Original Research

EVALUATION OF HEPATITIS C VIRUS GENOTYPING PROTOCOLS FOR USE IN A DIAGNOSTIC SETTING

Isaac Thom Shawa*, Mark Hopkins#

ABSTRACT

Background: Hepatitis C virus (HCV) has been reported to be the most common cause of chronic viral Hepatitis with increased prevalence rate worldwide. HCV genotype is an important predictive factor in the outcome of HCV treatment, therefore an essential component of clinical practice. Molecular methods are the most common diagnostic tools for HCV genotyping. HCV genotype might also have relevance for newer antiviral therapies in development.

Objectives: To develop and evaluate new cost-effective molecular methods for HCV genotyping using analytical instruments which were in place at Royal Liverpool University Hospital (RLUH) and to assess their sensitivity and specificity.

Methods: The plasma or serum RNA purification was done by automated QIAsymphony and QIAcube equipment. PCR assays capable of detecting and distinguishing HCV genotype by nucleotide probe or melt analysis were compared. The two-step PCR and one-step real-time PCR methods were assessed. The NS5B PCR was used to amplify the 5'NTR region of HCV for sequencing using Qiagen RT-PCT kit. The method was evaluated using HCV genomic sequence data as the gold standard.

Results: In preliminary PCR assessments, two-step real-time PCR amplified the products but could not be continued as efforts were made to assess and improve the one-step RT-PCR method for diagnostic purposes. Out a total of 71 samples 65 gave definitive genotype results, 59 were concordant and 6 mismatched. The discordant samples were repeated to confirm the genotypes but the results were similar. 6 samples could not be amplified. A Kappa value of 0.869 ($p<0.001$) indicating a 90.8 percent agreement was obtained. The sensitivity and specificity were 91.5% and 100% respectively.

Conclusions: The PCR amplification of NS5B HCV regions showed that the method is capable of detecting HCV genotypes, however it is unable to discriminate mixed genotypes. Whereas real-time PCR method is capable of detecting mixed genotypes, the genotype call may be less reliable. On the other hand, the sequencing method used in this study has an inherent limitation of giving definitive genotypes one at a time. Further efforts should be made to develop the real-time PCR to detect mixed genotypes.

Keywords: Hepatitis C Virus (HCV); Sequencing; Genotyping; Polymerase Chain Reaction (PCR); Malawi; Liverpool

*University of Malawi College of Medicine, Pathology department, P/Bag 360 Chichiri, Blantyre 3, Malawi; #Royal Liverpool University Hospital (RLUH), Virology department Duncan Building, Prescot Street, Liverpool L7 8XP

Correspondence: Isaac Thom Shawa, email:ishawa@medcol.mw

INTRODUCTION

Hepatitis is simply the inflammation of the Liver caused by factors such toxins, chemicals, drugs, heavy alcohol use and other genetic and metabolic disorders. These can damage the liver and hence are called non-infectious hepatitis because they cannot spread from one person to another.[1] Hepatitis infection can result in acute or chronic disease which leads to destruction of liver cells, a condition known as cirrhosis.[2]

Hepatitis C virus (HCV) has been reported to be the most common cause of chronic viral Hepatitis with increased prevalence rate worldwide. 150 million people were reported to be infected with HCV by 1997 worldwide.[3] It is dubbed the "silent epidemic".[1] Dryer, Limketai et al.[5] report estimated that 170 million people are infected with HCV worldwide.

Hepatitis C virus is a positive-strand RNA virus that contains a genome of about 10,000 nucleotides with a single, large continuous open reading frame[5] flanked by 5' and 3' untranslated regions[6] and belongs to the blood-borne flaviviridae family, and the genus hepacivirus.[7] Hepatitis C is considered as a major global public health problem. "Throughout the world, blood transfusion from infectious donors, unsafe therapeutic injection practices, and illegal

injection drug use have been the predominant modes of spread for HCV, with substantial geographic differences in their relative contributions to disease burden". [5] There are more than 60 known HCV genotypes and subtypes distributed worldwide. Six major genotypes (1,2,3,4,5 and 6) are reported to be of great significance. [8] Antiviral therapy for HCV consists of pegylated interferon and ribavirin. However, the therapeutic response rates differ from one HCV genotype to another. Therefore, accurate HCV genotyping is an important predictor for treatment.[9,10] HCV genotyping is important in clinical practice as it offers new avenues for antiviral therapy development. The genotypes have varied geographical distribution.[10] In the United Kingdom, genotype 1 is the most common (40-50%) followed by genotypes 2 and 3 which account for another 40-50% of HCV cases and finally genotypes 4, 5 and 6. [11]

Figure 1 below; represent the HCV genome organisation, the structural and non-structural proteins expressed within the polyprotein precursor encoding region.

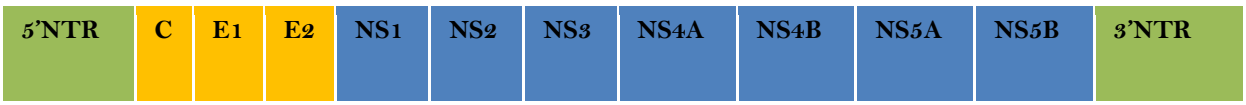

Fig 1: HCV model genome organisation: Drawn by Isaac Thom Shawa; adopted from Cambridge University press

During the time of this study, there was no virology laboratory that genotypes HCV onsite in Liverpool. The Virology department at the Royal Liverpool University Hospital (RLUH) was referring samples for HCV genotype testing to an external reference laboratory. This had a negative effect on results 'turn-around time' and possibly the cost. It was the aim of this study to evaluate the three methods of HCV genotyping using analytical instruments that were in place at RLUH.

Conventional and Real-time Polymerase Chain Reaction (PCR) was used to detect and distinguish HCV genotypes on surplus known HCV positive blood samples referred to RLUH virology laboratory for HCV testing. The gold standard method for HCV genotyping was HCV genomic sequence data for the viral non-structural 5b (NS5b) gene.

MATERIALS AND METHODS

Patient Samples

A total of 71 plasma and serum samples were collected from surplus HCV positive samples stored at the Royal Liverpool University Hospital virology department which were kept in the freezer at -70°C. Individual laboratory numbers were used to store samples in virology department. The samples were identified and the patient information was checked on Telepath information system. No patient names were used in the study except special study numbers.

The HCV genotype panel, Quality control for molecular diagnostics (QCMD) and the NIBSC panels were used in the assay evaluation. The external quality assurance (EQA) panel consisted of blind (QCMD) and known (NIBSC) genotypes.

Nucleic acid isolation

The plasma or serum samples were defrosted at room temperature, vortex for 5 seconds and centrifuged at 13000rpm for 30 seconds. Total nucleic acid was extracted from 800µl of plasma or serum by use of automated QIAGEN-QIAsymphony using the QIAsymphony ® Virus/Bacteria Midi Kit 96 following the manufacturer's instructions. RNA extraction was estimated on QIAsymphony using 'pathogen complex 800 V3' or 'virus Cellfree 1000 V3 default IC' protocols following the manufacturer's instructions. The elution volume was 85µl. A proportion of samples were also processed using QIAGEN-QIAcube using QIAamp® MiniElute® Virus kit following the manufacturer's instructions. A volume of 400µl of plasma or serum was used on QIAcube for the extraction of RNA under the large body fluid sample protocol. The elution volume was 60µl. All the purified nucleic acid preparations were stored in the freezer at -70°C.

Primers and Probes

Primer sets targeting the 5'Nontranslated region (5'NTR) and NS5b regions of HCV were used for the real-time PCR and sequencing PCR respectively. The Primers and probes were purchased from Metabion GmbH and Eurofins (Germany) in 100µM concentrations. The lyophilized primers were firstly reconstituted with the given amount of molecular water to make 100mM stocks. The working primer solutions were generated by mixing the forward and reverse primers to form different working primer mixes e.g. NS5b(A), NS5b(B), NS5b(C) and NS5b(D).

The real-time RT-PCR was performed based on the hybridization probe assay designed by (Bullock, Bruns et al. 2002)[3] using two primers with complementary sequences that are located in the 5'-NTR of the HCV genome conserved among major HCV genotypes. The forward and reverse primers were modified in order to improve the assay

performance. Different primer sets were tried in order to optimise the reactions. Table 1 below summarizes all the primer and probe sets which were modified for optimisation of the 5' NTR PCR assay.

Table 1: shows primers and probes sets for optimisation of the 5' NTR PCR assay

Primers	
Oligo Name	**Sequence (5'to 3')**
HCV_250_F	CTAGCCGAGTAGYGTTGGGT
HCV_395_R	GCGWCGGTTGGTGTTACGT
HCV_85_F	ATGGCGTTAGTAYGAGTGTYG
HCV_NAR3	CCCTATCAGGCAGTACCACAA
Probes	
HCV_312_FL	TCCCCCGGGAGGTCTCGTAGAC-Fluo
HCV_337_Red705	LCRed-705-GCACCATGAGCACGAATCCTAAACCTC-Pho
HCV_G_FL	GCCATAGTGGTCTGCGGAACCGGT-Fluo
HCV_G_LC640	LCRed-640-AGTACACCGGAATTGCCAGGACGACC-Pho

Sequencing

The NS5B sequencing PCR protocol for the region of HCV NS5b gene as described by (Rolfe, Wreghitt et al. 2009)[13] was assessed using primer sets indicated in table 2 below.

Table 2: Represents primer sets for NS5B sequencing PCR protocol

Primers	
Oligo Name	**Sequence (5'to 3')**
NS5b_F2	TATGAYACCCGCTGYTTYGACTC
NS5b_F2_pdc	TATGAYA8CCGCTGYTTYGAYTC
NS5b_R	TACCTNGTCATRGCYTCC GTG AA
NS5b_R-inosine	TACCTXGT8ATRGCYTCCGTRAA (X=inosine)
HCV_250_F	CTAGCCGAGTAGYGTTGGGT
HCV_395_R	GCGWCGGTTGGTGTTACGT
HCV_85_F	ATGGCGTTAGTAYGAGTGTYG
HCV_NAR3	CCCTATCAGGCAGTACCACAA
HCV_250_F2	CTAGCCGAGTAGTGTTGGGT (20)
HCV_395_R2	GCGTCGGTTGGTGTTACG (18)
HCV_394_R3	CGTCGGTTGGTGTTACGTT (19)
HCV_393_R4	GTCGGTTGGTGTTACGTTT (19)
HCV_394_R5	CGTCGGTTGGTGTTACGTTT (20)

The ability of the published primers (NS5bF2 and NS5bR) (Rolfe, Wreghitt et al. 2009) and the new primer sets F2+R2, F2+R3, F2+R4 and F2+R5; to amplify HCV NS5b gene was initially tested using three different kits; QuantiTect SYBR® Green RT-PCR Kit (Qiagen Cat No. 204243) and two other one-step PCR kits (Invitrogen and Qiagen) following the manufacturers' instructions. The HCV genotype sequencing reactions were performed on HITACHI 3130 GENETIC ANALYZER. The NCBI database web tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) was utilized to infer the HCV genotypes from the submitted sequences.

Real-Time PCR

The real-time RT-PCR was performed based on the hybridization probe assay designed by (Bullock, Bruns et al. 2002)[3] in a two-step real-time PCR reaction on LightCycler® FastStart DNA Master Hybridization probe kit (Roche) using two primers with complementary sequences that are located in the 5'-NTR of the HCV genome conserved among major HCV genotypes. The forward and reverse primers were modified in order to improve the assay performance. Different primer sets were tried in order to optimise the reactions. The hybridization probes were based on the resonance energy transfer (FRET) method and they contained oligonucleotides labelled with different fluorescent dyes.

In order to improve the laboratory protocol, the 5'-NTR assay was assessed as a one-step PCR protocol using different kits (i.e. Realtime ready RNA virus Master and Master HybProbe kits) following the Manufacturer's instructions. Realtime ready RNA virus Master kit (Roche) cat. No. 05619416001 was initially used as a one-step PCR on Roche-

LightCycler® LC480 system. The primers HCV 1+4 F+R; HCV F+R and probes 312+337; were initially used in this assay. The following PCR parameters were optimized for a LightCycler® carousel based protocol; RT-PCR at 50°C for 20 min, initial denaturation at 95°C for 30 sec. There were 45 cycles of amplification at 95°C for 3 sec; 56°C for 10 sec and 72°C for 12sec. Since the RNA could not be amplified (less signal), different reaction volumes were tried. The cDNA synthesis procedure (Reverse Ttranscription-PCR) was performed using Transcriptor High Fidelity cDNA Synthesis kit (Roche) Cat.no. 05091284001.

For confirmation of amplification and size of PCR products, 1.5% of agarose gel was prepared for separation of DNA fragments. PCR products (5µl) were loaded with an equal volume of Invitrogen blue juice dye into the gel wells. The electrophoresis running conditions were set at 130V, 122mA and 16W for 25min. The Hyperladder II (molecular marker) was used to compare the gel bands.

RESULTS

Nucleic acid extraction

The RNA was properly extracted with QIAGEN-QIAsymphony and QIAcube automated equipment. The QCMD panel was initially extracted using QIAsymphony but two samples were negative. When the same samples were re-extracted on QIAcube, all the samples were positive. This showed that QIAcube extracts produced better PCR products than QIAsymphony (which could be less sensitive on low viral loads). Of the 8 QCMD panel samples, 7 projects results matched with the reference genotypes except 1 sample.

HCV 5' NTR and Sequencing PCRs

SYBR® Green Assay

The primer mixes were analysed using QuantiTect SYBR® Green RT-PCR Kit. Since there were less differences in gel band sizes, one primer set (Primer NS5b(A) was chosen to be used for the rest of the project. We needed F2+R2 primer to specifically amplify genotype 1. When 5µl of RNA was used in the initial SYBR® Green RT-PCR reaction, there were many primer-dimer formations. The primer-dimers were less when the RNA was increased to 20µl in each SYBR® Green RT-PCR reaction. Increasing the RNA in the reaction also increased the PCR sensitivity. However, changing the annealing temperatures did not improve anything. Denaturing the RNA at 95°C on block heater increased the PCR products yield.

Two-step hybridization probe (5' NTR PCR)

The two-step real-time PCR using Roche-Transcriptor High Fidelity cDNA Synthesis and Roche-Lightcycler® FastStart DNA Master Hybridization probe kits gave good results but could not be continued since the main objective was to assess a one-step PCR method for diagnostic purposes. The reverse transcription and PCR done as separate procedures (two tubes) is tedious and subjected to contaminations.

One-Step Hybridization Probe

The In-house primer and probes could bind well especially on genotype 1 but there was amplification in some genotypes. There were many primer-dimer formations. The melting temperature for some few samples were low (49.36°C and 57.12°C) instead of being above 80°C. For this reason the probes could probably not bind at such low temperatures, hence the inability to amplify some samples.

Hybridization probe modifications

Modifying the reaction volumes and changing the primer sets only genotype 1 could be amplified on In-house mastermix but the band sizes on the gel were not impressive.

LightCycler® RNA Master HybProbe kit

The Real-Time ready RNA virus reagents cycling parameters were not compatible with the manufacturer's instructions and the probes could not bind well. Doubling the primer volume did not improve anything. The new LightCycler® RNA Master HybProbe reagents did not work. Modifying the RT time and doubling the primer and probe volumes did not improve anything as well.

NS5B Sequencing

Comparison of 3 different Kits

The initial primer sets tested on SYBR Green indicated that primer NS5b(A) was better and was tried on the other two kits; Invitrogen and Qiagen. Primer NS5b(A) worked on all the three reagents however, Qiagen produced better gel bands than SYBR Green and Invitrogen. All the subsequent runs were done using Qiagen® one-step RT-PCR kit. Doubling the purified RNA volume to 20µl in each Qiagen reaction volume increased the sensitivity and reduced the primer-dimer formations.

HCV sequence PCR

In initial PCR products purification where 10µl was eluted, the quality of sequence was good. When the elution volume was increased to 50µl, there were mixed sequence qualities possibly due to dilution errors. Out of 71 samples only 65 gave definitive results. 6 were not amplified. Table 3 summaries all the project HCV results.

Table 3: Summary of project results

Project genotype	HCV Genotype							
	1	2	3	4	5	Neg	Missing	Total
1	22	0	0	0	5	0	0	27
2	0	9	0	0	0	0	0	9
3	0	0	23	0	0	0	0	23
4	0	0	0	3	0	0	0	3
5	0	0	0	0	1	0	0	1
Negative	1	0	0	0	0	1	0	2
Not amplified	0	1	1	0	0	0	4	6
Total	23	10	24	3	6	1	4	71

Statistical analysis

Out of the 71 samples, 65 were analysed using SPSS in order to measure the agreement between the two methods (i.e. The project and Reference HCV genotyping) using Kappa statistical test. The remaining 6 samples were not amplified, hence their exclusion from Kappa analysis. Table 4 below gives a Kappa value of 0.869 ($p<0.001$) indicating a 90.8 percent agreement. Out of 65 definitive results, 59 showed agreement with minor discordants in subtypes and 6 were mismatches.

Table 4: Symmetric Measures - Kappa estimate

	Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Measure of Agreement - Kappa	.869	.050	11.791	.000
N of Valid Cases	65			

Not assuming the null hypothesis; Using the asymptotic standard error assuming the null hypothesis

DISCUSSION

HCV nucleotide sequence is the gold standard[12] and can be used for the detection of viruses that are difficult to cultivate.

The study demonstrated that NS5B PCR was capable of producing reliable HCV genotype results using Qiagen RT-PCR protocol with considerable confidence especially on samples with high viral loads.

However, the method could not discriminate HCV genotype 5 against 1. One sample of genotype 1a was reported as negative. This could have a great effect on treatment as genotype 1 therapy is different from other genotypes. RT-PCR and sequencing were repeated on all the discrepant samples and the results were the same. However, samples which were reported as genotype 1a instead of genotype 5, an original report was requested form the reference laboratory. The report stated that, results for genotype 5 were questionable; therefore the report confirms that our method gave reliable genotypes. Despite that, our study final report still indicated genotype discrimination errors (especially 5 versus 1a) subtypes (1b versus 1a) and mixed genotypes. Genotype 1b isolates that were identified as 1a, could not be an issue (i.e. is of less significance) as subtype identification is useful in epidemiological studies and outbreak investigation.

We also participated in the external quality assurance programme with unknown and known HCV genotype samples from QCMD and NIBSC respectively. Of the 8 plasma samples (QCMD panel), results for 7 samples were concordant with the expected reference results and 1 was discordant. Therefore, of the 6 known HCV genotype NIBSC quality assurance panel, 3 were concordant with the expected results, 1 was discordant and 2 could not be amplified by PCR, hence no genotype was determined. This could be due to low viral load.

The study published by Rolfe, Wreghitt et al.[13] assessed both real-time PCR and the NS5B sequencing. Real-time PCR method is capable of detecting mixed genotypes, but the genotype call is less reliable. In contrast, the sequencing PCR gives definitive genotypes but one at a time, this explains why we missed some genotypes on samples with mixed genotypes as noticed from the QCMD external reference HCV panel. Since the real-time PCR genotyping required further improvements, we did not have another method to compare with the sequencing method.

In this project, we attempted to optimize the one-step real-time PCR for HCV genotyping for comparison with the NS5b HCV PCR. The HCV 5-UTR region is not reliable to discriminate subtypes as it is based on single variations as compared to HCV NS5b gene region which provides better subtype discrimination since it is based on more variability than single variation. [13]

One-step real-time PCR is better because it is simple to interpret and less time consuming. [14] This was the

reason why more effort was put in trying to develop the one-step real-time PCR for HCV genotyping in this project, but could not be successfully developed for comparison with the NS5B sequencing. Few more modifications on the assay were required. Real-time PCR is increasingly becoming an HCV genotyping method in diagnostic laboratories. [15]

CONCLUSION

In Conclusion, the study managed to demonstrate that the PCR amplification of HCV NS5B region could assist in detecting HCV genotypes despite its inability to discriminate mixed genotypes. Such mixed genotypes could easily be detected by Real-Time PCR, hence the need to develop the One-Step Real-Time PCR. On the other hand, the sequencing method used in this study has an inherent limitation of giving definitive genotypes one at a time.

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