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A pan-genotypic Hepatitis C Virus NS5A amplification method for reliable genotyping and resistance testing

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ABSTRACT

Background: Chronic infection with the Hepatitis C Virus (HCV) is associated with the risk of progressive liver disease. Although, HCV treatment options and viral cure rates have tremendously increased over the last decade, all currently licensed combination therapies contain inhibitors of the replication complex NS5A. Resistance-associated substitutions (RAS) in NS5A can limit the efficacy of therapy; however, resistance testing is routinely not recommended for all patients. Notably, pan-genotypic combinations have been approved, however the correct identification of the HCV genotype is still required for treatment decisions and is a good predictor for treatment success.

Objective: The aim of this study was the establishment of a pan-genotypic NS5A amplification method for reliable genotyping and simultaneous resistance testing in a fast and cheap routine diagnostic setup.

Study design: Pan-genotypic degenerated nested PCR primer were designed and tested in 262 HCV-patients. The collection included samples from genotypes 1–7 and the median viral load was 1.07×10^6 IU/ml (range 248-21×10^6 IU/ml).

Results: Amplification of the expected 747bp fragment was successful in 257 of 262 (98.1%) samples including samples < 1000 IU/ml. The direct comparison of the genotype information obtained with core sequencing to those obtained by NS5A prediction showed high concordance (97.3%) and discrepancies occurred only for relatively rare subtypes. Resistance analysis using Geno2Pheno$_{[HCV]}$ showed NS5A-RAS in 23 of 257 (8.9%) of samples.

Conclusions: We successfully developed a routine diagnostic method for pan-genotypic amplification of NS5A. This amplicon can be used for simultaneous genotyping and resistance testing for enhancing and improving routine HCV diagnostic.

1. Background

Worldwide about 70 million people are chronically infected with the Hepatitis C Virus (HCV) associated with the risk of progressive liver disease [1,2]. HCV treatment options and viral cure rates (sustained virological response, SVR) have tremendously increased over the last decade, developing from Interferon-based therapies (50% SVR) to Interferon free combinations of direct acting antivirals (DAA) with SVR rates > 95% [3,4]. Current DAA targets are the NS3/4 A protease, the viral NS5A replication-complex and the RNA-dependent RNA-polymerase encoded in NS5B. Meanwhile, pan-genotypic treatment regimens with improved safety profiles and relatively high resistance-barrier are available [5–9]. Notably, inhibitors of the NS5A replication-complex are included in all currently licensed combination therapies.

Resistance-associated substitutions (RAS) can limit the efficacy of DAA therapy and have been associated with increased risk for therapy failure [10–13]. The majority of patients with therapy failure after treatment with NS5A inhibitors have selected RAS in NS5A [14,15]. These RAS persist over prolonged time after therapy and possibly impair SVR rates upon re-therapy [16–18]. In addition to RAS selected during treatment, they are also detected in treatment-naïve patients in relevant frequencies. In studies where viral resistance genotyping was performed, NS5A-RAS were detected in up to 16% at baseline [11,19,20]. Hence, resistance testing for the presence of virus with NS5A-RASbefore therapy is recommended for selected DAA combinations [5,21,22] and may help to optimize treatment decisions in patients with negative predictors for SVR [22] (e.g. patients with cirrhosis or DAA therapy failure or those infected with genotype 3).

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2. Objective

Although treatment combinations with pan-genotypic activity have been approved, the determination of the HCV genotype is still recommended before initiating antiviral therapy [23,24]. Depending on the regimen even correct identification of the viral subtype (genotype 1a or 1b) is still required for treatment decisions and is a predictor for treatment success [24]. Although commercial HCV genotyping assays are available and have a good test performance with a reported specificity up to 93–99% [25–27], sequencing of highly discriminating fragments either from the core or the NS5B region is still considered as the gold standard for HCV genotyping and also permits identification of recombinants [28,29]. Genotyping by sequencing however, requires multiple steps including generation of amplicons, sequencing and genotype prediction and is therefore time consuming and expensive.

As the HCV genotype is required and the NS5A resistance information would be beneficial for treatment decisions we aimed to generate a pan-genotypic NS5A PCR for simultaneous genotyping and resistance testing of NS5A in a fast, reliable and cheap routine diagnostic setup.

3. Study design

3.1. Patient samples

Plasma samples of patients with HCV-infection were obtained in the framework of the German PEPSI study for the surveillance of HCV baseline resistance from Dusseldorf (D) and Cologne (K). Written informed consent was obtained from all study participants and the study was approved by the ethics committee of the Medical Faculties of Dusseldorf (study number 5945R) and Cologne (#2012048). Viral RNA from 400 µl plasma was extracted automatically using the EZ1 Virus Mini Kit v2.0 on an EZ1 Advanced XL robot or manually with the QIAamp Viral RNA Mini Kit (both Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA was eluted in a volume of 60 µl and stored at −80 °C.

3.2. Reverse transcription

A "primer-mix" containing 1 µl reverse primer Oligo d(A) (50 pmol/µl), 1 µl dNTPs (10 mM each) and 1 µl water were aliquoted in 8-strips with hinged-caps (Eppendorf #951010022) and stored at −20 °C until usage. For reverse transcription, an appropriate number of tubes was thawed and 10 µl RNA was added to the "primer-mix". Secondary RNA-structures were reduced by melting for 5 min at 65 °C before cooling down to 25 °C. RNA was next reverse transcribed in vitro with Superscript III (Invitrogen) as previously described [30] by addition of 7 µl/well reverse transcription mix (4 µl SSIII-Buffer, 1 µl DTT, 1 µl RNase Inhibitor (NEB) and 1 µl SSIII) with the previously described conditions: 10 min at 25 °C, 60 min at 42 °C, 30 min at 50 °C, 30 min 55 °C, 15 min at 75 °C and 4 °C [30,31].

3.3. Nested PCR

Two-step nested PCRs were performed with primer combinations as shown in Table 1 and TaKaRa Ex Taq® DNA Polymerase Hot-Start (TaKaRa) according to the manufacturer's protocol. To reduce hands on time all PCR mixes were prepared in large batches and frozen ("frozen-PCR mixes"). Per well 45 µl PCRI mixture containing 1x TaKaRa Ex Taq polymerase buffer, 200 µM dNTPs (TaKaRa), 0.5 µM each Primer, 1.25 Units Polymerase were aliquoted in 8-strips and stored at −20 °C until usage. PCRII mixes were identical to PCRI except the final volume of 47 µl. For the amplification step an appropriate number of tubes was thawed and five microliter of cDNA were used for the 1 st round of nested-PCR. PCR condition were 180 s at 94 °C followed by 35 cycles each 30 s 95 °C, 30 s 55 °C and 120 s 72 °C followed by 10 min at 72 °C and hold at 10 °C. Subsequently, three microliter of PCR-product from the first round was used for the second round of nested-PCR. PCR conditions were identical to the first round.

3.4. Genotyping and resistance testing

PCR-products from second round of PCR were purified with ExoSAP-IT (Thermo Fisher) or QIAquick PCR Purification Kit (Qiagen, Hilden) and Sanger sequenced (Eurofin Genomics) with sequencing primer NS5A-Seq-F and NS5A-Seq-R (see Table1). Sequences were aligned and a consensus sequence was generated with the software Geneious 10.1.3 (Biomatters, Auckland, New Zealand). The NS5A consensus sequences were then genotyped by phylogenetic analysis with reference sequences suggested by Smith et al. [32]. Moreover, genotype and resistance analysis was also performed using the prediction algorithm geno2pheno$_{[HCV]}$ version 0.92 (http://hcv.bioinf.mpi-inf.mpg.de/index.php) [33]. (32)Genotyping results based on the core region [34] served as a reference for comparisons.

4. Results

4.1. Amplification strategy

To develop an amplification strategy that permits simultaneously genotyping and identification of resistance-associated variants in the NS5A region, degenerate primers covering the N-terminal part of NS5A were designed (Fig. 1A). Therefore, 488 HCV sequences from the Los Alamos Sequence database [35] were aligned and three conserved regions around nt 6069–6146, nt 6810–6851, nt7500-7541 (H77 numbering) were identified. We have previously observed [34] that longer primers consisting of a conserved part without degenerate nucleotides followed by 3′ variable region with degenerate nucleotides have better binding characteristics than shorter primers. Therefore, all primers were designed with a 5′ conserved part consisting of 18–23 nucleotides derived from GT1a followed by a pan-genotypic variable 3′ part with up to 8 degenerate bases (Table 1). The conserved 5′ part enhances primer binding and allows direct sequencing of the amplicon. Due to low amplification efficiency of GT2 (Suppl. Fig. 1) with the original primer set we generated additional PCR-II primers for GT2 (6102-GT2-F and 6854-GT2-R) and included them into the PCR-II mix (Table 1)

4.2. Polymerase selection

Inosine containing primers are not equally accepted by all polymerases [36]. Therefore, we first analyzed the amplification efficiency of different commercially available polymerases. To address this, RNA from GT1a and GT1b infected patients was reverse transcribed with primer Oligo d(A) and the amplification efficiency was analyzed by nested PCRs with the pan-genotypic NS5A primers using four different hot start polymerases; GoTaq Hot Start (Promega), Qiagen HotStar Taq (Qiagen), Platinum HotStart (Thermo Scientific) and TaKaRa Ex Taq (TaKaRa) (Fig. 1B). As seen in Fig. 1B, all polymerases were able to amplify the 747 bp fragment with differences in the performance. Notably, the GoTaq Hot Start and the TaKaRa Ex taq polymerase were the most efficient. Because the TaKaRa Ex Taq polymerase provides the advantage of proofreading activity, this polymerase was used throughout all further experiments.

4.3. Düsseldorf cohort

After validation of the primers and polymerase combinations, 262 HCV-RNA positive patient samples with known genotype from the routine diagnostic were amplified with the pan-genotypic protocol. The genotype was determined by core sequencing, the collection included samples from genotypes 1–7 and the median viral load was 1.07×10 [6] IU/ml (range 248-21×10 [6] IU/ml). Amplification of the

Table 1
Primer combination used for amplification of NS5A.

Reaction	Primer Name	direction	sequence 5'- > 3'	genotypes
RT	Oligo d(A)	reverse	AAAAAAAAAAAAAAAAAAAA	1-7
PCR I	6069-F	forward	GAGGGGGCAGTGCAATGGATGAAYMGIYTIATIGCITTYGC	1-7
	7542-R	reverse	GAGATCCGGATCCCCAGGYTCICCYTCIAGIGGIGGCATIGA	1,2,3,5,6,7
	7541-R	reverse	AAGTCCGGGTCACCGGGGTCCCCYTCWARYGRAGGCWTTGA	4
PCR II	6105-F	forward	*TTCGCCTCCCGGGGGAAC*CAYGTITCICCIACICAYTAYGT	1,3-7
	6102-GT2-F	forward	GCC*TTCGCCTCCCGGGGGAAC*CAYGTYGCCCCIACICAYTAYGT	2
	6852-R	reverse	*CACGGCTACGTCCGGTTC*IGGITCRCAIGGIAGYTGIGAYCC	1,3-7
	6854-GT2-R	reverse	CAA*CACGGCTACGTCCGGTTC*AGGITCRCARGGRAGYTGIGAICC	2
Sequencing	NS5A-Seq-F	forward	TTCGCCTCCCGGGGGAAC	na
	NS5A-Seq-R	reverse	CACGGCTACGTCCGGTTC	na

Sequencing sites in PCR II primers are indicated in italic.

expected 747 bp fragment was successful in 257 of 262 (98.1%) samples. The five negative samples had all very low viral loads (< 3000IU/ml) and were not enriched for a specific genotype (Table 2).

Notably, a similar pan-genotypic one-step amplification strategy of NS5A was recently published [37], using slightly different primer binding sites. To directly compare our procedure to the published protocol, we first analyzed the amplification efficiency testing titration series of the viral RNA. As seen in Fig. 2, both protocols were able to amplify the NS5A fragment, however, the amplification efficiency and DNA yield was higher with our nested PCR protocol compared to the one-step protocol. For a head-to head comparison on clinical samples a collection of rare genotypes (Suppl. Table 1) and samples < 20.000 IU/ml were amplified side to side. While no differences were observed for the amplification of rare genotypes, amplification and sequencing of samples < 20.000 IU/ml was more effective with our novel protocol compared to the published protocol. With our protocol amplification

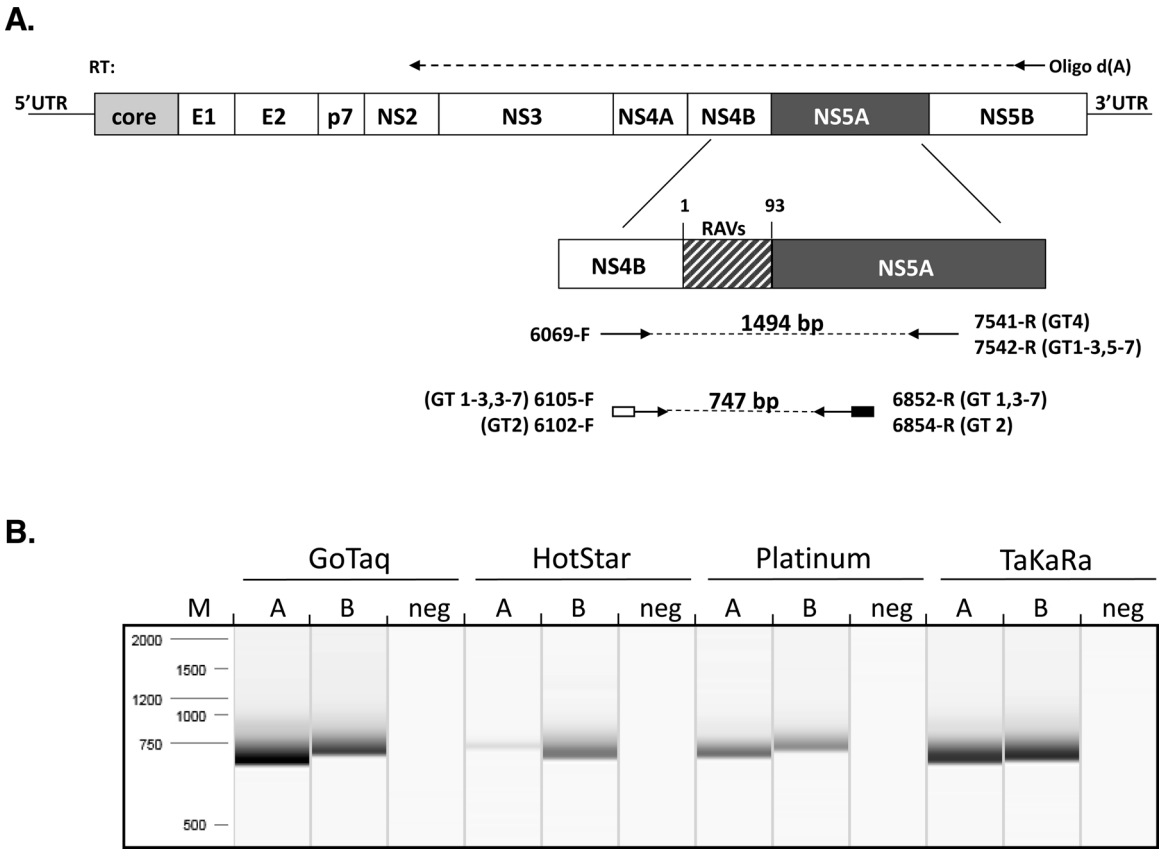

Fig. 1. Rationale for the pan-genotypic NS5A approach.
(A) Scheme of the HCV genome based on the H77 reference genome. The location of reverse transcription primer is depicted above and the nested-PCR primers for genotyping and resistance analysis are depicted below the HCV genome. The NS5A RAV containing region is depicted by horizontal stripes. For enhancement of binding and direct sequencing of the amplicon all primers were designed with a pan-genotypic variable 3′ part with up to 8 degenerate or Inosin bases followed by 18–23 nucleotides derived from GT1a. Amplicons are directly sequenced with primer Seq-F and Seq-R (depicted as white and black box on PCR-II primer, respectively) binding to the GT1a sequence in the pan-genotype primers. (B) Amplification efficiency of commercially available HotStart polymerases. RNA from a GT1a and a GT1b infected patient was extracted using the EZ1 automatic extraction device and reverse transcribed with primer Oligo d(A). cDNA was amplified by nested PCRs with the pan-genotypic NS5A primers using four different Taq polymerases; GoTaq Hot Start (Promega), HotStar Taq (Qiagen), Platinum HotStart (Thermo Scientific) and TaKaRa Ex Taq (TaKaRa). PCR products were separated on a QIAxcel Advanced capillary electrophoresis device using a DNA screening cartridge with a 250bp 4 kb DNA ladder (Qiagen). The expected fragment is 747 bp. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Efficiency of different pan-genotypic NS5A amplification protocols.

Patienten-ID	core Genotyp	viral load	Walker et al.	Andre-Garnier et al.
D16-36079	4a	248	–	–
D17-23546	1b	715	–	–
D17-36276	3a	752	Yes	–
D17-20294	1b	852	–	–
D16-50221	4	948	Yes	Yes
D16-47267	3a	1970	Yes	Yes
K16-15343	4a	2288	–	–
D16-57092	3a	2405	Yes	–
D17-17585	1a	2797	–	–
D16-41860	3a	3477	Yes	Yes
D17-21569	1b	4568	Yes	Yes
D17-24705	1b	6213	Yes	Yes
D17-38540	1a	9978	Yes	–
D17-2015	1b	11254	Yes	Yes
D16-53895	3a	11675	Yes	–
D17-37507	2a	14464	Yes	Yes

was successful in 11 out of 16 (68.75%) samples, while the one-step protocol was only able to amplify 7 out of 16 (43.75%) samples (Table 2).

4.4. Genotyping

In principle, sequencing of NS5A would allow both genotyping and resistance prediction, provided that genotyping by NS5A is as reliable as core sequencing. To analyze this, NS5A amplicons were bulk sequenced with primers listed in Table 1, aligned with the HCV reference dataset [32] and the genotype was resolved by a phylogenetic analysis (Fig. 3). For comparison, the core sequences from the routine diagnostic were also aligned and resolved by a phylogenetic tree. The comparison showed identical genotypes in 250 of 257 (97.3%) (Table 3). Three samples with discrepant results were true 2k/1b variants with 2k in core and 1b in NS5A. The remaining four discrepant samples were assigned with the same genotype but the subtypes were predicted differently (core/NS5A: 1i/1d, 2b/2k, 2q/2f and 2k/2c). All discrepancies occurred for relatively rare subtypes with limited data available in sequence databases. With the exception of correct identification of recombinants, genotyping based on NS5A seemed to be reliable and as good as genotyping based on core.

Since simultaneous genotyping and resistance prediction using a prediction algorithm like Geno2Pheno$_{[HCV]}$ [33] would be faster and more comfortable, the NS5A sequences were also used to predict the viral genotype with Geno2Pheno$_{[HCV]}$. Compared to the phylogenetic analysis of the core fragment, Geno2Pheno$_{[HCV]}$ predicted for the NS5A fragment correct genotypes in 248 of 257 (96.5%). The discrepancies included again the three recombinant 2k/1b variants and the four samples that were already discrepant in the NS5A tree. Two additional samples were 1 h in the phylogenetic analysis of the core and the NS5A fragment but predicted as 1a by Geno2Pheno$_{[HCV]}$. Notably, for both samples a low sequence homology to the reference strain was reported by Geno2Pheno$_{[HCV]}$ (79% and 80%). Taken together, genotype prediction based on NS5A is reliable and prediction by Geno2Pheno is nearly as reliable as phylogenetic analysis. In case of low sequence identity with the reference sequence reported by Geno2Pheno$_{[HCV]}$, correct genotyping needs validation by phylogenetic analysis.

Resistance associated substitutions (RAS) Prediction of RAS was performed with Geno2Pheno$_{[HCV]}$. Overall, the frequency of RAS was low with 23 of 257 (8.9%) patients harboring RAS. RAS were detected in different genotypes including GT1a/b, GT3a, GT4a/d and a GT1 and a GT3 isolate with unassigned subtype. Notably, four of the 23 patients carrying RAS were previously treated with a DAA combination containing an NS5A inhibitor.

5. Discussion

Even in the era of pan-genotypic HCV treatment, the HCV genotype is still an important diagnostic parameter. Genotyping by sequencing is the most reliable genotyping method, especially for rare genotypes and recombinants [28,38]. A major challenge for a NS5A pan-genotypic PCR protocols is the enormous sequence diversity between genotypes and subtypes that hinders identification of ideal primer binding sites for efficient and unbiased amplification of all genotypes. Here, we developed and optimized a nested PCR that allows successful amplification of all genotypes and subtypes even from samples with low viral HCV-RNA concentrations.

Sequencing of the NS5A region produces highly reliable genotype information as seen by the high concordance of our NS5A genotype prediction with the core genotyping. This is in line with previous finding [37,39]. The four samples with different subtypes in core/NS5A all belonged to rare or untyped sub-genotypes and most likely present genotyping inaccuracy rather than real discrepancies. Genotype prediction by Geno2Pheno was also highly specific. Again, discrepancies were only detected for rare subtypes and were marked by low sequence identity with the reference sequence (Table 3). Thus, genotyping by NS5A allows precise genotyping and additionally has the advantage, that recombinant viral variants are correctly genotyped in the relevant DAA target region. Genotyping with Geno2Pheno has the advantage of being fast, comfortable and simultaneously determines RAS.

The clinical relevance of resistance mutations for treatment of HCV genotype with DAAs is still not clear [40]. RAS are frequently detected in treatment naïve patients, however, lower SVR rates of patients with RAS compared to patients without RAS are only detected in GT1a and GT3 infected patients [41,42] whereas baseline RAS in GT1b are not of clinical importance [43,44]. The overall frequency of baseline RAS (8.9%) was lower compared to other studies [11,45,46]; however the frequency of RAS in GT3 was comparable to data from other West-

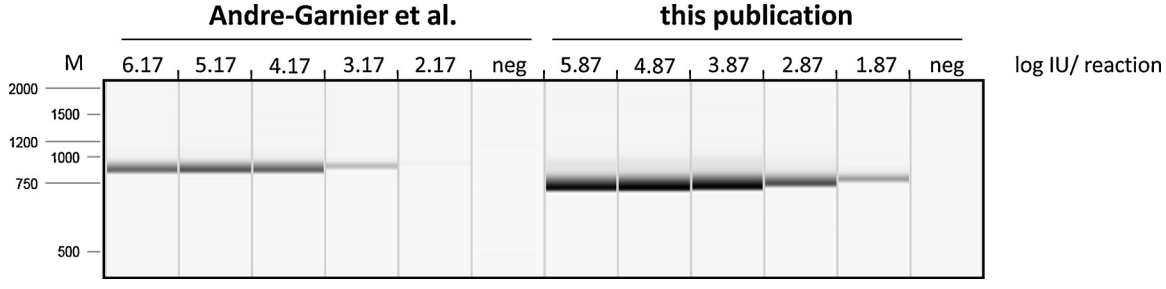

Fig. 2. Comparison of different pan-genotypic approach for NS5A amplification.
Efficiency of NS5A amplification was analyzed by serial dilution of RNA (22 × 10 [7] IU/ml) and amplification with the indicated protocols. PCR-aliquots were separated on a QIAxcel Advanced System. M. QX DNA Size Marker 250 bp – 4 kb (Qiagen). As the protocols use different amounts of input RNA the viral load per reaction is indicated for better estimation of amplification efficiency. Of note, both protocols amplify the lowest dilution (equal to 1000 IU/ml plasma), however with different efficacy.

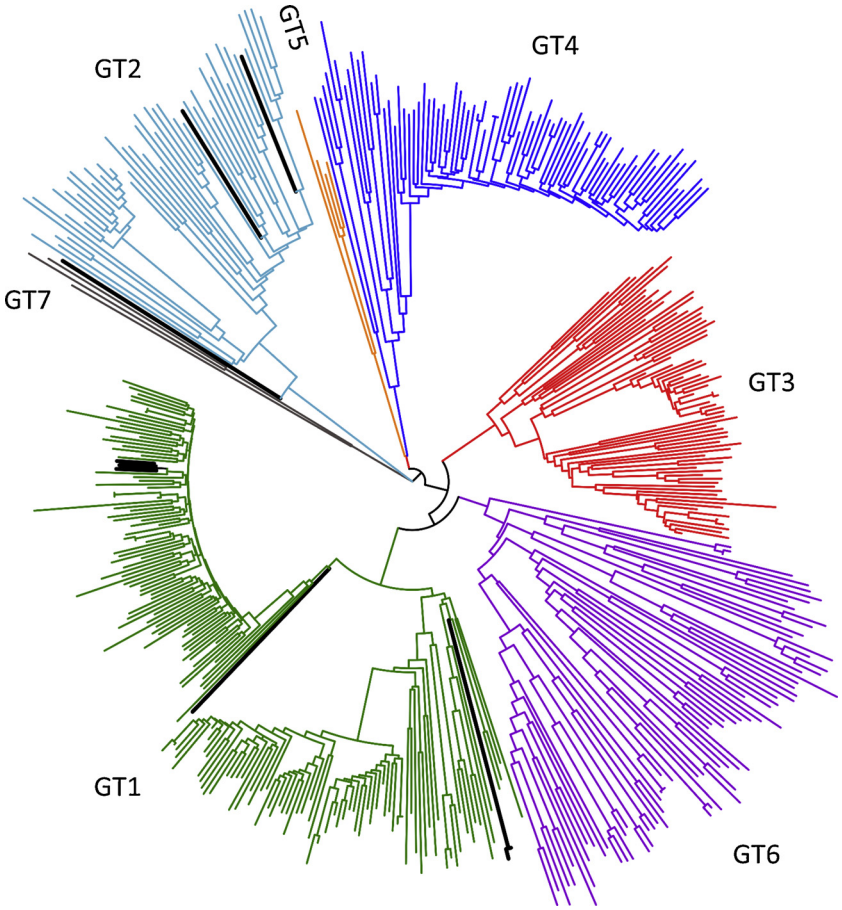

Fig. 3. Phylogenetic tree of NS5A sequences.
The NS5A sequences were aligned with the reference genomes provided by Smith et al [32] and a phylogenetic tree was generated by the Neighbor-Joining method using the Tamura-Nei genetic distance model. The tree was generated using Geneious R.10 and visulized using iTOL3 [47]. The branches of disconcordant sequences are marked in **black**. The tree is color coded according to the genotype.

Table 3
Discrepant samples.

sample number	core tree	NS5A tree	Geno2Pheno
D16-56229	2k	1b	1b (91%)
D17-32430	2k	1b	1b (90%)
D17-44030	2k	1b	1b (91%)
D17-55572	1i	1d	1b (81%)
D17-17662	1h	1h	1a (79%)
D17-51295	1h	1h	1a (80%)
D16-28550	2b	2t	2f (74%)
D17-32482	2q	2f	2f (85 %)
K15-15437	2k	2c	2c (83%)

German cohorts [19]. Most polymorphisms were found in GT1b and here the tyrosine to histidine at position 93 alone or in combination with polymorphisms at position 31 was predominant.

Recently, another pan-genotypic approach for NS5A genotyping [37] with slightly different primer binding sites using a modified one-step PCR protocol was published. Both protocols showed comparable results for samples with high viremia, however the two-step PCR was more efficient in amplification of samples < 10.000 IU/ml. Notably, the one-step protocol by Andre-Garnier requires the expensive T4 gene32 in the RT-reaction for efficient amplification of samples with low virus concentrations. Finally, with medium to low virus concentrations the one-step protocol yielded only low amounts of PCR product that did not permit sequencing.

In this study we only analyzed RAS from amplicons amplified with the pangenotypic protocol. For formally correct analysis, RAS detection should be compared to the detection rate with genotype specific amplification. However, resistance analysis was not the primary focus of this work and from the 10 out of 23 patients with RAS where we have genotype specific amplicons there was no discrepancy. The other limitation is that we had only access to a limited number of GT5, 6 and 7 samples. Since the binding site of the pangenotypic primer are relative conserved between the different sub/genotypes, we anticipate that this protocol will also work for other genotypes.

Taken together this protocol is an improvement to the currently available protocols and permits genotyping and resistance prediction in the most important RAV region NS5A. The pan-genotypic approach reduces hands on time and costs by 50%. Moreover, besides genotype information the clinician also gets information on baseline RAS even in patients that normally would not be screened for RAS. Finally, reverse transcription with the poly d(A) primer yield whole HCV genome cDNA [30] that also allows cheap and easy re-amplification of other HCV regions.

Conflict of interest

The authors have no conflict of interest.

Author contributions

Acquisition of samples and data (AW, KSE, NL, RK), analysis and interpretation of data, drafting of the manuscript (AW, JT) critical revision of the manuscript (AW, NL, JT), study concept, design and supervision (AW, JT).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2019.01.012.

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