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HCV NS3 sequencing as a reliable and clinically useful tool for the assessment of genotype and resistance mutations for clinical samples with different HCV-RNA levels

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Objectives: This study aims to evaluate the reliability and clinical utility of NS3 sequencing in hepatitis C virus (HCV) 1-infected patients who were candidates to start a PI-containing regimen.

Methods: NS3 protease sequencing was performed by in-house-developed HCV-1 subtype-specific protocols. Phylogenetic analysis was used to test sequencing reliability and concordance with previous genotype/subtype assignment by commercial genotyping assays.

Results: Five hundred and sixty-seven HCV plasma samples with quantifiable HCV-RNA from 326 HCV-infected patients were collected between 2011 and 2014. Overall, the success rate of NS3 sequencing was 88.9%. The success rate between the two subtype protocols (HCV-1a/HCV-1b) was similarly high for samples with HCV-RNA >3 log IU/mL (>92% success rate), while it was slightly lower for HCV-1a samples with HCV-RNA ≤3 log IU/mL compared with HCV-1b samples. Phylogenetic analysis confirmed the genotype/subtype given by commercial genotyping assays in 92.9% (303/326) of cases analysed. In the remaining 23 cases (7.1%), 1 was HCV-1g (previously defined as subtype 1a), 1 was HCV-4d (previously defined as genotype 1b) and 1 was HCV-1b (previously defined as genotype 2a/2c). In the other cases, NS3 sequencing precisely resolved the either previous undetermined/discordant subtype 1 or double genotype/subtype assignment by commercial genotyping assays. Resistance-associated variants (RAVs) to PI were detected in 31.0% of samples. This prevalence changed according to PI experience (17.1% in PI-naive patients versus 79.2% in boceprevir/telaprevir/simeprevir-failing patients). Among 96 patients with available virological outcome following boceprevir/telaprevir treatment, a trend of association between baseline

NS3 RAVs and virological failure was observed (particularly for HCV-1a-infected patients: 3/21 failing patients versus 0/22 achieving sustained virological response; $P=0.11$).

Conclusions: HCV-NS3 sequencing provides reliable results and at the same time gives two clinically relevant pieces of information: a correct subtype/genotype assignment and the detection of variants that may interfere with the efficacy of PI.

Introduction

In recent years, treatment of hepatitis C virus (HCV) infection has been characterized by significant changes, especially for genotype 1. This genotype has the broadest geographical distribution, with a higher prevalence of HCV-1b in Europe and HCV-1a in the USA[1] and a low rate of sustained virological response (SVR) with the standard of care (peg-IFN-α and ribavirin) compared with other genotypes.[2,3] In 2011, two NS3 PIs, boceprevir and telaprevir, were the first direct-acting antiviral agents (DAAs) approved for the treatment of chronic HCV infection in genotype 1, by both the FDA and the EMA.

In 2013 and 2014, the standard of care for treatment of genotype 1 consisted of peg-IFN-α and ribavirin plus either sofosbuvir (a pan-genotypic nucleotide polymerase inhibitor) or simeprevir (a second-wave PI, active in genotypes 1 and 4). More recently, three new all-oral regimens were also approved by the FDA and EMA: sofosbuvir/ledipasvir (NS5b/NS5a inhibitors) for genotypes 1, 4, 5 and 6; simeprevir plus sofosbuvir for genotypes 1 and 4; and paritaprevir/ombitasvir + ritonavir + dasabuvir (NS3/NS5a inhibitors + non-nucleotide polymerase inhibitor) for genotype 1, and paritaprevir/ombitasivir + ritonavir for genotype 4. In 2014, the pan-genotypic NS5a inhibitor daclatasvir was also approved in Europe for all genotypes.

These new all-oral regimens based on the combination of more DAAs from different classes are safe, highly effective and require relatively short durations of therapy. Some of them can also be used as a component of a triple combination regimen with peg-IFN-α and ribavirin. For example, the PIs still represent an important drug class involved in the treatment of HCV infection for genotypes 1 and 4, within either an IFN-containing regimen (with simeprevir)[4] and/or within IFN-free regimens (with simeprevir or paritaprevir).[4,5]

Overall, the new antiviral regimens show high, but variable, SVR rates (60%–100%) due to the specific DAA/DAA combination used, HCV genotype, presence of detectable pre-existing amino acid substitutions conferring resistance and severity of liver disease.[4]

Notably, the majority of the new DAAs are not pan-genotypic, but they show a multi- or limited genotypic coverage, with different antiviral sensitivity according to genotype and subtype.[6] For this reason, correct determination of the HCV genotype and subtype remains a relevant recommendation prior to treatment initiation and genotyping/subtyping should be performed with an assay that accurately discriminates subtype 1a from 1b.[4] For example, HCV-1a and HCV-1b subtypes have shown different responses to DAA-based regimens, with different patterns of resistance mutations. The HCV-1b subtype has been associated with better response to triple therapy and IFN-free regimens compared with HCV-1a.[7–12] For these reasons in the new treatment recommendations for patients infected with chronic HCV-1 the use of the fixed-dose combination of ombitasvir/paritaprevir/ritonavir + dasabuvir is different according to subtype infection: without ribavirin or shorter for subtype 1b versus with ribavirin or longer for subtype 1a-infected patients without/with cirrhosis, respectively.[4] Therefore, the accurate assessment of HCV genotype and subtype prior to treatment initiation is mandatory, to reduce the risk of failure driven by inappropriate genotype/subtype characterization.[13]

Regarding resistance development, different patterns of resistance mutations have been observed for these two major HCV-1 subtypes for PIs. Usually, with PIs, HCV-1b is associated with a lower rate of resistance-associated variants (RAVs) and also shorter persistence of resistance after treatment failure.[9,14,15] Baseline RAVs have also been observed with a different distribution among HCV genotypes and subtypes.[16–18] For example, the NS3 Q80K RAV, specifically associated with resistance to simeprevir (mostly in combination with IFN and ribavirin), is frequently found in HCV-1a (prevalence of 30%–48% in the USA and ~20% in Europe) while it is rare in genotype 1b.[19–23] For this reason, American and European guidelines recommend the performance of NS3 sequencing to evaluate the presence of this variant in patients with HCV-1a infection before starting treatment with simeprevir.[4,5,24]

However, several other natural polymorphisms associated with resistance to NS5a (i.e. L31M and Y93H) and NS5b (i.e. L159F and C316N) inhibitors show high prevalence worldwide[25] and in the specific setting of patients, their presence at baseline has been associated with lower response to IFN-free regimens.[25–30] Moreover, despite the overall high SVR rates reported in recent clinical trials, the few DAA-failing patients, with relapse or breakthrough or non-response, show in the majority of cases RAVs at failure.[7,11,29,31–37]

In these patients, drug resistance development could represent an important issue for retreatment options. Indeed, a broad cross-resistance profile exists between compounds of the same class (particularly evident for PIs and NS5a inhibitors). In this context, drug resistance testing prior to retreatment is mandatory to make a therapeutic decision.[5]

Genotypic resistance testing could therefore represent an important tool of potential clinical relevance, especially in patients with advanced disease and/or who previously failed NS3 or NS5a inhibitor-containing regimens. The aim of our study was to evaluate the reliability and clinical utility of full-length NS3 protease in-house-developed sequencing protocols for the analysis of HCV genotype and the presence of RAVs in patients who were candidates to start a PI-containing regimen.

Methods

Samples and patients

This retrospective study included plasma samples with detectable HCV-RNA from HCV-1-infected patients who were candidates for antiviral treatment that were sequenced during 2011–14 in our laboratory, for

routine clinical purposes. Approval by ethics committee was deemed unnecessary according to Italian law, because this was not an hypothesis of clinical trial on medicinal products for clinical use (art. 6 and art. 9, leg. decree 211/2003), and the research was conducted on anonymized samples (leg. decree 196/2003). Sample information (date of sampling, final results of sequencing, nucleotide sequences obtained and mutations found in each sequence), together with full clinical and therapy data, were recorded in an anonymous database.

HCV-RNA quantification

HCV-RNA was quantified in each sample using the commercial assays COBAS Ampliprep/COBAS TaqMan HCV Quantitative Test v2.0 (Roche Diagnostics, Mannheim, Germany) or Abbott RealTime HCV assay (Abbott Laboratories, Abbott Park, IL, USA). The lower limit of detection was 15 and 12 IU/mL, respectively, and the lower limit of quantification (LLOQ) was 25 and 12 IU/mL, respectively.

Population sequencing of the NS3 protease gene

HCV sequencing of NS3 protease (181 amino acids) was performed by using two different in-house-developed protocols, specifically designed for subtypes 1a and 1b.

HCV-RNA was extracted using a standard commercial silica gel membrane-binding method (QIAamp Viral RNA Mini Kit; Qiagen, Valencia, CA, USA). According to viraemia levels, for samples with an HCV-RNA <6 log IU/mL, 1 mL of plasma was before concentrated by ultracentrifugation at 25 000 **g** for 1.30 h at 4°C, while for samples with an HCV-RNA ≥6 log IU/mL, 140 μL of plasma was directly used to perform RNA extraction.

Synthesis and amplification of cDNA were performed in a single step by using the commercial SuperScript III One-Step RT–PCR System with Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA, USA) and specific primers designed for each HCV-1 subtype (Table 1).

The first-round PCR was performed as follows: 10 μL of RNA was added to 40 μL of PCR mixture containing 25 μL of (2×) reaction buffer, 8 μL of 5 mM Mg^{2+}, 3 μL of DNase/RNase-free water, 1 μL of each primer at a concentration of 10 μM, 1 μL of RNaseOUT (40 U/μL) and 1 μL of reverse transcriptase/*Taq*. Reverse transcription was performed for 30 min at 45°C, followed by denaturation for 2 min at 94°C and amplification over 40 cycles at 94°C for 30 s, 55–56°C for 30 s, 68°C for 90 s and a final 10 min extension step at 68°C. The amplified product was run on a 1% agarose gel. When the product was not visible, a nested PCR was performed. In particular, according to HCV-RNA level, two different nested-PCR protocols were performed. For samples with an HCV-RNA >4 log IU/mL, 5 μL of amplified product was denatured at 94°C for 12 min and amplified with 35 cycles at 94°C for 30 s, 55–56°C for 30 s and 68°C for 90 s, by using the following reaction mixture with Amplitaq Gold DNA polymerase: 5 μL of 10× *Taq* buffer, 4 μL of 25 mM Mg^{2+}, 32.2 μL of DNase/RNase-free water, 0.9 μL of each primer (Table 1) at a concentration of 10 μM, 1 μL of 10 mM deoxynucleotides and 1 μL of *Taq* (5 U/μL) for a total of 45 μL.

For samples with HCV-RNA ≤4 log IU/mL, nested PCR based on two overlapping NS3 amplicons was performed by using the Fast Start HiFi PCR system (Roche Diagnostics, Mannheim, Germany) as follows: 5 μL of cDNA was added to 32.5 μL of PCR mixture containing 3.75 μL of (10×) reaction buffer with $MgCl_2$ added, 0.75 μL of deoxynucleotides, 0.75 μL of each primer (Table 1) at a concentration of 10 μM, 0.4 μL of Fast Start HiFi enzyme and 26.1 μL of DNase/RNase-free water. The nested PCR amplification reaction was performed under the following conditions: 94°C for 30 s, 55–56°C for 30 s, 72°C for 90s and 72°C for 7 min.

Finally, NS3 protease amplified products were sequenced by an automated sequencer (ABI-3130) in sense and antisense orientation using four different overlapping sequence-specific primers for each subtype (see Table 1), using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences having a mixture of WT and mutant residues at a single position were considered to have the mutant(s) at that position.

Furthermore, in our standardized sequencing procedure, positive control plasma samples were reprocessed over time. Complete concordance in the detection of mutations between the repeated sequences was always found.

Table 1. HCV-specific oligonucleotide sequences used for amplification of NS3 protease (181 amino acids) for each HCV-1-specific subtype (HCV-1a and HCV-1b)

HCV-1 subtype	Reaction type	Primer name	Polarity	Sequence (5′–3′)
1a	RT–PCR	F1_V1a	forward	GATCTGGCCGTGGCTGTAGAG
		R1N_V1a	reverse	AGCACCTTGTAGCCCTGAGC
	nested PCR[a]	F1N_V1a	forward	GCGGTGACATCATCAACGGCT
		R3_1a	reverse	GGGACCTCATGGTTGTCTCTAGG
	nested PCR[b]	F1NV1a	forward	GCGGTGACATCATCAACGGCT
		R2_1a	reverse	CCTTGGGTGATGCGATGGTCC
		F2_1a	forward	GGACCATCGCATCACCCAAGG
		R3_1a	reverse	GGGACCTCATGGTTGTCTCTAGG
1b	RT–PCR	VF1b	forward	CTTGCGGTGGCAGTTGAGC
		VR1b	reverse	CCGTGGTGATGGTCCTTACCC
	nested PCR[a]	F2_1b	forward	GCTGCATCATCACCAGCCTCAC
		VR2b	reverse	GGCACCTTAGTGCTCTTGCC
	nested PCR[b]	F2_1b	forward	GCTGCATCATCACCAGCCTCAC
		R2_1b	reverse	GCGCACCGGAATGACATCAG
		F3_1b	forward	CAATGTAGACCAGGACCTCGTCG
		VR2b	reverse	GGCACCTTAGTGCTCTTGCC

[a]The oligonucleotides were used for the amplification of samples with HCV-RNA >4 log IU/mL.
[b]The oligonucleotides were used for the amplification of samples with HCV-RNA ≤4 log IU/mL.

Phylogenetic analysis of NS3 protease

Phylogenetic analysis was performed to test sequencing reliability and possibility of cross-contamination or sample mix-up during laboratory procedures and to evaluate concordance with previous subtype assignment by commercial genotyping assays.

NS3 protease sequences were aligned using the Clustal W algorithm integrated into BioEdit software. Then, all sequences were compared with reference strains of genotypes 1–7 (GenBank accession numbers: HCV-1a, M62321; HCV-1a, EU482831; HCV-1b, D90208; HCV-1c, D14853; HCV-1g, AM910652; HCV-2a, D00944; HCV-2b, D10988; HCV-2c, D50409; HCV-3a, D17763; HCV-3b, D49374; HCV-4a, Y11604; HCV-4b, FJ025854; HCV-4c, FJ462436; HCV-4d, FJ462437; HCV-4f, EF589160; HCV-5, NC_009826; HCV-6, NC_009827; and HCV-7, EF108306) using the neighbour-joining method[38] and the Kimura two-parameter distance estimation approach[39] in MEGA v5.1.[40]

The reliability of the phylogenetic clustering was evaluated using bootstrap analysis with 1000 replicates. The significance of the group was assumed when bootstrap values were >70%.

NS3 sequencing success rate

The NS3 protease sequencing success rate was determined on the overall population and according to different HCV-RNA level groups (≤2, 2.1–3, 3.1–4, 4.1–6 and >6 log IU/mL). For this analysis, only samples with detectable and quantifiable HCV-RNA (above the LLOQ of the assay used, cut-off >12 or >25 IU/mL) were considered.

All analyses were performed using the SPSS software package (version 19.0) for Windows® (SPSS, Chicago, IL, USA).

HCV resistance mutation analysis

Prevalence of NS3 protease RAVs was analysed at baseline and at PI failure. Thirty-seven mutations associated with PI drug resistance were considered (36AGLM, 43ISV, 54AS, 55AI, 56H, 80KLR, 122AR, 155IKMCGQT, 156GSTV, 168AEGNTVY and 170AT).[41–44]

Results

Sample collection

A total of 630 HCV plasma samples with detectable HCV-RNA from 326 HCV-1-infected patients, who were candidates to start a new treatment regimen containing a PI, were collected between 2011 and 2014 (Figure 1). The distribution of samples based on HCV-RNA level changed during the years, according to the introduction of PI use. Indeed, from 2012, 224 patients started a PI and were prospectively followed during treatment. Notably, the percentage of samples tested with viraemia <4 log IU/mL increased from 9.9% in 2011 to 34.7% in 2014.

NS3 protease sequencing success rate

Among 567 samples with quantifiable HCV-RNA (above the LLOQ), the overall NS3 protease sequencing success rate was 88.9%.

In particular, it was 99.4% for samples with HCV-RNA >6 log IU/mL and 99.1% and 93.9% for those with HCV-RNA of 4.1–6 and 3.1–4 log IU/mL, respectively. The success rate was

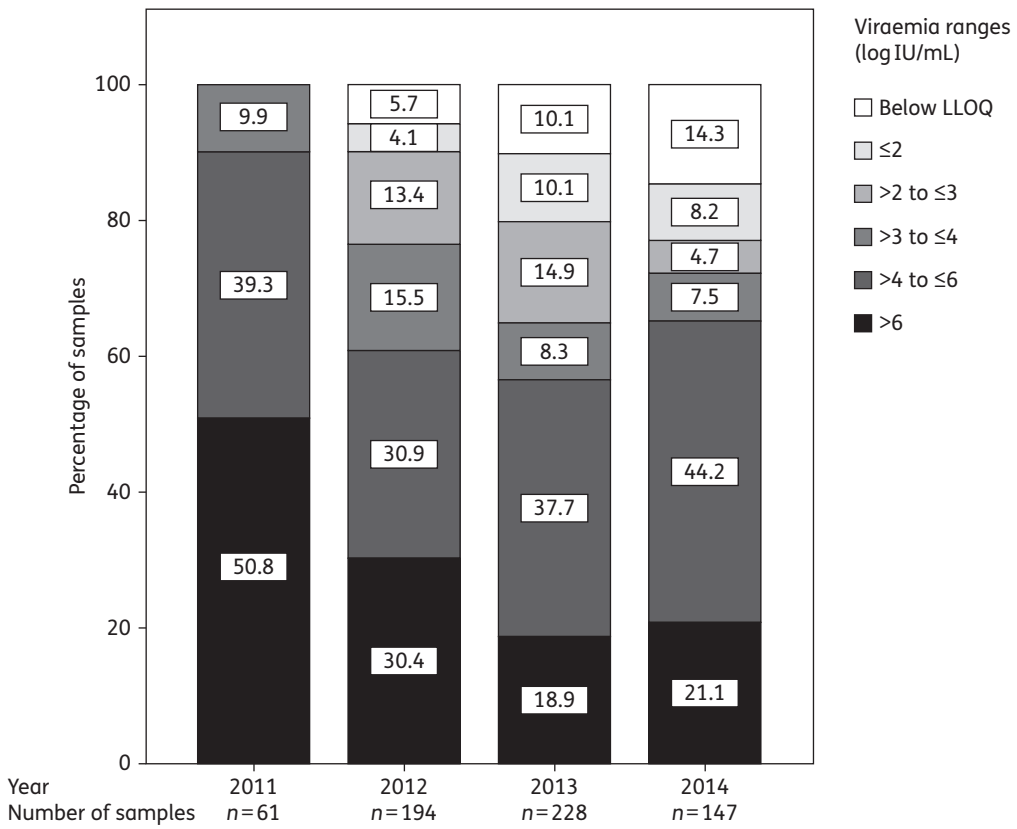

Figure 1. Genotypic requests for 630 samples from 326 HCV-infected patients over the years 2011–14. All samples were stratified according to different HCV-RNA levels. Each viraemia range is represented by a different shade.

still relevant for samples with HCV-RNA of 2.1–3 log IU/mL (61.2%), while it was lower when HCV-RNA was ≤2 log IU/mL (14.3%) (Figure 2a).

Interestingly, the amplification rate for samples with HCV-RNA >3 log IU/mL was quite similar for HCV-1b and HCV-1a samples (>92% in both cases), while it was slightly lower for HCV-1a samples with HCV-RNA <3 log IU/mL (37.8%) in comparison with HCV-1b samples in the same viraemia range (49.2%; $P=0.30$) (Figure 2b).

NS3 sequencing reliability and genotype/subtype analysis

In order to test the reliability of NS3 sequencing and exclude cross-contamination or sample mix-up during laboratory procedures, we performed a phylogenetic analysis of all 504 NS3 sequences obtained.

All sequences belonging to the same subject always showed high homology, also where obtained from samples with different HCV-RNA levels (bootstrap value >70%; Figure 3). Two illustrative clusters, from two HCV-1-infected patients prospectively followed, are reported in Figure 3.

Phylogenetic analyses of NS3 sequences confirmed the genotype and subtype assigned by commercial genotyping assays in 92.9% (303/326) of cases analysed.

Discordances were identified in 23 cases (Figure 4). In particular, one discordant case previously indicated as HCV-1a by a commercial genotyping assay was found by sequencing to be HCV-1g (see ID_32 in Figure 4). Another case previously misclassified as HCV-1b was found to be HCV-4d (ID_325) and another previously HCV-2a/2c was HCV-1b (ID_481).

Furthermore, NS3 sequencing allowed better characterization of 20/23 cases with previously undetermined subtype 1 ($n=7$), discordant results by different commercial assays ($n=6$) or double genotype/subtype assignment ($n=7$) (Figure 4).

Prevalence of RAVs in NS3 protease

PI RAVs were detected in 156/504 (31.0%) samples by NS3 sequencing. The prevalence of NS3 protease RAVs changed according to PI experience. Indeed, as expected, RAVs were less frequently detected in PI-naive patients (48/280, 17.1%) than in boceprevir/telaprevir/simeprevir-failing patients (38/48, 79.2%; $P<0.001$).

Q80K was the most frequent mutation detected in PI-naive patients (6.4%) followed by Q80L (4.6%) and T54S (2.1%). NS3 RAVs in PI-naive patients were more common in subtype 1a (29.2%) compared with subtype 1b (8.4%; $P<0.001$) (Figure 5a). As expected, the polymorphism Q80K was more common in subtype 1a (15.0%) compared with subtype 1b (0.6%; $P<0.001$).

At virological failure, RAVs were detected in 78.3% of boceprevir/telaprevir-failing patients and in 100% of simeprevir-failing patients. The distribution of RAVs changed according to the HCV subtype (Figure 5b). Indeed, the V36M+R155K pattern was the most frequent observed in HCV-1a boceprevir/telaprevir-failing patients, while T54A/S, A156T and V170A RAVs (alone or in combination) were the most frequently selected in HCV-1b failing patients.

The two HCV-1a-infected patients failing a simeprevir-based regimen both failed with the R155K mutation.

Baseline RAVs and virological outcome

A total of 96 patients infected with HCV genotype 1, treated with telaprevir- or boceprevir-based triple therapy, with available baseline NS3 sequencing and virological outcome were also characterized.

In this set of patients, a trend of association between baseline telaprevir or boceprevir RAVs and virological failure was observed, particularly for HCV-1a-infected patients. Indeed, 3/21 HCV-1a failing patients (14.3%) showed baseline RAVs (1=V36L+Q80K and 2=T54S) while 0/22 (0%) HCV-1a patients achieving SVR showed baseline RAVs ($P=0.11$, Fisher's exact test). Interestingly, the only HCV-1g-infected patient showed the T54S mutation at baseline and was a null responder to a boceprevir-containing regimen. Regarding HCV-1b subtype, only two patients showed telaprevir or boceprevir RAVs at baseline (1=V36L and 1=T54S) and both reached SVR.

At baseline, 11 patients (treated with telaprevir- or boceprevir-based triple therapy) showed Q80K as the only RAV (HCV-1a=10 and HCV-1b=1). Among them, six achieved SVR and five experienced virological failure (all HCV-1a, three with boceprevir and two with telaprevir; data not shown).

Finally, among the two failing patients treated with simeprevir, only one had baseline NS3 sequencing available and showed the R155K mutation at baseline. This patient later failed with the V36M+R155K resistance pattern.

Discussion

The aim of this study was to evaluate the reliability and clinical utility of an in-house-developed HCV NS3 sequencing protocol in a large number of HCV samples. HCV NS3 sequencing may allow a precise subtype and genotype assignment along with the evaluation of resistance to PIs, thus identifying patients with higher risk of failure, especially those with advanced liver disease.

Due to the genetic variability observed in the regions flanking the NS3 protease gene, we developed two specific HCV-1 subtype (HCV-1a and HCV-1b) protocols. Overall, our results showed that, among 567 samples with quantifiable HCV-RNA, the NS3 sequencing success rate was 88.9%. It was particularly high for samples with HCV-RNA >4 log IU/mL (99.2%) and with HCV-RNA 3.1–4 log IU/mL (93.9%).

Reasonable results in terms of success rate (61.2%) were also obtained for samples with HCV-RNA 2.1–3 log IU/mL. Furthermore, the NS3 protease success rate was quite similar for HCV-1b and HCV-1a samples with HCV-RNA >3 log IU/mL, suggesting that subtype diversity does not represent a limitation when HCV-RNA levels are high.

Phylogenetic analysis confirmed the reliability of NS3 sequencing performed at different HCV-RNA levels. Indeed, all sequences belonging to the same subject (regardless of HCV-RNA level) always clustered together and the subtype assignment was always confirmed.

Phylogenetic analysis of NS3 sequences confirmed the previous information on genotype and subtype given by commercial genotyping assays in 92.9% of patients analysed. Furthermore, NS3 sequencing was helpful to characterize 20 cases with previous undetermined subtype 1, discordant results or double genotype/subtype assignment by commercial genotyping assays. Notably, three samples collected from one patient, previously

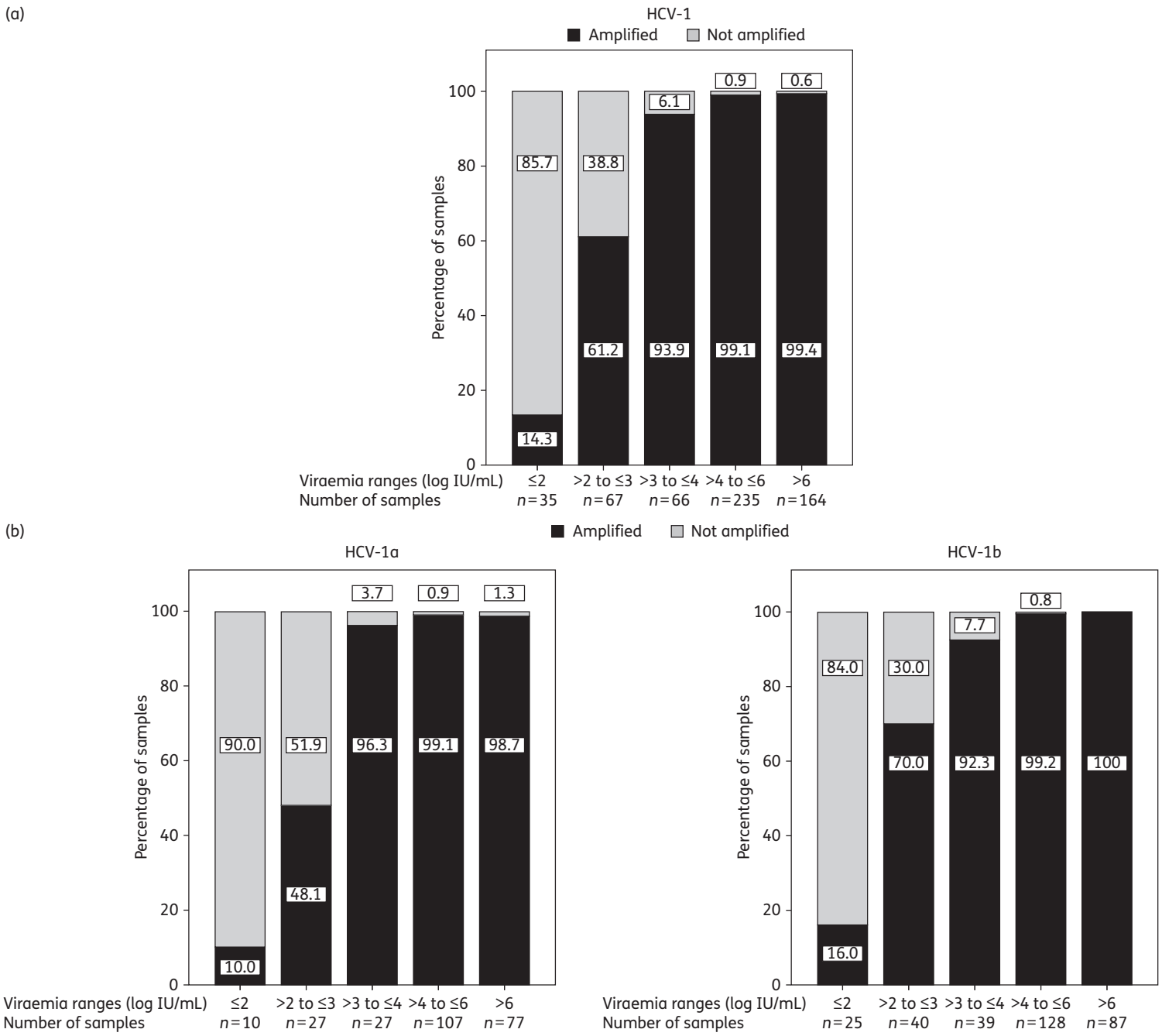

Figure 2. NS3 protease sequencing success rate stratified according to different HCV-RNA levels. (a) All HCV-1 samples were stratified according to amplified and not amplified and according to HCV-RNA levels. (b) Samples were also stratified according to HCV-1a and HCV-1b subtypes.

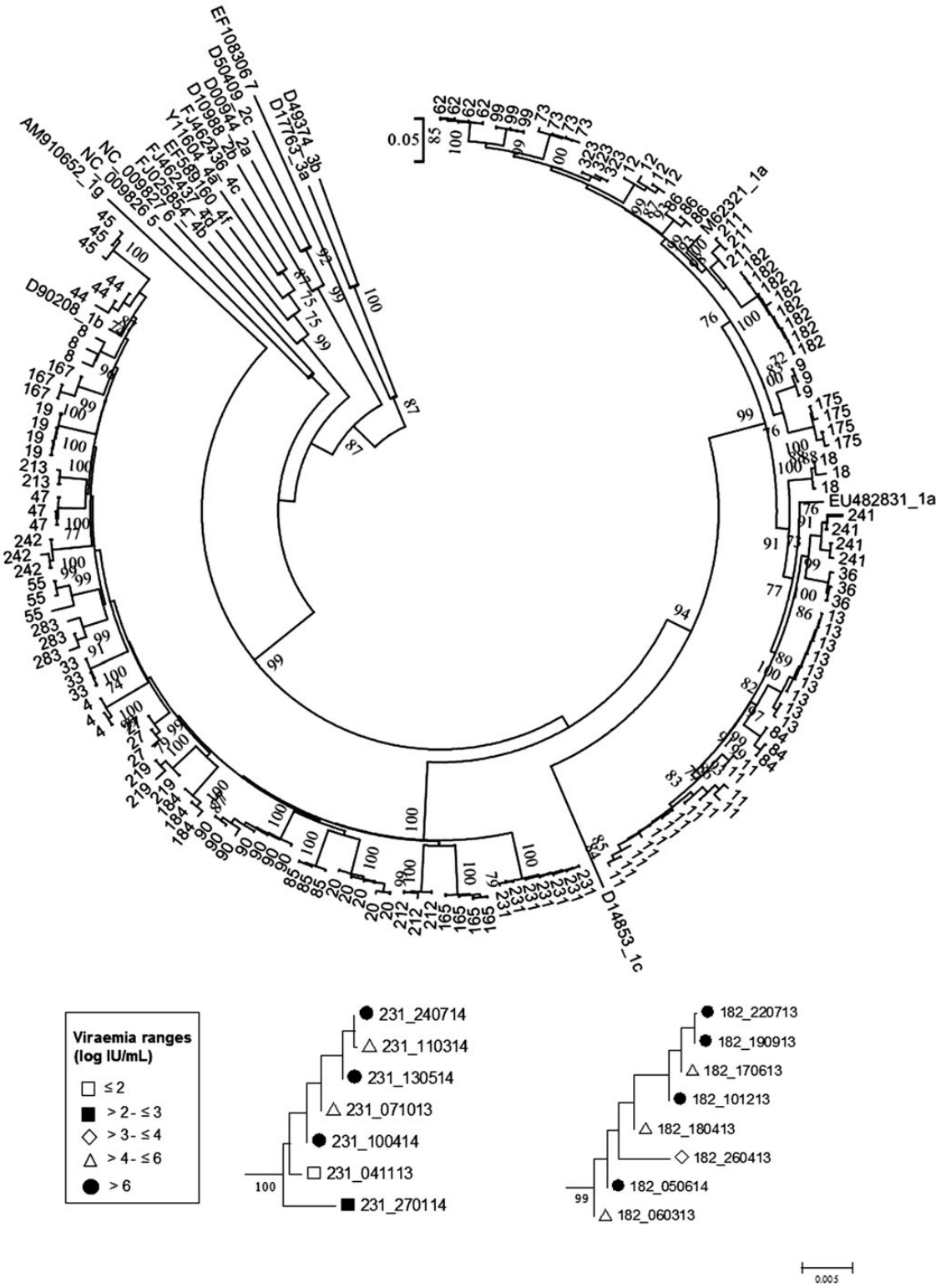

Figure 3. Phylogenetic tree of HCV NS3 sequences. The phylogenetic tree shows 149 sequences (from 37 patients, where ≥3 sequences per patient were obtained). Clinical samples are reported with a number, which represents the patient identifier. The reliability of the branching patterns was evaluated by bootstrapping (1000 replicates). Only bootstrap values >70 are reported. Genetic distances were calculated using the Kimura two-parameter model of MEGA v5.1. The scale bar indicates 5% nucleotide sequence divergence. Reference sequences are reported with the relative GenBank accession number. Two illustrative clusters of sequences from two infected patients are also shown in more detail: the first number is the patient identifier and the second number is the sample collection date; the symbols represent the viraemia ranges.

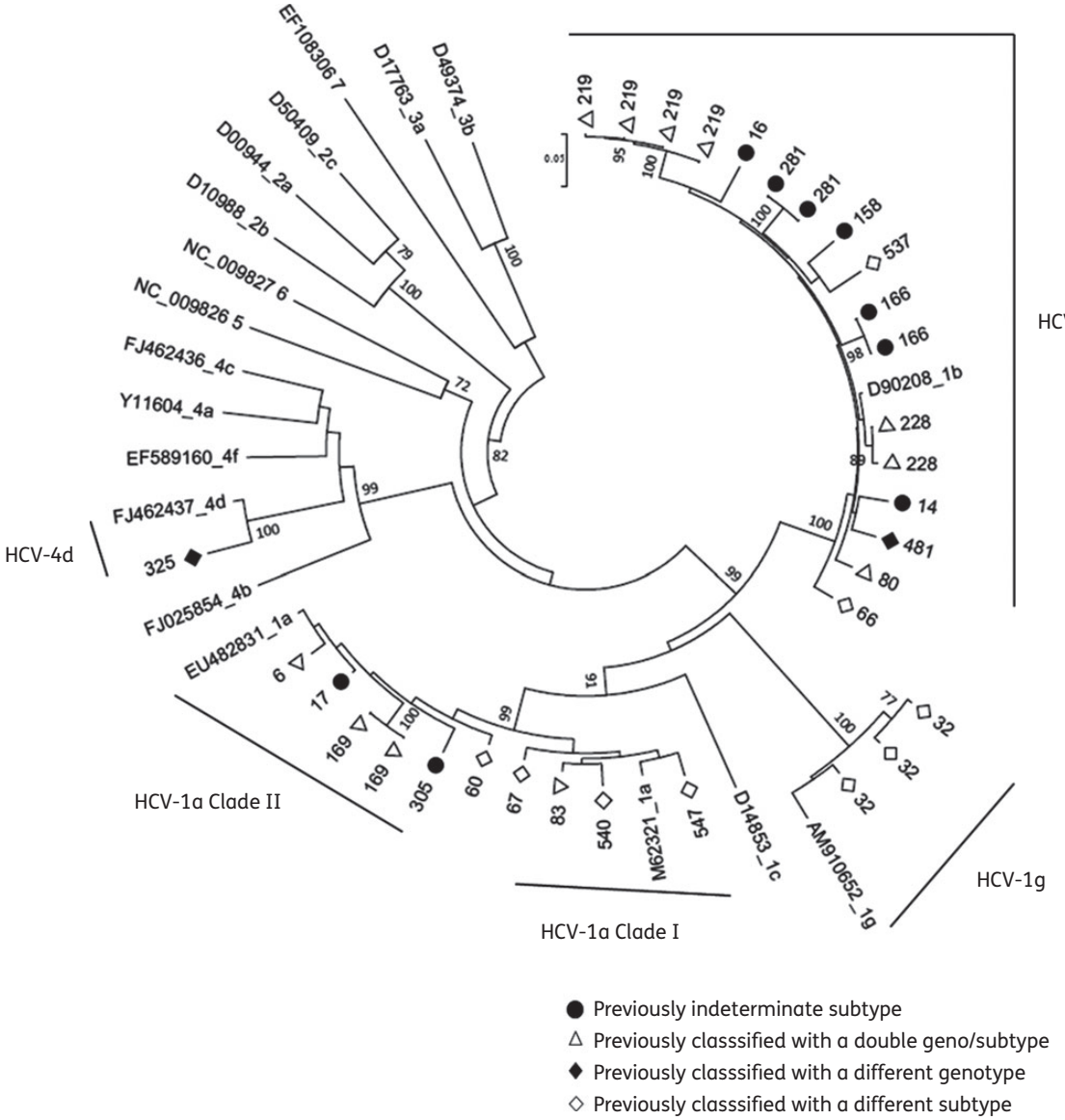

Figure 4. Phylogenetic tree of clinical samples with discrepant or not defined subtyping results by commercial genotyping assays. The reliability of the branching patterns was evaluated by bootstrapping (1000 replicates). Only bootstrap values >70 are reported. Genetic distances were calculated using the Kimura two-parameter model of MEGA v5.1. The scale bar indicates 5% nucleotide sequence divergence. Reference sequences are reported with the relative GenBank accession number. Clinical samples are reported with a number, which represents the patient identifier. The symbols represent the types of discordance between sequencing and commercial genotyping.

misclassified as subtype 1a by two commercial real-time assays, were found to be infected with HCV-1g, as we previously described.[45] Interestingly, two other cases of discordant genotype with the assignment given by line probe (LiPA) assay were identified. In particular, one sample was from a patient infected with HCV-4d, previously misclassified in 1998 as genotype 1b;[46] the other derived from a patient infected with an HCV-1b, previously misclassified as 2a/2c in 2005.

Our rate of discordance in genotype assignment by NS3 sequencing and commercial assays is in line with other results.[13,22,47–51] It is known that some genotyping assays used in clinical practice have limited ability to correctly identify genotype 1 subtypes.[52] In particular, first-generation assays, which analyse only the 5′-non-coding region (NCR) (Trugene and Siemens InnoLiPA Versant version 1.0), are able to discriminate subtypes 1a and 1b in 70%–77% and 90%–91% of cases, respectively.[48] Concordance of the Trugene assay with NS3/4a or NS5b sequence-based genotyping/subtyping was recently showed to be 79.6%.[22] Second-generation assays (Siemens LiPA Versant line probe assay 2.0 and Abbott RealTime HCV Genotype II assay) correctly identify HCV-1a/b in 93%–98% and 89%–98% of cases, respectively.[52]

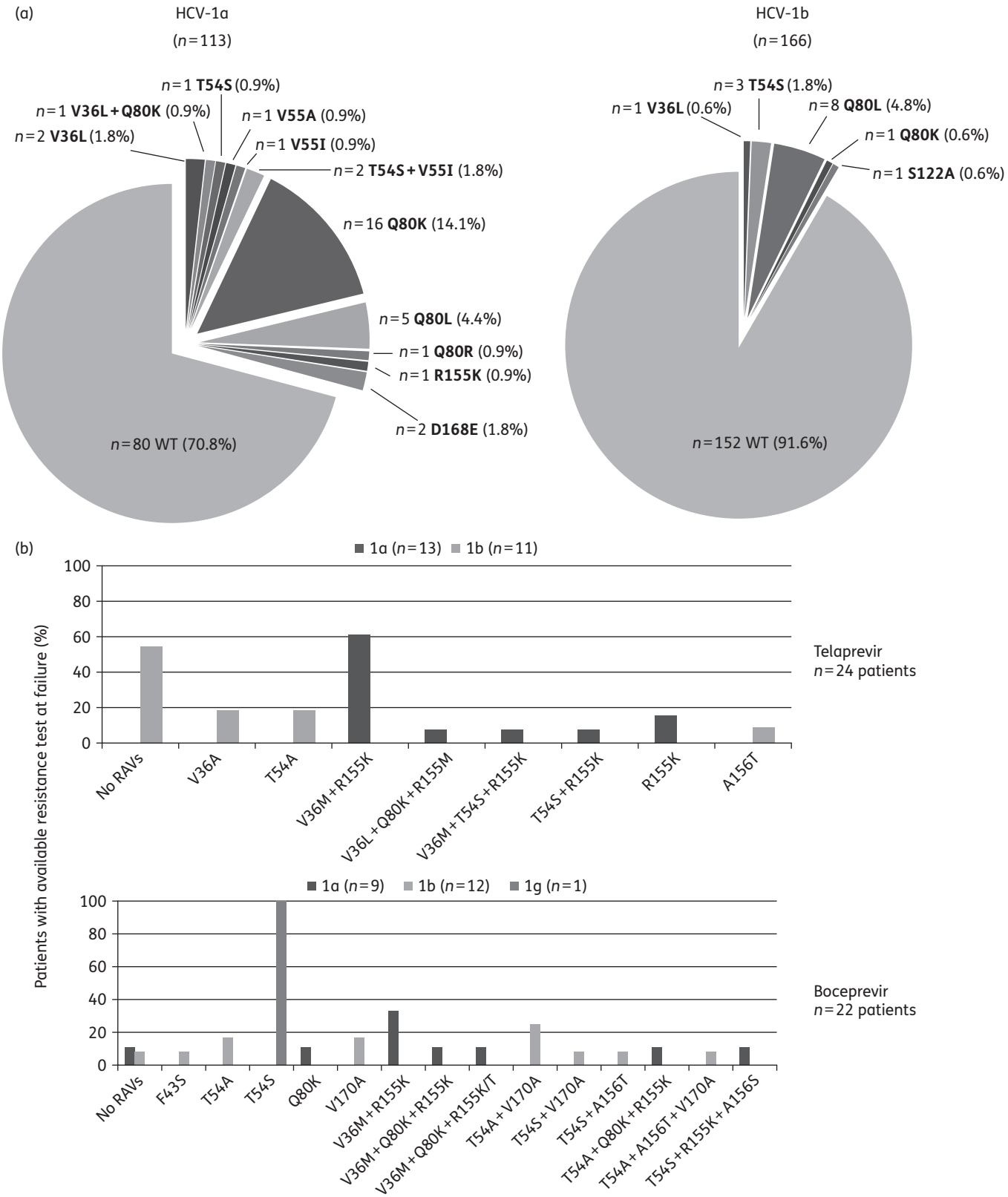

Figure 5. Prevalence of NS3 RAVs in samples detected at baseline or at virological failure. (a) Prevalence of natural RAVs detected by NS3 population sequencing in samples from DAA-naive patients. (b) NS3 protease RAVs detected in samples from telaprevir- or boceprevir-failing patients.

Therefore, even if it is more time-consuming, direct sequencing followed by phylogenetic analysis is currently the most reliable tool for the identification of HCV genotype and subtype. This is particularly relevant in view of the different protocols for treatment of different HCV genotypes/subtypes currently available. The major difference in terms of cost between diagnostic procedures (100-fold below) and anti-HCV drugs suggests a cost–benefit advantage of performing this procedure accurately before treatment decision. However, future cost-effectiveness analyses may help to determine whether HCV sequencing is in fact cost-effective.

Regarding resistance, NS3 RAVs were detected in our population with an overall prevalence of 31.0%. As expected, this prevalence changed according to PI experience. The rate of natural resistance in our HCV-1-infected PI-naive patients (17.1%) was similar to that observed in other studies.[16,21,53–55] In particular, the most prevalent NS3 polymorphism detected at baseline was Q80K (6.4%). The distribution of this polymorphism changed according to HCV-1 subtype (15.0% in HCV-1a and 0.6% in HCV-1b), in agreement with previous findings.[19,23,55] Interestingly, 6/12 telaprevir/boceprevir-treated patients with a baseline Q80K polymorphism experienced virological failure. Further studies are necessary to evaluate whether this effect of Q80K, as well as of other baseline polymorphisms, also occurs with newer antivirals.

In patients experiencing virological failure to PIs, the prevalence of RAVs increased from 31% to 79.2%. The resistance profile of telaprevir and boceprevir differed according to HCV-1 subtype. Indeed, while V36M and R155K were the most common RAVs detected in genotype 1a, virological failure in genotype 1b was associated with RAVs mainly involving positions 54, 156 and 170. This result is in concordance with previous studies[9,56] and can be attributed to a different genetic barrier for resistance development between the two subtypes, as a consequence of different genetic backgrounds and codon usage.[16,57]

Currently, there is no indication for a resistance test before starting HCV treatment, except for the detection of the Q80K NS3 mutation, for patients infected with HCV-1a genotype in which a treatment with simeprevir is considered.[4,5] At present, the clinical relevance of a genotypic resistance test at baseline in DAA-naive patients is not well defined. Nevertheless, in select cases of more difficult-to-treat patients (i.e. cirrhotics) in which a low-genetic-barrier DAA is considered,[58] it could provide critical information to guide and optimize anti-HCV treatment. The relatively low cost of sequencing, compared with a failing therapy, should encourage studies aimed at better defining the advantage of its use.

The NS3 sequencing test is particularly helpful in patients who previously failed a PI treatment. Indeed, since RAVs could persist after treatment failure for a variable time in many patients,[9,15,59] resistance testing might help to decide new treatment options and reconsider a potential PI use, especially in light of new PIs in the pipeline (such as grazoprevir and asunaprevir). The latest version of the American Association for the Study of Liver Diseases recommendations suggests performing NS3 and NS5a resistance testing also in cirrhotic/advanced patients with prior failure to an NS5a regimen.[5]

Overall, our results suggest that HCV NS3 sequencing provides reliable and useful results. Since the large majority of current DAAs are not pan-genotypic and have exceptionally high costs, HCV sequencing is an efficient and relatively low-cost assay. HCV NS3 sequencing can provide at the same time two important pieces of virological information: an accurate assessment of HCV genotype/subtype and detection of variants harboured by potential non-responders to therapy, thus reducing the risk of failure especially in cirrhotic and difficult-to-treat patients.

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