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Cross Reactivity Values in Hepatitis C Infection and a Solution to Detect True Positive Serums by Third Generation of ELISA Test

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ABSTRACT

A large number of healthy donors being falsely labeled as carriers of HCV (false-positive) due to the cross reaction. The low specificity of the enzyme immunoassay (EIA) tests for the anti-HCV antibodies is responsible for this event. To examine the cross reactivity values of different Hepatitis C Virus antigens and purpose a solution to detect true positive results by third generation of ELISA. A total of 988 blood samples were participated in this study and two kinds of ELISA tests were used in all of the samples: single-antigen ELISA and third generation of ELISA. Tests set up procedures were perfomed by appropriate concentrations of antigens and conjugated titer for each test. Thereafter, all true positive samples were examined to determine IgG subtypes. The highest antibody response was against core HCV protein (85%) followed by NS4, NS5 and NS3 respectively. It was observed that high percentage (95%) of samples with S/Co > 4 in third generation of ELISA had positive RIBA tests (p value < 0.001). It was also demonstrated that NS3 and NS4 antigens were diagnosed simultaneously more (in compare with NS5 and Core24) in S/Co > 4 samples (p value = 0.006). The highest produced antibody during HCV infection was IgG1, significantly (P=0.023). In conclusion, if the S/Co of third generation of ELISA is more than four or the samples were positive in NS3 and NS4 single antigen ELISA tests, the RIBA test will be positive which indicated true positive results.

Keywords: Hepatitis C virus; Third-generation HCV antibody enzyme; immunoassay; RIBA; Cross reaction

INTRODUCTION

Hepatitis C virus [HCV] is a highly heterogeneous RNA virus which is one of the most important causes of liver cirrhosis and hepatocellular carcinoma [1, 2]. HCV is able to persist for a virtually indefinite period of time in the host [3] due to the ability of the virus to undergo rapid and substantial sequence modifications [4-6]. The anti-HCV antibodies can be detected 7-8 weeks after infection and usually persist for life [7, 8]. Diagnosis of HCV infection can be established readily by sensitive and specific serological assays which incorporate a mixture of viral polypeptides on the solid phase. The initial test that is typically used is called an enzyme-linked immunosorbent assay or ELISA. It looks for the antibodies to HCV that the immune system develops in response to an infection [9-12]. The HCV polyprotein is processed by the host and viral proteinases to produce at least ten functional viral proteins including [from the amino to the carboxyl terminal]: core [C], envelope [E] 1, E2, p7, non-structural antigens [NS] 2, NS3, NS4A, NS4B, NS5A, and NS5B [11, 13-15]. Therefore, the third generation of enzyme-linked immunosorbent assay [ELISA] includes four recombinant proteins derived from the core and three nonstructural regions [NS3, NS4, and NS5] of HCV [16, 17]. In fact, both structural proteins, such as the putative nucleocapsid protein, and nonstructural region polypeptides, such as NS3, NS4, and NS5 can be established readily by serological assays. However, HCV envelope proteins have never been included in commercial immunoassays, due to the considerable sequence heterogeneity among viral isolates [10, 18, 19]. There is evidence that a high

percentage of patients who are positive for anti-hepatitis C virus [HCV] antibody have hepatitis C infectious, but a large number of healthy donors being falsely labeled as carriers of HCV [false-positive] due to the relatively low specificity of the enzyme immunoassay [EIA] tests for anti-HCV antibodies [20-24]. False-negative results also may arise in immunocompromised patients, such as those with human immunodeficiency virus [HIV] infection or uremia. In the context of HIV infection, the HCV seroconversion delay is often prolonged and leads to the failure of reactivity of current anti-HCV antibody detection tests [8, 25, 26]. Therefore, results obtained by EIA need to be confirmed by additional testing. The diagnosis of HCV infection can be supported or confirmed by the recombinant immunoblot assay [RIBA] or tests for HCV RNA which is too laborious and expensive to perform regularly as a confirmatory assay. Thus, the RIBA test defined as a confirmatory assay. It consists of recombinant proteins identical to those in the EIA test [27-29]. There is evidence of cross-reaction of the ELISA test with antibodies against other viruses, for example the cross-reactivity of HCV epitopes with the Flu-NA peptide was observed in several studies [30-32]. Infact, an influenza virus peptide derived from neuraminidase 231-239 [Flu-NA] and an HCV CTL epitope from NS3 1073-1081 [HCV-NS3], both of which are HLA-A2 restricted. This similarity was responsible for cross reaction of HCV proteins with antibodies against influenza virus. Thus, cross-reaction of HCV proteins caused false-positive results in serological tests, particularly ELISA [33]. Mondelli et al. and Puntoriero et al. indicated an important role of hypervariable region 1 [HVR1], which is located at the N terminus of envelope protein E2, in cross reactions of HCV ELISA tests [34, 35]. In a recent study, Scarselli et al. also provided supplementary evidences for the existence of cross-reactive antibodies to HVR1. They observed this cross reaction in a significant proportion of patients with documented chronic HCV infection [36].

Objectives

In present study, we evaluated cross reactivity values of different Hepatitis C Virus antigens and purposed a solution to detect true positive results by third generation of ELISA.

MATERIALS AND METHODS

Patients and Serums

A total of 988 blood samples were consecutively collected from three patient groups which referred to the Tehran Blood Transfusion Organization in 2015: healthy controls, false positives and HCV patients. The 475 healthy individuals in the control group were seronegative for the HCV, HIV, and hepatitis B surface antigen (HBsAg) ELISA tests, and had liver aminotransferases (ALT and AST) values within normal limits. One hundred sixty eight participants in false-positive group were seropositive for the HCV ELISA test and seronegative for the RIBA test (HCV blot 3.0; Genelabs Diagnostics®, Singapore) which is routinely used to confirm positive results of ELISA in the Tehran Blood Transfusion Organization. The 345 patients in the HCV group were positive for both the HCV ELISA test and RIBA test, negative for HIV infection and HBsAg. Experiments in this study were based on the third-generation HCV antibody enzyme immunoassay (ELISA 3) which was followed by RIBA test as a confirmatory assay. In fact, subjects were screened for the presence of anti-HCV antibodies by third-generation enzyme immunoassay and positive results were confirmed by third generation immunoblot assay (RIBA 3). An immunoblot test is considered positive if two or more proteins react and considered indeterminate if only one positive band is detected. All participants provided written informed consent before enrolment and all information were kept confidential and those not consenting to participate in the study were excluded.

Tests Set up Procedure

Two kinds of ELISA tests were used in this study: single-antigen ELISA and third generation of ELISA (EIA 3). Both of these tests were employed for all samples. In single-antigen ELISA, only one type of HCV antigens (NS3, NS4, NS5, Core24) was coated in a polystyrene flat-bottom ELISA plate, however in EIA 3 all four antigens simultaneously were coated. We used IgG conjugated to HRP as conjugate, which can detect all subtypes of human IgG antibodies. For each test, appropriate concentration of antigens and suitable conjugated titer (titer in which it has the highest OD in positive controls and lower than 0.1 OD in negative controls) were determined by Checker Board. The appropriate conjugated titer was 1:35000 for all single-antigen tests and 1:25000 for EIA 3. Thereafter, cut off value of each test was evaluated by testing all negative samples. It was calculated for single-antigen test by the sum of mean OD of negative samples and 2 × standard deviation of OD in negative samples as well as EIA 3 test by sum of mean OD of negative samples and 3 × standard deviation of OD in negative samples. Finally, S/Co ratios (the ratio of the actual EIA test reading (sample = S) to the cutoff (C) value for the test) for third generation of ELISA test were calculated. Single-antigen ELISA also was performed on all false positive and true positive samples to detect the cross reaction and antibody response against of each HCV antigens, respectively. Antigens were coated

with concentration obtained above, for each antigen to examine IgG subtypes. However, anti-human IgG1, anti human IgG2, anti human IgG3 and anti human IgG4 which were conjugated to HRP used as conjugates, separately. Titration was conducted as mentioned above to determine the proper titers for these conjugates and it was calculated 1:10000, 1:8000, 1:10000 and 1:5000, respectively. Then, cut off value of each conjugate was examined by testing all negative samples as mentioned for single-antigen tests and all true positive samples were examined to determine IgG subtypes. All of the mentioned antigens were produced by RPC Russian Company (Table 1).

Table 1. Recombinant antigens

Recombinant antigens	Concentration (μg/ml)
E.coli derived recombinant Core 24 (genotype 1b, amino acids 2-119)	2
E.coli derived recombinant NS3 (genotype 1a, amino acids 1192-1459)	0.5
E.coli derived recombinant NS3 (genotype 1b, amino acids 1356-1459)	0.5
E.coli derived recombinant NS4 (Mosaic amino acids, 1691-1710, 1712-1733, 1921-1940 from genotypes 1,2,3,5)	0.5
E.coli derived recombinant NS5 (genotype 1b, amino acids 2061-2302)	0.5

Coating and ELISA Procedure

Antigens were dissolved in methanol at a concentration of 1.0 mg/ml, and then diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6, in different concentrations (0.5-5μg/ml) and 100 μl was added to each well of a polystyrene flat-bottom ELISA plate (96 well MicroWell™ MaxiSorp™ flat bottom plate). Plates were incubated overnight at 4°C and then washed three times with 350 μl Phosphate Buffered Saline (PBS) containing 0.05% Tween 20, pH 7.4. The nonspecific binding of immunoglobulins was prevented by adding a mixture of 1.5% bovine serum albumin (BSA), 3% threhalose and 1.5% gelatin in PBS, and then incubating for 1 h at room temperature. After the incubation period, plates were emptied and allowed to dry completely at room temperature. Until use in the test, plates were stored in the zip-lock plastic bags in a desiccator for more than 6 months at 4 ° C. Table 2 shows the buffers used in this study.

Table 2. Buffers and diluents

Buffers and diluents	Contents
Coating Buffer	Carbonate buffer, PH=9.6
Blocking Buffer	PBS buffer + %2 proteinase free BSA + threhalose %3+ 1.5% gelatin
Washing Buffer	%0.02 tween 20+PBS
Sample Diluent	50 mM Tris, 0.14M NaCl, %1 BSA, 0.05% Tween 20, %2 normal Goat serum, Ethylen Glycol %0.01, pH= 8
Conjugate Diluent	50 mM Tris base, 0.14 M NaCl, %1 BSA, %0.05 tween 20, %0.01 mouse serum, pH=8
Stop solution	0.1M HCL

In the next step, 200 μl of the sample diluent was added per well, followed by addition of 10 μl of samples or positive and negative controls to the respective wells, shaking for 10 seconds and incubation at 37 °C for 1 hour. Thereafter, plates were washed five times as in the above and 100 μl of the appropriate diluted conjugate in conjugate diluent was added per well and incubated at 37 °C for 30 minutes. Mouse polyclonal type specific anti human IgG (types 1, 2, 3, 4) were used in the conjugate sample to determine the type of produced antibodies. They were again washed for five times as above and received 100 μl of the TMB reagent per well with a multichannel pipette. Then, they were incubated in dark at room temperature for 15 minutes. Finally, 100 μl of 0.1M HCL (stop solution) was added to the wells and the optical density (O.D.) was read at 450 nm and 630 nm by means of ELISA Reader (BioTech).

Statistical analysis

The results were analyzed using SPSS for windows® release 12.0 (SPSS, Chicago, IL, USA). Student t test or Manne Whitney test was used for the baseline characteristics of patients. Data were also analyzed with Chi-square test or the Fisher's exact test and correlations were determined by means of Pearson's test. Differences were considered significant if the p value was less than 0.05. GraphPad Prism for Windows® release 6.0 was also used for graphs.

RESULTS

Of the 988 subjects in the study, 257 (26%) were women and 731 (74%) were men. We evaluated antibody responses against core, NS3, NS4, and NS5 HCV proteins. Our findings showed that the highest antibody response was against core HCV protein (85%) followed by NS4, NS5 and NS3 (54%, 50%, and 41%, respectively) as shown in figure 1.

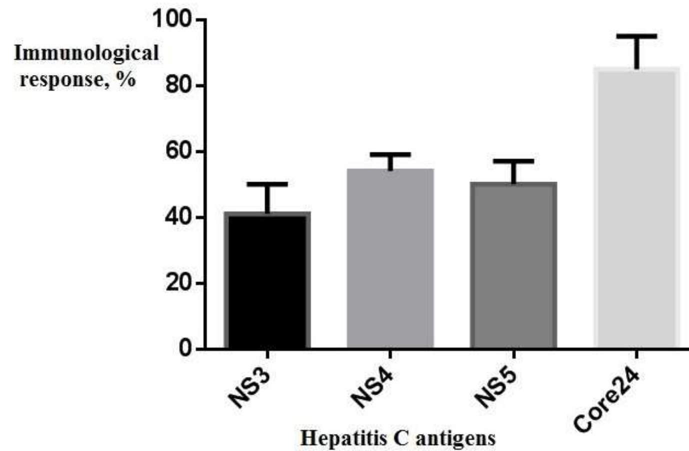

Figure 1. Humoral antibody response against HCV proteins indicated high sensitivity of human immunological system in response to the Core 24 protein, 85% followed by, NS4 (54%), NS5 (50%) and NS3 (40%)

It was observed that high percentage (95%) of samples with S/Co > 4 in third generation of ELISA had at least two positive single antigens ELISA tests (*p* value < 0.001). These findings approve a true correlation between ELISA test results with S/Co over four and RIBA positive results, since RIBA test is considered positive when two or more antigens are detected. In fact, we can conclude that, if the S/Co of third generation of ELISA is more than four, the RIBA test will be positive. It was also demonstrated that NS3 and NS4 antigens were diagnosed simultaneously more (in compare with NS5 and Core24) in S/Co > 4 samples (*p* value=0.006). Thus, the samples which were positive in NS3 and NS4 single antigen ELISA tests, will also have positive RIBA test, almost certainly.

To evaluate the cross reactivity of false positive samples, different hepatitis C Virus antigens including Core24, NS3, NS4, and NS5 were employed in the experiment as a single antigen coating in which observed cross reactions were 18%, 24%, 29%, and 41%, respectively. In addition 12% of samples had cross-react with both NS4 and NS5. Therefore, most of the cross reaction was shown with non-structural proteins, included NS5, NS4 and NS3, respectively and the lowest cross reaction was shown with the core structural protein (Figure 2).

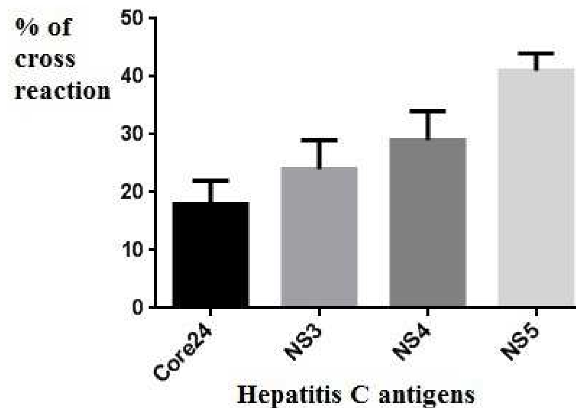

Figure 2. Single-antigen ELISA was performed separately on all positive samples to detect the cross reaction among HCV antigens. It recognized NS5 as the main protein in cross reactions which is responsible to detect false positives

Type of produced antibodies was determined using mouse polyclonal anti human IgG in the conjugate. We observed the highest produced antibody during HCV infection was IgG1 significantly, as shown in figure 3 (P=0.023). In fact, according to table 3, produced IgG1 against NS5, NS4, NS3 and core antigen were %72, %68, %80 and %95, respectively.

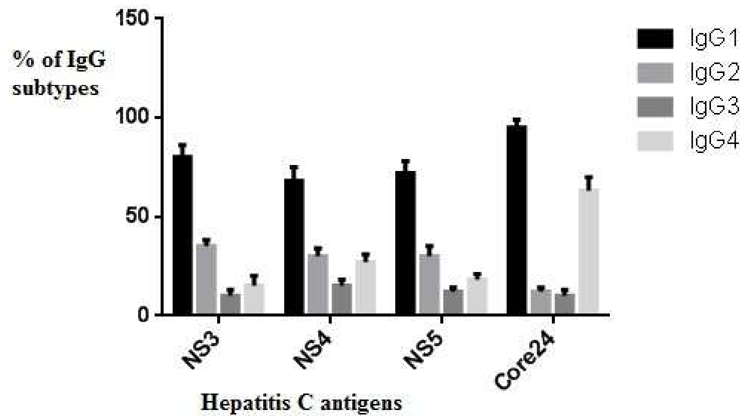

Figure 3. Anti-human IgG1, anti human IgG2, anti human IgG3 and anti human IgG4 which were conjugated to HRP used as conjugates to determine IgG subtypes in all true positive samples. The graph shows IgG1 as the main subtype in response to all four HCV antigens which is followed differently in various HCV antigens

Table 3. Percentage of IgG subtypes antibodies against HCV antigens

IgG subtypes	% of antibodies against HCV antigens			
	Core24	NS3	NS4	NS5
IgG 1	95%	80%	68%	72%
IgG 2	12%	35%	30%	30%
IgG 3	10%	10%	15%	12%
IgG 4	63%	15%	27%	18%

DISCUSSION

Various antibodies in serum of an HCV infected patient become evident at different intervals from the time of initial inoculation. Anti-core antibodies against the nucleocapsid protein [as the first antibody to appear] can be detected by the time ALT is peaking [37]. Infected patients produce antibodies reactive with the core [C] protein as well as nonstructural proteins of HCV within days to weeks after beginning of clinical symptoms. However, protective antibodies have not yet been recognized [38-40]. Findings of this study demonstrated that the percentage of antibody response against core HCV protein was the highest that followed by NS3, NS4 and NS5 proteins, respectively. Kanesaki T et al. conducted a similar action in which they examined immune response against different antigens of HCV in Japanese children with hemophilia and indicated that the percentage of children with seropositivity for C100, core, NS3, and NS5 proteins in one or more specimens was 82%, 91%, 91%, and 89%, respectively. They also found, antibodies to the core and NS3 antigens were detected earlier and persisted longer than others after HCV infection [41]. In continue to features of core protein, Medhi et.al, indicated that the HCV core antigen assay is more sensitive and may be useful in the early phase of infection before seroconversion [42] which was also confirmed by the findings of Lorenzo et.al [43]. In fact, the serology of HCV infection does not follow the classical pattern of IgM response observed in other viral infections, since it may be absent, late or persistent after HCV infection and does not correlate with the histologic activity [37]. In contrast with these results, Netski et al. evaluated immunological responses in acute infection [44]. Probably, these contradictions are related to different types of antigens or different immunological responses in various populations and also immunological status or infection course of patients. Thus, further studies on more people in other communities are required to shed light on this claim.

In this study, we observed that high probability [95%] of positive RIBA test was in samples with S/Co>4 in ELISA test, which was also reported by Bar-Shany S et al. They demonstrated that subjects with positive RIBA results are donors with high ratios [S/C > 3] [20]. On the other hand, findings of Y. Lazizi et.al study indicated that ELISA is more sensitive than RIBA II for the detection of anti-HCV antibodies [45]. As regards the ELISA test is more sensitive compared to RIBA, Cheng et.al showed that ELISA based on the antigen-sandwich [core, NS3, NS4 and NS5 antigens] principle had higher sensitivity and specificity [46]. The RIBA test is commonly interpreted as positive when 2 or more antigens from different gens, indeterminate when 1 antigen, or negative when 0 antigens had positive band [47]. Interestingly, results of our study illustrated that when 2 or more antigens are positive in ELISA test for single antigen, S/Co ratio of sample in the third generation ELISA test for all antigens is over four.

This suggests that there is a true correlation between ELISA and RIBA tests, in fact RIBA tests can be considered positive whenever S/Co of EIA 3 is more than four.

There are many studies associated with false-positive results by RIBA which has lower sensitivity and higher specificity in compare with third generation of ELISA. These studies indicated that cross reaction in false-positive results are more focused on non-structural [NS3 and NS5] antigens [48-50] which were approximately found in present study since it was observed in NS5, NS4 and NS3 antigens, respectively. However, in our study, single antigen ELISA tests were performed on each serum to increase specificity of ELISA to make our findings more reliable in compare with previous studies. This high cross reaction with non-structural proteins may be due to similarity between functional domains of these proteins with other similar proteins in infectious or cellular genes. Interestingly, several studies reported that one reason of false-positive results in serological tests special for HCV ELISA is vaccination or infection with influenza virus. Also, vaccination or infection with influenza virus considerably is responsible for false-positive results in other ELISA tests for HIV or HTLV-I [51-53]. In this regard, other factors contributing to the false-positive results of HCV ELISA are infection with parasitic worm such as schistosoma and the presence of autoimmune markers such as ANA, AMA, SMA and LKM [54]. Fasciola infection [55], syphilis [56-58], and autoimmune hepatitis in response to steroid treatment can also increase the rate of false-positive results in the HCV ELISA test [59]. In addition to mentioned results, we found that between all IgG subclasses [IgG1, 2, 3, 4], IgG1 was the highest produced antibody against HCV infection which is similar to the findings of Netski et al., that reported IgG1 as major response in the HCV infection [44, 60]. Generally in contrast to HCV [since isotype switching occurred only in core antigen], most of chronic human viral infections, mainly extract both IgG1 and IgG3 isotypes with lower production of IgG2 and IgG4 antibodies [61]. Similar to HCV, IgG isotype restricted antibody responses were observed in a group of asymptomatic chronic HBV infections [62], Epstein–Barr virus infection [63], and visna virus infection in sheep [64]. This restriction of antibody responses against HCV antigens may be due to a skewing of T-helper subsets, as suggested in some asymptomatic HBV carriers [62]. However, unlike HBV infection, no correlation was identified between symptomology and IgG1 restriction in chronic HCV infection. Therefore, the lag of IgG switching in HCV infection can cause limited T-helper cell function specific for the HCV antigens instead of skewing of T-helper cell subsets. Biological functions such as binding to macrophages and other phagocytes, the output of activation of the classical and alternative complement pathways and placental transfer varies among the human IgG isotypes. Therefore, a variety of IgG isotypes required to intercede a complete spectrum of biological actions. Unfortunately, an antiviral humoral response largely limited to a single IgG isotype, not be expected to mediate efficient viral neutralization and clearance mechanisms. This suppression pattern of host immune response may be due to the core protein [65-67]. However, further investigations are required to illustrate this fact. In conclusion, the data presented in this study suggest further studies on seroconversion samples with acute HCV infection and generated IgM antibody against different HCV antigens. Indeed, generated antibody classes may reveal the probable direction of serological and immunological response in HCV infection and also the antigen by which acute HCV infection could be diagnosed.

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