Journal of Clinical Virology 155 (2022) 105252

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Full-genome next-generation sequencing of hepatitis C virus to assess the accuracy of genotyping by the commercial assay LiPA and the prevalence of resistance-associated substitutions in a Belgian cohort

Kasper T. Christensen [a,*], Florian Pierard [a], Kurt Beuselinck [b], David Bonsall [c,d], Rory Bowden [d], Katrien Lagrou [b,e], Frederik Nevens [f], Yoeri Schrooten [a,b], Peter Simmonds [g], Anne-Mieke Vandamme [a,h,i], Eric Van Wijngaerden [j], Tim Dierckx [a], Lize Cuypers [a,b], Kristel Van Laethem [a,b]

[a] Laboratory of Clinical and Epidemiological Virology, Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, KU Leuven, Leuven, 3000, Belgium
[b] Department of Laboratory Medicine, University Hospitals Leuven, Leuven, 3000, Belgium
[c] Big Data Institute, Li Ka Shing Centre for Health Information and Discovery, Nuffield Department of Medicine, University of Oxford, Old Road Campus, Oxford, OX3 7LF, United Kingdom
[d] The Wellcome Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford, OX3 7BN, United Kingdom
[e] Laboratory of Clinical Microbiology, Department of Microbiology, Immunology and Transplantation, KU Leuven, Leuven, 3000, Belgium
[f] Department of Gastroenterology and Hepatology, University Hospitals, Leuven, 3000, Belgium
[g] Nuffield Department of Medicine, University of Oxford, Henry Wellcome Building for Molecular Physiology, Old Road Campus, Headington, Oxford, Oxford, OX3 7BN, United Kingdom
[h] Global Health and Tropical Medicine, Institute of Hygiene and Tropical Medicine, Universidade Nova de Lisboa, Rua da Junqueira, 100, Lisbon,1349-008, Portugal
[i] Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, Clinical and Epidemiological Virology, Institute for the Future, KU Leuven, Leuven, 3000, Belgium
[j] Department of General Internal Medicine, University Hospitals Leuven, Leuven, 3000, Belgium

ARTICLE INFO

Keywords:
Hepatitis C virus
Full genome
Next-generation sequencing
LiPA
Antiviral resistance
Treatment failure

ABSTRACT

Background: Although most currently used regimens for Hepatitis C virus (HCV) infections can be initiated without prior knowledge of genotype and subtype, genotyping is still useful to identify patients who might benefit from a personalized treatment due to resistance to direct-acting antivirals (DAA).
Objectives: To assess the utility of full-genome next-generation sequencing (FG-NGS) for HCV genotyping.
Study design: 138 HCV plasma samples previously genotyped by VERSANT HCV Genotype Assay (LiPA) were subjected to FG-NGS and phylogenetically genotyped Genome Detective. Consensuses were analysed by HCV-GLUE for resistance-associated substitutions (RASs) and their impact on treatment response was investigated.
Results: 102/138 (73.9%) samples were sequenced to a genome coverage and depth of >90% of the HCV open reading frame covered by >100 reads/site. Concordant genotype and subtype results were assigned in 97.1% and 79.4% of samples, respectively. FG-NGS resolved the subtype of 13.7% samples that had ambiguous calls by LiPA and identified one dual infection and one recombinant strain. At least one RAS was found for the HCV genes NS3, NS5A, and NS5B in 2.91%, 36.98% and 27.3% samples, respectively. Irrespective of the observed RAS, all patients responded well to DAA treatment, except for HCV1b-infected patients treated with Zepatier (33.3% failure rate (5/15)).
Conclusion: While LiPA and FG-NGS showed overall good concordance, FG-NGS improved specificity for subtypes, recombinant and mixed infections. FG-NGS enabled the detection of RAS, but its predictive value for treatment outcome in DAA-naïve patients remains uncertain. With additional refinements, FG-NGS may be the way forward for HCV genotyping.

Abbreviations: HCV, hepatitis C virus; FG-NGS, full-genome next-generation sequencing; LiPA, VERSANT HCV Genotype Assay; DAA, direct-acting antiviral; RAS, resistance-associated substitution; IQR, interquartile range.

* Corresponding author.
E-mail address: kasperthorhauge.christensen@kuleuven.be (K.T. Christensen).

https://doi.org/10.1016/j.jcv.2022.105252
Received 16 May 2022; Received in revised form 26 July 2022;
Available online 4 August 2022

1. Introduction

The hepatitis C virus (HCV) is a blood-borne pathogen causing an inflammation of the liver that can lead to liver damage, cancer, and death. Globally, 58 million persons have a chronic HCV infection with an estimated 1.5 million new infections and 290.000 deaths every year [1]. The extreme diversity of HCV is facilitated by the high error rate of the NS5B polymerase of 3.5×10^{-5} per base and has resulted in eight recognized genotypes and more than 90 subtypes classified by a nucleotide diversity of >30% and >15% across the HCV open reading frame (ORF), respectively [2,3]. The subtypes exhibit different patterns in disease progression and prognosis. While modern combination therapies of direct-acting antivirals (DAA) can achieve sustained virologic response (SVR) rates higher than 95% across sub- and genotypes, the European Association for the Study of the Liver (EASL) still recommends HCV sub- and genotype determination, where available and affordable, to optimize the virological results of HCV therapy [4]. According to EASL, persons living in or migrating from certain regions in Asia and Africa or other areas where HCV subtypes naturally harbour RASs in the NS5A gene (e.g. 93H), may benefit from sequencing to identify strains resistant to first-line regimens.

One of the most widely used commercial genotyping assays is the VERSANT HCV Genotype Assay (LiPA) that uses reverse-hybridization of probes to fragments of the HCV genome to produce unique patterns on an interpretation chart. The 2.0 version of LiPA released in 2008 uses probes against the core region in addition to the 5′ untranslated region (UTR) of the 1.0 version. Commercial tools in genotyping are generally benchmarked for their accuracy against Sanger sequencing of a small fragment of the NS5B gene (~350 bp) [5] due to its relatively high level of conservation across genotypes, simplifying primer design. While only 0.5% of LiPA analyses yield indeterminate results [6], studies estimating the concordance in interpretable genotypes of LiPA and NS5B sequencing, vary from as low as 80.4% to 100% [7–12]. Increasing sequence information for a sample beyond a single, conserved NS5B fragment of the HCV genome can contribute to more accurate genotyping using phylogenetic methods. Here, we compare the ability of genotyping by LiPA 1.0 and 2.0 to phylogeny using full-genome (FG) sequences obtained through next-generation sequencing (NGS). Additionally, we examine the presence of resistance-associated substitutions (RAS) in the genes encoding the NS3 protease, the NS5A protein and the NS5B polymerase that are targeted by DAAs and their impact on DAA treatment response.

2. Methods

2.1. Sample collection

Plasma samples were collected from DAA-naïve people living with HCV while attending the University Hospitals Leuven. The samples were genotyped by VERSANT HCV Genotype Assay (LiPA) between 2001 and 2017 as part of routine clinical care, with the clinic switching from LiPA 1.0 to LiPA 2.0 in 2008. If possible, NGS was performed on the left-over sample used for LiPA. Otherwise, the leftover from a sample used for routine viral load testing closest in time to genotyping by LiPA and before initiation of DAA therapy was chosen. Clinical and demographic characteristics were collected from medical records. This study was approved by the Ethical Committee Research UZ/KU Leuven (S61339).

2.2. Sample preparation, sequencing, and phylogenetic genotyping

All sequencing libraries were prepared with a modified version of the ve-SEQ protocol [13] as presented in Supplementary Materials. Sequence reads were aligned to a sample-specific reference and consensus sequences generated using an *in-house* bioinformatics pipeline. Only samples that resulted in >90% of the HCV ORF covered to a depth >100 reads/site were considered for further analysis to ensure proper identification of variants. Read alignments were used to generate consensus sequences with a threshold of calling ambiguity codes set to a nucleotide frequency of >15%. The consensus sequence generated with the pipeline was submitted to the Genome Detective Virus Tool for phylogenetic subtyping and concordance was determined according to the level of agreement between Genome Detective and LiPA.

2.3. Assessing resistance associated substitutions

Consensus sequences were submitted to HCV-GLUE v.1.1.108 [14] for detection of RASs. HCV-GLUE scans consensus sequences against a catalogue of 533 RASs and assigns levels of evidence of the presence of RASs in twelve different DAAs. For this study, only RASs of the two categories with the strongest evidence (category I and II) were considered. Category I RASs are defined to have either EC_{50} fold change in resistance ≥ 5 *in* vitro and found at either baseline or after treatment in vivo or have been found both at baseline and after treatment in vivo. A category II RASs is supported by only one of the two parameters.

2.4. Analysis on potential recombination

One sample showed evidence of being a HCV2k1b recombinant with FG-NGS and not LiPA. Its consensus sequence was submitted to a bootscan analysis [15] using a 2k and 1b reference (JX227953, D90208) in Recombination Detection Program 4 [16]. The same consensus sequence was also examined phylogenetically by aligning it with one representative reference per known subtype and splitting the alignment at the established breakpoint for 2k1b (nt. 3186 relative to AF009606) using MUSCLE [17] with the gap open penalty set to −800. A phylogenetic tree was constructed using IQ-TREE [18] with automatic detection of substitution model (GTR-F-R7) and 1000 bootstraps.

3. Results

3.1. LiPA and genotyping making use of full-genome NGS and phylogenetic methods are largely concordant

The patient characteristics of this study cohort are outlined in Table 1. Of 138 plasma samples, 36 (26.1%) failed to reach our inclusion criteria of >90% of the HCV ORF covered to a sequencing depth of >100, and therefore only 102 samples had their genotype determined by both LiPA and FG phylogeny. All 102 samples were sequenced prior to initiation of treatment with a median time difference of 147 days (interquartile range (IQR): 49 – 336).

At genotype resolution, 99 of these 102 samples (98.0%), were determined to be a single infection with a concordant genotype identification by FG phylogeny and LiPA (Fig. 1). At subtype resolution,

Table 1
Characteristics of the samples used to study the concordance of INNO-LiPA to full-genome phylogeny.

	Total Cohort (n = 138)	Comparison Cohort (n = 102)
Male	109 (79.0%)	77 (75.5%)
HIV co-infected	58 (42.0%)	27 (26.5%)
HIV-coinfected reporting sex with same or either gender	38/58 (65.5%)	17/27 (63.0%)
History with IVDU	28 (20.3%)	20 (19.6%)
Country of birth not Belgium	41 (29.7%)	49 (48.0%)
Median age (IQR)	48 (41.25 – 58)	49.5 (42 – 60)
Median viral load (IQR)	851,138 (208,930 – 2137,962) IU/mL	1175,208.5 (309,323 – 2802,344) IU/mL
LiPA 1.0	54 (39.1%)	50 (49.0%)
LiPA 2.0	84 (60.9%)	52 (51.0%)

IVDU: intravenous drug use. Age was determined from year of birth relative to year of sampling for NGS.

concordance was slightly lower. Of the 102 analysed samples, 81 (79.4%) were determined to be single infections with concordant subtype calls by LiPA and FG-NGS: 1b ($n = 48$), 1a ($n = 24$), 3a ($n = 9$). LiPA provided a range of subtypes (e.g. 4a/c/d) or a genotype without any subtype prediction (e.g. 2) of which one was correct in 14/102 samples (13.7%) and predicted the correct genotype but the wrong subtype in 4/102 samples (3.9%). Three and four samples showed discordant results for their genotype and subtype, respectively.

Of the seven discordant results, four were sequenced using a sample drawn on the same date as the LiPA genotyping. For the remaining three, time between LiPA genotyping and the drawing of the sequenced sample were 226, 3689 days, and 3794 days.

One sample identified as HCV4a/c/d by LiPA had FG coverage of both contigs and reads for two subtypes indicating a mixed infection of a strain classified as HCV3a and a strain classified as HCV4d (see Fig. 2). Another sample identified by LiPA as HCV2a/c was flagged in the bioinformatic pipeline as a 2k1b recombinant. Genome Detective does not include recombinants for phylogenetic genotyping but manual analysis using phylogeny and bootscan analyses confidently identified this as a 2k1b recombinant (see Fig. 3).

3.2. High frequency of resistance-associated substitutions against first-line regimens in subtypes 1b and 3a

The frequency of all identified category I RASs can be found in Supplementary Table 3. A total of 21/91 (23.1%) sequences from patients who were subsequently treated with DAA exhibited category I resistance to at least one of the drugs in the regimen given to that patient. All these sequences were either HCV1b (16/48, 33.3%) or HCV3a (5/10, 50.0%). Only 6/91 (6.6%) sequences had category I RASs against every drug in their regimen. A total of 49/103 sequences (47.6%) showed category I resistance to at least one of the twelve DAAs regardless of regimen given. Category I RASs were most common for the NS5A inhibitor daclatasvir (28/103, 27.2%) and least common for NS3 inhibitor voxilaprevir and glecaprevir (both 0/103) (Table 2).

3.3. High rate of treatment failures for subtype 1b samples treated with Zepatier

Of the 102 samples 84/102 (82.4%) originated from patients that subsequently were treated with only an IFN-free DAA regimen, and 6/102 (5.9%) were treated with IFN and DAA on separate occasions. Of the 90 DAA treatments, 85/90 (94.4%) were successful in achieving SVR and 5/90 (5.6%) failed to achieve SVR. All these five treatment failures were from a group of 15 HCV1b patients treated with Zepatier. Of the subgroups of these patients with treatment success and failure, 2/10 (20.0%) and 3/5 (60.0%) had at least one category I RAS, and 5/10 (50%) and 5/5 (100%) had at least one RAS of any category, respectively. The majority of RASs (8/12 (66.6%)) were against elbasvir with the remaining 4/12 (33.3%) all being category II RASs against grazoprevir in patients who responded to treatment.

4. Discussion

To the best of our knowledge, this is the largest study to date of the concordance between a probe-based HCV genotyping assay and assignment of HCV subtypes making use of full-length genomes. Surprisingly, the older and newer versions of the assay showed similar concordance profiles, and while the frequency, and types of RASs were similar to those previously reported [19,20], rates of treatment failure with Zepatier were considerably higher [21].

The rates of concordance at the subtype level between LiPA and

A

LiPA→NGS	LiPA 1.0	LiPA 2.0	Classification
1a→1a	8	16	Concordant subtype
1b→1b	27	21	Concordant subtype
3a→3a	5	4	Concordant subtype
2a/c→2a	1	0	Concordant genotype
2→2c	1	0	Concordant genotype
4a/c/d→4d	0	5	Concordant genotype
4c/d→4c	1	0	Concordant genotype
4→4d	1	0	Concordant genotype
4→4k	2	0	Concordant genotype
4→4q	1	0	Concordant genotype
4→4r	0	2	Concordant genotype
1b→1a	1	1	Discordant subtype
1a→1c	0	1	Discordant subtype
2a/c→2i	1	0	Discordant subtype
1a→4d	0	1	Discordant genotype
4a/c/d→3a, 4d*	0	1	Concordant genotype, missed mixed infection
2a/c→2k1b	1	0	Discordant genotype
	50	52	

B

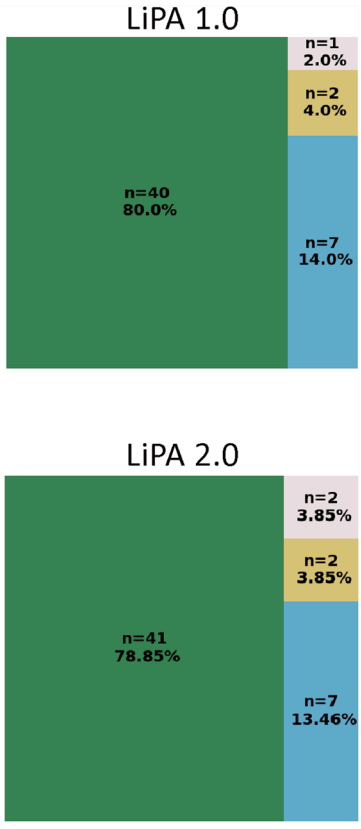

Fig. 1. Concordance for HCV genotype and subtype determination by LiPA and full-genome (FG) phylogeny. Panel A: Subtype reported by LiPA compared to subtype as determined by Genome Detective on FG consensus sequences obtained through NGS. Panel B: Concordance classification treemaps shown for LiPA 1.0 and LiPA 2.0. *phylogeny based on FG-NGS identified this sample as a mixed infection.

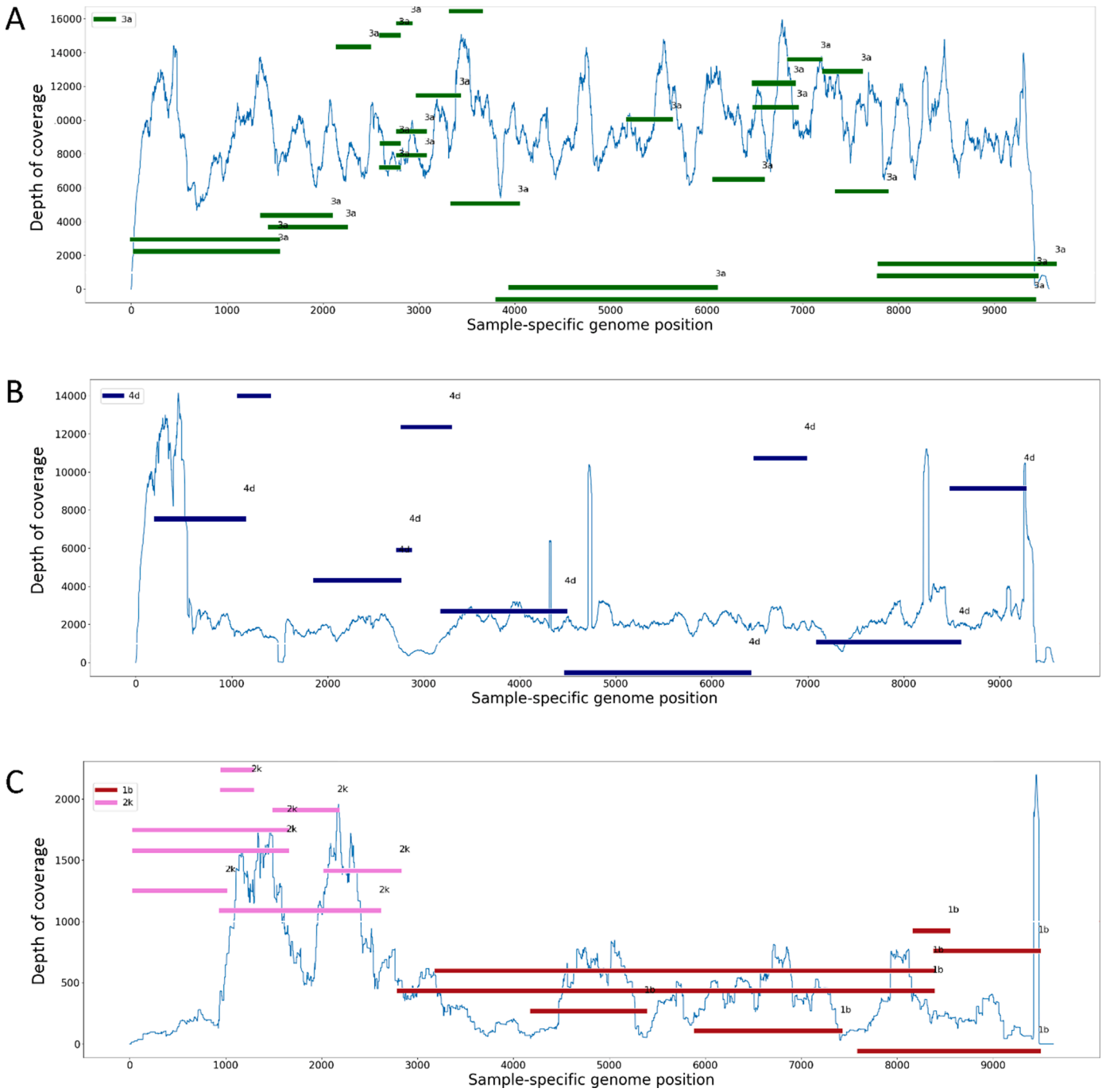

Fig. 2. Coverage graphs for the samples with a mixed infection and a recombinant strain. Graphs show the depth of coverage in reads/site across the HCV genome (continuous blue line), and the best aligning position for each contig using BLAST (horizontal bars, green: 3a, blue: 4d, pink: 2k, red: 1b). Panels A and B: The read and contig coverage from two separate analyses for the sample with a potential mixed infection: one using only HCV3a contigs (A) and one using only HCV4d contigs (B). Panel C: The read and contig coverage from the sample with a potential 2k1b recombinant strain. All contigs generated were included for formation of the sample-specific reference.

sequencing are generally higher than reported previously (see Supplementary Table 2). One explanation is the high frequency in our cohort towards samples of subtypes 1a and 1b which are more easily identified by LiPA than subtypes of genotype 2, 4, and 6.

The inability of LiPA 2.0 to accurately identify mixed infections has been documented before [22,23] and several studies have used sequencing to retroactively correct misidentified 2k1b recombinants by LiPA in a clinical setting [24,25]. The reported prevalence of mixed infections with HCV has been inconsistent and likely underestimated in part due to the limitations of the reverse hybridization methods routinely used in the clinic [26]. The prevalence of mixed infections using Sanger sequencing varies between studies with estimates ranging from 1.0% to 25.3% [27,28]. In a previous study, 6/84 (7.14%) patients had mixed infections identified through deep sequencing of NS5B all of which were entirely missed by LiPA [22]. Taken together, this data shows that while the prevalence and implications of mixed infections and recombinants are poorly understood, high-throughput sequencing is an invaluable tool to identify patients with complex infections that could potentially benefit from customized care.

For a 12-week ribavirin-free Zepatier treatment in patients with a

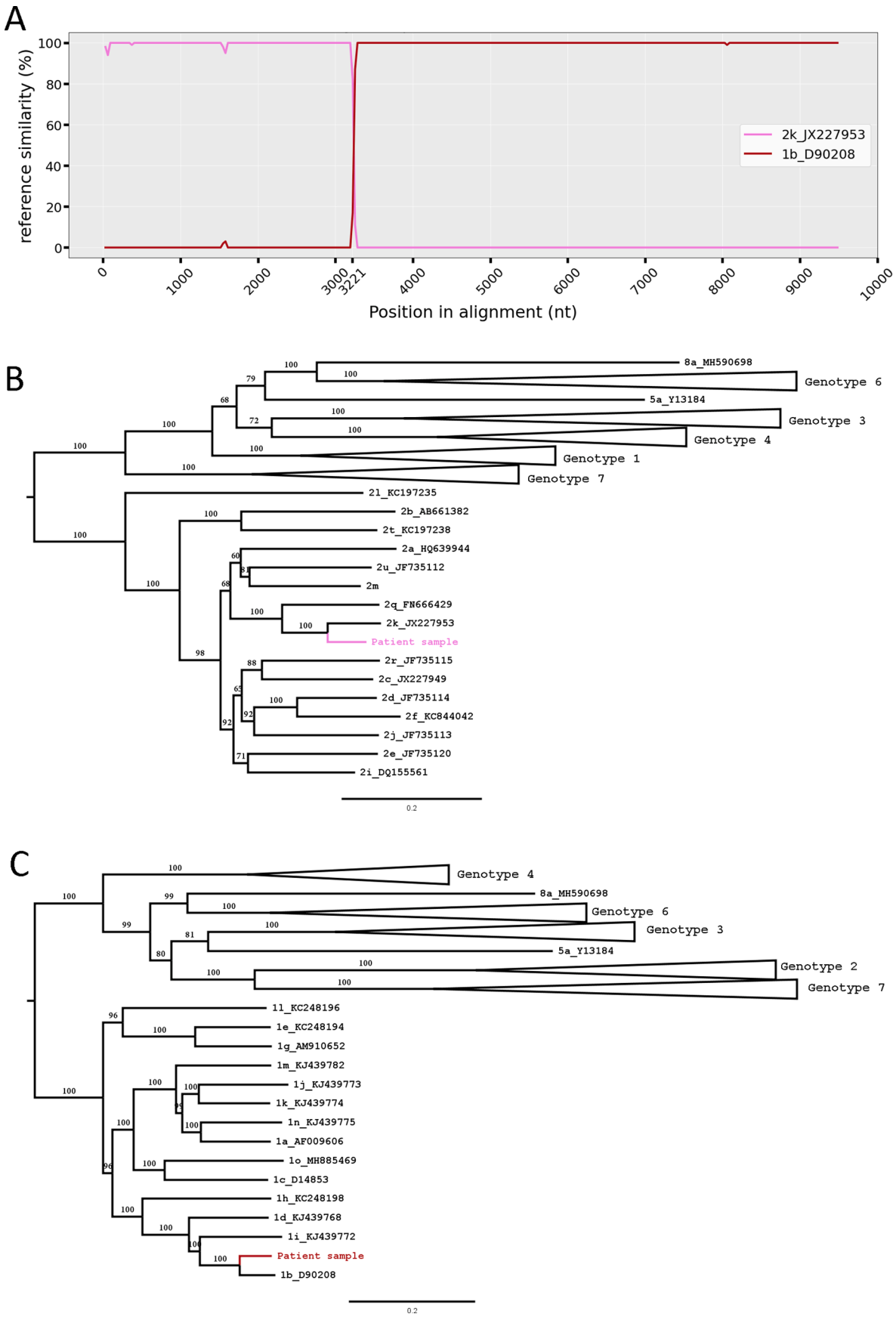

Fig. 3. Confirmation of the breakpoint located in the NS2 gene in HCV 2k1b strain. Panel A: Bootscan analysis of the patient consensus sequence in an alignment with a 2k and a 1b reference. The relative position of the breakpoint nt (3221) is indicated on the 1st axis. Panel B and C: Maximum Likelihood trees of consensus subgenomic regions delimited by breakpoint 3221.

Table 2
Frequency of resistance-associated substitutions in consensus sequences from FG-NGS.

	Directing acting antiviral											
	NS3				**NS5A**						**NS5B**	
	GLE	**GZR**	**PTV**	**VOX**	DCV	**EBR**	**LDV**	**OBV**	**PIB**	**VEL**	**DSV**	**SOF**
	ANY subtype (n=103)											
n	0	3	2	0	28	13	15	13	2	10	20	16
%	0	2.9	1.9	0	27.2	12.6	14.6	12.6	1.9	9.7	19.4	15.5
	HCV1a (n=26)											
n	0	3	2	0	0	0	0	4	0	0	0	0
%	0	11.5	7.7	0	0	0	0	15.4	0	0	0	0
	HCV1b (n=48)											
n	0	0	0	0	22	13	14	8	0	8	19	11
%	0	0	0	0	45.8	27.1	29.2	16.7	0	16.7	39.6	22.9
	HCV1c (n=1)											
n	0	0	0	0	1	0	0	1	0	0	0	0
%	0	0	0	0	100	0	0	100	0	0	0	0
	HCV2 (n=3)											
n	0	0	0	0	2	0	0	0	0	0	0	0
%	0	0	0	0	66.7	0	0	0	0	0	0	0
	HCV2k1b (n=1)											
n	0	0	0	0	0	0	0	0	0	0	1	0
%	0	0	0	0	0	0	0	0	0	0	100	0
	HCV3 (n=10)											
n	0	0	0	0	2	0	0	0	2	2	0	5
%	0	0	0	0	20	0	0	0	20	20	0	50
	HCV4 (n=14)											
n	0	0	0	0	1	0	1	0	0	0	0	0
%	0	0	0	0	7.1	0	7.1	0	0	0	0	0

Resistance according to HCV-GLUE and including only category I RAS. GLE: glecaprevir, GZR: grazoprevir, PTV: paritaprevir, VOX: voxilaprevir, DCV: daclatasvir, EBR: elbasvir, LDV: ledipasvir, OBV: ombitasvir, PIB: pibrentasvir, VEL: velpatasvir, DSV: dasabuvir, SOF: sofosbuvir.

HCV1b infection, the presence of NS5A RASs has been reported to only lower the rate of SVR from 99.6% to 94.7% [29]. Here we report a much higher rate of treatment failures for patients with HCV1b infections treated with Zepatier. We designed a simple algorithm for predicting treatment outcome based on the RAS present at treatment initiation (see Supplementary Materials) but the association between a patient's susceptibility score and their treatment outcome failed to reach statistical significance.

One limitation of our study is the rate of failure to produce an appropriately wide and deep read alignment for 36/138 (26.1%) samples. The relatively long median time of 1031 days from the time the plasma samples were collected in the clinic to time of sequencing could have influenced the yield and depth of coverage. Additionally, 22/138 (15.9%) were HCV4 samples of which a rather high share of 8/22 (36.4%) failed to cover >90% of the HCV ORF to a depth >100 reads/site. The authors of the ve-SEQ protocol used only HCV4a references in their probe design to cover the diversity of HCV4 but Belgium has a much higher proportion of HCV4 than other countries in Western Europe [30]. Indeed, of the 14 HCV4 sequences identified here, a total of five distinct HCV4 subtypes were identified (4c, 4d, 4k, 4q, and 4r). We hypothesize that this bias against HCV4 comes from the underrepresentation of HCV4 references used in probe design. The rate of failure to meet the sequencing inclusion criteria was similarly high for HCV1a. Many of our HCV1a samples were sequenced before the protocol had been finalized which may have led to insufficient coverage. This left HCV1a with a sequencing failure rate of 21/47 (44.7%). Disregarding the HCV4 and HCV1a samples, the sequencing failure rate was only 6/69 (8.7%) which could be expected for the analysis of leftover samples with a median age of 1031 days for all samples (744 days for failed samples).

Second, the median time difference between plasma samples used for genotyping by LiPA and the samples used for genotyping by FG-NGS was 832 days (IQR: 0 – 3088) with only 20/102 (19.6%) sequenced samples drawn on the same date as their counterparts used for genotyping by LiPA. Although we cannot completely rule out sample mix-up and cross-contamination, we have implemented standard measures to prevent and control for these technical problems in our laboratory. Throughout our experiments, negative controls were never able to generate a single HCV contig due to extremely low read counts and phylogenetic analysis of all HCV consensus sequences obtained in this study, also including the ones that did not reach our inclusion criteria, did not indicate cross-contaminations.

Finally, the distribution of reads across samples was uneven with the 10% of samples with the highest read counts accounting for 70.2% of all allocated reads across the 138 samples. Modifying calculated pooling volumes by viral load or determining HCV concentrations through qPCR [31] could contribute to a more even distribution of reads.

In 2018 only 12 countries were on track to achieve the targets for HCV elimination set out by WHO and only three of these were low- to middle-income countries. [32,33]. Global elimination efforts were further set back during the COVID-19 pandemic with 46/54 (85.2%) of countries experiencing an average of −49% decline in DAA utilization [34]. With the majority of infected unaware of their status, and in the absence of a vaccine, the most viable strategy for HCV elimination is the upscaling of screening and tailored treatment with DAAs. However, in order to accurately diagnose new patients, commercial genotyping assays used in routine clinical care to determine appropriate therapies must be as robust as possible. NGS as diagnostics remains prohibitively expensive in resource limited settings, however, and while the price and convenience of more sophisticated tests are improved, a broad adoption of rapid diagnostic tests is necessary to address the need of the 73% of HCV infections in low- and middle-income countries [35].

Our results indicate that LiPA 1.0 and 2.0 both have a good ability to determine the major geno- and subtypes in Belgium, but have issues in identifying rarer, foreign subtypes, mixed infections, and recombinant strains. As the cost and quality of sequencing assays improve, NGS is increasingly being used for routine clinical care. Two recent, commercial NGS assays are now in use for genotyping and detection of RASs [36, 37] and recently, England's National Health Service has been the first national health-care provider to adopt a FG-NGS pipeline into the clinical pathway of HCV treatment programs [31].

Declaration of Competing Interest

The authors declare no commercial or financial relationship that

could be construed as potential conflict of interest.

Acknowledgements

This work and KTC were supported by grants from the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO) (G069214, G0B2317N, 1S38819N). LC acknowledges FWO travel grant for a research visit at University of Oxford (V431117N). The authors thank the staff in Oxford in their support of the laboratory work and the donation of the probes used for enrichment of HCV.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2022.105252.

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