

Improvements in the QUALITATIVE HCV UMELOSA test to detect the RNA of the hepatitis C virus

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TECHNIQUE

ABSTRACT

QUALITATIVE HCV UMELOSA is a test used since 2005 to confirm HCV infection and the follow-up of the antiviral therapy in patients infected with the hepatitis C virus (HCV). The drawbacks of the assay are the need for two rounds of the polymerase chain reaction and that it does not detect amplification inhibitions, leading to false positive and negative results, respectively. Therefore, the general objective of this study was to modify the QUALITATIVE HCV UMELOSA test without affecting its analytical characteristics. RNA extraction, amplification and detection stages of HCV were standardized. An internal standard was incorporated in the extraction step and a specific primer was introduced for its detection, to detect the presence of PCR inhibitors. Appropriate primers were introduced that made it possible to eliminate the nested PCR and therefore decrease the probability of obtaining false positive results. Finally, the analytical characteristics of the new test gave good clinical and analytical results and it was 100 % consistent with the reference kit. The new standardized trial showed a sensitivity of 72.31 IU/mL, which was higher than that of QUALITATIVE HCV UMELOSA (200 IU/mL), thereby enabling its use as a confirmatory assay in therapeutic follow-up and for the screening of plasma mixtures for hemoderivative production. The modifications made on QUALITATIVE HCV UMELOSA make it socially possible to offer better care for the patient with economic benefits because of the reduction of the production cost of the kit and the cost of the Cuban health system.

Keywords: RNA, HCV, Qualitative HVC UMELOSA, PCR, assay sensitivity, internal standard, inhibitors

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RESUMEN

Mejoras al ensayo UMELOSA HCV CUALITATIVO para la detección del ARN del virus de la Hepatitis C. El ensayo UMELOSA HCV CUALITATIVO se emplea desde el año 2005 en la confirmación de la infección por el virus de la hepatitis C (VHC) y el seguimiento de la terapia antiviral. Tiene como desventajas el empleo de dos rondas de reacción en cadena de la polimerasa (PCR) y la no detección de inhibiciones de la amplificación, con resultados falsos positivos y negativos. Por ello, el objetivo de este trabajo fue la modificación del ensayo UMELOSA HCV CUALITATIVO, para incrementar su sensibilidad y sin afectar sus características analíticas. Se estandarizaron las etapas de extracción del ARN, su amplificación y la detección del VHC, se añadió un estándar interno en la extracción y una sonda específica para su detección, para detectar la presencia de inhibidores de la PCR. Además, se introdujeron cebadores específicos para disminuir la probabilidad de obtener resultados falsos positivos, y se evaluaron las características analíticas del ensayo modificado. Este mostró buena especificidad clínica, analítica y una concordancia del 100 % con el estuche de referencia, y una sensibilidad de 72.31 UI/mL, superior a la del UMELOSA HCV CUALITATIVO (200 UI/mL). Esto permite su uso como prueba confirmatoria en el seguimiento terapéutico, y para la pesquisa de mezclas de plasma para la producción de hemoderivados. Además, desde el punto de vista social, este ensayo permite una mejor atención al paciente y aporta un beneficio económico al disminuir el costo de producción del estuche y los costos para el sistema de salud cubano.

Palabras clave: RNA, VHC, UMELOSA VHC cualitativo, PCR, sensibilidad del ensayo, estándar interno, inhibidores

Introduction

The prevalence of the hepatitis C virus (HCV) infection was estimated by the World Health Organization (WHO) to be 3 %, which represents approximately 185 million persons infected in the world [1]. The infection by this virus may be expressed as acute hepatitis, frequently asymptomatic, which can lead to a chronic infection in 80 % of the cases [2]. Patients with chronic infection normally are asymptomatic for long periods of time. Approximately 20 % of these individuals develop hepatic cirrhosis after a 20 year evolution. Patients with cirrhosis, besides the risk of liver failure and the need of a transplant, have a

highly significant risk of developing hepatocellular carcinoma.

At present, the infection by HCV is the main cause of liver transplant and of hepatocarcinoma in the western world, representing the main cause of hepatic morbidity and mortality [3]. In Cuba, approximately 1.38 % of the blood donor population has positive antibodies to HCV [4]. Liver cirrhosis is the tenth cause of death in women and the eleventh in men; in these, HCV has a leading role [5]. It is therefore considered that HCV is a serious health problem in our country.

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The selected diagnostic method is the serological determination of anti-hepatitis C antibodies, which is highly sensitive [6]. Positive serological results should be confirmed with an additional test, which is now the most frequently used technique, i.e. the detection of viral ribonucleic acid (RNA) in circulation using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

QUALITATIVE HCV UMELOSA has been produced since 2005 (Center of Immunoassay, CIE, Cuba), which is the first Cuban molecular diagnosis test for the confirmation of HCV infection. Its advantages include the detection of the infection in the window period between the infection and seroconversion, enabling the diagnosis of immunodepressed persons, and the confirmation of the infectious cases resolved (seropositive patients without viral replication) [7].

The QUALITATIVE HCV UMELOSA test includes RT-PCR and later a nested PCR steps. The latter is a source of environmental contamination, leading therefore to false positive results. Furthermore, this test does not detect the inhibitions of PCR in samples containing inhibitory components, which may give rise to false negative results. Therefore, the aim of this study was to modify the QUALITATIVE HCV UMELOSA trial in order to eradicate these deficiencies.

Materials and methods

Extraction of RNA from the HCV using QUALITATIVE HCV UMELOSA

The extraction of the RNA by QUALITATIVE HCV UMELOSA from the human serum or plasma was made through modifications of the method described by Chomczynski and Sacchi in 1987 [8]. It was started by adding 500 μ L of the lysis buffer (0.3-0.6 mol/L ammonium thiocyanate (BDH, Poole, England); 0.6-1.0 mol/L guanidine thiocyanate (Sigma, Saint Louis, USA); phenol (v/v) 35-40 % (Merck, Darmstadt, Germany); 5-8 % v/v Glycerol (Sigma, Saint Louis, USA); 0.1-0.3 mol/L sodium acetate (Sigma, Saint Louis, USA); pH of 4 to 5 adjusted with glacial acetic acid (Merck, Darmstadt, Germany) and 0.05-0.1 mg/mL yeast RNA (BDH, Poole, England), water treated with diethylpyrocarbonate and sterilized (Sigma, Saint Louis, USA)) [9] to 150 μ L of human serum or plasma.

After vigorously mixing, 100 μ L of chloroform were added to each tube with shaking the mixture for 15 s, and centrifuged at 12 000 g for 15 min, at 23 °C. Then, the supernatant was collected and added to another tube containing isopropanol (Merck, Germany) in a proportion of 1:1. After homogenization by inversion, another centrifugation was carried out at 12000 g for 10 min and at 4 °C, giving rise to the RNA precipitate. A washing step of the precipitate was made with 75 % ethanol (Merck, Germany) and finally the precipitate was dried at 22-25 °C and resuspended in 20 μ L of ribonuclease-free water.

Modifications made in the extraction of the RNA

Selection of the sample volume

To select the volume of extraction of the serum or human plasma, we evaluated a negative control and four

positive samples at low concentrations of RNA of the HCV (2000, 500, 250 and 100 IU/mL), prepared from a secondary standard with 3.52×10^5 IU/mL. The RNA was extracted from two replicates of the negative control and from four replicates of each one of the positive samples, starting from different sample volumes: 150, 200 and 500 μ L.

Establishing the dilution of the internal standard (IS) in the RT-PCR assay

The RNA was isolated using four replicates of two negative controls and two replicates of two secondary standards: UMECONT-1 and UMECONT-2, with high viral loads, quantified by the Cobas Taq-Man HCV test v1.0 (Roche Diagnostics, Germany), resulting in 1.43×10^6 IU/mL and 3.52×10^5 IU/mL, respectively. A lot of the IS transcript (T-IS) was used as the internal quantification standard; it contained a sequence of the p64K protein from *Neisseria meningitidis* and from the same primer binding sites as the sequence of RNA from HCV, which was added to the lysis buffer in the dilutions of $1:4 \times 10^6$, $1:4 \times 10^7$ and $1:4 \times 10^8$ to determine the competing dilution.

Inverse-amplification transcription of the competitive RT-PCR

The synthesis of the complementing deoxyribonucleic acid through inverse transcription and its amplification by the polymerase chain reaction was made in the same vial using 25 μ L of a reaction mixture and 5 μ L of the isolated RNA, which had been resuspended in water free from RNases. The reaction mixture was standardized for a similar trial using the same primers [10], containing the buffer Go Taq; MgCl₂ at 1.5 mM, 250 μ M of each dNTP; 3.6 U of the inverse transcriptase AMV-RT, 7.5 U of the ribonuclease inhibitor RNasin and 1.05 U of the polymerase DNA Go Taq Flexi (all of which were from Promega, USA). The program for the amplification was the following: firstly, a step at 45 °C for 30 min, followed by a denaturation step at 95 °C for 3 min, 50 thermal cycles (95 °C, 30 s; 67 °C, 30 s; 72 °C, 30 s), and finally an extension step at 72 °C for 7 min. The RT-PCR was carried out in a thermocycler with a gradient (Eppendorf AG 22331, Germany) [11].

Modifications in the amplification step

Selection of the volume of the RT-PCR mixture and of the volume of purified RNA

The RNA was isolated using four replicates of the negative control and six replicates of four positive samples with low concentrations of RNA from the HCV (2000, 500, 250 and 100 IU/mL) prepared from the UMECONT-2. The extraction of the RNA from the HCV was made from a volume of the sample of human serum or plasma giving the best result in the trial, and in the RT-PCR the following combinations of the mixture of RT-PCR and purified RNA were assessed: (25 μ L of the mixture RT-PCR and 5 μ L of RNA; 20 μ L of the mixture RT-PCR and 10 μ L of RNA and 15 μ L of the mixture of RT-PCR and 15 μ L of RNA).

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Hybridization and detection of the amplified product through chemical denaturation

The qualitative detection of the amplified product was carried out through hybridization in the UMELOSA® (ultra micro-analytic enzyme-linked oligonucleotide-sorbent assay) format. The amplicons having the sequence of HCV and the internal standard (IS) were captured in duplicates on alternative strips of polystyrene ultramicroplates (Greiner Bio-One, Germany) coated with the primers: Q5 (5'NH₂-TCG TGC AGC CTC CAG GAC CCC TCC CGG 3') and S1 (5'NH₂-TAG TTG AAT TGA AAG TGC CCG ACA T 3'), respectively. Before application in the wells of the plates, we added the denaturalization solution containing NaOH in each test tube and after incubating for 10 min at room temperature (22-25 °C) the denaturalized amplified products were placed in the respective wells in the ultramicro-plate.

Later, the neutralization-hybridization solution formed by HCl, sodium salts and SDS [12] was applied and the plate was incubated for 1 h at 37 °C in a humid chamber. After that time, the microplate was washed and the conjugate of alkaline Streptavidine/Phosphatase, diluted at 1:500 000 was added; it was incubated at 37 °C in a humid chamber for 30 min and after washing the plate, the substrate 4-Metilumbeliferil phosphate was added. The fluorescence signals corresponding to the hydrolysis of the substrate by the conjugate bond to the biotinylated amplicons were detected by the plate reader PR-621 (TecnoSuma, CIE, Cuba).

Hybridization and detection of the amplified product using thermal denaturation

The qualitative detection of the amplified product using thermal denaturation was carried out according to the above description, but substituting the chemical denaturalization step. It was then added the neutralization-hybridization solution for the incubation of the amplified products at 95 °C for 10 min [13, 14] and the later inclusion of a combined hybridization solution with sterile purified water at the proportion of 1:1.

Modifications in hybridization and detection

Selection of the denaturation method of the amplified products

The RNA was isolated, starting from the four negative controls and the twenty replicates of a sample with 250 IU/mL of RNA of the HCV prepared from UMECONT-2. The extraction was made from 500 µL of the volume of the serum or human plasma sample and the amplification was done by RT-PCR according to the method described above, using 15 µL of the volume of the mixture of RT-PCR and 15 µL of the volume of isolated RNA. The hybridization and detection were carried out with chemical denaturation of the amplified products, with two replicates of the negative controls and ten replicates of a sample with 250 IU/mL of RNA of the HCV. Meanwhile, the hybridization and detection of the amplified products of the rest of the negative controls and the replicates of the sample with 250 IU/mL of RNA of the HCV was made using thermal denaturalization.

Evaluation of the analytical characteristics of the modified assay

For the evaluation of the analytical characteristics, HCV RNA was extracted from 500 µL of the volume of the sample of human serum or plasma and later amplified by RT-PCR using the combination of 15 µL of the sample of RT-PCR and 15 µL of purified RNA. The hybridization and detection of the amplified products was carried out using thermal denaturalization.

Clinical specificity

In order to validate clinical specificity of the new trial, we evaluated 146 samples that were seronegative to UMELOSA HCV and negative to QUALITATIVE UMELOSA HCV. Clinical specificity was calculated using the following equation:

$$\text{Clinical specificity (\%)} = \frac{\text{Negative samples}}{\text{True negative samples}} \times 100$$

Where the true negative samples are the negative samples plus the false negative samples, established by a reference method.

Analytical specificity

A test was made on five positive samples to the human immunodeficiency virus type 1 (HIV-1) and 2 (HIV-2), five positive samples to Chagas disease and five positive samples to the human hepatitis B virus.

Evaluation of sensitivity using an international standard of HCV RNA

The sensitivity of the modified trial was evaluated using the international standard (IS) (06/100), genotype 1a, with the concentration of 154 881 IU/mL of RNA of the HCV, from which the following concentrations were prepared: 200, 100, 50, 25, 12.5 and 6.25 IU/mL. The replicates were tested on different days and by different operators. The RNA was isolated with 24 replicates of each one of these concentrations. In this study, a sample was regarded as positive when the fluorescence value was above the mean of the negative control plus three times its standard deviation. The PROBIT analysis [15] was carried out to determine the detection limit of the assay [16].

Evaluation of genotypic specificity

The RNA was isolated from four replicates of genotypes 1 to 6 of the Genotyping Panel 08/264 of NIB-SC, with a concentration range of 500 to 1500 IU/mL.

Robustness

Alternately, 22 samples (11 negative and 11 positive samples) to the RNA of the HCV were evaluated to test the capacity of the test in preventing crossed contaminations. The positive samples with a concentration of 10 000 IU/mL were prepared from a secondary standard.

Evaluation of the capacity of the trial to detect inhibitory samples of RT-PCR

Several inhibition tests were designed, taking into account reports from the literature, to demonstrate that the modified test is able to detect the presence of inhibitory substances of the PCR. Several types of inhibitors were tested, such as: organic solvents

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(acetone, ethanol and isopropanol), salts (sodium acetate and sodium chloride) and hemolyzed blood, by freezing-thawing. In each test we included a negative control and a positive control to 7500 IU/mL of HCV RNA. Each inhibition variant was tested by triplicate. Total inhibition was considered when the fluorescence signals of HCV and IS were below 20 fluorescence units. The extraction of the RNA of the HCV was made from 500 μ L of the sample of serum or human plasma with the following features: volumes of 0.7; 1.0; 1.5; 3.0 and 5.0 μ L of the organic solvents: acetone, ethanol and isopropanol, were added to the 20 μ L of water in which the extracted RNA was resuspended. The saline solutions of sodium acetate at 0.006; 0.01; 0.02 and 0.04 mol/L and of sodium chloride at 0.03; 0.06 and 0.12 mol/L were evaluated by resuspending the RNA in 20 μ L of these solutions instead of using water free from ribonucleases. The hemolyzed blood used in this study was obtained by freezing-thawing of a sample of human blood that was negative to HCV, HIV and HBV. It was used as a diluent to prepare a sample that is positive to 7500 IU/mL of RNA of the HCV.

Comparison between modified QUALITATIVE HCV UMELOSA and QUALITATIVE HCV UMELOSA

RNA was extracted from 483 samples: 146 negative samples and 337 positive samples through the QUALITATIVE HCV UMELOSA using 150 μ L and the amplification was carried out by RT-PCR as described in the QUALITATIVE HCV UMELOSA kit, v. 2, 2012) [12], using 25 μ L of a reaction mixture of RT-PCR and 5 μ L of the purified RNA. The extraction of the HCV RNA using the new variant of the QUALITATIVE HCV UMELOSA test was made with the same samples and using 500 μ L of the volume of the human serum or plasma sample. The amplification through RT-PCR was carried out using 15 μ L of the RT-PCR mixture and 15 μ L of purified RNA. The hybridization and detection of the amplified products was carried out using thermal denaturalization. The percentage of coincidence between both tests was also calculated.

Statistical analysis

In the experiment made to determine the sensitivity of the modified test with the international standard (06-100), the statistical package MINITAB Release 14 (Minitab Inc. Pennsylvania, USA.) and the PROBIT analysis [15] were used to determine the detection limit at 95 % and its confidence interval [15].

In the experiment to select between the thermal and chemical denaturation, the means were compared using the Student's *t* test. The differences observed between the means were estimated at a 95 % confidence interval (CI), with a significance level (*p*) of 0.05, all these determined with the statistical package STATISTICA 6.0 (StatSoft Inc., 2001, Oklahoma, USA).

Results and discussion

Modifications in the RNA extraction

Selection of the sample volume

As observed in table 1, the volume of the sample for which the best percentages of positivity were obtained for all concentrations of RNA of the HCV evaluated

Table 1. Percentages of positivity obtained from different volumes and concentrations of samples that are positive to the RNA of the HCV by the QUALITATIVE HCV UMELOSA test

RNA concentration (IU/mL)	Positivity percentage per sample volume		
	150 μ L	200 μ L	500 μ L
2000	100	100	100
500	50	100	100
250	50	100	100
100	0	0	100

was 500 μ L. Both the volume of the sample used in the QUALITATIVE HCV UMELOSA test (150 μ L) and 200 μ L of the sample did not enable the detection of the lowest concentration of RNA of the HCV evaluated (100 IU/mL), which coincides with the detection limit of the QUALITATIVE HCV UMELOSA test using a secondary standard [17]. In all cases, the replicates of the negative control gave signs of fluorescence of the negative HCV.

Establishing the competition dilution of the IS in the RT-PCR assay

As shown in table 2, the optimum dilution of the T-IS as an internal standard was $1:4 \times 10^6$ since with this standard the highest fluorescence signals were obtained for the internal standard of the negative controls. This makes it possible for the trial to readily detect the presence of inhibitions before which the fluorescence signal corresponding to the internal standard would fall.

Furthermore, with the dilution of $1:4 \times 10^6$ of the T-IS as an internal standard, the high fluorescence signals of the HCV were obtained for the positive controls, which are values of interest for both controls (data not shown).

A dilution of less than T-IS was not evaluated because higher concentrations of the internal standard favor the fluorescence signal of the IS and significantly decrease the fluorescence signal of the HCV for positive samples.

Modifications in the amplification

Selection of the volume of the RT-PCR mixture and the volume of the purified RNA

As observed in table 3, the best combination was that of 15 μ L of the mixture of RT-PCR and 15 μ L of purified RNA since, with this combination, 100 % positivity was obtained for all concentrations evaluated.

Modifications in the hybridization and detection

Selection of the denaturation method of the amplified products

The value of the mean fluorescence corresponding to the replicates of 250 IU/mL that are thermally denatured, was significantly higher than the one

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Table 2. Selection of the competing dilution of the transcript T-IS 0309-1 corresponding to the internal standard used to validate the modified QUALITATIVE HCV UMELOSA test

Negative control	Dilution of the T-IS-0309-1					
	$1:4 \times 10^6$		$1:4 \times 10^7$		$1:4 \times 10^8$	
	FU HCV \pm SD	FU IS \pm SD	FU HCV \pm SD	FU IS \pm SD	FU HCV \pm SD	FU IS \pm SD
1	8.8 \pm 1.3	120.1 \pm 12.4	4.6 \pm 0.6	87.4 \pm 24.2	5.1 \pm 0.7	5.7 \pm 0.7
2	8.7 \pm 2.7	115.8 \pm 11.0	8.7 \pm 4.7	70.1 \pm 48.1	4.3 \pm 0.3	4.5 \pm 0.4

FU HCV: mean units of fluorescence of the wells coated with the HCV primer; FU IS: Mean fluorescence units of the wells coated with the internal standard (IS) primer; SD: standard deviation of the mean.

Table 3. Percentages of positive samples using different combinations of volumes of amplification mixtures and of purified RNA, during the modification of the QUALITATIVE HCV UMELOSA test

RNA concentration (IU/mL)	Positivity percentage of volume of RT-PCR mixture plus volume of purified RNA (μ L)		
	25 + 5	20 + 10	15 + 15
2000	100	100	100
500	66.6	100	100
250	33.3	100	100
100	66.6	66.6	100

corresponding to chemically denatured replicates ($p < 0.05$) and the confidence interval for the difference of the means does not contain the zero value and at the same time it is positive (Table 4).

The best variant selected was thermal denaturalization, because it detected 100 % of the ten replicates of the sample with 250 IU/mL of RNA of the HCV as observed in table 4. Furthermore, this method has the advantage of not requiring the addition of denaturation and neutralization solutions, with which there is a saving in time, reagents and others.

Clinical specificity

The clinical specificity of a TAN (technical amplification of nuclear acids) test consists of its capacity to determine unequivocally the presence of a specific DNA or RNA sequence within a large variety of other sequences. It depends, first of all, on the selection of the primers for the detection of the amplified product and the conditions of the amplification and detection processes. It is also defined as a proportion of the negative samples (non-reactive) that are correctly identified by the test used.

All samples evaluated were negative according to the MODIFIED QUALITATIVE HCV UMELOSA, obtaining 100 % clinical specificity for the new test.

Analytical specificity

On studying the analytical specificity of the MODIFIED QUALITATIVE HCV UMELOSA, all samples gave negative results. This is possible because the specificity of the PCR is based on the correct binding of the primer to the DNA target and in the ability of the polymerase to appropriately replicate the selected region.

Evaluation of the sensitivity using an international HCV RNA standard

In all cases, the expected results were obtained for the positive control and the negative control included in each setting (data not shown). The results obtained for each concentration of the IS (International Standard) evaluated are shown on table 5.

The PROBIT analysis showed that the 95 % detection limit was at 72.31 IU/mL, with a CI of 52.31 to 137.75 IU/mL (Table 6).

The sensitivity obtained with the IS (72.31 IU/mL) is better than that reported for the QUALITATIVE HCV UMELOSA with the IS (200 IU/mL) [17]. The differences may be due to the fact that both studies for sensitivity were carried out with different amounts of replicates, the international standards used do not have the same genotypes, or due to the changes made in the extraction, amplification and detection methods in the modified QUALITATIVE HCV UMELOSA.

Table 4. Comparison of positivity and fluorescence parameters of two denaturation methods of the amplified products of a sample ($n = 10$) with 250 IU/mL of RNA of the HCV, for the modification of the QUALITATIVE HCV UMELOSA test

Parameters	Positivity (%)	FU HCV \pm SD	FU IS \pm SD
Chemical denaturation	55	33.61 \pm 7.44	130.31 \pm 21.60
Thermal denaturation	100	44.09 \pm 11.30	112.10 \pm 19.32
p (Student's t test)	ND	0.001	ND
Dif. (CI 95 %)	ND	7.0; 21.7	ND

FU HCV: Mean fluorescence units of the wells coated with the HCV primer; FU IS: Mean fluorescence units of the wells coated with the internal standard (IS) primer; SD: standard deviation of the mean; p: probability of the hypothesis test; Dif: Difference. CI 95 %: 95 % confidence interval. ND: not determined.

Table 5. Sensitivity of the modified QUALITATIVE HCV UMELOSA test, using different concentrations of the World Health Organization (WHO) international standard for HCV RNA (internal standard, IS)

Concentration of IS RNA (IU/mL)	Replicates	Positive results	Positive results (%)
200	24	24	100
100	24	24	100
50	24	19	79.16
25	24	20	83.33
12.5	24	15	62.5
6.25	24	12	50

Table 6. Results of the PROBIT analysis run to determine the sensitivity of the modified QUALITATIVE HCV UMELOSA test

Percentage	Percentile	Error	Lower value	Upper value
1	-102.7330	36.7449	-266.4110	-56.3193
2	-90.7170	33.3419	-238.9970	-48.5337
3	-83.0933	31.1886	-221.6150	-43.5826
4	-77.3582	29.5723	-208.5470	-39.8508
5	-72.6932	28.2604	-197.9220	-36.8099
6	-68.7226	27.1459	-188.8830	-34.2171
7	-65.2411	26.1706	-180.9620	-31.9400
8	-62.1238	25.2990	-173.8720	-29.8978
9	-59.2888	24.5079	-167.4280	-28.0374
10	-56.6791	23.7810	-161.4990	-26.3220
20	-37.2874	18.4380	-117.557	-13.4576
30	-23.3045	14.6917	-86.0917	-3.9619
40	-11.3567	11.6354	-59.5146	4.4608
50	-0.1893	9.0274	-35.2348	12.8945
60	10.9780	6.9429	-12.2711	22.6442
70	22.9259	5.9496	8.5716	36.8013
80	36.9087	7.0962	25.2088	61.1252
90	56.3005	11.1603	41.0094	102.1310
91	58.9101	11.8016	42.9067	107.8780
92	61.7452	12.5120	44.9374	114.1520
93	64.8624	13.3070	47.1400	121.0810
94	68.3439	14.2091	49.5693	128.8500
95	72.3146	15.2529	52.3079	137.7430
96	76.9796	16.4957	55.4905	148.2260
97	82.7146	18.0428	59.3632	161.1540
98	90.3384	20.1242	64.4598	178.3900
99	102.3540	23.4453	72.4098	205.6390

FDA demands a sensitivity or limit of detection (LD) of 95 % of 5000 IU/mL [16] for the screening of the HCV in individual blood bags, while to evaluate mixtures of plasma in blood banks or for the use of hemoderivatives in Europe, a minimum sensitivity of

100 IU/mL or less is required [18, 19]. The sensitivity of the modified test (72.31 IU/mL) complies with the requirements of international regulations for the screening of blood or plasma bags for hemoderivatives, as long as the number of bags to be mixed is regulated according to the sensitivity of the trial. In our case, the mixtures would be of 50 bags, in theory, but in practice it has been demonstrated that it is best to work with mixtures of 24 bags, thus minimizing the risk that a positive bag, with a very low concentration, may escape the screening [20]. On evaluating the IS (Table 5), it is observed that 100 IU/mL (n = 24) are detected 100 % of the times.

At present, the producers of qualitative clinical trials compete to reach lower detection limits. Examples of this are the tests produced by Roche Molecular Systems (Pleasanton, USA), the leader in this market being AMPLICOR® HCV Test v 2.0, having a LD at 95 % of 60 IU/mL in the serum and of 50 IU/mL in the plasma [21]; the COBAS AMPLICOR® HCV Test v 2.0, with 60 IU/mL in the serum and 50 IU/mL in the plasma [22], and the COBAS Ampliprep/Cobas Taqman HCV Test (although it has another type of assay, real time PCR, and it is designed specifically for screening in blood banks) with 28 IU/mL [23]. These very low detection levels are not only for the earlier detection of a patient during the window phase (the acute phase of the disease), detecting the patient during screening in blood Banks, therefore declining to zero the transmission by this route, or adequately monitoring the treatment. They are also established to assure a very profitable market which is almost exclusive for the large diagnostic agents producing companies in the world (Roche, Siemens and Abbott), which makes it unaffordable for a developing country to use commercial assays having this 'quality' for the free of charge diagnosis and follow-up of the treatment in its population.

Evaluation of genotypic specificity

With the modifications made on the QUALITATIVE HCV UMELOSA, all the genotypes from 1 to 6 from the Genotyping Panel 08/264 (NIBSC) with a concentration range of 500 to 1500 IU/mL (four replicates of each genotype were detected at all times (100 %) Genotyping panel 08/264 (NIBSC). These results are in agreement with those obtained in the genotypic sensitivity study made on the QUANTITATIVE HCV UMELOSA having a similar format to ours, in which the six genotypes of the Genotyping panel 02/202 were detected at 1000 IU/mL in 100 % of the tests [9].

Recent studies carried out on Cuban patients with chronic HCV show that the subtype 1b of HCV is prevalent in Cuba and the subtype 1a has a low prevalence [24]. In that study, based on the analysis of the sequence of the region coding for the capsid protein of HCV, the viral genotype was determined in 58 Cuban patients who were positive for HCV RNA. Here we obtained a prevalence of 94.8 % for subtype 1b and of 5.2 % for subtype 1a. This study also revealed the mutability of subtype 1b in Cuba, making it prevalent, since this allows for its fast propagation, producing a negative impact on antiviral therapy in our country [24]. In another study carried out by Roca *et al.*, subtype 1b also had the highest prevalence in 33 Cuban

patients studied, constituting 42.4 %; subtypes 1a and 2b showed moderate prevalence (9.09 %) and subtype 3a was found in 35 % of Cuban patients and it was always found as a co infection with subtype 1b [25]. In 2012, another study carried out in 79 Cuban patients with HCV was published; 10 of them (12 %) had genotype 1a and 69 had genotype 1b (82 %). Five patients (6 %) had combined infections, among which two (2 %) had genotype 1a/3a and three had genotype 1b/3a, 1b/4a and 1a/1b/3a, respectively [26].

Robustness

The robustness of an analytical procedure offers a measure of its confidence for routine use. There was no cross contamination between the positive samples with high viral loads and the analyzed negative samples. All negative samples and the negative control of the trial were negative in the test, while the positive sample and the positive control were positive. Similar results were obtained with the QUALITATIVE HCV UMELOSA when evaluating fourteen alternate samples, seven were positive with high viral load and seven were negative, to test the capacity of the trial avoiding crossed contamination [17].

Evaluation of the capacity of the test to detect samples that inhibit RT-PCR

The samples can contain inhibitors such as hemoglobin, cloth, tissues and soils. Other important sources of inhibitors are the materials and reagents that get into contact with the sample during its processing. These include excess KCl, NaCl and other salts, and organic solvents such as ethanol, phenol and isopropanol.

Phenol at a concentration higher than 0.2 % inhibits the PCR reaction, and ethanol, isopropanol and acetone at concentrations higher than 1 %. Certain saline solutions such as sodium acetate at above 5 mmol/L and sodium chloride at above 25 mmol/L are also inhibitory [27].

As shown in table 7, when 1 µL of isopropanol and 5 µL of acetone and ethanol were added, a total inhibition of the PCR was produced (threshold set to less than 20 FU). Our test was more sensitive to the presence of small volumes of residual isopropanol. When using saline solutions, sodium acetate produced complete inhibition at 0.04 mol/L and sodium chloride at 0.12 mol/L. The use of hemolyzed blood by freezing-thawing also produced the complete inhibition of PCR (Table 7).

It is very important to detect inhibition to avoid false negative results. For this, we introduced an IS in the lysis buffer, to detect the inhibitions due to the fall of the fluorescence signals both of HCV and IS.

PCR is extremely sensitive and reproducible when analyzing samples of pure DNA. Nevertheless, the efficiency of amplification and the detection limit are frequently altered by the interference of different molecules with DNA polymerase, or with the nucleotides and nucleic acids.

The interactions between the proteins and DNA polymerase are the main cause of PCR inhibitions. The human blood contains more proteins than DNA; many of these proteins interfere with the polymerization process that occurs in the PCR. An example of

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Table 7. Inhibitory effect of organic solvents, salts and hemolyzed blood on the modified QUALITATIVE HCV UMELOSA test

Salts					Solvents								
Sample	Sodium acetate (mol/L)				Sample	Acetone (μ L)					Sample	Isopropanol (μ L)	
	0.006	0.01	0.02	0.04		0.7	1.0	1.5	3.0	5.0		0.7	1.0
HCV	209.10	176.60	24.00	19.70	HCV	210.00	210.00	210.00	210.00	3.90	HCV	93.50	16.90
IS	5.20	6.90	24.20	19.20	IS	97.90	160.70	150.10	5.50	3.60	IS	64.90	3.70
Sample	Sodium chloride (mol/L)			Sample	Ethanol (μ L)					Sample	Hemolyzed blood		
	0.03	0.06	0.12		0.7	1.0	1.5	3.0	5.0		HCV	IS	
HCV	209.60	191.90	5.60	HCV	188.30	174.20	140.30	73.10	3.70	HCV	5.50		
IS	4.50	4.10	5.40	IS	25.30	13.28	10.20	3.90	3.80	IS	12.60		

FU HCV: Mean fluorescence units of the wells coated with the HCV primer; FU IS, Mean fluorescence units of the wells coated with the internal standard (IS) primer. Each result represents the mean of three replicates. Values in grey boxes indicate the total inhibition for each substance (threshold set to less than 20 FU).

this is the Heme group, which acts as a human blood inhibitor because it frees the iron ions affecting the ionic balance and altering polymerase activity [28]. Isopropanol, ethanol and acetone produce the precipitation of nucleic acid, which does not favor its replication because the probability of interaction of the replicative machinery with nucleic acids is affected. The K^+ ions are frequently used in the PCR regulating solutions to ensure the appropriate ionic environment. Changing the K^+ content or introducing other ions such as those of Na^+ may produce inhibition [28].

Comparison of modified QUALITATIVE HCV UMELOSA and QUALITATIVE HCV UMELOSA

A complete agreement (100 %) was obtained between the results of both tests. A similar result was achieved on comparing QUALITATIVE HCV UMELOSA with the AMPLICOR HCV Test, v 2.0, which showed a 100 % agreement between both tests [17].

The new standardized test (modified QUALITATIVE HCV UMELOSA) was able to reduce in 3 h the assay period compared to QUALITATIVE HCV

UMELOSA, because it reduces hybridization time to 1 h and uses only one round of PCR. Modified QUALITATIVE HCV UMELOSA showed better sensitivity than QUALITATIVE HCV UMELOSA, while preserving its analytical characteristics. Furthermore, it uses lower amounts of reagents than the current test, thereby reducing its production cost. It also has the advantage of detecting samples that inhibit PCR, thereby preventing false negative results, and, on the other hand, the probability of obtaining false positives is decreased on eliminating the second amplification round, making it possible for the results to be issued in a more reliable and rapid manner. Both changes, from the social viewpoint, offer a better care for the patient and economic benefits because they decrease the production cost of the kit and the costs to the Cuban health system.

In summary, with this optimized test, under sustainable production, which is available for our national health programs, a very valuable working tool is available that has the sensitivity and quality to comply with the internationally established requirements for TAN assays.

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