IN-HOUSE ASSAY SET UP FOR GENOTYPING AND RESISTANCE PROFILE OF HEPATITIS C (HCV) BY SEQUENCING THE NS5B (CENTRAL REGION) AND NS3 USING A SINGLE METHOD

Jean-Claude UWIMBABAZI[1,2,3], Sébastien BONTEMS[1,2,3], Marie-Pierre HAYETTE[1,2,3], Léon MUTESA[4], Pierrette MELIN [1,2,3]

[1] Université de Liège; [2]CHU of Liege – Clinical Microbiology; [3]Center for Interdisciplinary Research on Medicines (CIRM) -ULiege; [4] Center for Human Genetics, CMHS-University of Rwanda

1. INTRODUCTION

Hepatitis C virus (HCV) infection remains a major public health concern affecting approximately 185 million people worldwide (Childs-Kean, L.M et al.). There is a high variability between circulating strains of hepatitis C virus. This variability determines and influences the response and duration of treatment (Petruzziello, A. et al.). Currently, there is limited data available on the HCV viral strains in Rwanda and there is no systematic laboratory genotyping and sequencing tools for optimal management of patients in the country. The purpose of this work is to develop and set up an in-house method within the Clinical Microbiology Laboratory in the CHU-Liege that allow simultaneous genotyping and Direct acting antivirals (DAAs) resistance profiling by direct sequencing the HCV NS3 and NS5B genes which is applicable to a wide range of genotypes. The developed tool will be subsequently transferred to Rwanda. This will aid in determining the HCV genotype among infected patients; ascertaining the epidemiological surveillance of circulating strains in Rwanda; and help in monitoring of patients under antiviral treatment in various health care facilities. The first objective was to validate the detection and identification of the common circulating HCV genotypes 1-6 using in-lab developed or literature cited primes. The second objective was to evaluate the sensitivity of the method and its capacity to monitor resistance of HCV to antiviral treatment especially to DAAs currently used in various clinical settings; and finally, to analyze clinical samples from patients routinely followed up in CHU of Liege.

2. MATERIALS AND METHODS

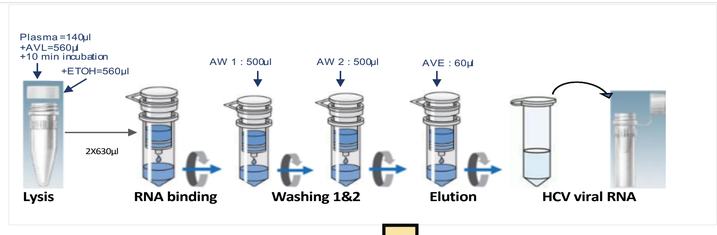
2.1. Clinical samples

Blood samples from HCV infected patients routinely followed up at CHU of Liege were used. Plasma HCV RNA viral load measurements were performed on the automated Abbott m2000sp/rt System with a lower detection limit of 15IU/ml. Samples from naïve and experienced DAAs patients were considered.

2.2. NS5B (Central region) and NS3 amplification and sequencing set up

1. Nucleic acid extraction and purification

Total nucleic acids were extracted manually from 140µl of plasma using QIAamp Viral RNA Mini Kit (Qiagen) and eluted in 60µl of AVE solution.



2. Oligonucleotide primer design

Primers previously designed within clinical microbiology laboratory and those defined in various literature were consulted. To increase sensitivity and specificity, forward and reverse identified and chosen primers, PR3 and PR4 for NS5B Central region and NS3-1F, NS3-2F, NS3-R and nestNS3-1F, nestNS3-2F, nestNS3-R for NS3 gene were adapted accordingly. For the later sequencing, M13 universal primers were added at the 5' end of each of all forward and reverse primers)..

3. Master Mix Préparation and RNA amplification by RT-PCR

a. Master Mix Preparation

RT and PCR were performed using SuperScript III Reverse transcription kit (Invitrogen) and One Step PrimeScript kit (TaKara, Otsu, Japan) separately with some modifications of the manufacturer's recommandations. The kit was used in two steps. First, RT perfomed in a 15µl RT mix containing 2,5µl of extracted RNA after which PCR was performed in a 30µl volume containing the 15µl cDNA and 15µl PCR Master Mix. Nested PCR were performed when necessary only for NS3 in a 25µl reaction mix containing 2,5µl of the 1st PCR products and 22,5µl nested PCR Master Mix containing two sense primers nestNS3-1F, nestNS3-2F and the nestNS3-R reverse primer.

b. RNA amplification by RT-PCR

RT Thermocycling conditions for both of 2 genes were 30°C for 5 min, 42°C for 5 min, 95°C min followed by 4°C cooling step. Then PCR cycling conditions were 94°C for 3 min, followed by 45 cycles of 94°C for 15s, 53°C for 1 min, 72°C for 1 min then 1 cycle of 72°C for 10 min followed by a 4°C cooling step. Nested PCR conditions were 94°C for 5 min, followed by 5 cycles of 94°C for 30s, 53°C for 1 min, 72°C for 1 min, then 35 cycle with 94°C for 15s, 67°C for 30s, 72°C for 30s followed by one cycle of 72°C for 10 min then by a 4°C cooling step..

4. Gel electrophoresis, read and photo

The amplified products were analysed by electrophoresis through 2% E-Gel

2.3. Direct sequencing of HCV NS5B central region and NS3

PCR products were purified using the enzymatic method ExoSAP-IT® (Amersham). Bidirectional sequence was performed using the fluorescent big dye terminator method (Applied Biosystems) with the M13 universal forward and reverse primers. Applied Biosystems ABI 3500XL Genetic Analzer was used for Sanger sequencing method. Nucleotide and amino acid sequences visualisation was performed using SeqScaner and then cleaned and analyzed using Vector NTI AdvanceTM 10 (Invitrogen) software. The obtained sequences were then submitted in the free access Geno2pheno HCV V1.0 analysis algoritm to determine the genotypes, subtypes as well as the mutation profiles responsible for inducing resistance to NS3 PIs and NS5B DAAs, particulaly Sofosbuvir used in the HCV treatment regim in Rwanda.

2.3.1. Méthode

1. ExoSapIT (ES) reaction

Within microplate, enzymatique PCR products cleaning was performed in 12µl mix containing 6µl of PCR products and 6µl of ES cycling conditions were 94°C for 3 min, followed by 45 cycles of 94°C for 15s, 53°C for 1 min, 72°C for 1 min then 1 cycle of 72°C for 10 min followed by a 4°C cooling step

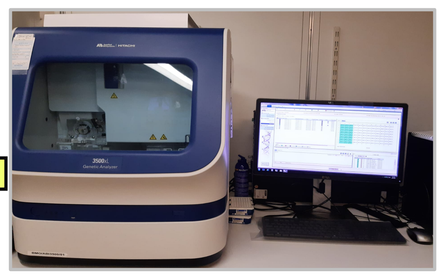
2. Sequencing reaction

Sequencing reaction was performed in 10µl mix consisting 4µl of PCR purified products and 6µl Mmix containing Primers (sens and reverse separtly)+ Big dye terminator. Cycling conditions: 1x96°C for 3 min; 25 cycles of 96°C for 15s, 50°C for 15s, 60°C for 4 min then 10°C cooling step.

3. Sequencing Products purfication

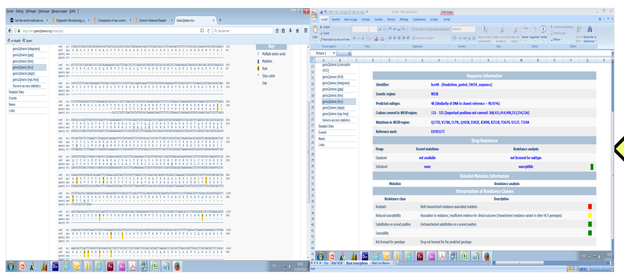
Within microplate (Micro Amp), CleanSeq Agencourt kit was used to purify sequencing products where ETOH , beads and H2O were used while EDTA was used for elution.

4. Capillary Sequencing using ABI-3500XL Genetic Analyzer



5. Sequence and amino acids cleaning and analysis: SeqScaner and Vector NTI Advance 10 (Invitrogen) software analysis

6. HCV viral genotypes and mutation profile détermination: Geno2pheno HCV V1.0 analysis algorithm



3. RESULTS

RESULT 1

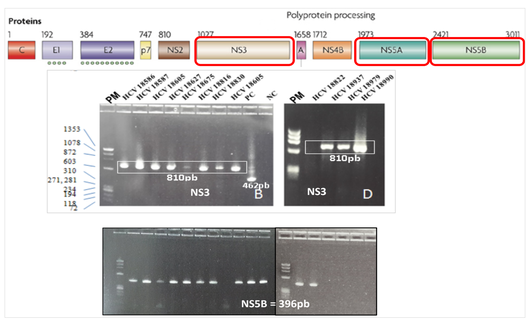


Figure 1: NS5B (Central region), and NS3 RT-PCR performance (some gel photos from RT-PCR method set up)

RESULT 2

N°	Samples used for method set up	HCV Genotyping by LIPA	HCV Genotyping and resistance profile by sequencing the NS5B central region		HCV Genotyping and resistance profile by sequencing the NS3	
			Genotypes/ subtypes	*Mutation associated to resistance to Sofosbuvir*	*Genotypes/ subtypes*	*Mutation associated to resistance to Simeprevir and other IPs*
1	HCV18586	3a	3a		3a	
2	HCV18587	1b	1b		1b	D168T, T54S,V55I
3	HCV18605	4a/c/d	4d		4d	
4	HCV18627	1b	1b		1b	
5	HCV18675	3a	3a		3a	
6	HCV18816	2a	2a		PCR failed	
7	HCV18830	1b	1b		1b	S122G
8	HCV18922	4h	4k		4k	
9	HVC18937	2	2i		2i	
10	HCV18979	6	6a		6a	
11	HCV18990	2b	2b	M289I	2b	
12	HCV18799	1b	1b		1b	S122GCR
13	HCV18970	1b	1b		1b	

Table 1 : Genotyping and mutation resistance profile determination by sequencing the NS5B (Central region) and NS3 from 13 HCV genotype known samples from the CHU of Liege

RESULT 3

Patient ID	Sex	HCV Genotyping LIPA	Sample ID	HCV Genotyping and resistance profile by sequencing the NS5B central region			HCV Genotyping and resistance profile by sequencing the NS3		
				Genotypes/ Subtypes	Mutation associated to resistance to Sofosbuvir		Genotypes/ subtypes	Mutation associated to resistance to Simeprevir and other IPs	
					Reported mutations as conferring resistance	*Unreported mutations as conferring resistance*		*Reported mutations as conferring resistance*	*Unreported mutations as conferring resistance*
E1	M	4	HCV17458	4d			4d		
			HCV18372	4d			4d	D168V	
E2	F	4	HCV18464	4k			Unanalyzable		
E3	M	4/a/c/d	HCV17880	4a		T282S	4a		V170IV, I153L
			HCV18524	4a		T282S	4a		D168E, V170I, I153L
S1	F	4	HCV17591	4l			4l		S174M
S2	M	4/a/c/d	HCV15372	4d			Unanalyzable		
S3	M	4	HCV17839	4k		C316N,	4k		V170I
S4	F	4a	HCV17681	4r		C316KN	4r		
S5	F	4	HCV13793	4k			4k		
			HCV15065	4k			Unanalyzable		
			HCV15225	4k			4k		
			HCV15300	4k			4k		
			HCV17713	4k			4k		
S6	F	4e	HCV14881	4r			4r		
			HCV15046	4r		L320ILV	4r		

Table 2 : Genotyping and mutation resistance profile determination by sequencing the NS5B (Central region) and NS3 from 16 CHU of Liege clinical samples.

4. CONCLUSION

An effecient and a high performance in-house assay was developed to amplify and sequence the NS3 and NS5B (central region) of HCV Genotypes 1-6. This tool allows to simultaneous genotyping and PI, anti-NS5B (especially DAAs) resistance profiling of HCV genotypes 1-6. This method is being tranfered & implemented in Rwanda and will be useful for virological monitoring and identification of varients that can influence the treatment of different DAAs.

Email et contacts Tél: jcuwimbabazi@doct.uliege.be, Tél : + 32 470 61 28 73