



Comparing RT-qPCR and Hepatitis C Virus Antigen Detection Assay for Detecting Active Infection in Blood Donors in Fars Province, Iran

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Abstract

Background: Immunoassay is still used to detect hepatitis C virus (HCV) antibodies in donated blood in many developing countries. However, an immunoblotting confirmation test is needed to confirm positive results.

Objectives: We compared the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of nucleic acid testing and HCV core antigen (HCVcAg) detection in the serum samples of blood donors with HCV antibodies to determine active infection.

Methods: Overall, 90 serum samples from blood donors referred to Fars Blood Transfusion Organization, Iran during March 2017-March 2019 and initially tested for HCV antibodies were included in the study. Enzyme immunoassays were used to detect the HCV antigen and anti-HCV antibody. A commercial reverse transcription-polymerase chain reaction (RT-PCR) kit was used to quantify HCV RNA. The HCV genotypes were also determined by DNA sequencing. In order to compare the HCVcAg detection method with the RT-qPCR reference method, sensitivity, specificity, performance, PPV, and NPV were calculated.

Results: Out of 90 serum samples, 73 were positive for anti-HCV antibody, and 17 sera were negative. The HCV RNA was detected in 60 (82%) of anti-HCV antibody-positive samples, whereas the HCVcAg test detected HCV antigen in 54 (74%) of the samples, indicating a significant correlation between the two assays ($r = 0.86$). The overall sensitivity and specificity for HCVcAg detection method were 93.85% [95% confidence interval (CI): 84.99 - 98.3%] and 100% (95% CI: 94.64 - 100%), respectively. Based on the statistical analysis, the accuracy of the antigen detection test was 94.83% (95% CI: 87.26 - 98.58%). Moreover, the agreement between HCV RNA detection using RT-qPCR and HCVcAg detection was 97.78% (kappa value: 0.94).

Conclusions: The sensitivity and specificity of HCVcAg detection in blood donors were ideal compared to the RT-qPCR reference method. However, the method should be tested on more HCV antibody-positive and -negative samples. Furthermore, our study revealed a significant association between the number of RT-qPCR-positive cases and the cases diagnosed by the HCVcAg detection method for screening and detecting active HCV infection in blood donors.

Keywords: HCV Core, Hepatitis C Virus, RT-qPCR, Sensitivity

1. Backgrounds

Modern health care requires safe and adequate blood supplies for transfusion therapy to be effective. Minimizing risk and optimizing clinical outcomes are the two biggest challenges of blood transfusion. Before blood transfusion, the donor's blood is subjected to laboratory testing to ensure that the patient receives the safest product. In addition, an infection-screening procedure should be followed before the release of whole blood and apheresis for clinical use.

Blood donation by people infected with human im-

munodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) poses the highest threat to transfusion safety due to asymptomatic periods (1). Therefore, a blood donation sample must be screened for infection with these viruses (2). For an infectious disease test, a window period refers to the interval between condition occurrence and when a test can determine disease presence. Window periods for antibody-based tests vary based on the time required for seroconversion (3).

Enzyme immunoassays (EIAs) serve as the primary screening tool in blood banks in developing countries.

Many developed countries perform nucleic acid testing (NAT) on the donated blood, resulting in dramatic reductions in transfusion-transmissible HCV (4). The current primary screening platform mainly targets anti-HCVcAg because it is genetically conserved and correlates with viral RNA levels (5-8). The result of the test is reported as positive or negative. A blood test to detect antibodies against HCV, known as recombinant immunoblot assay (RIBA), was used for many years as a secondary confirmation test for HCV if a first-line screening test presented indeterminate results. However, due to other tests being more sensitive and accurate, it has been discontinued as a method of detecting HCV and is now replaced by other methods (9).

The sensitivity/specificity of third-generation EIAs is approximately 99% (10). However, it is impossible to determine whether the infection is chronic, acute, or resolved based on the presence of anti-HCV antibody. Consequently, one should perform an HCV RNA test to confirm the presence of viremia (11). Fourth-generation testing for HCV antigens and antibodies (combination EIA) is more convenient because it detects two markers of HCV infections simultaneously. In addition, Ortho-Clinical Diagnostics has developed a new test that measures total HCVcAg in serum and plasma as well as anti-HCV antibodies (12-16). Several countries currently use this assay to screen blood donors.

HCVcAg is one of the definitive indicators of HCV infection as it appears earlier than HCV antibodies, is expressed only in patients with active infection, can distinguish between previous infection and current infection, and is not affected by immunosuppression. Therefore, HCV antigen tests can be used to diagnose active HCV infection using a one-step test (17). On the other hand, the RNA assay provides quantitative results, is highly sensitive and specific, has low detection limits, and is a reliable method with high sensitivity and specificity. However, it cannot be routinely used by low-income countries to screen blood donors due to the high technical skills needed, costs, and long turnaround times. Nevertheless, RNA levels and HCVcAg were found to be strongly correlated (18). Overall, the detection of HCVcAg can provide a valuable alternative to molecular testing and is of particular importance during the window period of HCV infection and before antibodies appear.

2. Objectives

In this study, we evaluated the sensitivity of HCV antigen, antibody, and genome detection tests used to identify the active disease cases among blood donors referred to Fars Blood Transfusion Organization.

3. Methods

The present study aimed to assess the sensitivity of HCV RNA and HCVcAg detection as screening tools for HCV active infections, along with antibody detection as a routine screening method for donated blood.

3.1. Patients and Samples

All blood samples were obtained from the blood donors with written consent. The Ethics Committee of Shiraz University of Medical Sciences formally approved the research protocol (IR-SUMS.REC.1396.S845). Our study included 90 serum samples from blood donors referred to Fars Blood Transfusion Organization, Iran, during March 2017-March 2019. We observed that 73 of the serum samples were initially positive for anti-HCV antibodies, while 17 were negative. All the tested donors were male and aged 18 - 59 years. None of the donors had been infected with HBV, HIV, or *Treponema pallidum*. Moreover, the participants had not received any medication for the treatment of HCV infection. From each participant, 5 mL of blood was collected. After 2 h of blood clotting at room temperature, serum was obtained. Next, blood clots were centrifuged at 12,000 g for 10 min. The serum samples were then collected, and aliquots in small sterile tubes were stored at -70°C until experimenting.

3.2. Anti-HCV Antibodies and HCV Core Antigen Detection

Anti-HCV antibody was detected with Cobas e-601 analyzer and Elecsys Anti-HCV II assay kit (Roche Diagnostics International Ltd., Rotkreuz, Switzerland) according to the manufacturer's instructions. Positive sera (S/Co ratio > 1) were evaluated with the HCV BLOT 3.0 (MP Biomedicals), which is a confirmatory qualitative enzyme immunoassay for the *in vitro* detection of antibodies against HCV in human serum or plasma. According to the manufacturer's specifications, the positive response was defined. These immunoblot strips contain recombinant HCV proteins from some regions of the HCV genome, including capsid, NS3, NS4, and NS5. HCVcAg was detected using Human HCVcAg ELISA kit, Orb4060 (Biorbyt, Cambridge, UK), according to the manufacturer's instructions. The cut-off value was calculated as the average of the negative control OD +0.1. As a result, the $OD_{\text{sample}}/\text{cut-off value} \geq 1$ was considered positive, and the samples with $OD_{\text{sample}}/\text{cut-off value} \leq 1$ were considered negative.

3.3. RNA Extraction

RNA was extracted from the serum specimens using the AccuZol™ Total RNA extraction kit (Bioneer, Korea), following the manufacturer's instructions. The nanodrop

UV- spectrophotometer device measured RNA purity and estimated RNA concentration. Afterwards, the extracted RNA was stored at -70°C until further analysis.

3.4. cDNA Synthesis and Nested RT-PCR

HCV RNA was detected in serum samples by nested reverse transcription-polymerase chain reaction (RT-PCR) with primers corresponding to the 5' non-coding region of the viral RNA. Each complementary DNA (cDNA) sample was prepared according to the manufacturer's instructions using AddScript cDNA Synthesis Kit (Addbio, Korea). HCV cDNA was amplified by nested PCR using two sets of primers (19) shown in Table 1. For the PCR amplification, the AccuPower®PCRPreMix kit (Bioneer, Korea) was used according to the manufacturer's protocol with the following conditions: 40 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 40 s for the first PCR, and 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s for the second PCR. A 1% agarose gel stained with GelRed (Biotium) was run at 70 volts for 60 min to visualize the DNA samples. Amplified PCR products were purified using a commercial PCR Purification Kit (Addbio®, Korea).

3.5. DNA Sequencing and Identification of HCV Genotypes

Microgen Co. Ltd. (South Korea) sequenced purified DNA samples. In order to determine HCV genotypes, the nucleotide data sequences obtained by direct sequencing were submitted to the HCV sequence database at Los Alamos National Laboratory, USA (hcv.lanl.gov/content/sequence).

3.6. Quantification of HCV RNA by Real-time PCR

The RNA levels of the HCV genome were quantified using a one-step AccuPower® HCV Quantitative RT-PCR Kit (Bioneer, Korea) using the instructions provided by the manufacturer. Real-time PCR was performed using genomic RNA extracted from previously tested samples.

3.7. Statistical Analysis

An analysis of the data was carried out using SPSS version 18. The degree of agreement between the results of HCVcAg and HCV RNA molecular tests for the diagnosis of HCV infection was determined by Cohen's kappa coefficient (κ). P-value < 0.05 was considered statistically significant. Real-time PCR was used as a reference method to calculate the sensitivity, specificity, NPV, and NPV of antigen detection.

4. Results

4.1. HCV Core Antigens and Nested PCR

To compare the sensitivity and specificity of HCVcAg detection assays with PCR assays, we examined 73 HCV antibody-positive and 17 HCV antibody-negative samples. Out of 73 anti-HCV antibody-positive samples, 61 (83.6%) were confirmed by western blotting assay. The blotting assay revealed that 8 (11%) and 4 (5.4%) out of 73 samples were negative and indeterminate (IND), respectively. Regardless of western blotting results, all 73 HCV antibody-positive blood samples were analyzed using the HCVcAg assay, showing that 54 of 73 samples (74%) were positive, while 19 tested negative (26%). None of the serum specimens in the control group (anti-HCV-negative) were positive for HCVcAg. All HCVcAg-positive and -negative cases were examined for HCV RNA using either nested or real-time PCR techniques. Among 73 samples tested, 60 tested positive for HCV RNA (82%). However, HCV RNA was not detected in 13 of 73 (17.8%) HCV antibody-positive serum samples.

4.2. HCV Genotyping

DNA sequencing was used to determine which genotype is prevalent among blood donors in our region. With reference sequences obtained from the GenBank HCV database, the sequences showed a 97-100% identity. Therefore, all the positive PCR samples were subjected to sequence analysis. Data analysis indicated that 43.8%, 29.4%, 13.1%, and 11.6% of the samples belonged to genotypes 1a, 3b, 1b, and 3a, respectively. However, HCV genotypes were not determined in 2.1% of the samples.

4.3. Real-time PCR

Out of 73 serum samples positive for HCV antibodies, 61 were found to contain HCV RNA by real-time PCR and nested PCR. In 54 samples with positive HCVcAg results, viral loads had a range of 1.18×10^3 - 3.3×10^6 IU/mL. Real-time PCR gave positive results for four samples with negative HCVcAg testing. Tables 2 and 3 show the results of HCVcAg detection and HCV RNA PCR, as well as the results of western blotting in relation to both antigen detection and RNA detection. Using RT-qPCR as a reference test, no significant difference was found between HCVcAg detection and PCR for the diagnosis of HCV active infection (Pearson's correlation coefficient $r = 0.86$). In addition, compared to the 100% sensitivity of PCR as a gold standard reference test, the antigen detection test in the blood samples of donors yielded a sensitivity of 93.85% (95% CI: 84.99 - 98.30%). However, the specificity of HCV antigen detection was 100% (95% CI:

Table 1. Primers Used for Nested PCR

Primers	Sequence	Product Size
Outer		272 bp
Forward	5'-ACTGTCTTCACGCAGAAAGCGTCTAGCCAT-3'	
Reverse	5'-CGAGACCTCCCGGGGCACTCGAAGCACCC-3'	
Inner		256 bp
Forward	5'-ACGCAGAAAGCGTCTAGCCATGGCGTTAGT-3'	
Reverse	5'-TCCCGGGGCACTCGAAGCACCCATCA-3'	

94.64 - 100%). Based on the statistical analysis, the accuracy of the antigen detection test was 94.83% (95% CI: 87.26 - 98.58%).

Table 2. Comparison Between HCV Core Antigen and RNA PCR

HCVcAg	HCV RNA		Total
	Positive	Negative	
Positive	54	0	54
Negative	6	13	19
Total	60	13	73

Abbreviation: HCVcAg, HCV core antigen.

5. Discussion

In the present study, to demonstrate whether the HCVcAg screening test can be compared to the virus nucleic acid detection test in donors' blood samples, the two methods were tested on 73 positive and 17 negative serum samples for anti-HCV antibodies by EIA. Our findings revealed that the HCVcAg assay performed well and correlated closely with HCV RNA detection tests. The typical assessment of active HCV infection includes an anti-HCV antibody screening test followed by NAT confirmation. However, due to high prices and the requirement of equipped laboratories, HCV RNA testing is limited in developing nations. Serum HCVcAg has been proved to be a viable alternative to NAT because it is more practical and less expensive. When NAT is unavailable, World Health Organization advises using HCVcAg as an alternate test to identify HCV viremia in low- and middle-income countries (20).

HCV infection in blood transfusions is screened using the anti-HCV tests according to a protocol. However, anti-HCV antibodies may not always indicate current HCV infection even when verified by an immunoblotting test (21, 22). HCV infection can also be detected by its core antigen. This protein is produced during viral assembly and

indicates active disease (23). In addition, the blood of infected individuals contains circulating HCVcAg and bound antibody/antigen complexes (24). Therefore, it is recommended to test for HCVcAg to detect active viral infection. Moreover, NAT is performed in some developed countries on donated blood as a highly sensitive and specific test (25).

The presence of HCVcAg in 54 and HCV RNA in 60 HCV antibody-positive samples despite all blood donors being asymptomatic, shows that they were in an active stage of the disease. However, undetectable HCVcAg and HCV RNA in HCV antibody-positive samples may indicate complete recovery from the disease. During chronic HCV infection, core antigen production is closely related to HCV RNA replication. Therefore, HCVcAg levels correlate well with the level of HCV RNA (26). Our results of blood donors with active infection showed a statistically significant correlation between HCVcAg positivity and HCV PCR results. However, the study had some limitations, including a limited sample size for test performance evaluation, especially in blood donors with negative HCV antibodies.

Several studies have used the antigen detection method in serum samples collected from various individuals to diagnose an active infection or track treatment for HCV infection. The results were then compared with the method for detecting nucleic acids. All studies found a strong association between HCVcAg and HCV RNA testing (27-32). RNA was detected in six samples in our experiment, whereas HCVcAg was not found. HCV infections with viremia less than 3×10^3 IU/mL were not detected by the HCVcAg test. This is in line with the limits of detection reported by some other studies (33, 34).

The HCVcAg detection by ELISA and the RNA detection test in serum samples were both positive and consistent in all cases where the western blotting was reported to be 3⁺. However, HCV RNA was found in five samples where the western blotting for HCVcAg was negative or less than 3⁺ or at least two other HCV antigens were positive. One reason could be the sensitivity and specificity of the commercial kit employed in this study which used antibodies specific for HCVcAg. Furthermore, there is a likelihood that the re-

Table 3. Results of Western Blotting Assay Versus Real-time PCR and ELISA

	Western Blotting Assay			Total
	Positive	Negative	IND	
Real-time PCR				
Positive	58	0	2	60
Negative	3	8	2	13
ELISA for HCVcAg				
Positive	53	0	1	54
Negative	8	8	3	19
Total	61	8	4	73

Abbreviations: HCVcAg, HCV core antigen; IND, indeterminate.

sults might be false negative if the core antigen had a mutation. It is possible to produce HCV antigen detection kits containing monoclonal antibodies specific to several HCV epitopes with different molecular methods (35). As a result, antigen detection would be more sensitive and specific.

When compared to PCR, as the gold standard reference test, the HCVcAg detection test on donors' blood samples had a sensitivity and specificity of 93.85% and 100%, respectively. Accordingly, HCVcAg screening seems to be a reasonable replacement in situations where PCR is unavailable. In conclusion, HCVcAg detection by ELISA seems comparable to the real-time RT-PCR method for screening and detecting active HCV infection in blood donors due to its specificity, 100% PPV, accessibility, higher cost-effectiveness, and ease of use.

Footnotes

Authors' Contribution: A.B.B and A.F developed the original idea and protocol, abstracted and analyzed the data, and wrote the manuscript. L.K collected and interpreted the clinical data and revised the manuscript. G.R.D and M.M supported the study regarding administrative and technical aspects and material. S.S and A.B.B supervised the study. S.N helped to write and revise the manuscript grammatically.

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Data Reproducibility: The data presented in this study will be available to the reviewers or the EIC upon their re-

quest without any limitation as a part of the review process.

Ethical Approval: Shiraz University of Medical Sciences Ethics Committee formally approved the research protocol (IR-SUMS.REC.1396.S845).

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Informed Consent: All blood samples were obtained following obtaining written consent from the blood donors.

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