Original article

Automated Low Cost Method of Quantification of Hepatitis C Virus

Marikar FMMT[1], Senevirathna D[2], Fernandopulle N[2]

Abstract:

This paper describes the development of a Dig-dUTP based multiplex real time RT-PCR for the simultaneous detection of HCV viral amount in plasma samples. Viral genomes were identified in the same sample by Dig-dUTP PCR 216 bp region. Analysis of known scalar concentrations of reference plasma indicated that the multiplex procedure detects at least 500 copies/ml of HCV. In addition, we also assayed HCV viral load in eighty co-infected patients and in fifteen blood donors, confirming the sensitivity and specificity of the assay. This method may represent a useful alternative method for the detection of HCV co-infection, reliable for a rapid and relatively inexpensive screening of blood donors. The assay may be used to determine post-therapy viral clearance.

Keywords: Dig-dUTP; HCV; RT-PCR; PCR, viral load

Bangladesh Journal of Medical Science Vol.14(3) 2015 p.247-253
DOI: http://dx.doi.org/10.3329/bjms.v14i3.22270

Introduction:

Hepatitis C virus (HCV) is member of Flaviviridae family which infects over 170million people [1]. It is estimated that only 20% of infected individuals recover from this viral infection, while the rest become chronically infected [2-4]. Hepatitis C virus is a major cause of chronic liver disease, including chronic hepatitis, cirrhosis and hepatocellular carcinoma [5]. HCV viremia is correlated with viral replication and probably, with the progression of hepatitis. The quantification of HCV RNA in serum could provide a promising marker of viral replication and therefore, of the efficiency of the antiviral treatment [6]. Routine diagnosis of HCV infection is based first of all on screening assays for specific serum antibodies detection, which fail to distinguish between active and past infection. The use of molecular tests for HCV RNA detection, quantification and typing has become very important in the management of infected patients [7].

In the last two decades, quantitative RTPCR have been utilized mainly to measure the virus load[8]. The presence of HCV-RNA is assessed using qualitative assays and for quantification tests there are various amplification techniques are available. Commonly used commercially available assays for quantitation of HCV-RNA are the quantiplex HCV branched-DNA assay (bDNA; Chiron Corporation, Emmeryville, CA) [9] and the HCV AMPLICOR MONITOR assay (Roche Diagnostic Systems, Branchburg, NJ) [10]. The most commonly used methods for the determination of serum HCV viral load involve amplification of either (i) a direct HCV genome target (QPCR) or (ii) an associated signal (branched chain or bDNA assays) [11].

In this present study we describe the validation of a quantitative amplification assay for detection of HCVRNA, which is based on HCVRNA using Dig-dUTP. This mimic RNA was introduced at the very beginning of the extraction protocol. In the subsequent steps, the viral and competitive templates were co-processed, giving reliable quantitative results. The automated method described here provides a simplified procedure for assaying viral replication during antiviral therapy. Moreover, the methodology, which is described in this study, could be applied to the quantitation of other RNA viruses. It is developed as home-brew method and cost effective with low cost per reaction.

Materials and Methods:

Samples, labeling and storing:

Eighty untreated patients were enrolled in this study. Blood samples (5 ml each) were collected into plain sterile vials from patients referred to GENETECH

1. Faiz MMT Marikar, Department of Biochemistry, Faculty of Medicine, General Sir John Kotelawala Defence University, Kandawala Estate, Rathmalana, Sri Lanka
2. D Senevirathna
3. N Fernandopulle
Genetech Molecular Diagnostics and School of Gene Technology, Colombo, Sri Lanka

Corresponds to: Faiz MMT Marikar. Email: faiz.marikar@fulbrightmail.org

laboratory, Sri Lanka by physicians. Blood samples which were subjected to HCV antibodies tests were collected from National Blood Transfusion Center Colombo, STD clinic Colombo, National Hospital Colombo, Teaching Hospital Kandy, Sri Jayawardenapura Hospital, Apollo Hospital Colombo, Nawaloka Hospital Colombo and Durdans Hospital Colombo, Sri Lanka. Voluntary informed consent was obtained from the patients and from the parents or guardians in case of minors before the collection of samples. Ethical clearance was obtained from the University of Peradeniya, Sri Lanka. Soon after collection of specimens were labeled to remove all patient identifying information. A serial code number was assigned. The serial code number and the date of collection were written on the sample container. The patient identifying information has been stored separately. Serum was separated into a labeled tube, within 6 hours after collection of blood samples. Clotted blood samples were centrifuged at 2000 g for 5 minutes and serum was separated and stored at -70^{0}C.

Extraction of HCV virus:

Serum was separated from each blood sample at 3000 ×g for 5min and then labeled and stored deep frozen at −20^{0}C. RNA was extracted using RNA extraction Kit (Ultrascript, Anagen Technologies Inc., USA) as per manufacturer's instructions [12]. The solution (10 µl), which contains RNA was obtained from the supernatant after spinning for 3 minutes at 2000 g [13] and collected into RNA-se free collection tube. According to the calculated value, the original RNA contains 2.9 × 10^{12} copies/ µl. Therefore 2 µl from that was added in to a 98 µl of distilled water to make dilutions with a dilution factor of $10^{2.}$

In-house RT-PCR assay for the detection of HCV:

Extracted RNA was amplified by RT-PCR using both sense and anti sense primers forward (5'-GTC TAG CCA TGG CGT TAG TA-3') reverse (5'-TCT CGC GGG GGC ACG CCC AA-3'). RT-PCR was performed using the RNA reaction mixture (18 µl) containing 4 U of RNAsin, 0.27 µM of each forward and reverse primers (HCV 5UT F and R, Cha *et al.*, 1992) [13-14], and 15 µl of viral RNA, was prepared. The RNA mix was incubated for 5 minutes at 65^{0}C and 10 minutes at room temperature. Reverse Transcription mixture (RT mix) (5 µl), containing 1X RT buffer, 0.18 mM dNTP (dATP, dCTP, dGTP, and dTTP), 4 U of RNAsin and 25 U of MMV reverse transcriptase was added to the RNA reaction mix. The thermal cycler (PERKIN ELMER Gene Amp PCR System 2400) amplification vial and activated reactions are maintained in a chilled rack prior to being placed into a Thermal Cycler (Perkin Elmer 480). Competitive RT-PCR is performed using the following thermal cycling program: Reverse transcription (37 °C for 30 min, 94 ◦C for 2 min) in which the carbazole-labeled reverse primer initiates reverse transcription from HCV genomic RNA.

Mimic RNA Quantification:

An insertion of HCV was introduced within the target sequence defined for amplification. Serum from an HCV-positive patient provided the HCV sequence. After amplification, electrophoresis, and purification, the HCV were combined in a PCR vial and was cloned into the cloning site of pCR^{R}2.1-TOPO^R TA (Stratagene, La Jolla, Calif.) by the procedure described by Sambrook et al[15]. The insertion sequence was confirmed by a PCR method with a HCV and plasmid regions. A total of 50 mg of HCV was obtained; this RNA corresponded to the positive strand of the HCV genome. The integrity of the mimic RNA was checked by denaturing gel electrophoresis, and the mimic RNA was tested for contaminating DNA by PCR.

Quantitative real-time RT-PCR:

For each serum sample, several RNA extractions were performed with aliquots of GuSCN buffer containing the mimic RNA. After cDNA synthesis, PCRs were set up with the following modifications. The primer concentrations were 0.25 µM, and the labeling mixture was dig-dUTP (25, µM) and dlTP (175, µM). The final volume was 50 ml. Different concentrations of primers and nucleotides were tested, and these concentrations were selected for their specificities, sensitivities, and the optimum OD that was recorded. Standard HCV RNA for measurement of copy number was transcribed from plasmid pCR^{R}2.1-TOPO^R TA - HCV (Invitrogen, USA), using the AmpliScribe T7 High Yield Transcription Kit (Epicentre Biotechnologies, Madison, WI).

Probes and PCR with dig-dUTP:

Two 20-nucleotide probes were selected within HCV insertion sequences. The oligonucleotide corresponding to the patient amplification products obtained from the viral RNA and was subsequently named the viral probe. Conversely, the oligonucleotide corresponding to the insertion was the mimic probe. The probes were labelled with biotin at their 5' ends, and their sequences were as follows: viral probe, 5'-biotin- GAG AGC CAT AGT GGT CTG CG-3'; mimic probe, 5'-biotin-TGT TGG GAA GGG CGA TCG GT-3'. RNA extractions were done and calculated the 10^6 and 10^8

HCV viral RNA copies, normal serum sample and distilled water (2 extractions from each). Normal RT-PCR was done for one of the extracted RNA set. The other extracted RNA set was subjected to the RT-PCR with dig-dUTP with the viral RNA (10 µl) was added to 10 µl of Reverse Transcription mixture (RT mix) containing 1X RT buffer, 0.2 mM dNTP, 4 U of RNAsin, 0.5 µM reverse primer and 25 U of MMV- reverse transcriptase. The thermal cycler (PERKIN ELMER Gene Amp PCR System 2400) was programmed at 42^{0}C for 20 minutes and 95^{0}C for 2 minutes for the RT step. Then 30 µl of PCR mix containing 1X Taq polymerase buffer, 0.1 mM of each dATP, dCTP, dGTP, 0.09 mM of dTTP and 0.01 mM of dig-dUTP, 0.2 µM of each primer and 2.5 U of Taq DNA polymerase was added. The thermal cycler was programmed at 88^{0}C for 1 minute; 93^{0}C for 30 seconds, 60^{0}C for 40 seconds, and 72^{0}C for 40 seconds for 35 cycles; followed by a final extension for 10 minutes at 72^{0}C. The PCR products were run in 2% agarose gel and stained with ethidium bromide.

Detection of dig labeled PCR products:

Probe hybridization was done according to the probes (15 pmoles of each HCV- NP and HCV-CP,) were dissolved in 600 µl of diluents separately. From that 100 µl was added per each well as in micro titer plate. The micro titer plate was incubated at 37^{0}C for 30 minutes for the hybridization of probe to the wells. The wells were washed with 250 µl of wash buffer 1, 4 times to remove excess probes. The PCR product (50 µl) was incubated at 94 ^{0}C for 10 minutes and cooled down on ice immediately to denature procedure. Product hybridization was done with Dig-labeled, denatured PCR product (10 µl) was added into each well with Control Normal RT PCR, extracted PCR, and wild type. The PCR amplification product from the mimic RNA was captured by the mimic probe and the products from the viral RNA were captured by the viral probe. After development of colours, absorbance was taken at wave length of 450 nm. Analysis of unknown specimen of series of competitor RNA with constant amount of serum was subjected to RNA extraction, cDNA synthesis, PCR amplification and detection of product with each biotinilated probe.

Data analysis:

All of the following data were corrected if not otherwise indicated. The ODs from the mimic and viral probes were plotted as a function of the log 10 of the number of mimic RNA copies. In the competitive assay, the point where the two curves crossed was the equivalence point. It was convenient to plot only one curve by calculating the ratio of the mimic OD by the viral OD. The equivalence point was then defined by a ratio equal to 1. The correlation of the ratios was analyzed by linear regression.

Results:

Sensitivity of the assay:

The mimic RNA templates included in the GuSCN buffer were extracted, reverse transcribed, and amplified for 20, 25, 30, and 35 cycles. At 35 cycles, we obtained 1.2 OD units with 20 copies of the targets in the reaction tube, while the mean of the negative controls was 0.06 OD units. The sensitivities were lower for the other conditions. The plateau was reached at 35 cycles with 2 x 10^6 RNA copies in the sample. These conditions permitted a working copy number range of 10 to 10^6 (Figure 1).

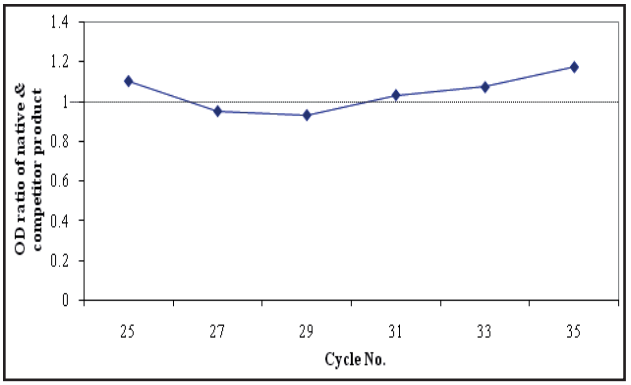

Figure 1: The absorbance ratio of viral & competitor RNAs against the cycle number

The hybridization assays between mimic probe and six different HCV positive samples (representing each genotype 1a, 1b, 2a, 2b, 3 and, 4) were carried out to find out whether there is any cross reactions. It was found that all the OD values were less than negative cutoff point showing that there were no cross reactions between mimic probe and HCV wild type viral RNA. The curve drawn with the ratio of competitor RNA and viral RNA were close to 1(Fig. 1), showing that the assay yields of the two targets were identical.

Detection of positive samples:

To confirm that the assay in which the labeling was done during the PCR steps and the automated detection method could detect wild viral sequences, we compared the method with detection of the amplified products by electrophoresis. The efficiency of RNA recovery by this method was quantified with the DNA band at 216 bp was observed in 10^8 and 10^6 HCV wild RNA copies in both normal and dig PCRs (Fig. 2). But there was a slight size difference between normal and Dig products. Because of the

size difference it was confirmed that dig-dUTP had been incorporated into the PCR products. Readings of experiment was given in Table 1. The expected results were obtained.

All the readings were less than normal control readings (less than 0.5000). The sequence of the probe

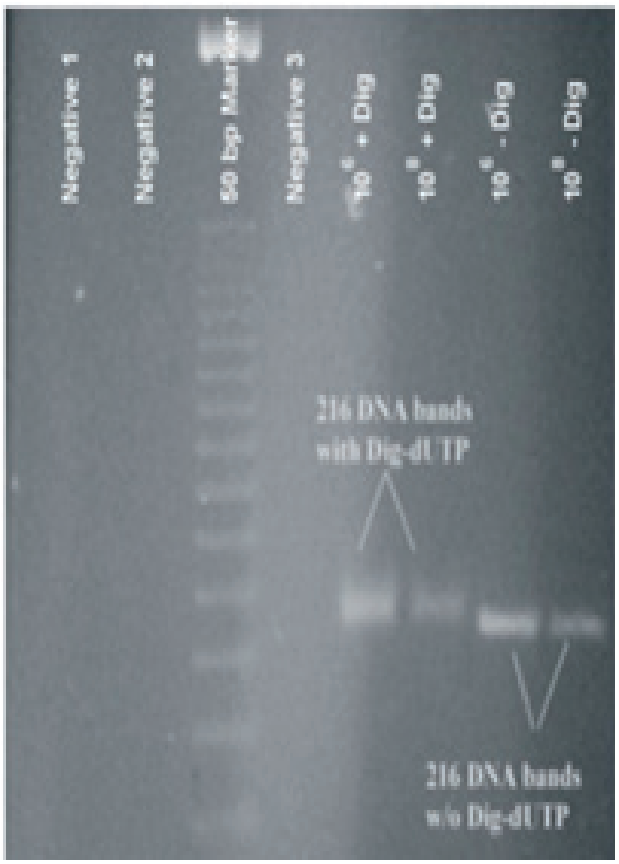

Figure 02: 2% agarose gel stained with ethidium bromide which shows difference between the Dig-dUTP incorporated products and un-incorporated products. Negative 1, 2 and 3 represent distilled water PCR, extraction and normal sera specimen respectively

is a highly conserved region of HCV genome, and is found in sequences of all HCV strains deposited in GenBank. The ability of this viral probe to recognize wild viral sequence from clinical samples has been assessed by comparing the results obtained by PCR labeling and automated detection and the results obtained by classical PCR and Southern blotting and hybridization.

Standardization and lower limit of detection, and quantitation assay:

Nine replicates each of the HCV-RNA standards International Standard were quantitated on the same assay run, and a conversion factor to compare the two standards was determined with the impurities when it RNA extraction procedure. According to the MEGA short script ™ the maximum expected value for the positive control is about 90 µg. In this experiment it was 103.65 µg (Table 2). But the absorbance ratio between 260 nm and 280 nm was 1.64 (for the pure RNA it should be 2.00 – Table 1). This explains presence of impurities with the transcribed RNA and that may be the reason to get a slightly higher value. The lower limit of detection of the assay was assessed using HCV native RNA, HCV competitor RNA and the HCV negative specimens (distilled water). The results which we obtained in this study were 61.35 µg, 121.9 µg, and zero µg respectively (Table 3). The assay RNA amounts of detection were calculated for each reagent lot and instrument pair, and were defined as the concentration of HCV where 95% of the results were above the International standard (Table 3).

Quantitative assay:

Figure 3, illustrates the principle of the quantitative automated reverse capture assay with the one probe. HCV RNA was extracted from one HCV positive serum sample with an unknown viral copy number, reverse transcribed into cDNA, and amplified five times along with an increasing number of mimic RNAs ranging from 50 to 5 x 10^6 copies. As expected, the OD of the mimic RNA increased with an increasing number of mimic templates. At the same time, the OD of the viral RNA was constant at first and then decreased when high mimic RNA copy numbers were present. As a result, there was a crossing point between the two curves, which is the graphic representation of the equivalence point at which the mimic and viral RNAs were present at equal concentrations (Fig. 3B). The low OD observed for the vial containing 5 x 10^3 mimic RNA copies gave a ratio which fit in the ratio curve well. Three quantitative assays were performed on 3 different days starting with one sample to test the reproducibility's of the assays. Within the equivalence zone, the three curves showed very similar shapes and the linear regression analysis displayed very close intersections for a ratio of 1 and numerical calculation was shown in table 4.

Figure 3. Representative results of the quantitative RT-PCR assay. A- ODs obtained with either the mimic or the viral probe. B- OD ratios were plotted over the log of the mimic RNA copy numbers, and a linear regression was calculated for the equivalence. Three quantitative assays were performed on 3 different days starting with same specimen no.1 to test the reproducibility of the assay. Within the equivalence zone, the three curves showed very

Table 1: Spectrophotometer absorbance of transcribed RNA

Type of RNA	Absorbance at 260 nm	Absorbance at 280 nm
Positive control	0.3455	0.2117
HCV native RNA	0.2046	0.1013
HCV competitor RNA	0.4063	0.2681
Negative control	-0.0606	-0.0375

Table 2: Calculation of RNA amount

	Positive control	**HCV native RNA**	**HCV competitor RNA**
Concentration of RNA corresponding to absorbance 1	40 µg/ml	40 µg/ml	40 µg/ml
Concentration of RNA corresponding to absorbance	40 µg/ml / 1 × 0.3455 =13.82 µg/ml	40 µg/ml / 1 × 0.2046 =8.18 µg/ml	40 µg/ml / 1 × 0.4063 =16.25 µg/ml
Amount of RNA in 1.5 ml	13.82 µg / 1 ml × 1.5 ml 20.73 µg	8.18 µg / 1 ml × 1.5 ml 12.27 µg	16.25 µg / 1 ml × 1.5 ml 24.38 µg
Amount of RNA present in 20 µl (original)	20.73 µg	12.27 µg	24.38 µg
Amount of RNA present in 100 µl (original)	20.73 µg / 20 µl × 100 µl 103.65 µg	12.27 µg / 20 µl × 100 µl 61.35 µg	24.38 µg / 20 µl × 100 µl 121.9 µg
The absorbance ratio between 260 nm and 280 nm	0.3455 / 0.2117 1.64	0.2046 / 0.1013 2.02	0.4063 / 0.2681 1.52

Table 3- Method of RNA quantification

Total molecular weight of RNA fragment	128,613.6 g mol^{-1}
No. of RNA molecules In 128,613.6 g	6.022×10^{23}
The mass of a single RNA molecule	$128613.6 / 6.022 \times 10^{23}) \times 10^{6}$ µg 2.135×10^{-13} µg
Amount of RNA in 100 µl (16/02/07, HCV wild)	61.35 µg
Amount of RNA in 1 µl	61.35 / 100 µg = 0.61 µg
No. of RNA molecules In 1 µl	$(1/ 2.135 \times 10^{-13}) \times 0.61$ 2.9×10^{12}

Table 4 – calculation of the viral load copies

Native OD/Competitor OD	1.853 – 0.1838 log 10 (competitor RNA copy No.)
When the ratio becomes 1 Competitor RNA copy No. = Native RNA copy No. 1.853 – 0.1838 log 10 (competitor RNA copy No.) = 1	
Log 10 (competitor RNA copy No.)	4.641
Competitor RNA copy No.	43752
Native RNA copy No.	43752
If one RNA particle is equal to one viral particle Viral copy No. of specimen 1 in 100 µl	43752
Viral load of specimen 1	437 520 copies/ml

similar shapes and the linear regression analysis displayed very close intersections for a ratio of 1. The mean viral load of the specimen 1 was 436 268 ± 3149. Reproducibility was evaluated by using replicates for each and every PCR product and by doing two or three different runs.

Discussion:

HCV is now becoming a major risk of liver associated diseases in the world. Because HCV can persist for many years without any symptoms, it has become a silent epidemic and lack of a vaccine increases susceptibility to HCV infection. The global prevalence rate of HCV infection varies with geographic region and in South East Asia 32.3 million (2.15 % of the total population) infected cases have been recorded (1). Sri Lanka is one of the few countries in Asia which does not have data related to the HCV. At the beginning of this study we assumed there

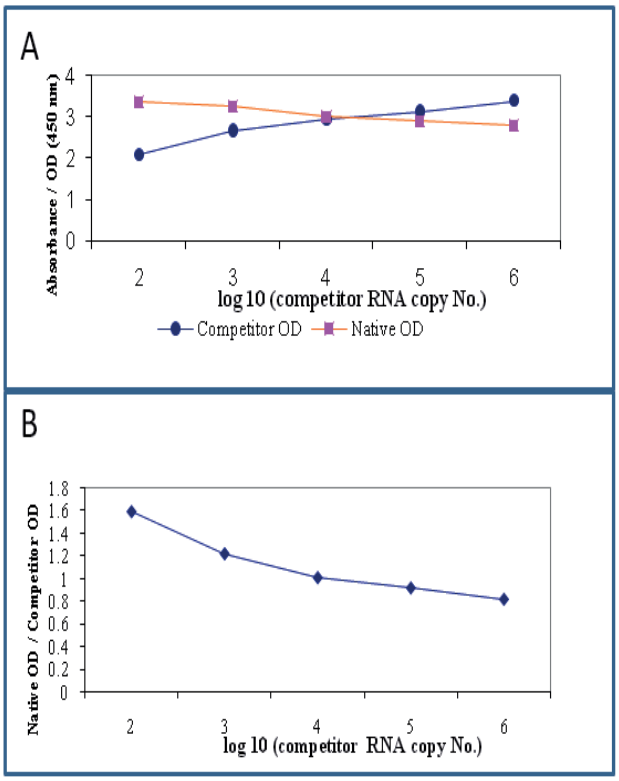

Figure 3. Representative results of the quantitative RT-PCR assay. A- ODs obtained with either the mimic or the viral probe. B- OD ratios were plotted over the log of the mimic RNA copy numbers, and a linear regression was calculated for the equivalence.

may be a significant patient enrollment of HCV for liver associated diseases in Sri Lanka, especially by considering the situation in India and Pakistan where high HCV prevalence has been recorded among the liver disease patients [16]. Therefore, it was necessary to first determine the prevalence of HCV in Sri Lanka. Detection of Anti-HCV antibodies was the existing method. However, it can be used only for screening purposes and cannot be used for the confirmation of HCV. The large number of false positive results obtained from antibody assays highlighted the importance of developing a confirmatory quantity assay for HCV. This study lead to the development and establishment of a QRT-PCR based confirmatory assay for HCV for the first time in Sri Lanka. A cohort of liver disease patients were then selected as the best group to study the prevalence of HCV and were subjected to the RT-PCR based testing. Of 1933 samples from liver disease patients only 54 (2.79%) were positive for HCV RNA (unpublished data). Although we expected higher prevalence at the beginning of the study, this data revealed comparatively low prevalence of HCV among liver disease patients in Sri Lanka.

Because the rates of response to the treatment vary according to the viral load present in blood of the patient and, it was important to test for viral load and genotype. But as a result of this study, PCR based quantification of HCV and genotyping was possible at low cost. The detection limits of the HCV Dig dUTP assay were determined using serial dilutions of in vitro RNA. The lower limit of reliable qualitative detection, defined as the 50% detection limit of the assay, was found to be $10^{2,3}$ copies per 100 ml input (i.e. 103.3 (2000) cps:ml). When a plasma input of 1 ml in 9 ml lysis buffer was used, the sensitivity improved by a factor 5–10-fold (results not shown). In a quality control study of HCV-RNA amplification assays it was shown that the 50% detection limit of qualitative cDNA-PCR assays is more than enough for proper treatment.

The Dig-dUTP RNA Quantitative assay was designed to produce accurate and reproducible quantitation results for HCV genotypes 1–6. The assay primers and probes were designed to hybridize to sequences within the 5' untranslated region of HCV, the most highly conserved region of the HCV genome. To help achieve reproducible genotype quantitation, the first five PCR thermal cycles are carried out at a lower stringency than the subsequent PCR cycles. The Dig-dUTP HCV assay quantitated genotypes 1–6 HCV RNA transcripts equally, and produced similar quantitation values as the other kits when used to assay 54 patient specimens containing representatives of genotypes 1–6. This is significant because the In house method test has been accurate and precise. The Dig-dUTP HCV RNA quantitative assay did not cross-react with high concentrations of microorganisms that might be present in a plasma or serum specimen, and produced a specificity of 100% when used to test HCV negative plasma samples. In combination, these data suggest that the Dig-dUTP HCV assay quantitates HCV genotypes 1–6 equally with excellent specificity. In conclusion, we developed a simple Dig dUTP HCV PCR system based assay that can be used for high-throughput screening of HCV in Serum with low stringency.

Acknowledgments

We like to thank Mr. D. N. Gunasekera and the staff of Genetech Molecular Diagnostics. We specially thank Prof. Aravinda De Silva for providing Dig-dUTP and the plasmids.

Conflict of interest : Nil

References:

1. Guyatt, G., A.D. Oxman, and E.A. Akl, GRADE guidelines: 1. Introduction–GRADE evidence profiles and summary of findings tables. *J Clin Epidemiol*, 2011; **64**: p. 383–94. http://dx.doi.org/10.1016/j.jclinepi.2010.04.026

2. Roblin, D.W., B.D. Smith, C.M. Weinbaum, and M. Sabin, Hepatitis C virus screening practices and prevalence in a managed care organization. *Am J Managed Care* 2011; **17:** p. 548–55.

3. Cohen, A.J., When HIV and HCV cohabitate. GMHC Treatment Issues 1999**. 13**(4): p. 5-7.

4. NIH. Management of Hepatitis C: 2002. 2002 [cited 2012 September 26]; Available from: http://consensus.nih.gov/2002/2002hepatitisc2002116html.htm.

5. Southern, W.N., M.L. Drainoni, and B.D. Smith, Hepatitis C testing practices and prevalence in a high-risk urban ambulatory care setting. *J Viral Hepat* 2011**; 18**: p. 474–81. http://dx.doi.org/10.1111/j.1365-2893.2010.01327.x

6. Lau, J.Y.N., G.L. Davis, J. Kniffen, K.P. Qian, M.S. Urdea, C.S. Chan, M. Mizokami, P.D. Neuwald, and J.C. Wilber, Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. *Lancet* 1993; **341**: p. 1501-1504. http://dx.doi.org/10.1016/0140-6736(93)90635-T

7. Zou, S., K.A. Dorsey, and E.P. Notari, Prevalence, incidence, and residual risk of human immunodeficiency virus and hepatitis C virus infections among United States blood donors since the introduction of nucleic acid testing. *Transfusion* 2010; **50**: p. 1495–504. http://dx.doi.org/10.1111/j.1537-2995.2010.02622.x

8. Bouaboula, M., P. Legoux, B. Pessegue, B. Delpech, X. Dumont, M. Piechaczyk, P. Casellas, and D. Shire, Standardization of mRNA titration using a polymerase chain reaction method involving co-amplification with a multispecific internal control. *J. Biol. Chem*. 1992**; 267:** p.:21830-21838.

9. Ghany, M.G., D.B. Strader, D.L. Thomas, and L.B. Seeff, An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases. *Hepatology* 2011; **54**: p. 1433–44. http://dx.doi.org/10.1002/hep.24641

10. Menezes, G.B., F.A. Pereira, C.A. Duarte, T.M. Carmo, H.P. da Silva Filho, M.A. Zarife, M.A. Krieger, E.A. Reis, and M.G. Reis, . Hepatitis C virus quantification in serum and saliva of HCV-infected patients. *Memórias do Instituto Oswaldo Cruz* 2012; **107(**5): p. 680-3. http://dx.doi.org/10.1590/S0074-02762012000500016

11. Jacobson, I., J. McHutchison, and G. Dusheiko, Telaprevir for previously untreated chronic hepatitis C virus infection. *New Engl J Med* 2011**; 364**: p. 2405–16. http://dx.doi.org/10.1056/NEJMoa1012912

12. Shamim. S., K. Anwar, U. Farman, K. Nasar, M. Asif, and K. Sanaullah, Prevalence of Hepatitis C Virus Genotypes in District Bannu, Khyber Pakhtunkhwa, Pakistan. *Hepatitis Research and Treatment* 2014(165826,): p. 1-5.

13. Bukh, J., R.H. Purcell, and R.H. Miller, Sequence analysis of the 5' noncoding region of hepatitis C virus. *Proc. Natl. Acad. Sci*. USA, 1992; **89**: p. 4942-4946. http://dx.doi.org/10.1073/pnas.89.11.4942

14. Kuntzen, T., J. Timm, A. Berical, L.L. Lewis-Ximenez, A. Jones, B. Nolan, J. Schulze zur Wiesch, B. Li, A. Schneidewind, A.Y. Kim, et al., Viral sequence evolution in acute hepatitis C virus infection. *Journal of Virology* 2007; **81**(21): p. 11658-68. http://dx.doi.org/10.1128/JVI.00995-07

15. Sambrook, J., E.F. Fritsch, and T. Maniatis, Molecular cloning: a laboratory manual, 3rd ed. 2003, NY: Cold Spring Harbor Laboratory Press, Cold Spring Harbor,

16. Afridi, S.Q., M.N. Zahid, and M.Z. Shabbir, Prevalence of HCV genotypes in district Mardan. *Virology Journal* 2013; **10:** p. 90. http://dx.doi.org/10.1186/1743-422X-10-90