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Original Article

Comparison of direct sequencing of the NS5B region with the Versant HCV genotype 2.0 assay for genotyping of viral isolates in Mexico

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ABSTRACT

Hepatitis C virus (HCV) infection affects an estimated 71 million people worldwide. HCV is classified into eight genotypes and >70 subtypes. Determination of HCV genotype is important for selection of type and duration of antiviral therapy, and genotype is also a predictor of treatment response. The most commonly used HCV genotyping method in clinical laboratories is a hybridization-based line probe assay (LiPA; Versant HCV Genotype 2.0). However, these methods have a lack of specificity in genotype identification and subtype assignment. Here, we compared the performance of Versant HCV Genotype 2.0 with the gold standard direct sequencing of the NS5B region, in 97 samples from Mexican patients. We found a genotypic concordance of 63.9% between these methods. While 68 samples (70%) were classified into HCV genotype 1 (GT1) by NS5B sequencing, it was not true for 17 samples (17.5%), which were not match HCV subtype by LiPA. Furthermore, nine of the 33 samples classified by NS5B sequencing as GT1a were not identified by LiPA. Use of direct sequencing could improve selection of the optimal therapy, avoid possible failures of therapy and avoid high costs resulting from incorrect genotyping tests in settings without broad access to pangenotypic regimens.

1. Introduction

Hepatitis C virus (HCV) is the aetiological agent of chronic hepatitis C, which is one of the main causes of cirrhosis [1]. The World Health Organization has estimated that 71 million people are infected with HCV worldwide [2]. In Mexico, between 400,000 and 1.4 million people are estimated to be serologically positive for HCV, and about 530,000 of these individuals show chronic replicative infection and require antiviral treatment [3,4]. HCV has a

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positive-sense, single-stranded RNA genome and is classified into eight genotypes (GT1–GT8) and >70 subtypes. These genotypes differ from each other by ~25–35% of the nucleotide sequence, and the subtypes differ by 15–25% at the nucleotide level [5,6]. The genotype distribution varies between countries; the GT1 and GT3 are the most frequent worldwide, GT2 and GT6 are found predominantly in east Asia, GT4 is most prevalent in the Middle East and northern Africa and GT5 is found in southern Africa. In Mexico, GT1a, GT1b and GT2b are most common genotypes [4,7].

In the absence of pangenotypic treatment for HCV infection, the genotype determination is crucial for selection of antiviral therapy and its duration. In addition, HCV genotyping represents an important predictor of the sustained viral response [5,8]. In the current era of treatment of chronic hepatitis C with Direct-Acting Antivirals (DAAs), the accuracy of genotype determination could be the key to understanding therapeutic failures [8,9]. Methods to determine the HCV genotype include direct sequencing, quantitative reverse-transcription PCR (RT-qPCR; for example, the Abbott Realtime Genotype II; Abbott Molecular, Des Plaines, IL, USA) and hybridization-based line probe assays (LiPAs, such as the Versant HCV Genotype 2.0 assay; Siemens Medical Solutions Diagnostics, Atlanta, GA, USA) [10]. The gold standard for HCV genotype and subtype identification is the direct sequencing of 5′ UTR, Core-E1 or NS5B regions [10,11]. However, due to the high degree of conservation in 5′ UTR region, methods that only target this region cannot accurately subtype genotypes 1a and 1b or differentiate between genotypes 1 and 6 [10]. For this reason, direct sequencing of NS5B or Core-E1 regions are more appropriate for that purpose.

Based on its genetic variability, the NS5B region is considered to distinguish the different genotypes and subtypes of HCV [12]. Nevertheless, the most commonly used HCV genotyping method in clinical laboratories is the Versant HCV Genotype 2.0 assay, which uses the 5′ UTR and Core sequences [13,14]. This assay has been approved by the USA Food and Drug Administration for the identification of HCV GT1–GT6 and subtypes GT1a and GT1b. However, several studies have demonstrated that this method has a lack of specificity for the differentiation of some genotypes and for correct subtype assignment [15–19]. Due to the high genetic diversity in the HCV genomes from isolates in different Latin American countries [20–22], the accuracy of certain genotyping and subtyping assays can be reduced [23,24]. In the present study, the effectiveness of Versant HCV Genotype 2.0 method was compared to direct sequencing of the NS5B region from HCV isolates of Mexican patients infected with HCV.

2. Materials and methods

2.1. Study design

A cross-sectional study design was performed for comparison of HCV genotype/subtype identification by direct sequencing of the NS5B region and by Versant HCV Genotype 2.0 analysis of 97 plasma samples obtained from HCV-infected Mexican patients receiving clinical care at the Hospital de Infectología, Centro Medico Nacional La Raza in Mexico City. HCV-infected adults were enrolled from July 2015 to March 2018, and according to international regulatory guidelines and the principles of the Declaration of Helsinki, all patients provided written informed consent for the use of their blood samples.

2.2. Participants

The recruited individuals were >16 years old, with HCV infection confirmed by a Chemiluminescent Microparticle Immunoassay (CMIA); these analyses were performed on an ARCHITECT i2000SR system (Abbott Laboratories, Abbott Park, IL, USA) with the ARCHITECT Anti-HCV Reagent kit (Abbott Laboratories), following the manufacturer's instructions. This method has sensitivity and specificity >99%. Confirmation of infection was obtained by positive reactivity with the Nucleic Acid Test and the Procleix Tigris System (Grifols, Los Angeles, CA, USA), following the manufacturer's instructions. All participants were negative for HIV and HBV infection.

2.3. RNA isolation and RT-PCR

A total of 16 ml of peripheral venous blood was obtained from each individual by venipuncture into four Vacutainer EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Plasma was separated from cells by centrifugation at 2000× *g* for 15 min at 20–22 °C. Viral RNA was purified from plasma samples with a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and stored at −80 °C in RNAase-free containers. The cDNA was obtained by RT-PCR using the AMV Reverse Transcriptase kit and Random Hexamer Primers (Promega, Fitchburg, WI, USA), following the manufacturer's instructions.

2.4. Versant HCV genotype 2.0

HCV RNA was amplified by RT-PCR with a Versant HCV amplification 2.0 kit (Siemens Medical Solutions Diagnostics, Munich, Germany), following the manufacturer's instructions. The 5′ UTR and Core fragments of the viral genome were co-amplified. Subsequently, denaturing, hybridization, washing and visualization were performed with Auto-LiPA System equipment and the Versant HCV Genotype 2.0 kit. The results were analysed with the Interpretation LiPA 2.0 kit, which lists line patterns and genotype patterns.

2.5. Amplification of the NS5B region

The HCV genome region NS5B was amplified from cDNA by semi-nested PCR using the MyTaq DNA Polymerase kit (Bioline, London, UK) and primers N–S: 5′-TATGAYACCCGYTGCTTTGAC-3′ and N-A: 5′-GAGGAGCAAGATGTTATCAGCTC-3′ for the first amplification. The PCR conditions were as follows: initial denaturation at 95 °C for 1 min followed by 28 cycles of denaturation at 95 °C for 15 s, annealing at 56 °C for 20 s and an extension at 72 °C for 15 s, followed by a final extension for 10 min. In the second round of amplification, the initiator N–S and N–N: 5′-GAATACCTGGTCATAGCCTCC-3′ were used for semi-nested PCR, with the same PCR programme. The amplicons of 385 bp from the NS5B region, corresponding to nucleotide numbering of 8256–8641 in the H77 reference HCV genome (GenBank accession no. AF009606), were obtained and then purified with a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions.

2.6. Sequencing and phylogenetic analysis

Amplification products were purified and sequenced bidirectionally at the Molecular Biology Unit, Instituto de Fisiología Celular, Universidad Autónoma de México, Mexico City, Mexico; on a 3500 Genetic Analyzer with the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA). Sequences corresponding to the HCV NS5B region were manually edited with BioEdit v7.0.9.0 [25]. Edited sequences were aligned to 96 reference sequences representing different subtypes of the HCV genotypes [26] (GenBank access codes are listed in supplementary table 1), using the Clustal W programme [27]. Likelihood mapping analyses were performed using the TREE-PUZZLE program [28]. For

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each analysis, 20 000 random quartets were evaluated. A best-fit nucleotide-substitution model for the HCV NS5B region was determined with jModelTest v2 software [29]. Maximum-likelihood phylogenetic trees were constructed with the MEGA v7 programme [30]. Evolutionary distances were calculated by the GTR + G + I model, robustness was estimated from 1000 bootstrap replicates, and branches with >70% support were considered significant. All sequences were subjected to confirmation with the Genotyping tool of the National Center for Biotechnology Information (NCBI) [31].

2.7. Statistical analysis

The data were summarized by median and interquartile range (IQR) values for continuous variables, and proportions for categorical variables; were conducted with SPSS software (version 22; IBM, Armonk, NJ, USA). The specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) for the determination of HCV GT1 subtype were conducted with OpenEpi online software [32].

3. Results

The study included 101 participants, four of whom were excluded from the analysis because the collected samples were insufficient. Among the 97 individuals who were included in the analysis, 59 (60.8%) were men. The median age was 45 years. The median HCV-RNA was 4.5×10^6 IU/mL (IQR 2.7–6.4). The main risk factors for HCV infections in these individuals were blood transfusion, nosocomial transmission and tattoos and/or piercings (Supplementary Table 2).

3.1. Genotyping results obtained with the Versant HCV genotype assay 2.0

HCV genotype was analysed in 97 samples with the Versant HCV Genotype assay 2.0; 64 samples (65.9%) were identified as GT1 (58 samples were subtype 1a and 1b), 26 (26.8%) as GT2 and three (3.1%) as GT3. In this study population, 19 isolates (19.5%) could not be assigned to a subtype, four (4.1%) were incompletely classified in subtypes 2a/2c, and four (4.1%) could not be assigned to a genotype or subtype by the Versant HCV Genotype assay 2.0 (Table 1).

3.2. Determination of the HCV genotype by sequence analysis

The phylogenetic analysis showed grouping in six clusters corresponding to GT1a, GT1b, GT2a, GT2b, GT2j and GT3a, according to comparison with the reference sequences (Fig. 1). The NS5B region was considered a high-confidence region, because the likelihood mapping results confirmed that over 80% of the trees rendered fully resolved topologies (Supplementary Figure 1), and phylogenetic analysis resulted in identical subtyping to the NCBI Genotyping analysis, with strong bootstrap supports >85% (Supplementary Table 2).

These analyses identified 68 samples (70.1%) as GT1, 26 (26.8%) as GT2, and three (3.5%) as GT3. Among the GT2 subtypes, four samples (4.1%) were identified as subtype 2j (Table 1). Regarding the reference method, we found genotypic concordance in 62/97 (63.9%) with the Versant HCV Genotype assay 2.0. The values of standard Versant HCV Genotype 2.0 assay that included specificity, sensitivity, PPV, and NPV were 25% (IC 95%: 4.55–69.94), 75% (IC 95%: 63.56–83.77), 94.44 (IC 95%: 84.89–98.09), and 5.55% (IC 95%: 0.98–25.76), respectively for identification HCV GT1.

4. Discussion

In this study, we compared HCV genotyping by the standard Versant HCV Genotype 2.0 assay with sequencing of the HCV NS5B region. We found discrepancies between the genotype assignments by the two methods in 11 individuals (11.3%). Overall, incomplete or mismatched genotype/subtype assignment by the Versant assay (with sequencing as the reference method) was observed in 35 individuals (36.1%), including failures in identification of GT1a and GT1b, and an inability to identify low-frequency subtypes, such as GT2j.

Accurate genotyping of HCV is essential for selection of the appropriate type and duration of DAA, as treatment response changes according to HCV genotype [33,34]. Our results are similar to those reported by Avó et al. and Cai et al., who demonstrated that Versant HCV Genotype 2.0 assay was less accurate than sequencing of NS5B, with only 47.2% and 66.7% concordance in Portuguese and Chinese populations, respectively [17,35]. However, in a Spanish population, Chueca et al. found an accuracy of 85% for the Versant assay [18], suggesting that its accuracy depends on the prevalence of different genotypes and subtypes in each geographic region and population.

The international recommendations for HCV treatment have emphasized the importance of differentiation of subtypes GT1a and GT1b prior to initiation of antiviral therapy by DAA, as well as the need for ribavirin in some treatment regimens [33,34]. In our study population, 25% of patients with GT1 classification by NS5B sequencing were not assigned to the accurate genotype and/or subtype; 27.3% of these GT1 samples were not identified as subtype GT1a (which is considered more difficult to treat than GT1b [33]) by

Table 1
Comparison of genotyping by reverse hybridization and by NS5B sequencing.

	Subtype by NS5B sequencing						Total, *n*, (%)
	1a	1b	2a	2b	2j	3a	
Genotype/subtype by reverse hybridization							
Unassigned	**2**	**1**		**1**			4 (4.1)
1	**3**	**2**	**1**				6 (6.1)
1a	24	**4**			**1**		29 (29.8)
1b	**1**	27		**1**			29 (29.8)
2	**1**	**1**	**2**	**6**	**2**		12 (12.3)
2a/2c	**2**			**2**			4 (4.1)
2b				9	**1**		10 (10.3)
3						**1**	1 (1)
3a						2	2 (2.5)
Total, *n* (%)	33 (34)	35 (36.1)	3 (3.1)	19 (19.6)	4 (4.1)	3 (3.1)	97 (100)

Numbers of isolates with discrepant results at subtype levels are bold.

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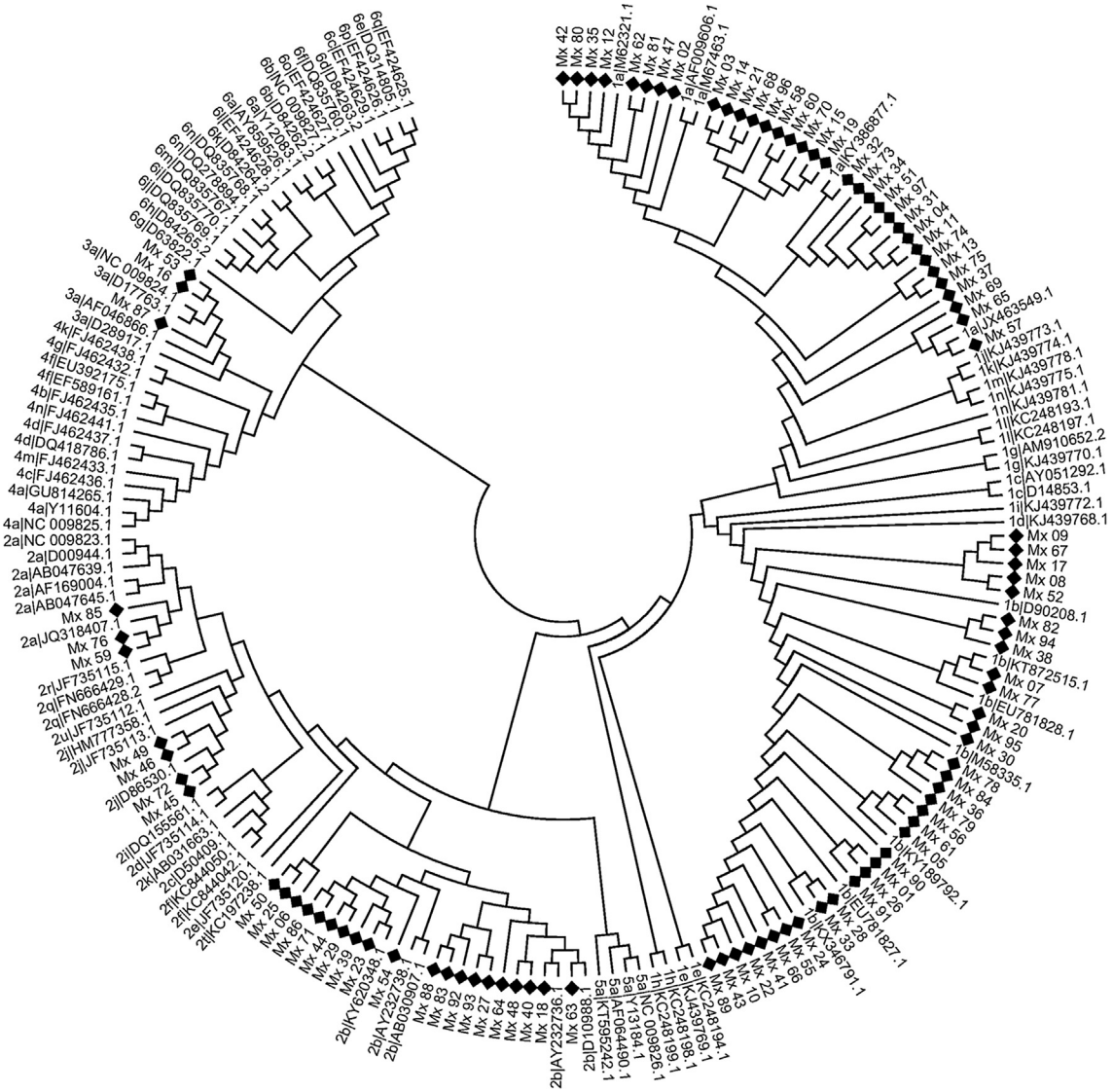

Fig. 1. Maximum-likelihood phylogenetic tree of the NS5B region. The diagram shows estimated maximum-likelihood phylogeny of reference and study-generated sub-genomic NS5B sequences. identified 68 samples (70.1%) as GT1, 26 (26.8%) as GT2, and three (3.5%) as GT3. Among the GT2 subtypes, four samples (4.1%) were identified as subtype 2j. Sequences generated in this study are prefixed with "◆". Reference sequences are labelled with their subtypes and accession numbers. The tree was constructed using MEGA v.7 software, by the maximum-likelihood method. The tree was inferred by the GTR + G + I model for nucleotide substitutions. Sequences obtained were stored in GenBank: NS5B Region (GenBank accession no. MK689245 to MK689341).

the Versant assay. Jiménez-Méndez et al. found that 32.9% of individuals with a GT1 classification could not be assigned to a specific subtype with the Versant HCV Genotype 2.0 assay in an analysis of 8802 individuals from a Mexican population [36]. Guelfo et al. found that 11% of 101 isolates were misclassified by Versant HCV Genotype 2.0 (compared with the reference method of sequencing of the HCV Core region), with 10 GT1b identifications being reclassified as GT1a by Core sequencing [37]. In the DAA treatment era, assignment of the accurate subtype, especially in individuals with GT1 HCV, will help to enable better decision-making, selection of optimal personalized therapies, avoidance of treatment failures and prevention of the generation or selection of resistance-associated substitutions.

In most middle-income countries: Argentina, China, Dominican Republic, Morocco, Thailand, Iran, Peru, Turkey, Ukraine and Mexico have been excluded from "voluntary licenses" to pan-genotypic DAAs: Glecaprevir/pibrentasvir (Gle/Pib), Sofosbuvir/velpatasvir (Sof/Vel) and Sofosbuvir/velpatasvir/Voxilaprevir (Sof/Vel/Vox) that would enable cost reduction and increase availability [38–41]. In this context, there is a need to perform exact genotyping to increase SVRs in the absence of pangenotypic DAA regimens. This approach would also limit resistance-associated substitutions. In this study, we identified the presence of GT2j in four individuals by NS5B sequencing; notably, by hybridization these individuals were classified as having GT1a, GT2 or GT2b infections. GT2j HCV has previously been identified by sequencing in Mexico, and has a substantial prevalence in South American countries, such as Argentina (5%) and Venezuela (21%) [38,42,43]. In rare subtypes, a high RAS prevalence has been observed in the NS3, NS5A and NS5B regions, causing varied SVR rates under different DAA regimens [9]. Future studies are needed in order to analyze if there differential response to DAAs regimens among GT 2j from GT 2.

The discrepancies found between the Versant HCV Genotype 2.0 and direct sequencing of the NS5B region found in twenty-six samples, suggest two possible explanations. First, direct

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sequencing of the NS5B region allows only the identification of the most prevalent and dominant HCV variants [44]. Second, although the recombination is considered to be a rare event for HCV, this phenomenon is thought to be underestimated due to limitations of the currently applied genotyping strategies [41]. Importantly, the 5′UTR-Core and NS5B regions are two conserved regions, located at both ends of the HCV genomes, whereas the recombination breakpoint are located in NS2/NS3 region [45]. Therefore, there is a possibility that there are HCV circulating recombinant forms (CRFs). Future studies based on deep sequencing tecnonologies of HCV genome would be necessary to identify a recombination breakpoint [41]. Thus, it provide a proper understanding of HCV molecular epidemiology, avoiding misclassifications of recombinant forms that can lead to DAA treatment failure [46,47].

While, the Versant HCV Genotype 2.0 assay is the most frequently used HCV genotyping method worldwide, their use do not allow the detection of recombinant strains or can lead to indeterminate genotyping results in samples from the high-diversity areas [48]. In fact there is a GT1 genetic lineage in South American countries (Bolivia, Colombia and Uruguay) that differs from lineages elsewhere [15–20,49–51].

The speed of technological advancement of sequencing methodology suggests that these techniques will soon be an affordable method for routine HCV genotyping to replace currently used methods. The Versant HCV Genotype 2.0 assay is a relatively expensive method. In Mexico, the conventional assay used for genotyping costs USD $500 per sample, in contrast to sequencing, which costs USD $350 per test. However, sequencing, chromatography and phylogenetic analyses must be performed by experienced personnel to obtain accurate results, similar to Versant HCV Genotype 2.0 assay. Next-generation sequencing (NGS) has been proposed as alternative and efficient technique in HCV genotyping, due its increased speed, relatively reduced costs, and accuracy to detect mixed Infections by HCV [52]. However, NGS is still expensive compared to routine methods, and a cost effectiveness analysis is required before its implementation in clinical practice.

This study described the HCV genotypes identified by direct sequencing and analysed phylogenetically in the NS5B region from HCV, isolated from Mexican Patients. Although, discordance with results obtained by hybridization based LiPA, was observed; future studies based on larger samples are necessary in order to estimate the magnitude of this discordance.

While the HCV strains with new genotype or subtype do not seem to be a barrier to achieving SVR, in the era of pangenotypic DAAs; the panoramic genotyping result might benefit the retreatment of the difficult-to-treat patients, who fails to be cured using the potent DAAs combination regimen, and thus promote the goal of global HCV elimination [48]. Importantly, safety and efficacy of DAAs regimens (ranging from 90–100%) are depending on the pangenotypic combination, the HCV genotype and the stage of liver disease [53]. Therefore, the identification of accurate HCV genotype and subtype not only contributes to understand the molecular epidemiology of HCV in a specific population [38], but also is critical factor that might determine efficacy of DAA regimens.

In conclusion, the comparison of Versant HCV Genotype 2.0 method with direct sequencing of NS5B in samples from 97 Mexican patients, with HCV infection, showed a genotypic concordance of 63.9%. These findings emphasize the need to provide more accuracy in HCV genotyping tests, in order to enable physicians to select the most appropriate treatment.

Authorship statement

We declare that all the authors meet the ICMJE authorship criteria, do not have any conflicts of interest, and consent to the publication of this manuscript. We confirm that this work is original and has not been published elsewhere and, if accepted, it will not be published in another place or in any other language, without prior consent in writing from the copyright holder.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jiac.2019.08.009.

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