QUANTITATION OF HEPATITIS C VIRUS RNA USING REAL-TIME PCR AND DIG-DUTP

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Hepatitis C virus (HCV) is a flavivirus, which has infected over 170 million people[1]. It is estimated that only 20% of infected individuals will be recovered from this viral infection, while the rest become chronically infected[2]. Hepatitis C virus was a major cause of chronic liver disease, including chronic hepatitis, cirrhosis and hepatocellular carcinoma[3]. The use of molecular tests for HCV RNA detection, quantification and typing has become very important in the management of infected patients[4]. Viral load has determined before initiation of anti-HCV therapy, as well as 36 months after therapy initiation to determine if therapy should continue. Therapy will be continued if viral load was fallen by 2 log10 IU/ml from the baseline viral load[5]. At the end of therapy, and periodically thereafter, viral load was measured to determine if HCV has been cleared, with clearance defined as <50 IU/ml.[5] Many laboratories have developed 'home-brew' assays based on these methodologies. Because each commercially available viral load assay has been developed using proprietary HCV RNA standards, the units of measure in each assay are specific to that method, making comparison of results between different assays difficult. As a consequence, a patient's HCV viral load can be accurately compared from one time point to another, only if the same assay is used for analysis each time. In this study the validation of a quantitative amplification assay is described for detection of HCVRNA, which was based on HCVRNA using Dig-dUTP. It is developed as a home-brew method and low cost per reaction in Sri Lanka.

Eighty untreated patients were enrolled in this study. Blood samples (5 ml each) were collected into plain sterile vials from patients referred to GENETECH laboratory, Sri Lanka by physicians. Blood samples, which were subjected to HCV antibody tests were collected from; National Blood Transfusion Center Colombo, STD clinic Colombo, National Hospital Colombo, Teaching Hospital Kandy, Sri Jayewardenepura 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. 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°C. Extracted RNA was amplified by RT-PCR using both sense and anti sense primers (HCV5UT, F and R) at a portion of the 5' Un-translated Region (UTR) of the HCV genome. 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, and 15 µl of viral RNA) was prepared[6]. The RNA mix was incubated for 5 minutes at 65°C and 10 minutes at room temperature. Reverse Transcription mixture (RT mix) (5 µl), containing 1X RT buffer, 0.18 mM dNTP, 4 U of RNAsin and 25 U of MMV reverse transcriptase was added to the RNA reaction mix.

The other extracted RNA set was subjected to the RT-PCR with Dig-dUTP with the viral RNA (10 µl) 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 PCR products were run in 2% agarose gel and stained with ethidium bromide. Product hybridization was done with Dig-labeled, denatured PCR product (10 µl) and substrate (TMB 100 µl) was added and kept 10 minutes at room temperature under dark condition. Stop solution (100 µl) was added and the absorbance was measured at wave length of 450 nm. Dig UTP was used.

To establish an efficient RT-PCR-based screen for HCV agents, searched for methods that could be carried out without labour-intensive RNA isolation by Silica with GuSCN method. A Lysis buffer solution (40 mM Tris, pH 6.4, 17 mM EDTA, 4 M GuSCN, 1% Triton X-100) and 10 µl of size-fractionated silica the solution was mixed well (vortexed) with 100 µl of serum and was incubated for 10 minutes at 70°C 10 minutes and cooled at room temperature. The solution was spun for 1 minute at 2000 g to pellet the silica. The pellet was washed and dissolved in nuclear free water, performed by direct addition of the one-step RT-PCR reaction mixture. 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. 01). 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. However, it is necessary to check the cross reactivity between viral probe with competitor PCR product and competitor probe with viral product. All the readings were less than normal control readings (less than 0.50). 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 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. All the OD values were taken as an average of triplicates and the whole PCR-ELISA was repeated 3 times on 3 different days for the unknown.

In conclusion, a simple Dig dUTP HCVPCR system based assay was developed, that can be used for high-throughput screening of HCV in Serum with low stringency.

REFERENCES:

1. Biggins, S.W., Bambha, K.M., Terrault, N.A.,, Inadomi, J., Shiboski, S., Dodge, J.L., Gralla, J., Rosen, H.R., Roberts, J.P.,*Projected future increase in aging HCV-infected liver transplant candidates: A potential effect of HCC.* Liver Transplantation, 2012.
2. Cohen, A.J., *When HIV and HCV cohabitate.* GMHC Treatment Issues, 1999. **13**(4): p. 5-7.
3. Lau, J.Y.N., *Methods in Molecular Medicine*. 2002, Totowa, NJ: Humana press.
4. Menezes, G.B., Pereira, F.A., Duarte, C.A., Carmo, T.M., da Silva Filho, H.P., Zarife, M.A., Krieger, M.A., Reis, E.A., *Hepatitis C virus quantification in serum and saliva of HCV-infected patients.* Memórias do Instituto Oswaldo Cruz, 2012. **107**(5): p. 680-3.
5. NIH. *Management of Hepatitis C: 2002.* 2002 [cited 2012 September 26]; Available from: http://consensus.nih.gov/2002/2002hepatitisc2002116html.htm.
6. Kuntzen, T., Timm, J., Berical, A., Lewis-Ximenez, L.L., Jones, A., Nolan, B., Schulze zur Wiesch, J., Li, B., Schneidewind, A., Kim, A.Y., Chung, R.T., Lauer, G.M., Allen, T.M., *Viral sequence evolution in acute hepatitis C virus infection.* Journal of Virology, 2007. **81**(21): p. 11658-68.