Development of ELISA Method for primary Detection of HCV using core Antigen

Shirin Nourollahi[1], Seyed Mehdi Boutorabi[2], Ali Mirjalili[3], Mahmoud Mahmoudian Shooshtari[4], Maryam Razaghi[1], Masoomeh Hashemi[5], Mehrdad Jalalian[6, 7]

[1.] Department of Microbiology, Faculty of basic Sciences, Islamic Azad University-Zanjan branch.Iran
[2.] Director of Research Lab., Pishtaz Teb Inc. Tehran, Iran.
[3.] Department of Biotechnology, Razi Vaccine and Serum Research Institute, Tehran, Iran.
[4.] Research Center of Iranian Blood Transfusion Organization, Tehran, Iran.
[5.] Department of Biochemistry, Payamenour University of Tehran, Tehran, Iran.
[6.] Khorasan Razavi Blood Center, Mashhad, Iran.
[7.] Department of Community Health, faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor D.E., Malaysia.
shirin_nourollahi@yahoo.com

Abstract: Studies show that Hepatitis C Virus (HCV) antigens appear before antibody while the early days of infection. Therefore detecting antigens could lead us to diagnosing the infection on time. The aim of this study was to develop a simple and sensitive enzyme immunoassay for the detection of hepatitis C virus (HCV) core antigen in order to evaluate the role of core antigen as a marker of HCV infection. A total of 280 samples was tested by third generation anti-HCV, and the reverse transcription polymerase chain reaction (RT-PCR) was performed only when the anti-HCV enzyme immunoassay (EIA) was positive. All samples were tested with HCV core antigen using Elisa kits. Among the 280 samples, 95 samples were anti-HCV positive. Among those 95 samples, 75 samples were RT-PCR-positive. The cut-off value was set at 0.15 unit of optical density (equivalent to 2.5 pg/ml of core antigen based on the distribution of healthy subjects (anti-HCV-negative subjects). The difference between the mean optical density values of HCV-ribonucleic acid-positive (HCV-RNA-positive) samples and HCV-RNA-negative samples in the HCV core antigen assay was highly significant (1.4 us 0.08, $p < 0.005$). The sensitivity and specificity of the core antigen assay were 88% and 96%, respectively. The pretreatment of the anti-HCV-positive samples with a solution that contained 1.5 M glycin buffer (pH = 2) increased the sensitivity of the assay (from 57.3% to 88%). This assay is a simple, sensitive, and useful method for use as a screening strategy for HCV infection in anti-HCV-positive or anti-HCV-negative individuals.

[Shirin Nourollahi, Seyed Mehdi Boutorabi, Ali Mirjalili, Mahmoud Mahmoudian Shooshtari, Maryam Razaghi, Masoomeh Hashemi, Mehrdad Jalalian. **Development of ELISA Method for primary Detection of HCV using core Antigen.** Journal of American Science 2011; 7(2):303-307]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Hepatitis C virus (HCV); Core antigen; ELISA; Polymerase Chain Reaction (PCR)

1. Introduction

Hepatitis C virus (HCV) is a single-strand, ribonucleic acid (RNA) virus that contains approximately 9500 nucleotides for coding a polypeptide with a length of roughly 300 amino acids. This virus is the cause of hepatitis C disease, and it has been well established that the initial origin of this infection is the injection of contaminated blood or blood products. At present, the suggested method for recognizing when an individual is infected by the hepatitis C virus is to determine the antibody against HCV. This approach is problematic, because the test produces both false-negative and false-positive results. In addition, this test cannot distinguish between chronic and acute infections. So, Immunoblotting is applied in order to study false-positive results (1, 2). It is especially important to recognize the infection in the initial steps to prevent secondary infections in blood transfer (3). Therefore, a method is required that can recognize HCV in samples, especially in the initial phases of infection. Presently, molecular methods are used to determine the presence of viral RNA in samples. These methods use the polymerase chain reaction (PCR) technique, which has advantages and drawbacks, for investigating treatment efficiency. The application of monoclonal or polyclonal antibodies, especially against the viral core antigen, is under investigation (4, 5, and 6). The aims of the present study is to use the Elisa kit approach and demonstrate high sensitivity and specificity for recognition of this antigen and to compare the effectiveness of the approach with other related techniques. Based on the results of this study, the problems with other techniques mentioned earlier can be alleviated.

2. Material and Methods

Samples:

280 serum samples were collected from clients of the molecular biology sections of some medical laboratories of Tehran.

Testing the antibody against the hepatitis C virus: Using the kit from the Pishtaz Teb Zaman Company, all 280 samples were examined according to the procedure provided in the enclosed brochure. Among the 280 samples, 95 samples contained the antibody, while the optical density (OD) values of the remaining 185 samples that were less than the cut-off value specified in the kit, which was an average OD of +02 as the negative control. The kit uses antigens NS3, NS4, NS5, and the Core to identify the antibody, and its use has been approved by the Iranian Reference Laboratory of the Ministry of Health and Care.

RT-PCR for positive samples:
RNA was extracted using the QIAGEN Company's QIAamp Circulating Nucleic Acid Kit Quantitative RT-PCR was accomplished by an Artus 3000 device. The characteristics of the cycle's program of this device are provided in Table 1.

Table 1. Characteristics of the cycles of RT-PCR device

Cycle information	**Quantities**		
Cycle quantity	45		
Analysis mode	Quantitative		
Heat turns	First	Second	Third
Heat degree	95	55	72
Incubation time(min)	8	20	20

Testing Core Antigen by the Elisa method:
We utilized monoclonal antibodies from mice, which were provided by the American company Genwaybio. The monoclonal antibodies were available at various concentrations in a carbonate coating buffer with pH = 9.6. Coating was performed at a temperature of 4 o C, after which the specimens were stored at room temperature for 24 hours. Subsequent to washing the Elisa plate with a phosphate buffer solution containing Polysorbate 20 (Tween 20), the wells were blocked for one hour at room temperature with a blocker solution containing 1% bovine serum albumin (BSA) and carbohydrate. The contents of the wells were then removed and dried at room temperature for six hours. These plates were stored in foil, along with a desiccant, at 2 – 8 oC until it was time for the experiment. This antibody identifies a marker of the antigen in the amino acid range of 1 to 80.

Conjugate 1: A polyclonal antibody of goat origin, prepared by the American company Genwaybio, was conjugated by biotin using the following procedure. This antibody identifies an index of the antigen in amino acid range of 80 to 120. An antibody solution with a concentration of 1 mg/ml was prepared with a buffer containing 0.2 M bicarbonate and 0.15 M sodium chloride at pH = 8.8. Then, a biotin solution (Act Biotin LKB) was prepared at a concentration of 4 mg/ml in dimethy formamide (DMF). Subsequently, 200 µl of the biotin solution were added to 10 mg of the 1 mg/ml-antibody solution, and the mixture of the two solutions, at room temperature, was shaken for 15 minutes. After that, 100 µl of 1 M ammonium chloride were added to stop the reaction, and the resulting solution was thoroughly mixed. Then, the solution was incubated for 15 minutes at room temperature while the mixing continued. In order to bring out the free bioptin, dialysis was performed against 0.05 N PBS buffer using a dialysis bag with a cut-off value of 30. Dialysis was repeated after changing PBS buffer solution. Considering the initial volume of antibody and its final volume, the concentration of the antibody conjugated by bioptin was calculated to be about 2.5 mg/ml.
Conjugate 2: In order to identify the antibody-antigen complex, we utilized different concentrations of HRP-conjugated streptavidin prepared by Sigma Company. This conjugate was diluted with a stabilizer solution that is specifically used for maintaining HRP-conjugated streptavidin. The stabilizer solution was obtained from Pishtaz Teb Company.
Test Procedure: A 100-µl serum sample and 50 µl of conjugate 1 diluted in a solution of phosphate buffer containing 0.5% BSA were added to each well. For positive control, the Core recombinant antigen of hepatitis C virus with a concentration of 100 pg/ml in phosphate buffer containing 1% BSA was used. This recombinant antigen was prepared by the Russian RPC Company. The wells were incubated for one hour at a temperature of 37 o C in a water bath. After incubation, the wells were washed out five times by washing solution containing phosphate buffer and Tween 20. Subsequent to completely taking out the washing solution, 100 µl of conjugate 2 were added to each well, followed by incubation for 30 minutes at 37 oC. After incubation, the wells were washed five times with the buffer solution, and 100 µl of the

chromogen substrate solution were added to each well after each well was completely emptied. The chromogen substrate solution contained tetramethyl benzidine and H2O2, prepared by Pishtaz Teb Company. Fifteen minutes later, the color of the solution changed from colorless to blue, indicating that the reaction had taken place. When 100 µl of stopping solution (1 N hydrochloric acid) were added, the reaction stopped, and the color of the solution changed from blue to yellow. The OD value of the wells at a wavelength of 450 nm was measured to be 630 using a reference filter. To determine the cut-off point, we calculated the mean and standard deviation (S.D.) of optical density pertaining to negative samples. Afterward, a cut off was determined for the kit according to the formula (cut off = mean + 3*S.D.).

For excluding the probable interference effect of the presence of the antibody with the Core antigen from the samples, serum samples with positive antibody tests were pretreated by two different solutions, including 1.5 M glycine buffer with pH = 2, 0.5 N hydrochloric acid, and Triton X-100 with a concentration of 0.1%. Then, 100 µl of the sample was mixed with 100 µl of each of the different solutions and kept for 30, 60, 90, and 120 minutes at 37 o C. For acidic solutions, the pH of the sample pH was adjusted to 7 by adding Tris buffer with pH = 10.4. In order to study the probable effect of these solutions in causing background optical density, several negative samples were also investigated in addition to the positive samples. Also, to make sure that the effect of these solutions on the antibodies contained in the serum on the samples that were treated by the Triton X-100 and acidic solutions, the antibody test was repeated once more, and the results were compared to titration of the antibody in serum samples that were not treated by these solutions. With the aim of investigating the diagnostic sensitivity by BBI International panels, seroconversion panel numbers PHV906, PHV914, and PHV920 were selected for study. Evaluation of analytic sensitivity was then studied by serial dilution of recombinant core antigen (amino acids 2 - 119) in the HCV antibody negative sample.

Imprecision Test: To facilitate studying the imprecision of the assay, three samples with different concentrations of antigen were tested ten runs over a five-day period, and each test was performed as duplicate.

3. Results

Among the 280 samples, 95 samples had positive antibody tests, while 75 of these 95 samples had positive PCR results. Among the 75 samples with positive results in both PCR and antibody tests, 43 cases (57) had positive results in the Core antigen test; for the 20 samples with positive antibody tests and negative PCRs, one case was positive in the Core antigen test, whereas five cases were positive in the Core antigen test but had negative results in both the PCR and antibody tests (Table 2).

Table 2. Correlation of antigen test with PCR method

PCR results		Core Antigen test	
		+	-
+	75	43	32
-	205	8	197

Considering the results, the sensitivity and specificity of the identification test were estimated to be 57% and 96%, respectively. The mean OD value of samples with negative antibody tests and PCRs was calculated to be 0.080 with a standard deviation of 0.023. The cut-off of the OD value for discriminating negative samples from positive samples was determined by adding three times the S.D. to the mean OD value, resulting in 0.15. Therefore, samples that had OD values greater than 0.15 were considered positive. The OD value values for the positive samples ranged from 0.35 to 2.91 with mean value of 1.44

Table 3. The correlation between the antigen test and the PCR method after pretreatment of samples by glycine buffer

PCR results		Core Antigen test	
		+	-
+	75	66	9
-	205	8	197

Effect of pretreatment of serum samples to acidic solutions and Triton X-100:

The titration of antibodies present in the serums that were treated by acidic solutions had a considerable decrease compared to serum samples without this treatment. Even so, no such decreases were observed in samples that were treated by Triton X-100. In addition, antibody titration had a substantial decrease after treatment by acidic solutions in 22 samples with negative antigen test and positive PCR test with verified antibody presence (Fig. 1).

This pretreatment led to increases in optical density and positive antigen tests in 23 cases out of 32 samples. Regarding the comparison of optical density of samples with positive PCR tests and negative antigen tests, pretreatment by glycine buffer (pH = 2) for one hour at a temperature of 37 oC resulted in more increase in optical density than treatment by 1 N hydrochloric acid at 37 oC. On the other hand, treatment by Triton X-100 had no effect on increase

of optical density. Furthermore, optical density of samples with negative antibody tests, which were used as controls, showed no increase. This indicates that the increase in optical density in samples with positive antibody tests after pretreatment by acidic solutions for testing the antigen is a specific phenomenon caused by dissociation of the antibody from the antigen and subsequent identification of Core antigen using this evaluation technique. (Fig. 1)

Comparison of pretreatment effect of serum samples with different solutions on dissociation of antigen-antibody complex in HCV Core antigen test:

The correlation between the PCR method and antigen determination after pretreatment of serum by acidic solutions is depicted in Table 3. Considering the results, the sensitivity and specificity of the identification test were estimated to be 88% and 96%, respectively. We investigated the value of different markers of HCV in commercially-available, seroconversion panel samples. These panels include groups of serial samples that have been acquired from an individual at different time periods, and they show the time when the antibody can be identified in the samples. The kit provided by the Pishtaz Teb Company was used to measure data related to antibodies, but the data pertaining to HCV RNA were similar to panel insert.

Considering serial dilution of Core recombinant antigen in a negative serum sample for evaluating the analytical sensitivity of the test and regarding the defined cut off for negative samples, the least amount identifiable through this test was determined to be 2.5 pg/ml, which is equivalent to 20000 IU/ml RNA (Fig. 4).

Imprecision Test: The extrapolated precision results obtained for three samples with different concentrations of antigen are presented in Table 4.

4. Discussions

The ELISA method is practical for identifying Core antigen of hepatitis C virus via specific antibodies that capture the antigen. Although identification of viral RNA has been known as a gold standard in detection of HCV infection, studies reveal that the Core antigen of the virus can be identified in a one-day period after appearance of RNA (3-5). The sensitivity of the test before and after pretreatment of serum with 1.5 M glycine solution was 57% and 88%, respectively, which indicates that its sensitivity and specificity results are favored compared to the results of other studies. In 2005, Gaudy et al. reported sensitivity and specificity values of 96.7% and 100%, respectively, using the kit of Core antigen made by American Ortho Company. Eight out of 2,395 cases with antigen concentrations lower than 8.5 pg/ml were verified using the neutralization technique (2). In 2005, Fabrizi et al. reported a sensitivity value of 92.7% and a specificity of 97% by using the Ortho kit and comparing it to the PCR method. The population that was studied included 125 people with negative antibody titration and 167 people with positive antibody titration (6). In 2006, Reddy and his colleagues reported a sensitivity of 60% and a specificity of 83% by using the Ortho kit and comparing it to the PCR method. These researchers conducted their studies on a population of 111 patients with chronic renal failure (7).

An increase in test sensitivity subsequent to pretreatment by 1.5 M glycine buffer indicates the elimination of the interference of antibodies against Core antigen present in serum. These antibodies prevent the binding of antigen to antibodies captured in the wells by forming a complex with antigen and masking the antigenic epitopes. pretreatment by specific buffers has also been applied using the Ortho kit. In 1999, Aoyagi et al. verified that pretreatment before the test for identification of antigen is followed by an increase in optical density in the antigen test and a decrease in antibody titration against the virus. These results were produced using a buffer containing Triton X-100 with 0.3% concentration, Chaps with 1.5% concentration, and SDS with 15% concentration. These researchers suggested a incubation period of 30 minutes at 56 oC (1).

All of the nine cases with a negative antigen test, even after pretreatment, had high antibody titrations, and it is possible that in the pretreatment step, all antibodies don't dissociate completely. Another reason for negativeness of these samples may be the existence of incomplete antigenic particles that have not been identified by the antibodies used in test. Furthermore, the concentration of nucleic acid was determined similarly in HBV infection. In the PCR method, there may be no correlation between the concentrations of antigen in blood flow (10).

Heterogeneity of sequence has been reported in the HCV genome. Several studies have specified that there are differences in test sensitivity for determination of different genotypes, both in the PCR technique and in the identification of antigen and antibody (1). In order to minimize the heterogeneity effect in the amino acid sequence for test sensitivity, the antibodies utilized should be capable of identifying the conserved amino acid sequence in constant in core antigen.

Most studies that have used the kit from the Ortho Company have reported an analytical sensitivity of 1.5 µg/ml, which is equivalent to 1*104 IU/ml RNA (11). Regarding serial antigen dilutions in negative serum, the analytical sensitivity of the test

in current research has been shown to be approximately 2.5 pg/ml, which is equivalent to 2*104 IU/ml RNA. The imprecision index in the designed kit in the present research was based on a variation coefficient of 4 – 12%. In the same way, Havs et al., in 2004, reported the inaccuracy of interpolation and extrapolation as 6% - 25% 1% – 9%, respectively (12).

According to results of the seroconversion serum from the BBI Company used in the present study, the identification of the Core antigen was accompanied by a considerable decrease in the serologic window period. The ELISA method for identification of the Core antigen is a direct technique for detecting HCV infection. It is practical in most laboratories, is affordable, and there is no need for expert and experienced personnel. Cross-contamination problems of PCR do not exist in this test, and maintenance of the sample is simpler due to higher stability of antigen compared to RNA. In conclusion, the method is useful for identifying HCV infection in patients with deficiency of homoral immune systems or those who take immunosuppressive drugs for immune suppression.

Acknowledgements:

The authors would like to acknowledge the Islamic Azad University (Zanjan Branch), Mr. Behrouz Hajian Tehrani and all staff of the section of research and development of Pishtaz Teb Inc., Dr. Siamak Samiee (department of molecular biology, Day hospital, Tehran), and Dr. Keyvan (Dr. Keyvan virology Lab., Tehran) for their support and contribution to this study.

Corresponding Author:
Shirin Nourollahi
Department of Microbiology
Faculty of basic Sciences
Islamic Azad University of Zanjan, Zanjan, Iran
E-mail: shirin_nourollahi@yahoo.com

References

1. Aoyagi.K, Ohue.C, Iida.K, Kimura.T, Tanaka.E, Kiyosawa. K, and Yagi.Sh(1999). Development a simple and highly sensitive enzyme immunoassay for hepatits C virus core antigen. J Clin Microbiol;37(b): 1802-1808
2. Gaudy. C, Thevanas. C, Tichet. J, Morriote. N, Goudeau. A and Dubois. F (2005) Usefulness of the hepatits C virus core antigen assay for screening of a population undergoing routine medical checkup. J Clin Microbiol; 43(4): 1722-1726
3. Letowska. M, Brojer-E, Mikulska. M, Gronowska. A, Rosiek. A. (2004). Hepatits C core antigen in Polish blood donor. Trasfusion; 44: 1067-71
4. Peterson. J, Green. G, Iida. K, Caldwell. B, Kerrison. P, Bernich. S, and et al.(2000). Detection of hepapits C core antigen in the antibody negative window phase of hepatits C infection. Vox Sang; 78: 80-85
5. Courouce. AM, Le Marrec. N, Bouchardeau. F, Razer. A, Maiez. M, and et al (2000). Efficiency of HCV core antigen detection during the pre seroconversion period. Transfusio; 40: 1198-1202
6. Laperche. S, Le Matree. N, Simon. N, Boukhardeau. F, Defer. C, Maiez-Montrevil. M, et al. (2003). A new HCV core antigen assay based on disassociation of immune complexes, an alternative to molecular biology in the diagnosis of early HCV infection. Transfusion; 43:L958-62
7. Fabrizi. F, Lunghi. G, Aucella. F, Mangoo. S, Barbisoni. F, Bisegna. S, Vigilante. D and et al.(2005). Novel assay using total hepatits C virus (HCV) core antigen qualification for Diagnosis of HCV infection of HCV infection in dialysis patients. J Clin Microbiol; 43(1): 414-420
8. Reddy. AK, Dakshiamurty. KU, Lakshai. V (2006). Utility of HCV core antigen Elisa in the screening for hepatits C virus infection in patients on hemodialysis. Ind J Med Microbiol; 24(1): 55-7
9. Daniel. HDJ, Vive Kamandan. P, Raghurameen. S, Sridharam. G, Chady. GM, Abraham. P. (2007). Significant of the hepatits C virus core antigen as an alternative plasma marker of active HCV infection. Ind J Med Microbiol; 26(1):37-42
10. Ballard. Al, Boxall. EH. (1999). Assessing the infectivity of hepatits B carriers. Commun Dis Public Health; 2: 178-83
11. Bounvier-Alias. M, Pater.m, dahai. H, Beucourt. S, Laderie. P, Blatt. F, et al. (2002) Clinical utility of total HCV core antigen qualification: a new indirect marker of HCV replication. Hepatology; 36:211-218
12. Havs. I, Sterzi. E, Havs. J, Marth. E, Kessler. H.H. (2004) Evaluation of total HCV core antigen qualification. J Lab Med; 28(1): 97-100.

11/28/2010