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Development and evaluation of an automated hepatitis C virus NS5B sequence-based subtyping assay

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Abstract

Background: Hepatitis C virus (HCV) genotyping and accurate subtype determination is becoming increasingly important to better understand viral evolution, the development of resistance to STAT-C, and possibly even for the treatment and management of chronic HCV-infected patients.
Methods: A subtyping assay based on a 329-bp sequence of the NS5B region, together with an automated subtype interpretation tool was developed. Clinical samples of the six major genotypes were used to assess assay performance characteristics.
Results: The NS5B BLAST-based subtyping assay showed clinical sensitivity for amplification of 89% (n = 603 random samples). Assessment of analytical sensitivity of amplification for genotypes 1, 2, 3 and 4 revealed a suitable performance for high viral load samples and decreased only with low viral loads. The results were 100% and 99% accurate at the genotype and subtype level, respectively. A concordance of 97% on genotype level and 62% on subtype level was observed by comparison with subtype results from 5′ non-coding-based assays with a panel of 276 isolates.
Conclusions: The HCV NS5B subtyping assay has been validated for research use. Based on its performance, it is the method of choice in cases where subtype rather than genotype information is needed.
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Keywords: hepatitis C virus; NS5B; sequencing; subtype.

Introduction

Hepatitis C virus (HCV) is a positive-sense, single-stranded RNA virus of the genus Hepacivirus within the family *Flaviviridae*. Since its discovery in 1989 by Choo et al. (1), a large number of strains have been characterized worldwide. It soon became clear that HCV shows an amazingly high nucleotide sequence diversity caused mainly by its RNA-dependent RNA polymerase which lacks proofreading 3′–5′ exonuclease activity, resulting in high error rates during replication. Sequence analysis of the 5′ non-coding (5′NC) region and regions coding for the envelope (E1), core and the non-structural 5B (NS5B) proteins led to the identification of six major genotypes and various subtypes. In order to bring structure to the variety of HCV genotypes and subtypes, consensus nomenclature was published (2) and a numbering system for nucleotides and amino acids proposed (3).

Patients infected with different genotypes respond differently to standard of care (SOC) therapy (pegylated alpha-interferon combined with ribavirin). Therefore, HCV treatment guidelines make a distinction in duration of treatment for genotype 1 vs. non-1 genotypes. Thus, the identification of the infecting genotype has become important for choosing the appropriate treatment schedule for patients (4–6). The contribution of the genotype and subtype information in future treatment options for SOC + STAT-C will be even more important, because of differences in resistance development pathways depending on the subtype of infection [e.g., occurrence of R155K/T drug-resistance mutation in 1a but not in 1b samples; (7)].

Each genotype differs from the others by 30%–35% at the nucleotide level, whereas subtypes show a sequence diversity typically between 20% and 25% (2). However, the sequence variability is not evenly distributed throughout the genome and reaches its minimum in the 5′NC region. This makes this region a preferred target for many molecular diagnostic assays: e.g., direct nucleic acid sequencing (8, 9), a Line probe assays (LiPA) (10), subtype-specific reverse transcription (RT)-PCR (11), DNA restriction fragment length polymorphism (RFLP) (12), heteroduplex mobility analysis (HMA) (13), primer-specific and mispair extension analysis (14), melting curve analysis with fluorescence resonance energy transfer (FRET) probes (15), serologic genotyping (16) and the 5′NC-based Invader assay (17, 18). While this conserved 5′NC domain allows convenient assay development, it presents intrinsic limitations to the identification of subtypes in clinical isolates because of the common use of genetic signature motifs between different subtypes (19).

The aim of this study was to develop a reliable and robust assay for accurate HCV subtype determination. Since the NS5B domain has previously been used for classification and has proven to contain sufficient genetic information for reliable HCV subtype assignments (9, 20–23), we chose this region for subtype determination. The design of the NS5B-based subtyping assay and assessment of the performance characteristics will be discussed.

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Materials and methods

Assessment of clinical sensitivity for amplification and sequencing

Clinical sensitivity for amplification was defined as the ratio of the number of measured positive samples (amplicons) vs. the number of positive samples that were tested. Clinical sensitivity for sequencing describes the ratio of the number of amplicons for which sequencing succeeded.

Plasma samples (n = 603) from HCV-positive patients were randomly selected and obtained from different geographic areas (US, France and Portugal). In cases where viral load information was known, the majority of samples had high viral loads ($>5\log_{10}$ IU/mL). The genotypes assigned to these samples (available from the original collection site) were as follows: genotype 1 (approx. 51%), genotype 3 (approx. 23%) and genotype 4 (approx. 17%). Genotypes 2, 5 and 6 were present in a range from approximately 3% to 5%.

Assessment of analytical sensitivity of amplification

For genotype 1, samples with a broad viral load obtained from different visits of patients with subtype 1a and 1b were used. Samples with subtypes 2a, 2b, 3a, 4a and 4d with high viral loads were used to prepare a dilution series with negative plasma in order to cover the range from $5\log_{10}$ IU/mL to $1\log_{10}$ IU/mL. HCV-seronegative plasma for the dilution series was obtained from the Rode Kruis (Belgium). Samples were grouped according to viral load and amplification success rates per genotype.

Assessment of accuracy

A panel of 542 isolates covering all six genotypes and as many as available subtypes was used. Isolates were processed and analyzed using the BLAST NS5B sequence-based subtyping assay and the results compared with phylogenetic tree analysis.

Concordance to 5′NC-based genotyping assays

A panel of 276 isolates were available with geno/subtype information from 5′NC-based assays: n = 252 with results from the VERSANT® HCV Genotype 2.0 Assay (LiPA; Siemens HealthCare Diagnostics, Berkeley, CA, USA); n = 4 with results from the TRUGENE® HCV 5′NC Genotyping Kit (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) and n = 20 with results from the VERSANT® HCV Genotype Assay (LiPA) without version specification. All 5′NC-based genotyping were performed externally, results were provided by the sample supplier.

HCV RNA extraction

RNA was extracted from 250 μL or 500 μL of plasma using the EasyMAG platform and reagents (Biomérieux, Boxtel, The Netherlands). Samples were eluted in 25 or 60 μL elution buffer. RNA was used immediately or stored until further use at –80°C.

Primer and assay design

Reference sequences of different geno- and subtypes were downloaded from the Los Alamos and the European HCV database, and aligned. The alignment was used to design amplification primers which cover the major six genotypes and their various subtypes (Figure 1A, Table 1). Primers 4, 5 and 6 were used to sequence the 388 bp amplicon in both directions. A 329 nucleotide sequence [nucleotide positions 8282–8610 according to H77 (AF009606)] was obtained and used in the downstream analysis (Figure 1B).

Amplification and sequencing

Samples were reverse transcribed and amplified using the SuperScriptIII™ One-Step RT-PCR/Platinum Taq High Fidelity kit (Invitrogen, Merelbeke, Belgium) with 5 μL RNA as input and 0.2 μM forward primer (Pr1 + Pr2; primer ratio 1:1; Table 1) and 0.2 μM reverse primer Pr3. This was followed by nested PCR using 2.5 μL of the outer PCR product with an Expand High Fidelity kit (Roche, Basel, Switzerland) and 0.4 μM nested sense (Pr4 + Pr5; primer ratio 3:4; Table 1) and antisense primer Pr6 (Table 1). PCR products of the correct length were purified using the QIAGENquick PCR purification kit (Hilden, Germany). Amplicons were sequenced in both directions using the sense and antisense nested amplification primers. Sequence analysis and DNA fragment assembly were performed using SeqScape version 2.5 software (Applied Biosystems). The alignments were performed with the CLUSTAL_X software (24).

BLAST analysis

The 329 nucleotide sequence was analyzed with BLASTN (25) analysis using a reference panel of 246 reference sequences with the same nucleotide length downloaded from the Los Alamos and the European HCV database. The reference panel contained sequences of the nucleotide region 8282–8610 (according to H77 accession number AF009606) from the six major genotypes and their respective subtypes [confirmed and provisionary as defined in (2)] as published in the databases. The following subtypes were included in the reference panel (1a–1e, 1h–1m, 2a–2f, 2i, 2k, 3a–3d, 3f–3i, 3k, 4a–4h, 4k–4r, 4t, 5a, 6a–6s). The BLAST analysis was performed one by one and automated by creating a local BLAST database from the 246 reference sequences and running a local copy of the BLASTN software implemented in a Pipeline Pilot protocol (Accelrys Inc., San Diego, CA, USA; www.accelrys.com). One crucial criterion for the development of an automated BLAST analysis was the introduction of a homology threshold above which the subtyping result was considered valid. Since the purpose of the BLASTN-based subtyping tool is to reduce the number of samples that have to be subtyped using the more laborious phylogenetic tree method, the number of samples for which both methods are discordant had to be minimized without rejecting too many concordant samples from the automatic subtyping. In order to optimize this balance and allow us to develop the BLAST tool, we initially analyzed a test set of sample sequences and compared the subtype determined by BLASTN, i.e., the subtype of the highest scoring reference sequence, with the subtype determined by building a phylogenetic tree of the reference sequences and the sequence under study, i.e., the subtype of the reference sequence cluster to which the analyzed sequence belonged. A bitscore threshold of 350 was chosen. The bitscore was used rather than the percentage homology to avoid the problem of finding alignments with very high homology that are not aligned over the full length of the sequence. If a bitscore ≥ 350 was obtained, the subtype of the sample was assigned and automatically received the status of ''Valid''. However, if a sample had a bitscore <350, the subtype received the status ''Not Valid'' and the result was flagged by the software. Although the proposed subtype by the BLASTN analysis resembled the best match with the reference sequences based upon the ''Not valid'' flag, an operator would initiate further investigation by phylogenetic tree analysis and the original BLASTN result could be

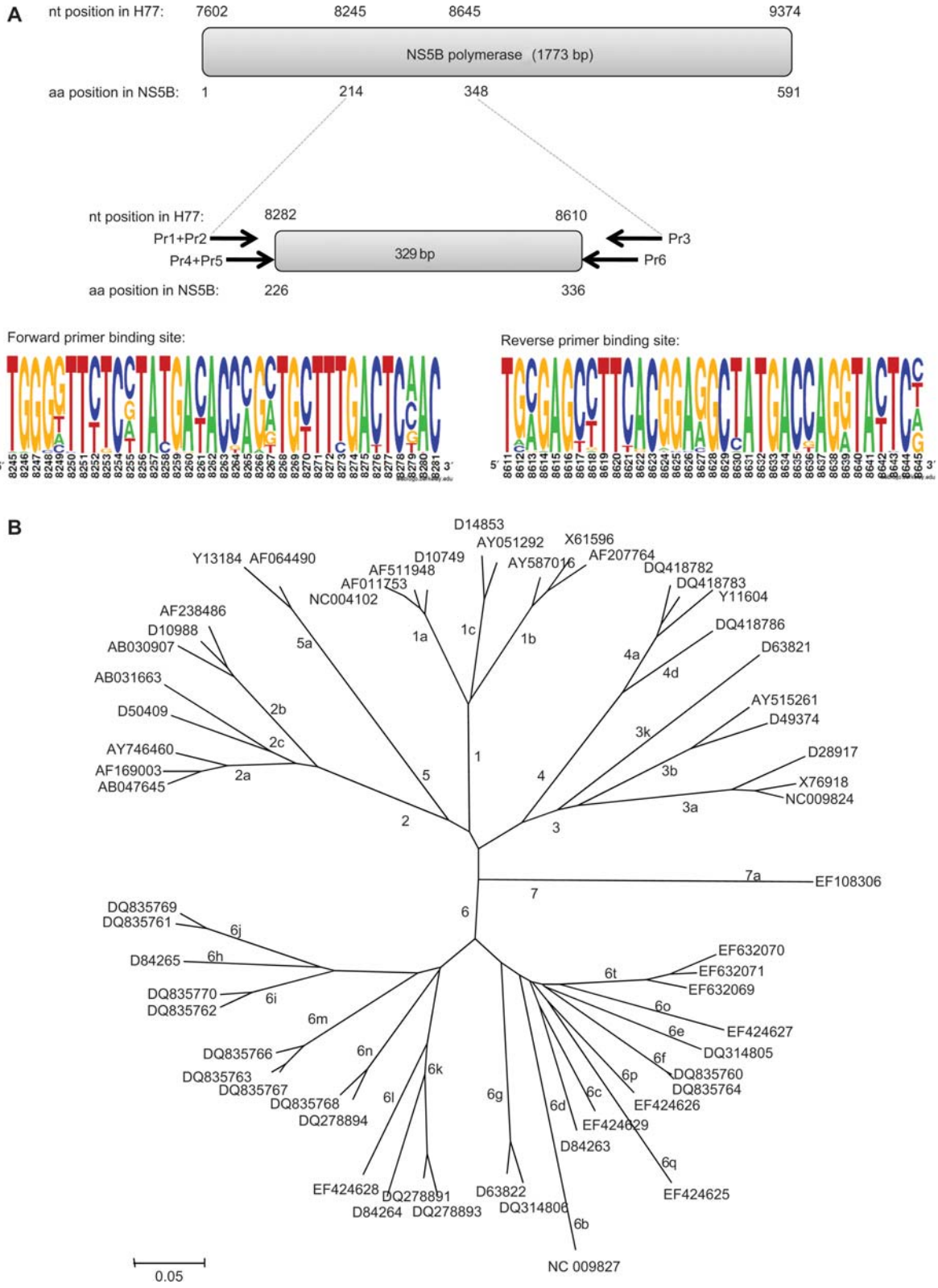

Figure 1 Design of the NS5B subtyping assay.
(A) Primer location and amplicon position within NS5B. The nucleotide (nt) positions are given according to reference strain H77 (AF009606), amino acid (aa) position is given according to annotation in H77 (NC004102) and numbering as suggested by Kuiken and Simmonds (3). The weblogo images (http://weblogo.berkeley.edu/logo.cgi) show the nucleotide variation within the primer binding region and give the binding position within NS5B. (B) The phylogenetic tree shows the discriminative power of the NS5B 329 bp fragment for subtype determination. The reference sequence panel was downloaded from the HCV Los Alamos database.

Table 1 PCR primers.

Name	Polarity	Sequence	Primer binding position[a]
Pr1	Sense	5′-TGGGGTTCGCGTATGATACCCGCTGCTTTGA-3′	8245–8275
Pr2	Sense	5′-TGGGGTTTTCTTACGACACCAGGTGCTTTGA-3′	8245–8275
Pr3	Antisense	5′-GGCGGAATTCCTGGTCATAGCCTCCGTGAA-3′	8616–8645
Pr4	Sense	5′-CCGTATGATACCCGCTGCTTTGACTCAAC-3′	8253–8281
Pr5	Sense	5′-TCCTACGACACCAGGTGCTTTGATTCAAC-3′	8253–8281
Pr6	Antisense	5′-AATTCCTGGTCATAGCCTCCGTGAAGACTC-3′	8611–8640

[a]Nucleotide position according to H77 (accession number AF009606).

overruled by the user. All sequences from the accuracy panel with a bitscore <350 (n=56) and the ''not accurate'' sequences with a bitscore >350 (n=3) are available from GenBank (accession numbers GU049343–GU049401).

Phylogenetic analysis

Phylogenetic analysis was performed with MEGA 4 software (26) using the Neighbor joining method. The evolutionary distances were computed using the Kimura 2-model. Pairwise deletion and bootstrapping were applied. Samples were analyzed in batches of approximately 100 sequences and combined with the reference panel (246 references) as used for the BLAST analysis. The phylogenetic tree was constructed. The subtype was immediately assigned if samples clustered with a (set of) reference(s). In cases where it did not, the phylogenetic analysis was repeated one by one with single sample sequences. If a sample clustered with a (set of) of reference(s) and had a bootstrap $>70\%$, the subtype was immediately assigned. If the sample did not cluster with a (set of) reference(s) or did cluster, but with a bootstrap $<70\%$, only the genotype was assigned.

Results

The amplification primers were designed to cover the six major genotypes (Figure 1A). This was achieved by using a mix of sense primers with varying nucleotide sequence (Table 1). The result was a single PCR product of 388 bp within the *NS5B* gene. As shown in Figure 1B, using a panel of 58 reference sequences from the HCV Los Alamos database, the 329 nucleotide sequence is of high discriminative power for subtype determination by phylogenetic tree analysis.

Clinical sensitivity (amplification and sequencing): all 603 samples were processed as described. A total of 538 amplicons were obtained, resulting in a clinical sensitivity for amplification of 89%. All obtained PCR products were further purified and sequenced. This resulted in a clinical sensitivity for sequencing of 100%. This result indicates that the selected primers and methodology generated amplicons in 89% of the cases.

The analytical sensitivity of the assay was determined by amplifying a dilution series of clinical samples from genotypes 1, 2, 3 and 4. Unfortunately, there was not enough sample material available to assess the sensitivity of the assay for genotype 5 and 6. The results are shown in Figure 2. For genotypes 1, 2 and 3, amplification success rate remains 100% for high and medium viral load samples and decreases at a viral load below $4\log_{10}$ IU/mL. The amplification success rate for genotype 4 dropped to 70% at viral loads below $5\log_{10}$ IU/mL (Figure 2).

Accuracy of the BLAST-based NS5B subtyping assay: a panel of 542 sample sequences was processed and analyzed with the BLAST-based NS5B assay and the results compared with those obtained by phylogenetic analysis. A result was defined *accurate* where concordance with the subtype result from phylogenetic analysis was achieved. In cases of discordance at the subtype level, the result was defined as *not accurate*. The sample panel contained the different genotypes in the following percentages: approximately 43%, 6%, 17%, 21%, 2% and 11% for genotype 1, 2, 3, 4, 5 and 6, respectively (Figure 3A). All results (n=542) were accurate at the genotype level. Those samples meeting the bitscore criteria of ≥ 350 (''Valid'' subtype result; 486 out of 542 samples) showed an accurate subtype result in 99% (n=483) of cases (Figure 3B). Only 1% (n=3) had no accurate subtype result since only genotype 4 assignment was possible by phylogenetic analysis. For the 56 samples with a subtype bitscore <350 (''Not valid'' subtype result), 25 had an accurate subtype determination. For the remaining 31 samples

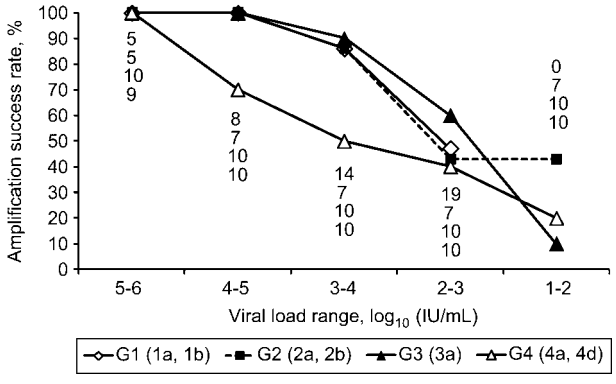

Figure 2 Analytical sensitivity of amplification.
Samples were grouped by viral load. Viral load is given in ranges from $\geq \log_{10}$ IU/mL to $<\log_{10}$ IU/mL. Numbers in columns give the number of tested sample per dilution step in the following order: genotype (G) 1, 2, 3 and 4.

that did not have an accurate subtype result, only a genotype could be assigned by phylogenetic tree analysis (n = 28), or the subtype assignment was not correct (n = 3). The latter resembled samples with a 4b subtype assignment by phylogenetic analysis. Accession numbers of sample sequences with a bitscore <350 (n = 56) and ≥ 350 but with a not accurate subtype result (n = 3) are given in the Materials and methods section.

Concordance with HCV 5′NC-based subtyping assays: a panel of 276 samples were used for the comparison (Table 2). Concordance at the genotype level was 97% (n = 267). Only 170 out of 276 samples (62%) showed concordant results at the subtype level (Table 2). The majority of discordant subtype results were detected within genotype 1 and genotype 4. Within genotype 1, 94 out of 134 samples (70%) showed a concordant subtype result with both methods. Only 41 out of 66 (62%) HCV-1b samples, as determined with a 5′NC-based method, showed concordant results with the BLAST NS5B sequence-based assay, with the majority of the 35% (n = 23) being re-typed as 1a. In contrast, 53 out of 54 (98%) 5′NC-based 1a samples showed a concordant subtype result by both methodologies. For subtype 3a, 48 of 52 (92%) samples showed concordance with the NS5B-based method, with the remaining 8% being re-typed by the NS5B-based method as 1a, 1b or 4d. Within genotype 4, for 67 out of 68 (99%) no subtype could be assigned by the 5′NC-based assay or the subtype result was discordant with the NS5B-based result.

Discussion

Since the HCV genotype is one of the most important factors guiding antiviral treatment and helps predict the outcome of this therapy (4, 27–30), most clinical laboratories routinely perform HCV genotyping assays. Nucleotide sequence analysis of different genomic regions (as the 5′NC, core, E1 and NS5B domain) in samples and comparison against a panel of known genotypes and subtypes is currently considered the ''gold standard'' for the identification of HCV geno- and subtypes (2). In practice, however, analysis of a single subgenomic region is widely used to determine the subtype of HCV. Since 5′NC amplification is regularly performed for HCV molecular diagnosis and viral load determination, genotyping methods, such as the VERSANT® HCV Genotype Assay (LiPA) or the TRUGENE® HCV 5′NC Genotyping kit, based on amplicons covering this region, are very convenient and thus have become widely used in clinical settings. However, one has to keep in mind that the 5′NC region is highly conserved (31), and what may be an advantage for consistent amplification over a broad range of genotypes for quantification of virus is without doubt a disadvantage for accurate subtype discrimination.

In order to design a robust HCV subtyping assay which covers all six major genotypes, the choice of the region to be analyzed is crucial. It must be sufficiently conserved to enable amplification primer design with broad genotype coverage, yet variable enough to allow discrimination of the

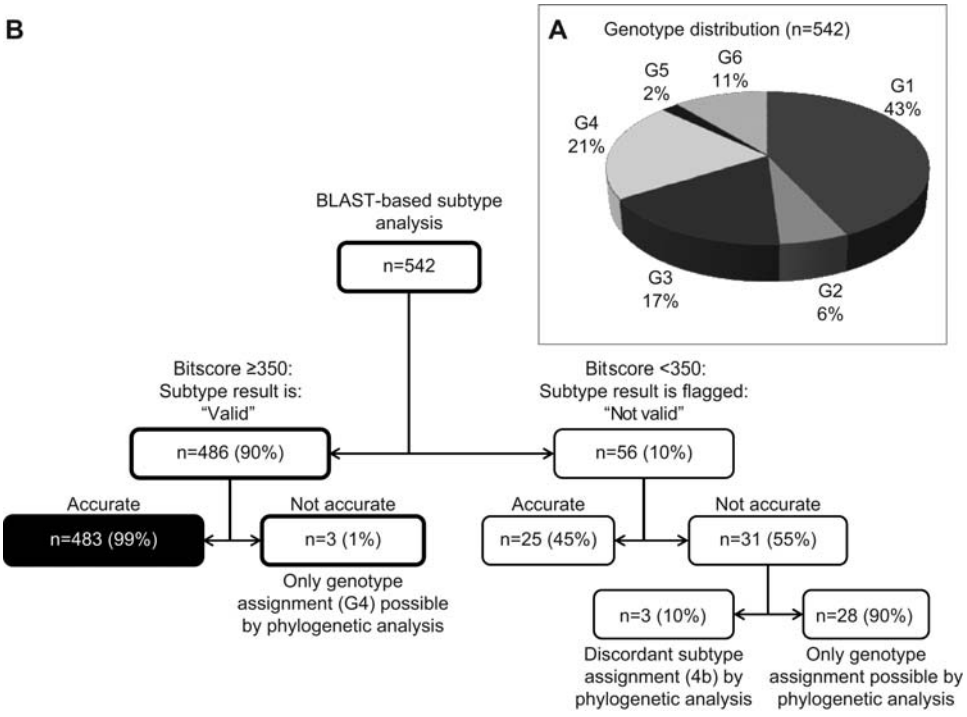

Figure 3 Determination of accuracy of the subtyping result by comparison with phylogenetic tree analysis.
(A) Genotype distribution of the sample sequences. n, number of sample sequences; G, genotype. (B) Results of the BLAST-based subtype analysis accuracy study. Accuracy was determined by comparing the subtype results of the BLAST-based NS5B subtype assay against the ''gold standard'' (phylogenetic tree analysis of the same region). A test result was considered ''accurate'' when it was concordant on subtype level to the result obtained by the ''gold standard''.

Table 2 Concordance to 5′NC-based assays.

5′NC-based	BLAST NS5B sequence-based										Total 5′NC
	1a	1b	1d	2b	3a	3b	4a	4d	4f	5a	
1	**9**	**1**	**1**		**1**						12
1a	**53**	**1**									54
1b	**23**	**41**			**2**						66
1a/1b	**2**										2
2				**2**							2
2a/2c					**1**						1
2b				**11**							11
3a	**2**	**1**			**48**			**1**			52
3b						**1**					1
4							**14**	**1**			15
4a/4c							**3**				3
4c/4d							**19**	**25**			44
4e							**1**	**4**			5
4f									**1**		1
1/4								**1**			1
5a										**6**	6
Total BLAST NS5B seq.	89	44	1	13	52	1	37	32	1	6	276

Concordant results on subtype level are highlighted with a gray background; discordant results on subtype or genotype level are marked with black boxes and white numbers. 5′NC subtype results separated by a slash means that the assay did not show a clear preference for the first or the second type. If no subtype could be assigned by 5′NC assays, only the genotype information was given. In total, a concordance of 97% (n=267) on genotype and 62% (n=170) on subtype level could be observed.

different subtypes. As numerous other authors have already demonstrated, phylogenetic analysis of the NS5B region is the method of choice for HCV subtype determination (8, 9, 20, 21, 23, 32, 33). Our primer design targets NS5B and was inspired by the early primer description by Enomoto and colleagues (32), and one primer from Sandres-Saune et al. (21), which we used unchanged. In addition, we knew that a 329-bp sequence of NS5B would be sufficient for subtype discrimination (20) which allows for a small amplicon and sequencing with one primer in each direction. We decided against degenerated primer synthesis, and opted instead for a mixture of two primers to ensure broad genotypic coverage during amplification and sequencing. We realized very early in assay development that phylogenetic analysis might be too challenging for a routine laboratory setting. Therefore, a more user-friendly analysis method was developed, an automated BLAST-based software tool that delivers validated subtype information based on a decision threshold.

We successfully demonstrated clinical sensitivity over a broad range of isolates from all six genotypes. However, we did not include samples with mixed HCV infection into the test panel. This assay is based on population sequencing, and although one may get an amplicon, the downstream sequence analysis is clearly not suitable to determine the different subtypes or genotypes in mixed HCV infections. Either cloning of the amplicon, which is very labor-intensive, or a hybridization-based method, as for instance the VERSANT® InnoLiPA HCV Genotyping kit, may be more suitable for detecting such infections. With the advent of next generation sequencing with sufficient read-lengths of around 400 bp [e.g., Roche (454), Titanium series reagents (run on a FLX Genome Sequencer)] one might speculate about even another approach for future subtyping.

We developed this assay as a module of an integrated HCV subtyping (i.e., subtype determination)-Genotyping (i.e., amplification and sequencing of a region of interest) platform (34), where the subtype of an isolate determines the primer selection for the downstream subtype-specific amplification and sequencing protocol of a region of interest. The approach was to use baseline samples to determine the subtype, and assume that samples of a later visit of this particular patient in a given time frame would belong to the same subtype, i.e., no additional infection has taken place. Although this assay does not reach the sensitivity of qualitative HCV assays which are based on amplicon generation in the 5′NC region (35), an analytical sensitivity of around 78% in the viral load range between $3\log_{10}$ IU/mL and $<4\log_{10}$ IU/mL was considered to be sufficient if using pretreatment plasma samples from a patient.

Accuracy of the NS5B BLAST-based method was studied for genotypes 1, 2, 3, 4, 5 and 6. Comparison of the automated BLAST method with phylogenetic analysis revealed a high level of concordance when following the bitscore threshold rule. Only in three cases were subtyping results with bitscore above the threshold discordant. All three belonged to genotype 4 (GenBank accession numbers GU049353–GU049355). We also observed that for a large fraction of subtyping results with a bitscore below the 350-threshold, the subtype was still concordant with the phylogenetic result. However, the majority of discordant results were caused by sequences for which we could not assign a subtype by phylogenetic analysis since they probably

belonged to new subtypes without a published reference sequence. All had a bitscore below 350. This is the downside of the BLAST algorithm, which will always assign the best matching reference subtype. Unfortunately, there is no rule to differentiate between these two groups. Bitscore values did not show a trend for such discrimination. We also looked at the number of reference sequences for the different subtypes, which were used for the analysis. When available, we included at least five published reference sequences into the reference panel. Not surprisingly, this makes the BLAST tool unsuitable for discovering new subtypes. As many other authors have described previously, the method of choice for identifying and assigning new subtypes is phylogenetic analysis of several genomic regions or the entire genome (2, 8, 9).

Concordance at the geno- and subtype level was studied for genotypes 1, 2, 3, 4 and 5. The one genotype level we observed a concordance between the NS5B BLAST method and the 5′NC-based assays of 97%. However, only 62% concordance was observed at the subtype level, with genotype 1 and 4 contributing predominantly to the discordant results. Unfortunately, we did not have access to samples with subtyping results from genotype 6. When looking at genotype 1, we observed a 2% discordance for subtype 1a, but 38% discordance for 1b, with the majority of samples (35%) being re-typed as 1a. This phenomenon within genotype 1 is not new, and has been described previously by several authors (9, 21–23, 33, 36–39). It is not surprising, as subtyping in the 5′NC is based primarily on a single variation (A/G) at position –99 for subtypes 1a/1b (19, 36). However, our finding is in contrast to Stelzl et al. (23) and Verbeeck et al. (40), who demonstrated better performance for subtype 1a and 1b discrimination using the HCV Genotype Assay (LiPA) 2.0 which includes probes for the core region. Within genotype 4, we observed a discordant subtype result in 99% of the cases. This seems to be higher than observed in other studies (21, 23), but this may be due to the larger panel of genotype 4 which we used in our study. However, based on our results we can conclude and confirm that subtype discrimination is not possible for genotype 4 within the 5′NC region (10). As already suggested by several authors (21, 23, 33, 37, 39, 41), and as confirmed with this report, NS5B is the region of choice for subtype determination, and 5′NC-based subtype results should be used carefully and with respect to the scientific or clinical purpose.

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Conflict of interest statement

Authors' conflict of interest disclosure: At the time of the study the authors were employees of Tibotec-Virco Virology BVBA. The company is involved in discovery and development of antiviral drugs and diagnostics for infectious diseases, mainly HIV and HCV. The work described in this study is a consequence of assay design in support of clinical trials and data submission to regulatory authorities.
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