

# High-Resolution Hepatitis C Virus Subtyping Using NS5B Deep Sequencing and Phylogeny, an Alternative to Current Methods

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**Hepatitis C virus (HCV) is classified into seven major genotypes and 67 subtypes. Recent studies have shown that in HCV genotype 1-infected patients, response rates to regimens containing direct-acting antivirals (DAAs) are subtype dependent. Currently available genotyping methods have limited subtyping accuracy. We have evaluated the performance of a deep-sequencing-based HCV subtyping assay, developed for the 454/GS-Junior platform, in comparison with those of two commercial assays (Versant HCV genotype 2.0 and Abbott Real-time HCV Genotype II) and using direct NS5B sequencing as a gold standard (direct sequencing), in 114 clinical specimens previously tested by first-generation hybridization assay (82 genotype 1 and 32 with uninterpretable results). Phylogenetic analysis of deep-sequencing reads matched subtype 1 calling by population Sanger sequencing (69% 1b, 31% 1a) in 81 specimens and identified a mixed-subtype infection (1b/3a/1a) in one sample. Similarly, among the 32 previously indeterminate specimens, identical genotype and subtype results were obtained by direct and deep sequencing in all but four samples with dual infection. In contrast, both Versant HCV Genotype 2.0 and Abbott Real-time HCV Genotype II failed subtype 1 calling in 13 (16%) samples each and were unable to identify the HCV genotype and/or subtype in more than half of the non-genotype 1 samples. We concluded that deep sequencing is more efficient for HCV subtyping than currently available methods and allows qualitative identification of mixed infections and may be more helpful with respect to informing treatment strategies with new DAA-containing regimens across all HCV subtypes.**

There are seven confirmed hepatitis C virus (HCV) genotypes, with whole-genome nucleotide sequences differing by >30%, and each can be further subdivided into related subtypes (67 confirmed), with nucleotide sequence divergence of between 15% and 30% (1).

Genotype identification has long been used in clinical practice, because major genotypes have different response rates and require different doses and durations of pegylated interferon and ribavirin (PR) treatment. In contrast, until recently, subtype identification was mainly used in epidemiological studies. However, both *in vitro* studies and clinical trials with different classes of direct-acting antiviral (DAA) agents (NS3 protease, NS5A-, and nucleos[t]ide and non-nucleos[t]ide NS5B-polymerase inhibitors), given with PR or in interferon-free combinations, have shown lower response rates for HCV genotype 1a than for HCV genotype 1b (2–8). Moreover, at least for HCV genotype 1, both the frequency and the pattern of resistance to different DAA classes are subtype specific (9). A striking example is the NS3-Q80K polymorphism, naturally found in >30% of naive subtype 1a patients but in <1% of subtype 1b patients (10), which conveys 30%-to-40%-lower sustained-virologic-response (SVR) rates to the macrocyclic protease inhibitor simeprevir (2). Similarly, all subtype 1g sequences identified naturally carry a mutation conferring resistance to linear NS3 protease inhibitors (11).

Subtype-specific differences in the genetic barrier to resistance appear to correlate to the RNA-dependent RNA polymerase mu-

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tational bias toward transition mutations and differences in codon usage characteristic of each subtype rather than to the degree of genetic diversity of the viral population (12–15). In addition, coinfection with two or more HCV strains of different genotypes or subtypes is a common finding in some high-risk groups. Researchers who performed several studies in persons who inject drugs (PWID) and among men who have sex with men (MSM) have reported the simultaneous presence of two or more HCV subtypes in 25% to 39% of incident infections (16–19). Taken together, the findings of the subtype-specific response to different classes of DAA and of the frequency of multiple infections among groups with the highest incidence and prevalence of HCV infection make accurate HCV subtyping, including detection of mixed infections, an essential tool to optimize current and future treatment regimens.

Currently available genotyping methods based on reverse hybridization with subtype-specific primers and probes targeting the 5' untranslated region (UTR) and core regions (Versant HCV Genotype 2.0 system; Siemens), and Real-time (Rt) PCR assays based on 5' UTR and NS5B sequencing (Abbott HCV Genotype II assay), accurately differentiate major HCV genotypes in the majority of cases and are widely used because of their technical simplicity. However, these assays have not been designed to confidently identify mixed infections, and their ability to accurately discriminate HCV subtypes other than 1a and 1b is very limited (20–27).

We have evaluated the analytical performance of a deep-sequencing-based HCV genotyping assay, targeting NS5B, using a readily available platform (454/GS-Junior; Roche), in 114 samples, in comparison with two commercially available techniques, using population Sanger sequencing and phylogenetic analysis of a 339-nucleotide (nt)-long NS5B fragment as a reference (28).

## MATERIALS AND METHODS

**Patients.** Coded serum samples from 114 HCV-infected patients previously tested by first-generation line probe assay (Inno-LiPA v1.0) were used without any patient-associated demographic and clinical information. Eighty-two were genotype 1, and 32 had no subtype calling by Inno-LiPA analysis. All specimens were collected and processed as recommended and stored at  $-80^{\circ}\text{C}$ . Patient samples were collected from different hospitals of the National Spanish Health System with the behest of selecting samples that are difficult to classify using commercial techniques. In addition to genotype 1 samples, we also asked for samples other than genotype 1 to cover as many subtypes as possible. Taking advantage of the Tropical Medicine outpatient clinics in Hospital Carlos III in Madrid, we received 26 samples from Spanish people, most of them of equatorial Guinean origin. The vast majority of them did not have a history of intravenous drug use (IDU).

**Genotyping specimens.** All specimens but two were subtyped using four methods, deep sequencing on a 454/GS-Junior platform, direct Sanger sequencing, and two commercial assays, Versant HCV Genotype 2.0 and Real-time HCV Genotype II. PCR-amplified products were sequenced in parallel by direct and deep sequencing. For subtyping using the commercially available procedures, serum samples were processed strictly following the manufacturing instructions.

**RNA extraction.** HCV RNA was extracted from 650  $\mu\text{l}$  of plasma or serum by automatic RNA extraction using a total nucleic acid isolation (TNAI) kit (AmpliPrep system; Roche Diagnostics, West Sussex, United Kingdom) or manual RNA extraction (140  $\mu\text{l}$ ) using a QIAamp viral RNA minikit (Qiagen, Hilden, Germany). The measures to prevent contamination suggested by Kwok and Higuchi were strictly applied (29).

**Target region and primers.** The NS5B target for HCV subtyping was a 339-nucleotide (nt)-long region spanning nt 8280 to 8618, according to the isolate H77 data available in GenBank under accession number AF009606 (30). Primer and PCR condition data have been deposited under patent number EP13382278 and are reported in the next sections.

In cases of low viral load or low RNA quality, when NS5B was impossible to amplify, we sequenced the 5' core region spanning nt 45 to 490 for reverse transcription-PCR (RT-PCR) and nt 146 to 490 for heminested PCR (Fig. 1). The specific primers for RT-PCR were forward primer UTR45 (5'CTGTGAGGAACTACTGTCTTCACGCAG3') and reverse primer Cor490 (5'CTAGTCGCGCGCACACCCA3'). The specific primers for heminested PCR were forward primer M13UTR146 (5'GTTGTA AAACGACGGCCAGTGTCTGCGGAACCGGTGAGTACA3') and reverse primer M13Core490 (5'CACAGAAAACAGCTATGACCCTAGTC GCGCGCACACCCA3'). The enzymes and amplification conditions were the same as for the NS5B amplicon (see below). Only 4 samples (P-IND-6, -13, -14, and -17) out of 114 were classified by the 5' core region.

**RT-PCR amplification for direct and deep sequencing.** The complete process is depicted in Fig. 1. Briefly, reverse transcription was performed using a Transcriptor One Step reverse RT-PCR kit (Roche Applied Science, Basel, Switzerland), 20 pmol of the downstream primer labeled 5Bo8707, and 20 pmol of the upstream primer labeled 5Bo8254 (Table 1). See the supplemental material for reaction details. A final product of 454 nucleotides is obtained.

Heminested PCR was performed using a FastStart High Fidelity PCR System, dNTPack (Roche Applied Science, Basel, Switzerland), 5  $\mu\text{l}$  from the PCR, and a pair of upstream (13N5Bo8254) and downstream (13N5Bo8641) primers (Table 1). Nested PCR was performed using the same conditions as were used for first PCR. A final product of 428 nucleotides (including primers) was obtained.

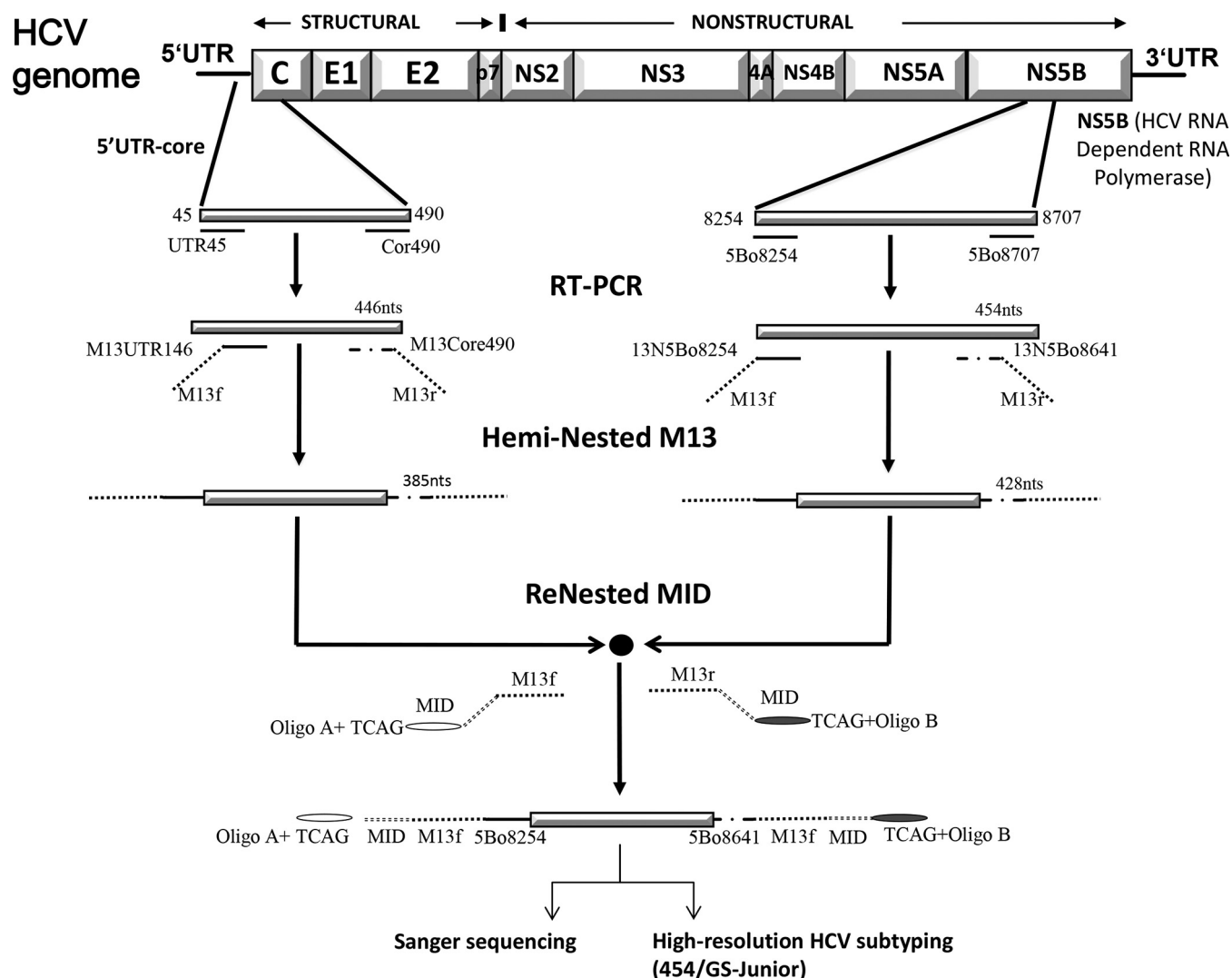
To identify every patient, the final product of heminested amplification was subjected to 15 cycles of reamplification using primers composed of a complementary universal M13 primer (either upstream or downstream) followed by a Roche's Validated Multiplex Identifier (MID) with oligonucleotide A or B at the 5' or 3' end of the upstream or downstream primer, respectively (Fig. 1). A final product of 498 nucleotides (including primers) was obtained.

Amplification products were analyzed by 1.8% agarose gel electrophoresis, and negative controls (amplifications in the absence of RNA) were included in parallel to ensure the absence of contamination by template nucleic acids. The final nested amplification yielded 498-nt fragments that were purified in agarose gel using a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA), quantified using the PicoGreen assay (Invitrogen, Carlsbad, CA, USA), and quality analyzed using a BioAnalyzer DNA 1000 LabChip (Agilent, Santa Clara, CA, USA) prior to sequencing.

One aliquot of each PCR product was subjected to Sanger sequencing and another to ultradeep pyrosequencing (UDPS) subtyping as follows. The sequences obtained by either one or the other of the technologies were subjected to phylogenetic analysis using recommended reference sequences (1).

**Direct Sanger sequencing.** The PCR product was directly sequenced using a BigDye Terminator v1.1 cycle sequencing kit and a capillary automated DNA-sequencing instrument (Applied Biosystems, Foster City, CA, USA). Consensus (average) sequences obtained by population Sanger sequencing were subjected to phylogenetic analysis (see below).

**Deep sequencing.** In order to facilitate the translation of deep sequencing from basic research to a health care routine diagnostic laboratory, most of the manual processing steps have been automated using a programmed Evo75 robot device (Tecan, Männedorf, Switzerland). Briefly, after nested PCR amplification, the final product is flanked by universal M13 sequences in both ends. The last PCR amplification is performed in a 96-well plate previously preloaded with a lyophilized universal primer pair composed of an M13 universal primer plus MID plus oligonucleotide A or B (454 sequencing) (M13-MID\_A/B primer) (TIB Molbiol, Berlin, Germany). Each patient sample requires a different MID sequence. The PCR product of this amplification is then automatically



**FIG 1** Scheme of the NS5B amplification protocol for HCV subtyping either using direct PCR Sanger sequencing or UDPS subtyping. Specific and universal primers are specified in each amplification step. NCR, noncoding region; nts, nucleotides; Oligo, oligonucleotide(s).

purified using Ampure Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA) and quantified using a fluorometric titration Quant-iT PicoGreen double-stranded DNA (dsDNA) kit (Life Technologies, Eugene, OR, USA) in an Infinity fluorometer (Tecan, Männedorf, Switzerland) and equimolecular pooling and a final enrichment step using an automated REMe STARlet Hamilton workstation (Hamilton Robotics, Reno, NV, USA).

Massive sequencing was performed in the 454/GS-Junior platform (Roche, Branford, CT, USA), using titanium chemistry (GS-Junior Titanium Sequencing kit), which enables sequencing of 400-to-500-nt fragments. An average of 1,250 sequences (reads) were obtained (minimum [min], 200; maximum [max], 5,240).

The fasta file from the GS-Junior is demultiplexed to obtain a fasta file for each sample and strand. Reads not identified by MID and/or primer

**TABLE 1** Primers used for RT-PCR and heminested amplifications

Primer type	Primer name	Primer sequence (5'–3')
RT-PCR		
Upstream	5Bo8254	CNTAYGAYACCMGNTGYTTTGACTC
Downstream	5Bo8707	TTNGADGAGCADGATGTWATBAGCTC
Heminested <sup>a</sup>		
Upstream	13N5Bo8254	GTTGTAAAACGACGGCCAGTTTNGADGAGCADGATGTWATBAGCTC
Downstream	13N5Bo8641	CACAGGAAACAGCTATGACCGARTAYCTGGTCATAGCNTCCGTGAA

<sup>a</sup> Heminested primers are composed of universal M13 forward (M13f) and M13 reverse (M13r) primers at the 5' ends followed by a specific fragment (italic typeface) complementary to the HCV PCR-amplified fragment.

are discarded. The allowances are one mismatch on the MID, two in the specific primer, and three on the universal primer M13, with no indels. Sequences not covering the full amplicon (positions 8280 to 8618) or showing more than 2 Ns or 3 gaps are discarded (31). MIDs and primers are trimmed. The reverse-strand sequences are reverse complemented and joined with the forward strand sequences of the same sample. Unique sequences (haplotypes) are identified and their frequencies computed as the number of observed reads.

Then follows a process of clustering of haplotypes to select those to be subtyped in each sample. The dominant haplotype is selected, and all those sequences with a pairwise identity above 90% with respect to this haplotype are clustered. The dominant haplotype is taken as the representative sequence of the cluster (the centroid) and the centroid frequency as representative of the results of the addition of the frequencies of all clustered haplotypes. This process is done recursively until no haplotypes are left. The result is a set of representative haplotypes (centroids) with their observed population frequencies. The threshold of 90% identity was selected on the basis of the minimum observed distance between pairs of reference sequences of different subtypes.

**Subtype calling.** Each UDPS haplotype centroid, or population Sanger sequence, is multiply aligned with the set of reference sequences (1) using Clustalo. This multiple alignment provides a matrix of genetic distances with the Kimura-80 model (K80) with a gamma parameter of 0.42. The query haplotype is classified as the subtype with the nearest reference sequence. For subtypes with multiple reference sequences, two other distances to the query haplotype are computed: the average and the farthest in each subtype. These distances are used as clustering quality scores. A high-quality clustering result is produced when the subtypings according to the shortest, the average, and the farthest distances coincide. A confidence score is produced by 200 cycles of bootstrap analysis on the multiple alignment obtained for each query haplotype and subtyping according to the shortest distance. Query haplotypes classified with discordant shortest-distance and average-distance subtyping or with bootstrap scores below 70 are tagged as dubious.

A minimum of 5 reads and of 1% abundance is imposed to infer a mixed infection by a minority subtype. As a further step, a fuzzy-logic-based expert system is being developed to infer recommendations based on the sequencing coverage, percentage of minority subtypes, and classification by the nearest distance and the average distance and by the bootstrap confidence level. This is the matter of a publication in preparation.

The data analysis system consists of four R modules, implementing the demultiplexing module, the quality filter and haplotype selection module, the phylogenetic analysis module, and the expert system module. The scripts use the Bioconductor packages Biostrings and ape.

**HCV genotyping using commercial available methods. (i) Versant.** The HCV Genotype 2.0 assay (LiPA HCV v.2.0; Siemens Healthcare Diagnostics, Eragny, France) is a line probe assay based on a reverse hybridization of biotinylated products targeting the 5' UTR and a fragment of the core region which are hybridized to immobilized oligonucleotide probes in nitrocellulose strips that are specific for the 5' -UTR core regions of the six genotypes.

**(i) Abbott Real-time.** The HCV Genotype II assay software, version 1.0 (Abbott Molecular, Des Plaines, IL, USA), is based on Real-time PCR targeting the 5' UTR and the NS5B gene for discrimination between genotypes 1a and 1b. The assay uses *rTth* DNA polymerase and four sets of PCR primers to amplify the 5' -UTR region, NS5B subtype 1a, NS5B subtype 1b, and an heterologous internal control, respectively. The assay uses fluorescence-labeled oligonucleotide probes specific for genotypes 1 to 6 and subtypes 1a and 1b, in three separate reactions, one to detect genotypes 1a and 3, the second for genotypes 2, 1b, and 1, and the third for genotypes 4, 5, and 6 (Abbott Rt HCV Genotype II insert package).

**Statistical analysis.** Rates and proportions were analyzed by the binomial and Fisher exact tests. A *P* value of 0.05 was considered statistically significant.

TABLE 2 Comparison of genotyping results obtained by the four methods in genotype 1 specimens

Patient code	Genotyping result			
	Direct NS5B sequencing	Versant HCV Genotype 2.0	Abbott Real time HCV genotype II	UDPS HCV Subtyping 454/GS-Junior <sup>d</sup>
P1–P17	1a	1a	1a	1a
P18–P61	1b	1b	1b	1b
P62	1a	1	1	1a
P63	1a	1a	1	1a
P64	1a	1	1a	1a
P65	1a	1a	1	1a
P66	1a	1	1	1a
P67	1a	1	1	1a
P68	1a	1	1	1a
P69	1a	1a	1	1a
P70	1b	1	1b	1b
P71	1b	1	1	1b
P72	1b	1	1b	1b
P73	1b	1	1b	1b
P74	1b	1	1b	1b
P75	1b	1	1	1b
P76	1b	1b	1	1b
P77	1b	1b	1b	1b + 3a + 1a <sup>b</sup>
P78	1b	1b	1	1b
P79	1b	1/1a	1b	1b
P80	1b	1	1b	1b
P81	1b	1b	1	1b
P82	1b	1b	1	1b

<sup>a</sup> *P* < 0.001 for the comparison of UDPS with Versant HCV Genotype 2.0 and Abbott Real time HCV genotype II techniques.

<sup>b</sup> Sample with mixed infection. Relative proportions of subtypes: 1b, 43%; 3a, 35%; 1a, 20%.

**Ethics statement.** The study has been approved by the Ethical Committees (CEIC) of Vall d'Hebron and of the clinical centers that have provided samples. All samples were collected from adults who provided the consent required to perform the study.

**Nucleotide sequence accession numbers.** The sequences obtained in this study have been deposited in GenBank under accession numbers SAMN03198770 (for patient P01) to SAMN03198883 (for patient PIND32). All sequences deposited in GenBank belong to NS5B except SAMN03198857, SAMN03198864, SAMN03198865, and SAMN03198868, which belong to the 5' core region (from nt 146 to nt 490).

**RESULTS**

As shown in Table 2, among the 82 HCV genotype 1 samples, 25 were subtyped as 1a and 57 as subtype 1b, by both direct and deep sequencing. The subtype could not be established in 13 (16%) samples by either the Versant HCV Genotype 2.0 or the Abbott Rt-HCV Genotype II method. Of the 25 subtype 1a samples, 5 (20%) and 7 (28%) could not be subtyped by the Versant/Siemens and Rt-HCV/Abbott methods, respectively. Similarly, of the 57 subtype 1b samples, 8 (14%) and 6 (10%) could not be subtyped by those techniques, respectively.

Interestingly, one sample which was classified as 1b by population Sanger sequencing and by both commercial techniques was found to be a mixed infection of HCV subtypes 1b (43%), 3a (35%), and 1a (22%) by our deep-sequencing-based high-resolution assay. This patient had no history of IDU.

Among the 32 untyped non-genotype 1 samples, 3 were found to be genotype 2, 4 to be genotype 3, 23 to be genotype 4, and 1

**TABLE 3** Comparison of genotyping results obtained by the four methods in 32 specimens with indeterminate genotypes by first-generation hybridization assay (LiPa 1.0)<sup>a</sup>

Patient code	Genotyping result		Abbott Real time HCV genotype II	UDPS HCV Subtyping 454/GS-Junior (% of total)
	Direct NS5B sequencing	Versant HCV Genotype 2.0		
P-IND-1	2c	2a/c	2	2c
P-IND-2	2c	2a/c	2	2c
P-IND-3	3a	3	3	3a
P-IND-4	3a	3a	ND	3a
P-IND-5	3a	3a	3	3a
P-IND-6	3a	3a	3	3a
P-IND-7	4a	4	4	4a
P-IND-8	4a	4a/c/d	4	4a
P-IND-9	4d	4	4	4d
P-IND-10	4d	4	4	4d
P-IND-11	4d	4a/c/d	4	4d
P-IND-12	4d	4a/c/d	4	4d
P-IND-13	4f	4f	4 + 5 <sup>b</sup>	4f
P-IND-14	4f	4f	4 + 5 <sup>b</sup>	4f
P-IND-15	4f	Ind	4 + 5 <sup>b</sup>	4f
P-IND-16	4f	Ind	4 + 5 <sup>b</sup>	4f
P-IND-17	4f	Ind	4 + 5 <sup>b</sup>	4f
P-IND-18	4f	Ind	4 + 5 <sup>b</sup>	4f
P-IND-19	4f	Ind	4 + 5 <sup>b</sup>	4f
P-IND-20	4f	Ind	4 + 5 <sup>b</sup>	4f
P-IND-21	4f	Ind	4 + 5 <sup>b</sup>	4f
P-IND-22	4f	Ind	5	4f
P-IND-23	4f	Ind	5	4f
P-IND-24	4f	Ind	5	4f
P-IND-25	4f	Ind	5	4f
P-IND-26	4r	Ind	1 + 4	4r
P-IND-27	5a	5a	5	5a
P-IND-28	6c	Neg	5	6c
P-IND-29	4d	Ind	4	4d (72) + 1a (28)
P-IND-30	2j	2	ND	2j (95) + 4f (5)
P-IND-31	4p	Ind	3 + 4	4p (53) + 3a (47)
P-IND-32	4d	Ind	1a + 4	4d (87) + 1a (13)

<sup>a</sup> P-IND, patient samples with an indeterminate genotype (by first-generation Inno-LiPA). Ind, indeterminate result. Such results can be interpretable by facultative analysis. ND, not done; Neg, no PCR amplification.

<sup>b</sup> 4 + 5, genotype 4 and reactivity with genotype 5.

each to be genotype 5 and genotype 6 by direct sequencing. [Table 3](#) shows the results obtained by each technique compared with direct sequencing for each sample. Again, absolute concordance in HCV subtype results was found between direct and deep sequencing in 28 (88%) of the 32 samples. In the four remaining samples, despite the fact that the population Sanger sequencing results correlated with the master sequence identified by deep sequencing, this new technology showed mixed infections by two different HCV subtypes. In this sense, it must be kept in mind that this method is not able to detect DNA mixtures. The Abbott Rt-HCV Genotype II technique was the other one that identified a mixed infection in two of the three cases (66%) whereas population Sanger sequencing and Versant HCV Genotype 2.0 did not.

The two commercial available genotyping methods used were unable to identify the HCV subtype of the majority of these samples. Versant HCV Genotype 2.0 matched the genotype in 14 of 32 samples (44%) and the subtype in 6 of the 32 samples (19%) and gave subtype results approaching identification (2a/c or 4a/c/d) in

5 of the 32 samples (16%), indeterminate subtype results in 15 of 32 samples (47%), and negative or misclassification results in 2 of the 32 samples (6%). In the remaining four samples (12%), it gave the genotyping result. Abbott Rt-HCV Genotype II, which is not designed to differentiate subtypes other than 1a/1b, correctly identified the HCV genotype in 12 of 30 samples (40%), none of the subtypes resulted in a misclassification result in 15 of the 30 samples (50%), and the assay was able to identify mixed infection in two of three samples tested.

It is interesting that 4 of the 32 indeterminate samples were mixed-infection samples as detected by deep HCV subtyping. Two of the four patients showing mixed infections had a history of IDU.

## DISCUSSION

The present report describes the performance of a subtyping assay based on phylogenetic analysis of reads obtained by deep sequencing of a NS5B fragment in samples poorly characterized by a first-generation hybridization assay, in comparison with two widely used commercial assays. The collection of samples used to validate the methodology described here does not necessarily represent the current distribution of HCV genotypes/subtypes in Spain, since samples that cover as many subtypes as possible and are difficult to subtype by first-generation Inno-LiPA were selected.

Similarly to what happens with Sanger sequencing or commercial techniques for genotyping, improper storage of serum samples or RNA manipulation may cause a decay in viral load and RNA degradation giving a negative amplification result. In cases of negative amplification from a chronically HCV-infected patient, the quality of the sample is studied by performing an RT-PCR-nested amplification of the 5'-UTR region. Even in cases of correct manipulation, we have observed that low viral loads (below 10<sup>4</sup> copies/ml) represent an important limitation with respect to successful PCR amplification and sequencing.

Our assay correctly identified the HCV subtype in all samples, matching the results of direct sequencing, and was able to identify the presence of different HCV subtypes, and their relative proportions, in five samples with mixed infections. In contrast, the two commercial assays, despite identifying all genotype 1 samples, failed HCV-1 subtype calling in 13 (16%) samples each. In addition, both assays failed genotype/subtype calling in half of the 32 samples with non-1 HCV genotypes. These results are similar to those reported in previous studies, in which uninterpretable or indeterminate genotype calls were observed in 2% to 45% of samples (especially in non-genotype 1 infections) and the HCV-1 subtype could not be assigned in 2% to 15% of samples ([20–25](#), [27](#), [32–34](#)).

Although the limitations of currently available genotyping assays for HCV-1 subtype classification may have not been a major problem with the first-wave DAAs, accurate HCV subtyping and identification of mixed infections are likely to play a major role in patient management in the immediate future for several reasons. First, under FDA guidance, indications for use of the recently approved protease inhibitor simeprevir (Olysio; Janssen Therapeutics, Titusville, NJ) on the package insert endorse baseline screening of HCV-1a-infected patients for the presence of the NS3 Q80K polymorphism, thus requiring prior knowledge of the HCV-1 subtype. A requirement for unequivocal subtype 1 calling before treatment might also be endorsed for some interferon-free regimens which are currently being investigated in clinical trials

restricted to HCV-1b-designated subjects only (ClinicalTrials.gov identifiers NCT01732796, NCT01728324, NCT01581 203, and NCT01767116).

Second, the striking differences between subtypes 1a and 1b in genetic barriers to resistance to most DAAs (4, 12, 35), and the presence of naturally occurring resistance mutations in other HCV-1 subtypes (11), strongly suggest that a similar phenomenon is likely to occur with other genotypes, especially for genotypes 2, 4, and 6 because of their high subtype diversity (11 subtypes for G2, 17 for G4, and 24 for G6). Unfortunately, subtype-specific resistance data for other genotypes remain scarce (36–39).

This limited knowledge is especially relevant for genotype 4 due to its high subtype diversity (1, 40) and because it accounts for most infections in Egypt (the country with the highest prevalence and incidence of HCV) (41), the Middle East, and sub-Saharan Africa. In this regard, in a recent study, Newman et al., using whole-genome deep sequencing, identified combined dominant NS3 and NS5B resistance mutations in a restricted subset of genotype 4 subtypes (4g, 4k-r, and 4v), further reinforcing the requirement for accurate subtype calling in clinical practice (40).

Third, reliable identification of mixed-type infections, frequently found in PWID and, to a lesser extent, in individuals undergoing frequent health care procedures in resource-limited areas, is beyond the capability of current assays. Since injecting-drug use is still the major driving force of the uncontrolled HCV epidemic throughout the world (42–44), any attempt at global eradication of HCV through interferon-sparing treatment will have to target PWID and deal with a high proportion of mixed infections. In this setting, accurate subtyping and identification of mixed infections by deep-sequencing methods will be of critical importance to enhance treatment success and minimize the development and rapid spread of drug-resistant viruses. Indeed, use of genotype-specific therapies might result in virological failure in individuals with mixed-type infection, which is relevant due to the cost of the new interferon-free regimens.

Finally, as HCV treatment shifts to more-effective and better-tolerated all-oral regimens and treatment uptake becomes widespread, suboptimal treatment adherence, rather than problems associated with potency and genetic barriers to resistance, may become a more frequent cause of treatment failure in clinical practice. Since even regimens containing potent agents with a high barrier to resistance, such as nucleos(t)ide NS5B polymerase inhibitors, have been shown to select subtype-dependent resistance (45, 46), in addition to fixed-dose formulations and reinforcement of adherence, reliable subtyping may help identify patients requiring closer surveillance and facilitate resistance testing in cases of treatment failure.

There were several limitations in our study. First, the limited number of samples with some HCV genotypes precluded evaluation of test performance in rare HCV subtypes. Second, since both our deep-sequencing assay and the reference method for final subtype assignment target only an NS5B fragment, the possibility that some discrepancies between NS5B and 5'-UTR/core-based assays in genotype/subtype calling were in fact examples of intergenotypic recombinants (47) cannot be ruled out. Although sequencing of an additional structural gene (such as core/E1) might suggest the presence of an HCV recombinant, full-genome sequencing would be required for its confirmation using a PCR-free technology (40, 48).

Despite these limitations, however, our results indicate that

deep sequencing of NS5B on a readily available platform, followed by a filtering process and a phylogenetic classification of sequence reads, is a very reliable method for HCV subtyping that could rapidly move from research to clinical diagnostic laboratories to replace classical sequencing methods in resolving indeterminate subtype results of existing assays. Furthermore, deep sequencing is likely to become the method of choice for HCV subtyping when interferon-sparing regimens reach high-risk population groups.

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