGGCAGAAACATCTG	55
GT6l1_NS3/4A_PCR1_For	TTGAGAGTCGGATCTTGGACGGGCAC	55
GT6l1_NS3/4A_PCR1_Rev	GTGGAACTTTGTGAGCGGCATTC	55
GT6l1_NS3/4A_PCR2_For	TGCTGACACTGCTGCATGTGGAGAC	55
GT6l1_NS3/4A_PCR2_Rev	GTCCATTCCGCCTGGCCCAAGCT	55
GT5a_NS5A_PCR1_For	GACCTAGTMAACCTCCTGCC	50
GT5a_NS5A_PCR1_Rev	TCAAGCAAGTCCTGCCACAC	50
GT5a_NS5A_PCR2_For	TCTCCGACRCACTACGTGCC	50
GT5a_NS5A_PCR2_Rev	TACACAAGATTGTGGTGGCG	50
GT5a_NS5A_PCR2_Rev	GTGACCTTCTTCTGCCT	50
GT6_NS5A_PCR1_For	GCYTTYAAGATCATGAGYGG	48
GT6_NS5A_PCR1_Rev	AGCAAGTCCTCCCACACGGAG	48
GT6_NS5A_PCR2_For	AYCAGTGGATGAAYAGGCT	48
GT6_NS5A_PCR2_Rev	AGCAGGGCCATTAACCACATC	48
GT5a_NS5B_PCR1_For	GCGGCTTCATATTCTTCCATGCC	50
GT5a_NS5B_PCR1_Rev	GGAGTGTTTAGCTCCCAGC	50
GT5a_NS5B_PCR2_For	GACCTTTCGTCAGGGTCATGGT	50
GT5a_NS5B_PCR2_Rev	GGGAGYAAAAAGATGCCTAC	50
GT6_NS5B_PCR1_For	GGCCYGAYTAYAAYCCRC	50
GT6_NS5B_PCR1_Rev	CTACCGAGCHGGYAGCA	50
GT6_NS5B_PCR2_For	TCNTAYAGYTCNATGCC	48
GT6_NS5B_PCR2_Rev	GGAAGCAGAAAGATGCCTAC	48
GT6a_NS5B_PCR1_For	CTCTCCCAATATGGGCCAGGCCYGAYTACAATCCACC	59
GT6a_NS5B_PCR1_Rev	ATGTTGCTAAGGCCGCTCGTCTATCGAGCGGGGAG	59
GT6a_NS5B_PCR2_For	CTCGGAAGCAGAGTCCTATAGCTCWATGCCCCC	59
GT6a_NS5B_PCR2_Rev	CGTCTACCGAGCGGGGAGCAAAAAGATGCCT	59
GT6e_NS5B_PCR1_For	CACTACCTATATGGGCGAGGCCAGATTACAATCCTCC	59
GT6e1_NS5B_PCR1_Rev	GCTTTGGGCTTCCCCCTTACCGAGCGGGAAGAA	59
GT6e2_NS5B_PCR1_Rev	GCTTTAGGCTTCCCGCTTACCGAGCGGGAAGAA	59
GT6e_NS5B_PCR2_For	CTCCGATGCTGGCTCATATAGCTCCATGCCCCC	59
GT6e_NS5B_PCR2_For	CTCCGATGCTGGTTCGTACAGCTCTATGCCCCC	59
GT6e_NS5B_PCR2_Rev	CGCTTACCGAGCGGGAAGAAGGAAGATGCCTAC	59
GT6l_NS5B_PCR1_For	CACTACCTATATGGGCGCGGCCTGATTACAACCCACC	59
GT6l_NS5B_PCR1_Rev	TGGAGTGTTATGCTCCCTGCCTACCGGGCAGGGAGCA	59
GT6l_NS5B_PCR2_For	TCCTATAGCTCCATGCCACC	59
GT6l_NS5B_PCR2_Rev	GGAGCAGGAAGATGCCTAC	59

New Zealand. The ethnicity for the United States and New Zealand patients was either non-Hispanic white or Asian; the ethnicity for the South African and Thai patients was unavailable. For the HCV-infected genotype 5 patients, the range for HCV RNA viral load was 65,000 to 9,300,000 IU/ml with a median of 550,000 IU/ml. For the HCV-infected genotype 6 patients, the range for HCV RNA viral load was 25,000 to 60,000,000 IU/ml with a median of 6,800,000 IU/ml.

Primer design. All available nucleotide sequences covering NS3/4A, NS5A, or NS5B were downloaded from the European HCV Database (26) and aligned in Clone Manager 9 (Scientific and Educational Software, Cary, NC) or BioEdit (Abbott, Carlsbad, CA). Nineteen nearly full-length genotype 5 sequences were aligned (Table 1). To account for the diversity of genotype 6, 19 full-length and near-full-length genotype 6 sequences, representing the HCV genotype 6 subtypes of 6b, 6c, 6d, 6e, 6f, 6 h, 6i, 6j, 6o, 6p, 6q, 6s, and 6t, were obtained and analyzed similarly. Genotype- and subtype-specific primers were designed (Table 1). Near-full-length sequences for HCV genotype 6 subtypes of 6a, 6g, 6k, 6l, 6m, 6n, 6r, 6u, and 6v were not available at the initiation of primer design. All primers were at least 18 nucleotides in length and synthesized by Integrated DNA Technologies (Coralville, IA).

cDNA synthesis and PCR amplification. Viral RNA was isolated with a QIAamp viral RNA minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions for the spin-column procedure. Using the MonsterScript 1st-strand cDNA synthesis kit (Illumina, Madison, WI),

cDNA was synthesized from the RNA on a Tprofessional basic thermocycler (Biometra, Göttingen, Germany) or an ABI Veriti 96-well thermal cycler (Life Technologies, Grand Island, NY) (Table 1). The cDNA primer was the reverse primer for the first PCR and was selected based on the available result from the LiPA 2.0 assay (Versant HCV genotype 2.0). Briefly, 10 μl of the RNA, isolated from 140 μl of plasma, and 0.125 μM primer were incubated at 70°C for 4 min and then immediately chilled for more than 1 min before proceeding. After adding 1 μl MonsterScript reverse transcriptase with RNase inhibitor and 4 μl MonsterScript 5× cDNA premix (Illumina, Madison, WI), the mixture was incubated at 50°C for 10 min and then at 60°C for 40 min. Subsequently, the reverse transcriptase was heat inactivated at 90°C for 2 min. The cDNA was used as a template for consecutive PCR amplification of NS3/4A, NS5A, or NS5B. Depending on the available LiPA 2.0 result, the NS3/4A, NS5A, or NS5B amplicons were amplified with either genotype- or subtype-specific primers (Table 1), which were then used as a template for a subsequent nested PCR. Each 50-μl PCR consisted of 1 μM each forward and reverse primer, a 200 μM deoxynucleoside triphosphate set (Bioline USA Inc., Taunton, MA), 2.5 mM $MgCl_2$ (Life Technologies, Grand Island, NY), 5 μl 10× PCR buffer (Life Technologies, Grand Island, NY), 2.5 U Platinum *Taq* DNA polymerase (Life Technologies, Grand Island, NY), and 4.5 μl cDNA, with the remaining volume being RNase-free water (Life Technologies, Grand Island, NY). The PCR parameters were 94°C for 2 min, 35 cycles at 94°C for 30 s or 48° to 59°C for 30 s, depending on the primers (Table 1), and then 72°C for 2 min. Nested PCR was performed next with 5 μl PCR product of each sample with the same PCR parameters mentioned above. PCR was set up at room temperature and performed on either of the aforementioned thermocyclers. All PCR products were run on a 0.8% precast E-Gel with ethidium bromide (Life Technologies, Grand Island, NY) and visualized with UV illumination to verify amplification.

Deep-sequencing-based consensus sequences. PCR products underwent Illumina MiSeq deep sequencing at WuXi AppTec (Shanghai, China) using a procedure previously described (18). The raw deep sequencing data were processed to generate consensus sequences with a 15% cutoff using methods previously described (27). Briefly, for each sample, contigs were generated with VICUNA (28) and aligned to subtype-specific references using MOSAIK (29) to generate an NS3/4A, NS5A, or NS5B assembly sequence. This sequence was used as a reference for aligning trimmed and quality score-filtered reads to generate a consensus sequence.

HCV genotyping using sequencing data. Using BLAST (basic alignment search tool), the HCV DNA nucleotide sequences were analyzed to determine the genotype and subtype classification (30). For each sequence, the genotype and subtype was assigned based on the highest BLAST identity percentage. The minimum BLAST identity percentage for genotype and subtype classification was 84%.

RAV analysis. For genotype 5 NS3 sequences, known resistance-associated variants (RAVs) were defined as L36A/G/M, Q41R, F43S/I/V/L, T54A/C/G/S, V55A/I, Y56H, K80H/R/L, T122R, R155any, A156any, D168any, and I170A/L/T, with "any" representing any amino acid. For genotype 6 NS3 sequences, known RAVs were defined as V36A/G/M, Q41R, F43S/I/V/L, T54A/C/G/S, V55A/I, F56H, L80H/R/K, S122R, R155any, A156any, D168any, and I170A/L/T. For genotype 5 NS5A sequences, known RAVs were defined as Q24G/N/R, L28A/G/T, Q30E/G/H/L/K/R/T, L31F/I/M/V, P32L, S38F, P58D/S, A92K/T, and Y93C/F/H/N/S. For genotype 6 NS5A sequences, known RAVs were defined as Q24G/N, F28A/G/M/T/V, R30E/G/H/L/K/T, L31F/I/M/V, P32L, S38F, T58A/D/G/S, A92K/T, and T93A/C/F/H/N/S. For genotype 5 NS5B sequences, known RAVs were defined as S96T, N142T, L159F, E237G, S282any, M289I/L, L320F/I/V, and V321A/I. For GT 6 NS5B sequences, known RAVs were defined as S96T, N142T, L159F, N237G, S282any, M289I/L, L320F/I/V, and V321A/I.

Nucleotide sequence accession numbers. All generated NS3/4A, NS5A, and NS5B consensus sequences were submitted to the NCBI GenBank database under accession numbers KX107848 to KX107897 and KX138233 to KX138249.

RESULTS

NS3/4A, NS5A, and NS5B PCR amplification from HCV-infected genotype 5 and 6 patients. All cDNA synthesis and PCR amplification attempts for 14/14 NS3/4A (6 genotype 5, 8 genotype 6), 27/27 NS5A (7 genotype 5, 20 genotype 6), and 26/26 NS5B (7 genotype 5, 19 genotype 6) genes from patient HCV plasma samples were successful using the designed primers (Table 1). A deep-sequencing-based or Sanger sequencing-based (population) consensus sequence was generated for each amplicon (Table 2).

For the 7 genotype 5 patients, NS3/4A, NS5A, and NS5B deep-sequencing-based consensus sequences were generated for 6/7, 7/7, and 6/7 patients, respectively. NS3/4A PCR amplification was not performed for one genotype 5 patient due to limited plasma sample availability. Additionally, NS5B Sanger population sequence was generated for one of the genotype 5 patients.

For the genotype 6 patients, the available LiPA 2.0 subtype result guided the selection of primers to use for the first amplification attempt. For patient samples without genotype 6 subtype information available, primers were designed with a 3′ end that was based on the most conserved region of the genotype 6 sequence alignment, including all subtypes; these primers then were used for the initial cDNA and PCR amplification attempt. For patient samples subtyped as genotype 6a/b, genotype 6a and 6b subtype-specific primers were tried individually. For patient samples subtyped as genotype 6c to 6l, subtype-specific primers for 6e and 6l were attempted first. For the 25 genotype 6 patients, NS3/4A, NS5A, and NS5B deep-sequencing-based consensus sequences were generated for 8/25, 20/25, and 11/25 patients, respectively. Due to limited plasma sample availability, NS3/4A, NS5A, and NS5B PCR amplification was not attempted for 17/25, 5/25, and 5/25 patients. NS5B population sequences were generated for 9/25 samples.

BLAST-based subtyping of NS3/4A, NS5A, and NS5B sequences. NS3/4A, NS5A, and NS5B sequences were used to determine concordance, discordance, or refinement of the LiPA 2.0 subtype classification (26) (Table 2). For the genotype 5 patients, patients were initially subtyped by LiPA 2.0 as genotype 5a for all 7/7 patients. BLAST-based subtyping of the available NS3/4A, NS5A, and NS5B sequences were in concordance, confirming the subtype as genotype 5a for all 7/7 patients (Table 2; also see Table S1 in the supplemental material). For the genotype 5 patients, the BLAST identity of their associated sequences ranged from 89% to 93% for NS3/4A, 87% to 93% for NS5A, and 92% to 94% for NS5B (Table 2; also see Table S1). For the genotype 6 patients, patients were initially subtyped by LiPA as genotype 6a/b for 4/25 (16%) patients, genotype 6c to 6l for 11/25 (44%) patients, and unknown subtype of genotype 6 for 10/25 (40%) patients. BLAST analysis of the NS3/4A, NS5A, and NS5B sequences, when available, resulted in subtype refinement for all 25 genotype 6 patients (Table 2; also see Table S1). Among the 25 genotype 6 patients, there were 6 of subtype 6a, 9 of subtype 6e, 1 of subtype 6f, 1 of subtype 6i, 1 of subtype 6j, 3 of subtype 6l, 2 of subtype 6n, 1 of subtype 6o, and 1 of subtype 6p. For the 14/25 genotype 6 patients with only two available sequences, concordance was observed for the BLAST-based subtyping of the sequences. For the 4/25 genotype 6 patients with all three sequences (NS3/4A, NS5A, and

TABLE 2 NS3/4A, NS5A, and NS5B PCR amplification results and comparison between LiPA 2.0 and BLAST-based subtyping methods

LiPA 2.0 subtype	Result for gene: NS3/4A Patients (*n*)	Nucleotide coverage (start position-end position)	Concordant[a] (no. [%])	Refinement[b] (no. [%])	BLAST identity range (%)	NS5A Patients (*n*)	Nucleotide coverage (start position-end position)	Concordant[a] (no. [%])	Refinement[b] (no. [%])	BLAST identity range (%)	NS5B Patients (*n*)	Nucleotide coverage (start position-end position)	Concordant[a] (no. [%])	Refinement[b] (no. [%])	BLAST identity range (%)
5a	6	1–685	6 (100)	0	89–93	7	1–450	7 (100)	0	87–93	7	1–591	7 (100)	0	92–94
6	1		0	1 (100)	95	9	1–456	0	9 (100)	89–96	9		0	9 (100)	93–97
6a/b	4		0	4 (100)	92–95	3	1–451	0	3 (100)	90–91	3		0	3 (100)	94–95
6c-l	3		0	3 (100)	89–93	8	1–456	0	8 (100)	84–94	7		0	7 (100)	89–95
Total	14		6 (42.9)	8 (57.1)		27		7 (25.9)	20 (74.1)		26		7 (26.9)	19 (73.1)	

[a] The same subtype was assigned by both methods.
[b] Indeterminate subtype from LiPA 2.0 was fully determined at subtype level by BLAST sequencing.

NS5B), concordance was observed for the BLAST-based subtyping of the sequences. For the genotype 6 patients, the BLAST identity of the available, associated sequences ranged from 89% to 95% for NS3/4A, 84% to 96% for NS5A, and 89% to 97% for NS5B (Table 2; also see Table S1). BLAST-based subtypes were confirmed in all cases using a phylogenetic analysis wherein a maximum-likelihood phylogeny was constructed using the consensus sequences of the samples described here and sequences from samples with subtypes previously described (5).

Sequence analysis for RAVs. Deep sequencing data with a 15% variant detection cutoff was analyzed for the presence of known NS3, NS5A, and NS5B RAVs (Tables 3, 4, and 5). Specific genotype 1a and 1b amino acid positions in NS3, NS5A, and NS5B have been well-characterized and documented as being associated with drug resistance. These amino acid positions are the basis for the genotype 5 and 6 NS3, NS5A, and NS5B RAV analysis in this study. Eleven amino acid positions in NS3, 9 amino acid positions in NS5A, and 8 amino acid positions in NS5B, described in Materials and Methods, were evaluated. For the genotype 5a patients, NS3/4A, NS5A, and NS5B deep sequencing data were available for 6/7, 7/7, and 6/7 patients, respectively. For the genotype 6 patients, NS3/4A, NS5A, and NS5B deep sequencing data were available for 8/25, 20/25, and 11/25 patients, respectively. The single NS3 RAV, D168E, was detected in 3/6 (50%) genotype 5a patients. The single NS3 RAV, Q80K, was detected in 4/8 (50%) genotype 6 patients who were all GT 6a subtype. No known NS5A RAVs were present in any of the 7 genotype 5a patients. For the genotype 6 patients, NS5A RAVs were detected at the NS5A amino acid positions 28, 58, and 93. Single NS5A RAVs were detected in 10/20 (50%) genotype 6 patients (F28M, $n = 2$; F28V, $n = 8$). Four of 20 (20%) genotype 6 patients had double NS5A RAVs (F28M and T93S, $n = 1$; F28V and T58A, $n = 1$; F28V and T93S, $n = 2$). NS5B RAVs were detected only in the genotype 6 patients, not the genotype 5 patients. For genotype 6, only the NS5B RAV M289L was identified and was observed in 7/11 (63.6%) patients.

DISCUSSION

Since the discovery of HCV in 1989 (31), research and drug development have been focused on the most prevalent and widespread genotypes, such as genotypes 1 and 2. In contrast, genotype 5 and 6 HCV remain poorly characterized. The recent development and approval of HCV DAAs efficacious against multiple genotypes has led to increased interest in these genotypes.

To enable resistance testing of genotype 5 and 6 HCV, a robust PCR amplification and sequencing assay of viral target genes, such as NS3/4A, NS5A, and NS5B, is necessary. In this study, primers were developed to amplify NS3/4A, NS5A, and NS5B from genotype 5 and genotype 6 samples. Successful PCR amplification of HCV genes, such as NS5B, and subsequent sequencing allows for BLAST analysis of sequences for patient genotype and subtype determination. Antiviral therapy response and optimal treatment duration may vary based on the genotype/subtype of infection. HCV-infected, genotype 1b treatment-naive patients treated for 12 weeks with either daclatasvir and simeprevir or daclatasvir, simeprevir, and ribavirin achieved a higher rate of sustained virologic response at posttreatment week 12 (SVR12) than HCV-infected genotype 1a treatment-naive patients with 24 weeks of daclatasvir, simeprevir, and ribavirin (32). For the TURQUOISE-II study, HCV-infected genotype 1a patients needed to be treated for 24 weeks, instead of 12 weeks, with ombitasvir, paritaprevir,

TABLE 3 NS3 amino acid changes at positions associated with drug resistance

Reference or patient group	GT[a]	Amino acid(s) at position[c]: 36	41	43	54	55	56	80	122	155	156	168	170
1a H77		V	Q	F	T	V	Y	Q	S	R	A	D	I
1b Con1		V	Q	F	T	V	Y	Q	S	R	A	D	V
5a SA13[b]		L36A/G/M	Q41R	F43S/I/V/L	T54A/C/G/S	V55A/I	F56H	K80H/R/L	T122R	R155any	A156any	D168any	I170A/L/T
Patients ($n = 6$)	5a								A ($n = 2$)			*E* ($n = 3$)	V ($n = 1$)
6a EUHK2[b]		V36A/G/M	Q41R	F43S/I/V/L	T54A/C/G/S	V55A/I	Y56H	L80H/R/K	S122R	R155any	A156any	D168any	I170A/L/T
Patients ($n = 4$)	6a							K ($n = 4$)	N ($n = 3$)				
Patients ($n = 2$)	6e							Q ($n = 2$)	A ($n = 1$)				V ($n = 2$)
									T ($n = 1$)				
Patients ($n = 2$)	6l							Q ($n = 2$)					V ($n = 2$)

[a] GT, genotype.
[b] Potential genotype-specific resistance-associated variants are shown.
[c] Amino acids in italics are potential resistance-associated variants. Blank cells indicate amino acids identical to the appropriate reference sequence.

ritonavir, and dasabuvir with ribavirin to achieve high SVR12 rates similar to those of the HCV-infected genotype 1b patients treated for 12 weeks with ombitasvir, paritaprevir, ritonavir, and dasabuvir with ribavirin (33). Therefore, accurate, specific genotyping and subtyping of a patient's HCV is needed. LiPA 2.0 is a reverse hybridization line probe assay that targets the 5′ untranslated region and core regions, and it is a commonly used commercial HCV genotyping method (34). For genotype 6 samples, this assay commonly reports the genotype as either genotype 6a/b or genotype 6c to 6l. Previous studies evaluating LiPA 2.0 reported genotype 6 samples as failing the assay or being misclassified as genotype 1 or a different subtype (35–37). In this study, a majority of the genotype 6 samples had either an unknown or not fully determined subtype by LiPA 2.0. Considering the diversity of genotype 6 and the difference between subtypes within this genotype, the subtype may be more accurately determined through BLAST analysis of the NS3/4A, NS5A, and NS5B sequences; sequencing and BLAST analysis allowed for subtype refinement of all genotype 6 samples. Although LiPA 2.0 has successfully genotyped genotype 5 samples, assay failures have been reported for genotype 5 samples (37). In this study, BLAST analysis of the available genotype 5 NS3/4A, NS5A, and NS5B sequences confirmed the subtype of these samples as subtype 5a. Since LiPA 2.0 may fail to classify, misclassify, or provide a vague subtype, especially for genotype 6, the sequencing of a PCR-amplified portion of the patient genome followed by BLAST analysis represents a robust alternative genotyping method.

Deep sequencing allowed for the detection of NS3, NS5A, and NS5B RAVs. For NS3, RAVs were detected in 3/6 (50%) genotype 5 and 4/8 (50%) genotype 6 patient samples. For genotype 5, no NS5A or NS5B RAVs were identified. In contrast, at least 1 NS5A RAV was detected in 14/20 (70%) genotype 6 samples, and at least 1 NS5B RAV was detected in 7/10 (70%) genotype 6 samples; in contrast, no NS5A RAVs were in the 7 genotype 5 samples. Further investigation is still needed to determine if there are genotype 5- and genotype 6-specific RAVs that contribute to reduced susceptibility to HCV DAAs.

This study has limitations. Due to sample availability, the primers were evaluated with a limited number of HCV-infected genotype 5 and genotype 6 patient samples. Considering the diversity of genotype 6, the primers should have been evaluated with a few samples for each genotype 6 subtype. Primer sensitivity was not assessed by attempting amplification from patient samples with low HCV RNA viral load. Further studies are needed to eval-

TABLE 4 NS5A amino acid changes at positions associated with drug resistance

Reference or patient group	GT[a]	Amino acid at position[c]: 24	28	30	31	32	38	58	92	93
1a H77		K	M	Q	L	P	S	H	A	Y
1b Con1		Q	L	R	L	P	S	P	A	Y
5a SA13[b]		Q24G/N/R	L28A/G/T	Q30E/G/H/L/K/R/T	L31F/I/M/V	P32L	S38F	P58D/S	A92K/T	Y93C/F/H/N/S
Patients ($n = 7$)	5a									
6a EUHK2[b]		Q24G/N	F28A/G/M/T/V	R30E/G/H/L/K/T	L31F/I/M/V	P32L	S38F	T58A/D/G/S	A92K/T	T93A/C/F/H/N/S
Patients ($n = 5$)	6a	R ($n = 1$)	L ($n = 5$)							
Patients ($n = 8$)	6e	K ($n = 6$)	*M* ($n = 3$)	S ($n = 8$)				H ($n = 1$)		*S* ($n = 1$)
		R ($n = 2$)	*V* ($n = 5$)					P ($n = 7$)		
Patients ($n = 1$)	6f	K ($n = 1$)	A ($n = 1$)	S ($n = 1$)				P ($n = 1$)		
Patients ($n = 1$)	6j	K ($n = 1$)	*V* ($n = 1$)	A ($n = 1$)				*A* ($n = 1$)		
Patients ($n = 1$)	6l	K ($n = 1$)	*V* ($n = 1$)	A ($n = 1$)				P ($n = 1$)		
Patients ($n = 2$)	6n	K ($n = 2$)	*V* ($n = 2$)	S ($n = 2$)						*S* ($n = 2$)
Patients ($n = 1$)	6o	K ($n = 1$)	L ($n = 1$)	A ($n = 1$)				*A* ($n = 1$)		
Patients ($n = 1$)	6p	K ($n = 1$)	*V* ($n = 1$)	S ($n = 1$)				P ($n = 1$)		

[a] GT, genotype.
[b] Potential genotype-specific resistance-associated variants are shown.
[c] Amino acids in italics are potential resistance-associated variants. Blank cells indicate amino acids identical to the appropriate reference sequence.

TABLE 5 NS5B amino acid changes at positions associated with drug resistance

Reference or patient group	GT[a]	Amino acid at position[c]: 96	142	159	237	282	289	320	321
1a H77		S	N	L	E	S	C	L	V
1b Con1		S	N	L	E	S	C	L	V
5a SA13[b]		S96T	N142T	L159F	E237G	S282any	M289I/L	L320F/I/V	V321A/I
Patients ($n = 6$)	5a								
6a EUHK2[b]		S96T	N142T	L159F	N237G	S282any	M289I/L	L320F/I/V	V321A/I
Patients ($n = 2$)	6a								
Patients ($n = 3$)	6e				H ($n = 2$) R ($n = 1$)		*L* ($n = 3$)		
Patients ($n = 1$)	6f				E ($n = 1$)		*L* ($n = 1$)		
Patients ($n = 1$)	6i				Q ($n = 1$)				
Patients ($n = 1$)	6l				R ($n = 1$)		*L* ($n = 1$)		
Patients ($n = 2$)	6n				H ($n = 2$)		*L* ($n = 2$)		

[a] GT, genotype.
[b] Potential genotype-specific resistance-associated variants are shown.
[c] Amino acids in italics are potential resistance-associated variants. Blank cells indicate amino acids identical to the appropriate reference sequence.

uate PCR amplification success with the described primers and primer sensitivity.

In summary, a deep sequencing assay for NS3/4A, NS5A, and NS5B amplification from plasma samples of HCV-infected genotype 5 and 6 patients was developed. The generated sequences enabled subtype refinement of these patient samples. Moreover, known NS3, NS5A, and NS5B RAVs were detected in some genotype 5 and 6 samples, but it is unclear if these RAVs will lead to reduced drug susceptibility, since genotype 5- and 6-specific RAVs still are not well-characterized. Genotype 5 and 6 sequence information has the potential to facilitate the better understanding of HCV genetic diversity and drug-associated resistance.

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