



## Nested-PCR based Detection of Hepatitis C Virus: Low-cost Strategy in Pakistan

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**Abstract:** One of the most common blood-borne illnesses is hepatitis C virus (HCV), Hepatitis C is referred to as the inflammation of the liver and caused by the HCV virus, HCV is estimated to cause 53000 fatalities per year over the world. The majority of HCV-infected patients are unaware of their infection. No vaccine is available for HCV although Interferon is used to treat HCV but effective only 20-38 %, but at present, only a minority of infected persons have been tested and are aware of their diagnosis. The expense of testing may play a substantial role in patients' ability to get rid of the hepatitis C virus (HCV). Costs in many low- and middle-income nations, including Pakistan, force the development of novel and economically advantageous testing methods. The major aim of this study is about the effective diagnostic procedure for detecting Hepatitis C in the samples obtained from Balochistan, for this purpose the samples were collected from the health organization BINUQ (Balochistan Institute of Nephrology and Urology Quetta). Twenty (20) HCV antibodies positive patients in the Molecular Laboratory Department of Biotechnology were processed and then subjected to RNA extraction. cDNA was synthesized by reverse transcriptase enzyme. cDNA was used for qualitative analysis of HCV-RNA through nested PCR. According to the study, 09 samples were detected as positive and 7 samples were HCV negative out of 16 patients' samples. The findings of the present study show comparison of the price for HCV- RNA tests per sample from patients with hepatitis C at various labs. When compared to the other five PCR-based tests in the laboratory-conducted anti-HCV, HCV qualitative, quantitative, and genotyping tests, Shoukat Khanum laboratory reported the most expensive costs for HCV -RNA tests. Dow laboratory HCV-RNA test is comparatively lower than Shoukat Khanum laboratory, while reported lowest and most cost-effective test of Molecular Diagnostic laboratory for anti-HCV. So, our molecular tests for HCV- RNA detection and quantitation showed very good diagnostic and clinical performance over all five public health laboratories.

**Keywords:** Hepatitis C, Comparison Diagnostic Test, Polymerase Chain Reaction

### 1. INTRODUCTION

Hepa means "liver", Hepatitis abolishes liver cells and also sources of inflammation of the liver, liver cirrhosis and carcinoma originated from various factors, such as drugs, viruses and alcohols. Hepatitis C is a thoughtful lethal challenging all over the world. Hepatitis C virus (HCV) is a blood-borne pathogen mostly affected through skin contact with the unwell individual. Hepatitis C is a considerable public health challenge worldwide

[1-4]. Physical conditions of Hepatitis C are yellowing of the skin, Enlargement of the liver and fluid in the abdomen. A, B, C, D, E, and G are the subtypes of the hepatitis C virus. These have an adverse effect on human livers and result in fatal infections. The hepatitis C (HCV) virus, which is the primary cause of (non-A non-B) hepatitis, was first discovered in 1980. It is an RNA virus and its complete genome has been recognized and sequenced [5]. HCV is a member of the flavivirus viral family.

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In Pakistan, the number of HCV-related chronic liver infections is increasing due to many associated risk factors. According to reports, around 60 % of Pakistani patients with liver cancer are anti-HCV positive [6] The incubation period of the HCV virus has ranging from 2 to 52 weeks.

According to HCV reported rate in the US national HCV prevalence from 2013 to 2016 was 0.93 % and varied by jurisdiction between 0.45 % and 2.34 % [7-8] The viral particle is mainly composed of a positive sense strand, single-stranded RNA genome with about 9,500 nucleotides, a nucleocapsid, and an RNA envelope made from host membranes into which virally encoded glycoproteins (E1 and E2) are inserted. Highly conserved untranslated regions (UTR) flank a sizable translational open reading frame that codes a polyprotein with 3,000 amino acids in the genome's 5 and 3 termini. [9 -10]. The burden of hepatitis C in Pakistan is the second-highest in the world. 80% fewer new cases of hepatitis C are expected by 2030, according to a strategy created by WHO to end the harm that it poses to public health [11]. Low- and middle-income countries (LMICs) are responsible for between 50 and 80 percent of the world's hepatitis C burden [12] due to high diagnosis rates of HCV. Hepatitis C can be caused by immune cells in the human body affecting the liver and enhancing autoimmunity, infections from bacteria and viruses like A, B, and C, parasites, liver damage from drugs like an acetaminophen overdose, which can be lethal. Hepatitis C positive patients can be screened by ELISA tests and more investigative approaches e.g. abdominal ultrasound, liver function test (LFT), etc. [13]. My research work emphasizes the collection of samples from the Balochistan Institute of Nephrology and Urology, Quetta (BINUQ) to validate the 'Hepatitis C' can be detected with an efficient and effective diagnostic test among the Balochistan population.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Blood Samples

BINUQ Balochistan Institute of Nephrology and Urology Quetta Pakistan collected 20 blood samples from adult patients (18-50 years) for HCV-RNA detection. Blood sample (5 mL) was obtained from each subject in disposable sterile syringes.

Then whole blood was shifted in the Molecular Lab, BUITEMS, Quetta, Department of Biotechnology. All patients signed or provided written informed consent forms. The expected time of infection and the patient's previous contacts were all included in the thorough history that was gathered. Exclusion criteria for this study was patients having any signs of infection disease causing other pathogens, such as HBV, HIV, or HDV were omitted from the study.

#### 2.1.1 Confirmatory Tests/Diagnosis

Anti-HCV antibodies were detected in suspected acute hepatitis C patients using the serological assay for entirely patient samples. Further testing was done on the positive samples.

Twenty HCV-positive patients The Molecular Laboratory Department of Biotechnology processed the Ab tests. Nucleospin For extraction of the viral genome (extraction Kit Macherey-Nagel, Germany) with slight modifications was used.

#### 2.1.2 Extraction of Viral RNA from Blood Samples

Following the manufacturer's instructions, 600 ul of lysis buffer with 150 ul of serum sample were used to extract the viral RNA. All of the samples were then incubated at 70 °C for 5–6 minutes in a water bath. After that 600 µL of pure alcohol was added to all samples. Collect the samples in the column tubes and centrifuged at 13000 rpm for one minute. RAW Buffer (500 µL) was added then again centrifuged for 6 minutes. After that, 200 µL RAV3 buffer was added to the samples, again centrifuged. After drying the samples, column tubes were incubated for 5 minutes at 70 °C. In the last and final step, 50 µl of elution buffer was added in column tubes for RNA elution for further processing.

### 2.2 Steps for Polymerase Chain Reaction (PCR)

For cDNA from extracted RNA Moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies Inc., USA) was used HCV RNA was detected by amplification through PCR. The primers were synthesized from the (5' Untranslated region) of the HCV genome, which was described by Chen and Weck [14]. Primer3 plus computer program was used to design the primers (<http://www.bioinformatics.nl/cgi-bin/primer3plus/>

primer3plus.cgi/).

RT PCR was performed using the following PCR ingredients as follows. The extracted RNA (10  $\mu$ L) was amplified by using HCV RT mix (5X first strand buffer (It includes 200 U of the MMLV reverse transcriptase enzyme along with 50 mM Tris-HCl (pH 8.3), 7.5 mM KCl, 3 mM MgCl<sub>2</sub>, 0.1 M DTT, and 10 mM dNTPs) and (1  $\mu$ L) HCV-antisense primer (10  $\mu$ mol/ $\mu$ L). A final volume of 20  $\mu$ L was used to conduct the RT-PCR experiments. PCR program was used for cDNA to be synthesized from extracted RNA for 50 min at a temperature of 37 °C with a primer specific for the core region and then for 3 minutes at 95 °C. It was spun down and kept at -20 °C until it was utilized for first-round PCR.

### 2.2.1 Nested Polymerase Chain Reaction of HCV cDNA

For qualitative investigation, the HCV 5' UTR cDNA was amplified. In the first step (round) the outer sense and antisense primers were used in the PCR process, followed by the second round of nested PCR with inner primers. To perform the first round of PCR, the following PCR ingredients were added 20  $\mu$ L of the PCR reaction mixture were inserted in a tube. Primers used during the study is given as exterior sense nucleotides sequence 695-718 (5'-CATGGTCCCAGCCTCCTCGCTGGC-3') and exterior antisense primer nucleotides sequences 873-896 (5'CCGCGAGGAGGTGGAGATGCCATG-3').

The cDNA RNA (2  $\mu$ L) was amplified by using (16  $\mu$ L) HCV PCR reaction mixture mix

(100  $\mu$ M of each of the four deoxynucleotides and 2.5 mM MgCl<sub>2</sub>) (dNTPs), 0.5  $\mu$ L forward primer (10 pM/ $\mu$ L), 0.3  $\mu$ L reverse primer (10 pM/ $\mu$ L) and (1  $\mu$ L) Taq DNA polymerase (200U). The overall volume for the PCR reactions was 20  $\mu$ L.

For amplification, the thermocycler (BIORAD PCR I cyclor version 3.021, USA) was programmed. Initial denaturation was done at 93 °C for 4 min, followed by 30 cycles, each of denaturation at 93 °C, 45 sec annealing at 55 °C, for 30 sec and 45 sec extension at 72 °C, with a final extension at 72 °C for 7 min. After completion of the first round, PCR bands were visualized on 2 % agarose gel. In the second round of nested PCR was carried out with the same reaction mix as taking the first round PCR as a template, but using different Inner sense and antisense primer sequences for amplification of PCR as given below (5'-CAACATTCCGAGGG GACCGT-3') and antisense primer (5'-GAAGGAAGGCCCTCGAGAACAAGA-3'). During the PCR, standard precautions were taken to avoid contamination. To check the cross-contamination negative control was also run in each round.

### 2.3 Agarose Gel Electrophoresis for Amplified Product Conformation

For conformation of PCR amplification, 2 % agarose gel was prepared in TBE buffer. According to the migration pattern of a 100-bp DNA ladder, the sizes of PCR products were determined (Fermentas Life Sciences). After the nested PCR 360 base pair was the size of the PCR products. Four of the twenty samples tested negative for HCV, while twelve tested positive. After qualitative analysis by PCR

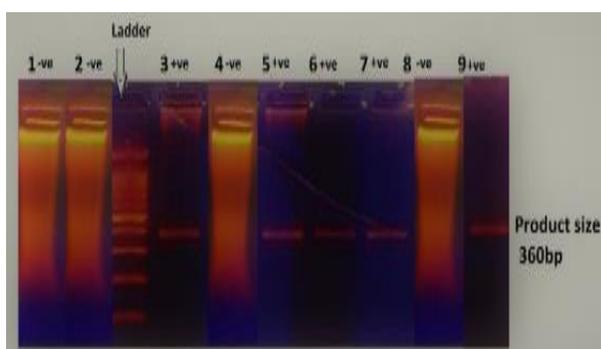


Fig. 1. Showing results of HVC on 2 % agarose gel.

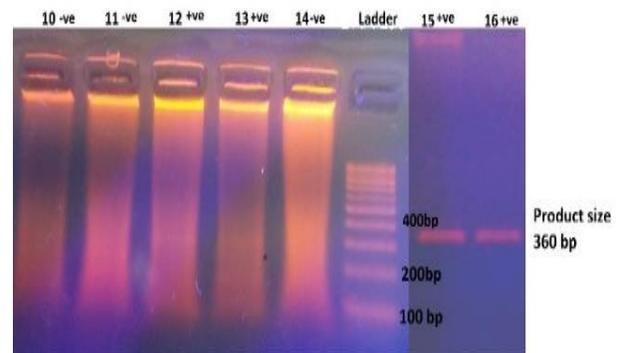


Fig. 2. Showing (HCV) RNA results after PCR amplification.

showing the figures 1 and 2.

### 3. RESULTS

To detect HCV-RNA, a total of 20 blood samples from adult patients (aged 18 to 50) were collected by the Baluchistan Institute of Neurology and Nephrology (BILNUQ) in Quetta, Pakistan. 5 mL of blood was extracted from each volunteer in disposable sterile needles. Anti-HCV antibodies were identified in all of the samples in patients having acute hepatitis C. HCV-RNA detection results in individuals. Table 1 shows a total 20 number of blood samples amongst 16 patients that tested positive for anti-HCV; 09 samples out of 16 were positive for HCV RNA. Following HCV RNA quantification, 16 samples with viral loads less than 500 IU/mL and 07 samples with viral loads greater than 500 IU/mL were examined.

**Table 1.** HCV-RNA detection in patients showing positive/negative PCR amplification.

Sample	PCR Results
1	PCR Negative
2	PCR Negative
3	<b>PCR Positive</b>
4	PCR Negative
5	<b>PCR Positive</b>
6	<b>PCR Positive</b>
7	<b>PCR Positive</b>
8	PCR Negative
9	<b>PCR Positive</b>
10	PCR Negative
11	PCR Negative
12	<b>PCR Positive</b>
13	PCR Negative
14	<b>PCR Positive</b>
15	<b>PCR Positive</b>
16	<b>PCR Positive</b>

#### 3.1 Serological Assays

Antibody tests for HCV are used to check for previous exposure and present infection. And for diagnosing a hepatitis C virus (HCV) infection, therapy guidance, or monitoring for the treatment of HCV. It looks for antibodies to the virus, which indicates HCV infection. EIA screening an enzyme immunoassay (EIA) for anti-HCV immunoglobulin G is used to diagnose HCV (IgG). Whole genome of the HCV codes 3,011 to 3,033 amino acids for

a polyprotein that is processed into 10 structural and nonstructural (NS) proteins [15]. This test will not tell you if you have an active or prior HCV infection.

There is some confirmation that a slightly positive test could be a false positive. According to the Centers for Disease Control and Prevention (CDC). An entirely positive antibody test should be followed by an HCV RNA test, which detects the presence of viral RNA in the blood to determine whether or not the subject is currently infected, The HCV antibody test can be used to establish which of the most prevalent hepatitis viruses is causing a person's symptoms as part of an acute viral hepatitis panel. The tests listed below can help identify an infection and suggest and monitor treatment:

RNA from HCV Qualitative tests are performed to determine whether an infection is present or past. If any HCV viral RNA is identified, the result is reported as positive or detected; otherwise, the result is reported as negative or not detected.

The quantitative test of Hepatitis C detects the number of international units of HCV RNA virus for one millimeter (mm) of HCV-positive patients' serum or plasma (IU/mL) [16]. This test can be used to check the virus's existence and detect a live infection. Quantitative assays (viral load) are used before and throughout treatments to compare the amount of virus present before and after treatment to measure cure response. HCV viral genotyping is used to define the genotype and kind of HCV present for assistance and monitoring of disease.

### 4. DISCUSSION

HCV is a single-stranded RNA virus that causes hepatitis C. RNA virus from the Flaviviridae family. HCV was initially recognized as Non-A, Non-B hepatitis virus in 1974 until the cloning of the etiologic agent in 1989. HCV infection affects about 3 % of the world's population (170 million people). 1.8 % of the population in the United States, or 3.9 million persons, are HCV seropositive. It is estimated that 10 million people in Pakistan are living with HCV infection [17]. Hepatitis C (HCV) is primarily transmitted by the use of contaminated needles in combination with a blood carrier after receiving infected blood from

impacted individuals. The majority of people are affected by sexual transmission in person, which can be extremely risky and lead to serious issues. [18-19]. Hepatitis C (HCV) virus is not transmitted by breast feeding, coughing, food, or water.

More than 50 subtypes have been identified, with genotype 1 accounting for roughly 75 % of occurrences in the United States. The medications chosen for treatment are influenced by the genotype of HCV viral infection. For the treatment of chronic viral hepatitis, molecular biology-based diagnostics are useful tools. They can be used to screen blood donations, diagnose active infection, determine prognosis, guide treatment options, and evaluate virological response to medication. The detection and quantification methods that usually are used in other labs are based on PCR and these are used in the management of hepatitis B and C virus infection. 20 serum samples from different individuals were evaluated simultaneously through active Anti- HCV.

The appearance of HCV RNA virus in plasma expresses active infection in patients and HCV-RNA can be detected 1 to 3 weeks post [20]. Different laboratories in Pakistan detect HCV-RNA. Due to the small amount of HCV- RNA in infected individuals, tests can only be performed using commercially available assay kits or in-house, home-made techniques in infected individuals. Reverse transcriptase enzyme (RT-PCR) are target amplification method for converting RNA to cDNA, which is then used as a template for the nested-PCR [21]. Primers whose sequences parallel to the 5' untranslated regions (5'UTR) are commonly

used because this is the most conserved region of the genome HCV-virus [22].

Table 2 shows an analysis to compare the price for HCV- RNA tests per sample in hepatitis C patients in different laboratories. Maximum expensive charges were reported from Shoukat Khanum laboratory for HCV -RNA tests when compared to other five PCR-based tests in laboratory-conducted Anti-HCV, HCV Qualitative, Quantitative and Genotyping tests. While on the other hand, average charges reported in the Alshifa laboratory were conducted for an HCV -RNA test for Anti-HCV (Rs. 2,300/-) Qualitative (Rs. 6,450/-) for Quantitative (Rs. 16,000/-and for Genotyping (Rs. 6,500/-). Dow laboratory HCV -RNA test is comparatively lower than Shoukat Khanum laboratory. The sample per-person test cost is highest for the Shoukat Khanum laboratory that has conducted HCV tests while reported lowest and most cost-effectiveness tests of the Molecular Diagnostic Laboratory for anti-HCV (Rs. 600/-). HCV - RNA for qualitative (Rs. 2000/-) for quantitative (Rs. 5000/-) and for Genotyping (Rs. 6000/-) per sample. So, our molecular tests for HCV- RNA detection and quantitation showed very good diagnostic and clinical performance over all five public health laboratories.

Daniel *et al.* 2005 described the RIA that was proposed for the recognition of anti-HCV antibodies. RIA proved 99.3 % sensitivity and 99.0 % specificity, individually [23]. In 2013, Firdaus *et al.* assessed the clinical effectiveness of RIA samples of HCV patients [24]. Around 15.74

**Table 2.** Shows analysis to compare the price for HCV RNA tests

Diagnostic Laboratories	Shoukat Khanum cost/sample	Agha Khan cost/sample	Chughtai cost/ sample	Dow cost/sample	Alshifa cost/sample	Molecular Diagnostic Lab (cost/sample)
Anti- HCV	2,100/-	2,400/-	2,200/-	800/-	2,300/-	600/-
HCV-RNA Qualitative	7,850/-	7,850/-	7,500/-	2,800/-	6,450/-	2000/-
HCV-RNA Quantitative	17,000/-	17,500/-	16500/-	5,500/-	16,000/-	5000/-
HCV- Genotyping	8500/-	8500/-	7000/-	7,500/-	6,500	6000/-

% of these samples were RNA-positive by nested RT-PCR, and 11.02 % were HCV seropositive by ELISA. Hence, the consequences of their study revealed that the RIA alone could not be relied on as an absolute diagnostic tool for screening HCV and besides this the nested PCR is a good implement for RNA detection [25] So, It is possible to successfully use the molecular diagnostic tests and reliable through different laboratories in Pakistan. In the HCV-RNA situation, our procedures can possess high sensitivity for detecting infections and good specificity over all other five laboratories. It offers rapid, accurate screening and diagnostic testing that improves human understanding of the disease, provides earlier detection and reduces the time to diagnosis. We have developed a method for the qualitative analysis of hepatitis HCV- RNA virus, at a lower cost as compared to other labs. This will help to control practicing modern molecular tools for disease diagnosis and encourage/contribute in the area of molecular diagnostics. All these insignificant expenses of our tests are all ready to be marketed.

## 5. CONCLUSION

The finding of our study identified a useful method for the rapid qualitative detection of HCV infection by comparing it with the price for HCV- RNA tests per sample in hepatitis C patients in different laboratories. Although our procedures provide accurate, reliable screening and diagnostic testing for the detection of diseases hepatitis C virus at low rates as compared to other laboratories for prevention of the further spread of HCV. This study also suggested that a one-step ELISA-based HCV-RNA screening method as well as a two-step PCR-based test technique would be the most cost-effective options when compared to the other strategies.

## 6. ACKNOWLEDGEMENTS

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## 7. CONFLICT OF INTEREST

The authors have no conflict interest.

## 8. REFERENCES

1. S.M. Afzal. Hepatitis C virus and interferon-free antiviral therapeutics revolution: implications for Pakistan. *Viral Immunology* 30:252–257 ( 2017).
2. M. Hellard, S.E .Schroeder, A. Pedrana, J. Doyle, and C.Aitken . The Elimination of Hepatitis C as a Public Health Threat. *Cold Spring Harb perspectives in medicine* 1:10(4) (2020)
3. R.H Miller, and R.H. Purcell. Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. *Proceedings of the National Academy of Sciences of the United States of America* 87:2057-61(2012).
4. M.N. Kamal, and M.H. El-Shabrawi. Burden of pediatric hepatitis C. *Asian Journal of Transfusion Science* 8(1): 19–25(2014).
5. N. Bhupinder, M.D. Bhandari, L. Teresa, and M.D.Wright. hepatitis C: An Overview 1995 *Annual Review of Medicine* 309-317(1995).
6. A. Bosan, H. Qureshi, K.M. Bile, I. Ahmad, and R. Hafiz. A review of hepatitis viral infections in Pakistan. *Journal of the Pakistan Medical Association* 60(12):1045–1058 (2010).
7. E.S. Rosenberg, E.M. Rosenthal, E.W. Hall, L.Barker, M.G. Hofmeister, P.S.Sullivan, P. Dietz, J. Mermin, and A.B. Ryerson. Prevalence of hepatitis C virus infection in US states and the District of Columbia, 2013 to 2016. *JAMA Network Open* 1(8): e186371-e186371 (2018).
8. A. Sohail, A.N. Jamal, and H. Andrew. The prevalence of hepatitis C virus infection in  $\beta$ -thalassemia patients in Pakistan: a systematic review and meta-analysis. *BMC Public Health* 20:587 (2020).
9. M.H. El-Shabrawi, and M.N. Kamal. Burden of pediatric hepatitis C. *Asian Journal of Transfusion Science* 8(1): 19–25 (2014).
10. A. Kane, Transmission of Hepatitis B, Hepatitis C and human immunodeficiency viruses through unsafe injections in the developing world: model-based regional estimates. *World Health Organization* 77:801-807 (2010).
11. WHO. Global health sector strategy on viral hepatitis 2016–2021. Towards ending viral hepatitis. *World Health Organization* (2016). <https://www.who.int/hepatitis/strategy2016-2021/ghss-hep/en/> (accessed Jan 21, 2020)
12. C.S. Graham, and T. Swan. A path to eradication of hepatitis C in low- and middle-income countries.

- Antiviral Research* 119: 89–96 (2015).
13. E. Stephen-Victor, H. Fickenscher, and J. Bayry. IL-26: An Emerging Proinflammatory Member of the IL-10 Cytokine Family with Multifaceted Actions in Antiviral, Antimicrobial, and Autoimmune Responses. *PLoS Pathogens* 12(6): (2016).
  14. Z. Chen, and K.E. Weck. Hepatitis C virus genotyping: interrogation of the 5' untranslated region cannot accurately distinguish genotypes 1a and 1b. *Journal of Clinical Microbiology* 40(9):3127–3134 (2002).
  15. S. Hamid, M. Umar, A. Alam, A. Siddiqui, H. Qureshi, and J. Butt. PSG consensus statement on management of hepatitis C virus infection--2003. *Journal of Pakistan Medical Association* 54:146–150 (2004).
  16. H.H. Hsu, and H.B. Greenberg. Hepatitis C. In: P.D. Hoepflich, M.C. Jordan, A.R. Ronald, eds. *Infectious Diseases. A treatise of infectious processes*, 5<sup>th</sup> ed. J.B. Lippincott Company. Philadelphia 820-825 (2012).
  17. A.O. Shakil, C. Conry-Cantilena, H.J. Alter, P. Hayashi, D.E. Kleiner, V. Tedeschi, K. Krawczynski, H.S. Conjeevaram, R. Sallie, and A.M. Di Bisceglie. Volunteer blood donors with antibody to hepatitis C virus: clinical, biochemical, virologic, and histologic features. *Annals of Internal Medicine* 123:330-7(1995).
  18. L. Blohm, C. Puttmann, S. Holz, G. Piechotta, J. Albers, C. Dammers, M. Kleines, A. Kurten, G. Melmer, J. Nahring, S. Barth, and E. Nebling. Rapid detection of different human anti-HCV immunoglobulins on electrical biochips. *Antibody Technology Journal* 4:23-32. (2014).
  19. C. Morishima, M. Chung, K.W. Ng, D.J. Brambilla, and D.R. Gretch. Strengths and limitations of commercial tests for hepatitis C virus RNA quantification. *Journal of Clinical Microbiology* 42: 421–425 (2004).
  20. J.P. Getchell, K.E. Wroblewski, A. DeMaria Jr, C.L. Bean, M.M. Parker, M. Pandori, D.R. Dufour, M.P. Busch, M.E. Brecher, W.A Meyer, and R.L. Pesano. Testing for HCV infection: an update of guidance for clinicians and laboratorians. *Morbidity and Mortality Weekly Report* 62(18): 362 (2013)
  21. Y. Mo, R. Wan, and Q. Zhang. Application of Reverse Transcription-PCR and Real-Time PCR in Nanotoxicity Research. *Methods in Molecular Biology* 926: 99–112 (2012).
  22. D.G Murphy, B.Willems, M. Deschenes, N. Hilzenrat, R. Mousseau, and S. Sabbah. Use of sequence analysis of the NS5B region for routine genotyping of hepatitis C virus with reference to C/ E1 and 5' untranslated region sequences. *Journal of Clinical Microbiology* 45(4):1102-1112 (2007).
  23. H.D.J. Daniel, P. Abraham, S. Raghuraman, P. Vivekanandan, T. Subramaniam, and G. Sridharan. Evaluation of a Rapid Assay as an Alternative to Conventional Enzyme Immunoassays for Detection of Hepatitis C Virus-Specific Antibodies. *Journal of Clinical Microbiology* 43: 1977–1978 (2005).
  24. R. Firdaus, K. Saha, and P.C. Sadhukhan. Rapid immunoassay alone is insufficient for the detection of hepatitis C virus infection among high-risk population. *Journal of Viral Hepatitis* 20: 290–293 (2013).