Research Article **Open Access**

Molecular Study of Human HCV RNA by Real Time-Polymerase Chain Reaction, Viral Kit and Robogene Quantification

Muhammad Zahoor ul Hassan Dogar[1,2], Taha Nazir[1,3]*, Humaira Akram[1], Alia Nasir-ud-Din[4] and Muhammad Farooq[5]

[1]*Biochemistry, Chemical Pathology, Molecular Biology Research Group, University of Sargodha, Pakistan*
[2]*Dean FMHS and Chairman Biochemistry Department SMC, University of Sargodha, Pakistan*
[3]*Scientific Executive, ICDTD Inc., Canada*
[4]*Fatima Jinnah Medical College, Shara-I-Fatima Jinnah, Queens Road, Lahore Pakistan*
[5]*Arqam Laboratory, Ibn-e-Sina Hospital, Sargodha, Pakistan*

Abstract

Hepatitis C virus (HCV) is one of the major causes of liver cirrhosis. It is highly mutable, circulates heterogeneously and potentially connected with diverse clinical phenotypes. Thus, we aim this study and collected blood samples from 183 HCV suspicious patients of age 31-58 years during March, 2013 to December, 2014. The prepared specimens were investigated for HCV RNA quantification by RT-PCR Robogene; isolation technique "Internal Virus Kit" (AJ Roboscreen, Germany). The subsequent samples were drawn after 4, 12 and 24 weeks. The reference range(s) of <15 IU/mL and <1.18 Log IU/mL were used to monitor therapy and/or disease progression. Levene's test for Equality of Variances, t-test for Equality of Means and Fisher's Exact tests were used for categorical variables to study the variance and measured association among variables. The specimens were collected from 183 HCV suspicious patients, age 31-58 years (Mean 44.83, SD 8.062). After amplification of the extracted RNA by RT- PCR using Bio-Rad's CFX 96 Machine, 68 patients (37.15%) were identified/ diagnosed as HCV positive, 108 (59.02%) found negative and 7 (3.83%) patients did not responded to therapy of peginterferon and ribavirin. Paired samples statistical analysis showed significant variance in the mean values of HCV RNA quantification (IU/ ml) before therapy and at wk 12 (p value 0.055); before therapy and at wk 24 (P value 0.053). however, there is no significant variance seen Before therapy and at wk 4 (P value 0.082); at wk 4 and wk 12 (P value 0.106); at wk 4 and wk 24 (P value 0.101) and at wk 12 and wk 24 (P value 0.118). The quantification of human HCV RNA by RT-PCR, internal viral kit and Robogene quantification can potential be used to to rationalize the treatment, enhance antiviral responses and mitigate mortalities because of HCV RNA.

Introduction

HCV belongs to the Flaviviridae family, bearing approximately 10 kb long positive sense ssRNA genome. Mostly, the acute, chronic, or resolved infection can't be differentiated by anti-HCV testing. Therefore, supplementary tests of anti-HCV immunoglobulin G activity index, reactivities of antibody to specific HCV component and identification of nonstructural proteins [1] are needed to confirm an anti-HCV positive result [2]. HCV RNA have high grade of genetic heterogeneity [3]. That allows the viral geneticist to classify it into distinct groups i.e. quasispecies genotypes, subtypes and isolates. Six major genotypes and >50 subtypes have been successfully identified. There is 31 to 34% genotypic difference in their nucleotide sequences, with 20 to 23% difference in their full-length genomic sequences. This extensive genetic heterogeneity and tendency for mutation has hindered the vaccine development [4]. Moreover, the infected patients respond in variety of the way because of different genotypes. The antiviral drug therapy designed on basis of identified infecting genotype is inevitable to guide the correct dose and duration of current combination therapy (pegylated alpha interferon plus ribavirin) [5]. The most detected HCV RNA 3a genotype especially in Pakistan [6]. It is important to amplify and quantify the genotypes of HCV RNA regarding the decision of the HCV related chronic hepatitis treatment and evaluate majority of the post-transfusion and sporadic hepatitis. We can avoid the substantial population of acute HCV infected patients to develop chronic illness associated with a high risk of liver cirrhosis and hepatocellular carcinoma. Hence, the quantification of HCV RNA by traditional molecular methods is unreliable. Northern blot and PCR products on a gel used to detect the mRNA. We run < 20-40 cycles of a tradition PCR to discover the quantity of DNA product that is not correlated with the quantity of target DNA. However; the nucleic acids can be analyzed quantitatively by two PCR methods; absolute and relative quantification [7]. We can compare the DNA standards using a calibration curve to get absolute quantification that provide an exact amount of target DNA molecules. Hence, the sample and standard should have the same amplification efficiency. Internal reference genes are correlated with the relative quantification to determine differences in target gene expression. The quantification expression is the modification in mRNA that is complementary DNA and interpreted as cDNA, produced by mRNA reverse transcriptase. Relative quantification does not require calibration curve and comparatively easier to carry.

The results of relative quantification can be compared with a number of different RT PCR expressions. The quantitative and qualitative differences in of HCV RNA can affect the whole PCR process and efficiency of reverse transcription. Therefore, the maintenance genes used to correct the non specific variations. Therefore, the stabilization of reference gene is most crucial aspect of the experiment [8].

*Corresponding author: Taha Nazir, Research Associate, Biochemistry, chemical pathology and Molecular Biology Research Group, University Medical & Diagnostic Centre, University of Sargodha, Pakistan, Tel: +92 321 222 0885; E-mail: tahanazir@yahoo.com

Moreover, the northern blot densitometry, visual examination of RNA gels, and starting/ semi quantitative PCR used for qualitative or semi-quantitative studies. The DNA microarrays can also be used for detailed examination of many organisms [9]. But, the quantification expression of mRNA varies in different experimental conditions that are evident by amplification of the majority of reference genes [10,11]. Therefore, an expert statistical review of overall methodology is necessary to obtain the reliable scientific result to select an appropriate reference gene [12]. There are certain statistical methods developed to establish a correlation and detect suitable gene under specified conditions. The geometric means can be compared by BestKeeper or geNORM to study different tissues and reference genes [13,14].

Material and Method

This study was conducted in Biochemistry, Chemical Pathology & Molecular Biology Research Lab, University Medical Complex & Research Centre (UMCRC), University of Sargodha and Arqam Lab, Ibne Sina Hospital, Sargodha Pakistan. The blood samples were drawn from median cubital or cephalic vein of 183 HCV suspicious patients of age 31-58 years during March, 2013 to December, 2014. The prepared specimens were then investigated for HCV RNA genotyping and quantification by RT-PCR Robogene; isolation technique "Internal Virus Kit" (AJ Roboscreen, Germany). 68 patients were identified/ diagnosed as HCV positive. The subsequent samples were drawn after four (Rapid Virologic Response -RVR) and 12 (early virologic response - EVR) and post therapy (after 24 weeks). The extracted RNA amplified by RT- PCR using Bio-Rad's CFX 96 Machine to see the rapid & early virologic response and after therapy results.

Patient preparation

Patient's selection criterion is as under;

1. Not be on Heparin therapy,

2. Diagnosed HCV RNA genotype 3a

3. Using the therapy plan of peginterferon and ribavirin.

Preferred specimen(s)

2 (1.5 mL) plasma collected in EDTA (lavender-top) tubes

Minimum volume

2 (0.4 mL)

Alternative specimen(s)

Serum • Plasma collected in: PPT potassium (white-top) tubes

Collection instructions

Separate plasma from whole blood within 24 hours of collection by centrifugation at 800 to 1600 x g for 20 minutes at room temperature. Transfer to a plastic screw-cap vial and freeze or refrigerate.

Specimen container

Plastic screw-cap vials

Transport temperature

Refrigerated (cold packs)

Specimen stability

Room temperature: 72 hours

Refrigerated: 14 days

Frozen: 42 days

Reject criteria

Unspun Serum Separator Tube (SST®) or red top tube, Unspun PPT tube

Methodology

Real-Time Polymerase Chain Reaction (RT-PCR)

Performing laboratory

1. Biochemistry, Chemical Pathology & Molecular Biology Research Lab, University Medical Complex & Research Centre (UMCRC), University of Sargodha, Sargodha 40100 Pakistan.

2. Arqem Laboratories, Ibne Sina Hospital, Sargodha Pakistan.

Reference range(s)

HCV RNA, Quantitative, PCR   <15 IU/mL

HCV RNA, Quantitative, PCR   <1.18 Log IU/mL

This test was performed using the "Internal Virus Kit" (AJ Roboscreen, Germany). The extracted RNA was then amplified by RT-PCR using Bio-Rad's CFX 96 Machine.

Clinical significance

Useful in monitoring therapy and/or disease progression. Reportable range is 15 to 100,000,000 IU/mL (1.18-8.00 Log IU/mL).

Clinical specimens and viral standards

Blood samples of candidate patients were collected, processed and centrifuged. The purified serum is used to extract the HCV RNA according to the instructions of Bio-Rad's CFX 96. These were then transferred to the Laboratory of molecular virology for further molecular and serological investigations or kept frozen. This study was approved by the institutional on human research publication and ethics. Working reagents for nucleic acid amplification techniques were used to establish the analytical sensitivity of the RT PCR. These calibrated reagents contained standard reference of HCV genotype 3a isolate.

Viral RNA isolation from body fluids

We open the extraction tube and add 450 μL Lysis Solution RL. Add 150 μL of the sample and mix vigorously by pulsed vortexing for 10 second. It was then incubated at room temperature for 15 minutes. The shaking plateform was used for continuous shaking of the sample. Alternatively, the sample was vortexed 3-4 times during incubation. Shaking did not reduce the lysis efficiency. After lysis, the Extraction Tube centrifuged shortly to remove condensate from the lid of the tube. Then we added 600 μL Binding Solution RBS to the lysed sample. That was mixed by vortexing or pipetting up and down several times. It was important that the sample and the Binding Solution RBS were mixed vigorously to get a homogenous solution. Then; 650 μL of sample was applied to the Spin Filter located in a 2 ml Receiver Tube. The cap was closed and centrifuged at 10,000 x g (12,000 rpm) for 1 minute. If the solution has not completely passed through the Spin Filter, we centrifuged it again at higher speed or prolong the centrifuge time. The Receiver Tube was discarded with the filtrate and the Spin Filter placed into a new 2 ml receiver tube. The Spin Filter was opened and added 500

μL Washing Solution HS. The cap was then closed and centrifuged at 10,000 x g (12,000 rpm) for 1 minute. The Receiver Tube discarded with the filtrate. The spin Filter placed into a new 2 ml Receiver Tube. The Spin Filter was opened again to add 650 μL Washing Solution LS. The cap is closed and centrifuged at 10,000 x g (12,000 rpm) for 1 minute. The Receiver Tube discarded with the filtrate and Spin Filter placed into a new 2 ml Receiver Tube. It was then centrifuged at maximum speed for 2 minute to remove all traces of ethanol. 2 ml Receiver Tube Discarded. We then place the Spin Filter into a 1.5 ml Elution Tube. The cap of the Spin Filter was opened carefully to add 30 ml RNase-free water. It was then incubated at room temperature for 2 minutes. Centrifuge at 6,000 x g (8000 rpm) for 1 minute. Add addition 30 μL RNase-free water to the Spin Filter and centrifuge again at 6,000 x g (8,000 rpm) for 1 minute. Discard the Spin Filter. Vortex the Elution Tube containing the combined eluates and centrifuge it shortly. The eluate contains the purified RNA. RNA is highly susceptible to degradation. Therefore, after the extraction the elution tube it was placed immediately on ice or make sure that the purified RNA is processed by the PCR PCR amplification within 20 minutes. For long time storage freeze the nucleic acid at -30º to -80ºC.

RT-PCR enzyme activity; pipetting steps

We have maintained the full activity of RT-PCR enzyme to perform all pipetting steps on ice or on an ice cold PCR rack. 25 x Reagent mix (HCV D4.25x) was prepared. 40 μL PCR grade water added to the vial containing lyophilized Reagent mix (HCV D4). The tub was closed and incubated at 37 ºC for 20 minutes; mixed by vortexing for 3 seconds and centrifuged for 5 second at full speed. Reagent mix dissolved and stored at 2-8 ºC. It was protected from light up to 30 days and avoided from freezing.

HCV RNA detection and quantitative PCR

HCV RNA was isolated form patient serum by "Internal Virus Kit" (AJ Roboscreen, Germany). Extracted RNA was amplified by "Robogene HCV RNA Quantification Kit" (AJ Roboscreen, Germany), and detected in Real Time using Bio-Rad's CFX 96 Real Time PCR Machine. Fluorophore – labeled oligonucleotide probes are used in Real Time PCR which binds specifically to the amplified product. Monitoring the fluorescence intensities during the PCR run allows the detection & quantification of the accumulating product without having to re-open the reaction tubes after the PCR run which minimizes the risk of cross contamination as compared to conventional PCR. Synthetic internal control was co-purified and amplified along with target, which excludes possibility of False Negative Results due to failed extraction or inhibition. Quantification standards were used for absolute quantification.

Robogene quantification analysis

The RoboGene® HCV RNA Quantification Kit is intended for real-time quantification of Hepatitis C Virus (HCV) RNA in human plasma or serum samples. The level of HCV RNA in serum and plasma can be used in conjunction with other clinical markers and clinical findings to distinguish between acute and chronic HCV infection and to assess the viral response to antiviral treatment.

During sample preparation a synthetic internal control is included via Extraction tubes RNA_D1 to control RNA extraction and to indicate for inhibitory effect on detection. Quantitation standard consists of 8 tubes coated with given amounts of synthetic HCV RNA, which must be amplified in parallel. Amplification of HCV RNA in samples and standards and of IC RNA is measured independently at different wavelengths due to probes labelling with different fluorescence reporter dyes.

Specifications

• Starting material: RNA from human blood or tissue samples

• Detection time: Standard qPCR cycler (e.g. TOptical, Rotor-Gene) approx. 3 hours

Analytical and diagnostic specificity

The analytical specificity was evaluated by analyzing 12 non-HCV positive specimens. Furthermore, 105 plasma samples from blood donors which have been tested negative for HCV RNA using the CobasTaqMan HCV kit were analysed to determine the diagnostic specificity. The RoboGene® HCV RNA Quantification Kit had a perfect analytical and diagnostic specificity. None of the analyzed samples gave positive test results for HCV RNA [14].

Prime PCR runs

We used the Prime PCR runs pathway or disease-specific assays. That were wet-lab validated and optimized from Bio-Rad. Pre-plated panels plates contain assays were specific for a biological pathway or disease. Custom configured plates set up in a user; define the layout with the option to choose assays for targets of interest, controls, and references. Individual assays tubes contain individual primer sets for use in real-time reactions. A Prime PCR runs from the Recent Runs list of the repeat run on the startup wizard.

Statistical analysis

The data was analyzed and the summary statistic was carried out by a statistical package, SPSS version 10.0 for window. Levene's test for Equality of Variances, t-test for Equality of Means and Fisher's Exact tests were used for categorical variables to study the variance and measured association among variables. All data presented as mean, %age or numbered values. P-values less than 0.05 were considered significant.

Result

The specimens were collected from 183 HCV suspicious patients, age 31-58 years (Mean 44.83; SD 8.062). After amplification of the extracted RNA by RT- PCR using Bio-Rad's CFX 96 Machine, 68 patients (37.15%) were identified/ diagnosed as HCV positive, 108 (59.02%) found negative and 7 (3.83%) patients did not responded to therapy of peginterferon and ribavirin (Table 1). The subsequent samples were drawn after 4, 12 and 24 weeks to monitor the RVR, EVR and DVR respectively.

P value ≤0.05 is considered statistical significant

Paired samples statistical analysis showed significant variance in the mean values of HCV RNA quantification (IU/ ml) before therapy and at wk 12 (p value 0.055); before therapy and at wk 24 (P value 0.053). However, there is no significant variance seen before therapy and at wk 4 (P value 0.082); at wk 4 and wk 12 (P value 0.106); at wk 4 and wk 24 (P value 0.101) and at wk 12 and wk 24 (P value 0.118) (Tables 2-5).

Discussion

Investigation of HCV-RNA of patients under chemotherapy provided information to evaluate the treatment response. That potentially helps the clinical virologists to select the correct drug regime, protocol and combinations to eradicate the virulent genome.

	Total	**HCV RNA +Ve**		**HCV RNA -Ve**	
		Patients	%	Patients	%
Male	128	49	72.058	79	68.695
Female	55	19	27.941	36	31.304
Total	183	68	100	115	100

Table 1: Screening of population for HCV RNA diagnosis.

Pair	HCV RNA Quantification	Paired Differences			t	df	P value
		Mean	**Std. Deviation**	**Std. Error Mean**			
1	Before therapy and at wk 4.	999465152.533	4661076898.471	565238599.457	1.768	67	0.082
3	Before therapy and at wk 12	1610871848.57	5797928206.09	819950870.27	1.965	49	0.055
4	Before therapy and at wk 24	1198239106.55	5007682416.28	607270692.41	1.973	67	0.053
5	At wk 4 and wk 12	266012435.82	1142572136.68	161584101.17	1.646	49	0.106
6	At wk 4 and wk 24	198773954.015	984559348.16	119395358.45	1.665	67	0.101
7	At wk 12 and wk 24	4320141.64	19178384.52	2712233.15	1.593	49	0.118

Table 2: Paired Samples statistical analysis of HCV RNA Quantitative (IU/ ml).

Drug combination/ response level	Abbreviation	Definition
PegIFN/RBV		
Rapid virological response	RVR	HCV RNA is undetectable at week 4
Early virological response	EVR	HCV RNA detectable at week 4 but undetectable at week 12 and should remained undetectable up to end of therapy
Delayed virological response	DVR	HCV RNA detectable at week 12, then undetectable at 24 wk and remained undetectable up to end of therapy
Null response	NR	HCV RNA level decrease ≤ 2 log10 IU/ml in from baseline at 12 wk of therapy
Partial response	PR	≥2 log10 IU/ml decrease in HCV RNA level from baseline at 12 wk of treatment. However, HCV RNA detectable at 24 wk
Breakthrough	BT	recurrence of HCV RNA at any time during therapy after a negative result or increase of 1 log10 IU/ml from nadir

Table 3: Therapeutical Monitoring of HCV patients; Virological response levels of PegIFN and Ribavirin.

	Patients	HCV RNA +ive	%age of total
		(N – 75)	(n-183)
Rapid Virologic Response	20	26.66%	10.93%
Early Virologic Response	38	50.67%	20.77%
Delayed Virologic Response	10	13.33%	5.46%
Non responder	7	9.33%	3.83%
HCR RNA -ive	108		59.02%
Total	183	100	100

Table 4: Therapeutical Monitoring of HCV patients; virological response levels of PegIFN and Ribavirin.

Group Statistics					
	Gender	N	Mean	Std. Deviation	Std. Error Mean
HCV RNA quantification IU/ml before therapy	Female	19	380524559.9199	1030200957.843	236344308.778
	Male	49	1515312094.0154	5851311832.560	835901690.357
HCV RNA or load IU/ml after 4 weeks	Female	19	155061002.3158	471851939.250	108250258.921
	Male	49	215723874.0612	1126285807.020	160897972.431
HCV RNA quantification IU/ml at 12 wk	Female	19	865988.3684	2162704.295	496158.393
	Male	49	4072516.3878	19388983.285	2769854.755
HCV RNA quantification IU/ml at 24 wk	Female	19	0.0000	0.00000[a]	0.00000
	Male	49	0.0000	0.00000[a]	0.00000
a. t cannot be computed because the standard deviations of both groups are 0.					

Table 5: Comparison of mean HCV RNA quantification IU/ ml (virologic response) of male and female during PegIFN and Ribavirin therapy.

The treatment of chronic HCV Genotype can be monitored by utilizing a quantitative Real-time RT-PCR assay with a Limit of Quantification (LOQ) and Limit of Detection (LOD). Real-time RT-PCR assay provide the HCV-RNA testing requirements with an LOQ and LOD of at least 12 IU/ mL [12]. HCV having variety of characteristics because of genetic subtypes that differentiate these from each other and a high molecular variability [15]. The genotypes have also been identified with divergence between the groups [16]. Thus, we have quantified the HCV RNA by internal virus kit, HCV RNA quantification kit and RT-PCR.

Meng et al. [17] reported the use of Robogen RT-PCR to detect the reference strains of HBV genotypes, HCV genotypes, and HIV subtypes with similar efficiency. An analogous range of detection of HBV, HCV and HIV genetic variants has also been reported. HBV DNA and HIV RNA can also be detected simultaneously, that confirm the results obtained by specific individual RT-PCR assays. But, the plasmas from HCV/HIV-1 dual-infected asymptomatic patients usually are not encouraged to test with the multiplex assay. Both of the viral genomes are detected by their respective molecular essays. The amplification data

obtained with calibrated standard reagents and dual-infected clinical samples demonstrated differences in primers, probes compatibility, and efficiency when simultaneously used for amplification. RNA virus genomic amplification showed higher intra- and inter-assay variations compared to DNA virus amplification. That suggests that the RT step was the main source of variability in this assay as previously reported for quantitative RT-PCR [18].

No major interference was observed between the simultaneous amplifications. Hence, a similar sensitivity was obtained for each virus when present alone or co-amplified with the other two. The data also showed the efficiency of DNA amplification was not affected by the simultaneous amplification of HCV RNA [19].

In addition of that, the animal model of the human hepatitis C virus (HCV) has impeded development of antiviral therapies against this epidemic infection. With chimeric human livers, the transplanted normal human hepatocytes can be generated into mice that carrying plasminogen activator transgene [20]. These mice developed prolonged HCV infections with high viral titers after inoculation with infected human serum. The viral load increased up to 1950 fold initially. That confirmed replication by detection of negative strand viral RNA in transplanted livers [21]. Moreover, the HCV viral proteins localized into the human hepatocyte nodules. Viral infection serially passed through generations to confirm synthesis and release of viral particles. The chimeric murine model present the suitable form to study the human hepatitis C virus *in vivo*.

In addition of that, Saglik et al. [22] reported no significant difference in patients infected with different genotypes. However, the determination of HCV genotypes is crucial importance regarding the treatment of chronic HCV infection. That also provides appropriate epidemiological HCV information to monitor of the outbreak. Although genotypes were determined as the most common HCV genotypes, the identification of other genomes also have important role in clinical investigation [23].

Conclusion

The quantification of human HCV RNA by RT-PCR, internal viral kit and Robogene quantification has high levels of scientific accuracy. The dynamic change during HCV therapy can potentially be investigated by this method. That helps the consultant virologists to rationalize the treatment, enhance antiviral responses and mitigate mortalities because of HCV RNA.

References

1. Araujo A.C., Astrakhantseva I.V., Fields H.A. & Kamili S.
Distinguishing acute from chronic hepatitis C virus (HCV) infection based on antibody reactivities to specific HCV structural and nonstructural proteins.
J. Clin. Microbiol., 2011, **49**: 54–57.

2. Bokharaei-Salim F., Keyvani H., Salehi-Vaziri M., Sadeghi F., Monavari S.H., Mehrnoush L. & Alavian S.M.
Mutations in the NS5A gene of hepatitis C virus subtype 1b and response to peg-IFNα-2a/RBV combination therapy in Azerbaijani patients.
Arch. Virol. 2014, **159**: 2893-2899.

3. Brunner A.M., Yakovlev I.A. & Strauss S.H.
Validating internal controls for quantitative plant gene expression studies.
BMC Plant Biol., 2004, **4**: 14-21.

4. Bukh J., Miller R.H. & Purcell R.H.
Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes.
Semin.
Liver Dis. 1995, **15**: 41-63.

5. Casanova Y.S., Boeira Tda R., Sisti E., Celmer A., Fonseca A.S., Ikuta N., Simon D. & Lunge V.R.
A complete molecular biology assay for hepatitis C virus detection, quantification and genotyping.
Rev. Soc. Bras. Med. Trop. 2014, **47**: 287-294.

6. Czechowski T., Stitt M., Altmann T., Udvardi M.K. & Scheible W.R.
Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis.
Plant Physiol., 2005, **139**: 5–17.

7. Dhanasekaran S., Doherty T.M. & Kenneth J. TB Trials Study Group.
Comparison of different standards for real-time PCR-based absolute quantification.
J. Immunol..Meth. 2010, **354**: 34-39.

8. Dheda K., Huggett J.F., Bustin S.A., Johnson M.A., Rook G. & Zumla A.
Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques. 2004, **37**: 112–119.

9. European Association for Study of Liver.
EASL Clinical Practice Guidelines: management of hepatitis C virus infection.
J. Hepatol. 2014, **60**: 392-420.

10. Freeman W.M., Walker S.J. & Vrana K.E.
Quantitative RT-PCR: pitfalls and potential.
Biotechniques. 1999, **26**: 112–125.

11. Irshad-ur Rehman, Idrees M., Ali M., Ali L. & Butt S.
Hepatitis C virus genotype 3a with phylogenetically distinct origin is circulating in Pakistan.
Gen. Vacc. Ther. 2011, **9**: 2 -9.

12. Janssens W., Buve A. & Nkengasong J.N.
The puzzle of HIV-1 subtypes in Africa.
AIDS. 1997, **11**: 705–712.

13. Kidd-Ljunggren K., Miyakawa Y. & Kidd A.H.
Genetic variability in hepatitis B viruses.
J. Gen. Virol. 2002, **83**: 1267–1280.

14. Meng Q., Wong C., Rangachari A., Tamatsukuri S., Sasaki M., Fiss E., Cheng L., Ramankutty T., Clarke D., Yawata H., Sakakura Y., Hirose T. & Impraim C.
Automated multiplex assay system for simultaneous detection of hepatitis B virus DNA, hepatitis C virus RNA, and human immunodeficiency virus type 1 RNA.
J. Clin. Microbiol. 2001, **39**: 2937–2945.

15. Mercier B., Burlot L. & Ferec C.
Simultaneous screening for HBV DNA and HCV RNA genomes in blood donations using a novel TaqMan PCR assay.
J. Virol. Meth.1999, **77**: 1–9.

16. Muir A.J, Poordad F.F., McHutchison J.G., Shiffman M.L., Berg T., Ferenci P., Heathcote E.J., Pawlotsky J.M., Zeuzem S., Reesink H.W., Dusheiko G., Martin E.C., George S., Kauffman R.S. & Adda N.
Retreatment with telaprevir combination therapy in hepatitis C patients with well-characterized prior treatment response.
Hepatology. 2011, **54**: 1538-1546.

17. Pawlotsky J.M.
Mechanisms of antiviral treatment efficacy and failure in chronic hepatitis C.
Antivir.l Res. 2003, **59**: 1-11.

18. Pfaffl M.W., Tichopad A., Prgomet C. & Neuvians T.P.
Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations.
Biotechnol. Lett. 2004, **26**: 509–515.

19. Radonic A., Thulke S., Mackay I.M., Landt O., Siegert W. & Nitsche A.
Guideline for reference gene selection for quantitative real-time PCR.
Biochem. Biophys. Res .Commun. 2004, **23**: 856-862.

20. Saglik I., Mutlu D., Ongut G., Inan D., Ogunc D., Can Sarinoglu R., Ozhak Baysan B., Gultekin M. & Colak D.
Distribution of hepatitis C virus genotypes among patients with chronic hepatitis C infection in Akdeniz University Hospital, Antalya, Turkey: a five-year evaluation.
Mikrobiyol. Bul. 2014, **48**: 429-437.

21. Strader D.B., Wright T., Thomas D.L. & Seeff L.B.
Diagnosis, management, and treatment of hepatitis C.
Hepatology. 2004, **39**: 1147–1171.

22. Stuyver L, van Arnhem W, Wyseur A, Hernandez F, Delaporte E, Maertens G.
Classification of hepatitis C viruses based on phylogenetic analysis of the envelope 1 and nonstructural 5B regions and identification of five additional subtypes.
Proc .Natl Acad Sci US A. 1994, **91**: 10134-10138.

23. Thellin O., Zorzi W., Lakaye B., De Borman B., Coumans B., Henne G., Grisar T., Igout A. & Heinen. E
Housekeeping genes as internal standards: use and limits.
J. Biotechnol. 1999, **75**: 197–200

24. Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A. & Speleman F.
Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.
Gen.Biol. 2002, **18**: 34-41.

26. Wu D., Liu F., Liu H., Dai L. & Tan D.
Detection of serum HCV RNA in patients with chronic hepatitis C by transcription mediated amplification and real-time reverse transcription polymerase chain reaction.
Zhong Nan Da Xue Xue Bao Yi Xue Ban. 2014, **39**: 664-72.