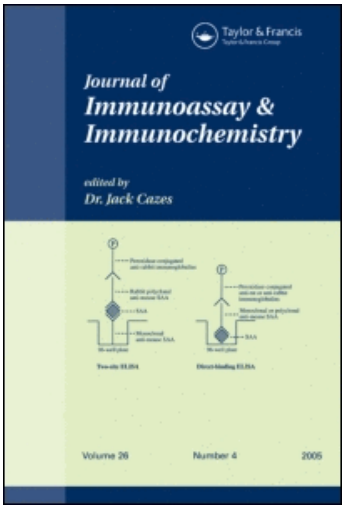

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A Novel Antigen Detection Immunoassay for Field Diagnosis of Hepatitis C Virus Infection

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ABSTRACT

The limitations of dominant methods-based on the detection of anti-HCV antibodies or HCV viremia currently used for the diagnosis of HCV infection enhance efforts to have a rapid, simple, sensitive, and specific alternative diagnostic approach to detect viral antigens. A highly reactive IgG antibody was raised to HCV-NS4 recombinant antigen. The produced antibody showed no cross-reactivity with the other HCV structural and nonstructural recombinant antigens (C1 + 2, C3 + 4, E2/NS1, NS3, NS5). The

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well established ELISA technique was adapted to detect the new target HCV-NS4 antigen in serum samples. Extremely high agreement was found between the results of ELISA and qualitative detection of HCV-RNA, using a RT-PCR test as a gold standard for the diagnosis of HCV infection. Based on these encouraging results, a novel enzyme immunoassay; dot-ELISA was developed for rapid (~5 min) and simple qualitative detection of the target HCV antigen in serum. The developed method detected the HCV target antigen in 95% of serum samples from HCV infected individuals, with a specificity of 97% using sera of noninfected individuals in comparison with PCR test. The antigen detection method showed high predictive values of positive (99%) and negative (90%). Moreover, the dot-ELISA could detect the HCV target antigen in sera negative for anti-HCV Abs, but positive for HCV-RNA, and in sera of HCV infected individuals with low viremia, as well as those with high viremia, using quantitative RT-PCR. Accordingly, the developed highly sensitive and specific HCV antigen detection method could be applied for mass screening of HCV infection.

Key Words: HCV; Diagnosis; HCV-NS4 antigen; Serum; Dot-ELISA; Hepatitis C.

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of nonA, nonB hepatitis, and its infections often progress to chronic hepatitis, cirrhosis, and hepatocellular carcinoma.[1] Histological examination of liver biopsy specimens is still the gold standard for the diagnosis of chronic hepatitis C, pathogenesis of liver injury, and assessment of anti-viral treatment.[2] However, liver biopsy is invasive and examination is required by a professional histopathologist. The alternative methods currently used for the diagnosis of HCV infection are based on the detection of anti-HCV Abs e.g., ELISA and RIBA. The anti-HCV Abs alone cannot discriminate patients who are infectious from those who have resolved the infection. In addition, anti-HCV Abs immunoassays remain suboptimal for detection of acute HCV infection.[3,4] Nucleic acid amplification technology-based tests (RT-PCR, branched DNA) could identify viremic samples in which antibodies are not yet present. However, the contamination potential and long time required to perform PCR limit its clinical application. In addition, a substantial variability has been observed among different laboratories performing PCR on the same serum samples.[5] Therefore, it would be advantageous to have a

rapid, simple, noninvasive, sensitive, and specific assay for the diagnostic testing of HCV infection in the developing countries. Recently, several groups have demonstrated evidence of HCV antigens in liver tissue,[6–9] serum samples,[10,11] and plasma samples.[12] In the present study, we evaluate the simple and rapid detection of a serum HCV-NS4 antigen using a novel dot-ELISA in comparison with HCV-RNA detection using PCR.

EXPERIMENTAL

Serum Samples

Serum samples of 325 Egyptian individuals (235 males and 90 females aged 12–57 years; mean age of 38.35 years) from Internal Medicine Dept., Mansoura University Hospitals, Mansoura, Egypt, and 50 healthy volunteers (35 males and 15 females aged 15–58 years; mean age of 36.6 years) were included in the present study. An informed consent was obtained from all individuals participating in the present study, and they were fully informed concerning the nature of the disease and the diagnostic procedures involved. Anti-HCV antibodies (Ortho HCV Ab ELISA test III kit, Ortho Clinical Diagnostics Systems, Raritan, NJ) and HBsAg (Biomedica, Sorin, Italy) were detected according to the manufacturer's instructions. The COBAS AMPLICOR HCV TEST, version 2.0 (Roche Diagnostics, Branchburg, NJ) was used as a gold standard to detect HCV-RNA in all serum samples, according to the manufacturer's instructions. This qualitative PCR test is able to detect HCV-RNA at a concentration of 50 IU/mL, with a positive rate of 95% or greater.

Quantification of HCV-RNA in Serum

The HCV-RNA was quantified in a group of 72 positive HCV-RNA serum samples by using the COBAS AMPLICOR HCV MONITOR TEST, version 2.0 (Roche Diagnostics) on the COBAS AMPLICOR Analyzer according to the manufacturer's instructions. The test permits simultaneous reverse transcription and PCR amplification of HCV target RNA and HCV Quantitation Standard RNA. The analyzer calculates the HCV-RNA levels in the test specimens by comparing the HCV signal to the quantitation standard signal for each specimen and the HCV-RNA titer is expressed in international units per milliliter (IU/mL). The levels of HCV viremia in serum were high ($>800 \times 10^3$ IU/mL), moderate

(100×10^3 to 800×10^3 IU/mL), low (600 to 100×10^3 IU/mL), and no detected viremia (<600 IU/mL).

Production of Anti-HCV-NS4 Antibody

Five New Zealand rabbits were immunized subcutaneously in three different inoculation sites with 500 μg of HCV-NS4 recombinant antigen[13] diluted (v/v) with Freund's complete adjuvant. On day 15, the rabbits were immunized again with the same dose of antigen emulsified with incomplete Freund's. On day 28, the rabbits were immunized with one more dose of antigen with incomplete adjuvant and sacrificed four days later. Blood samples were collected from all rabbits at 0, 28, and 32 days of immunization and sera were separated. The reactivity of the collected sera was tested using ELISA (Ortho Clinical Diagnostics Systems) with the use of anti-rabbit IgG conjugated with alkaline phosphatase (Sigma). The specificity of reactive rabbit sera was tested against multiple HCV antigens by RIBA test (INNO-LIA HCV Ab III, Innogenetics, Zwijnaarde, Belgium), with the use of anti-rabbit IgG conjugated with alkaline phosphatase (Sigma). Rabbit sera were incubated with the multiple HCV antigens coated RIBA strip and, subsequently, anti-rabbit IgG conjugate was added. Incubation with the enzyme substrate produced a color in proportion to the amount of anti-HCV-NS4 antibody present.

Enzyme Linked Immunosorbent Assay (ELISA) for HCV-NS4 Antigen Detection in Serum

After optimization of ELISA conditions, polystyrene microtiter plates were coated with 50 μL/well of tested serum sample diluted in carbonate/bicarbonate buffer (pH 9.6). The plates were incubated overnight at room temperature and washed three times using 0.05% (v/v) PBS-T20 (pH 7.2), and then free active sites were blocked using 0.2% (w/v) nonfat milk in carbonate/bicarbonate buffer. After washing, 50 μL/well of anti-HCV-NS4 antibody, diluted 1:100 in PBS-T20, were added, and incubated at 37°C for 2 h. After washing, 50 μL/well of anti-rabbit IgG alkaline phosphatase conjugate diluted in 0.2% (w/v) nonfat milk in PBS-T20, was added and incubated at 37°C for 1 h. The amount of coupled conjugate was determined by incubation with 50 μL/well *p*-Nitrophenyl phosphate substrate for 30 min at 37°C. The reaction stopped using 3 M NaOH and absorbance was read at 405 nm.

The cutoff level of ELISA above or below where the tested sample is considered positive or negative, was calculated as the mean ELISA optical densities of 36 serum samples from healthy volunteers ± 3 standard deviation (i.e., $0.224 \pm [3 \times 0.025]) = 0.299$.

Dot-ELISA for Simple and Rapid Detection of HCV-NS4 Antigen in Serum

All the assay steps were carried out on the surface of a nitrocellulose (NC) membrane fixed in a plastic cartridge (Device), and each reagent was completely absorbed into the NC membrane within 30 s (incubation time). After optimization of reaction conditions, 200 μL of serum sample diluted 1:20 in 50 mM EDTA were added per dot. Different concentrations (0.25, 0.1, 0.05 mg) of the HCV-NS4 antigen and an irrelevant protein (e.g., bovine serum albumin) diluted per 1 mL serum sample of HCV free healthy individual, were used as positive and negative controls, respectively. Blocking of the nonspecific binding sites on the NC membrane was done with 5% (w/v) BSA in PBS, pH 7.2. After washing three times using 100 μL/wash of PBS pH 7.2, 200 μL of anti-HCV antibody diluted 1:300 in PBS was added. After washing, 200 μL of the diluted anti-rabbit IgG alkaline phosphatase conjugate (Sigma) was added. After more washing, 200 μL of premixed NBT/BCIP alkaline phosphatase substrate in 0.1 M Tris buffer (ABC Diagnostics, New Damietta, Egypt) were added. Two minutes later, the reaction was stopped by adding 100 μL distilled H_2O and the color development observed. The development of a violet color indicate antigen detection (positive test). The developed purple color varied in its intensity from weak (1+, 2+) to strong (3+, 4+). The color of the tested serum sample was related to one of these color levels.

RESULTS

Reactivity and Specificity of the Anti-HCV-NS4 Antibody

Sera collected from the five immunized rabbits at 28 and 32 days after immunization showed increased levels of anti-HCV antibodies using ELISA. Sera collected from five rabbits at zero time as a control sera showed no anti-HCV antibodies. No significant batch-to-batch variation ($p > 0.05$) was shown in the reactivity of various bleeds from the various rabbits using ELISA. The developed anti-HCV antibody showed specific reactivity to the NS4 region and showed no reactivity to other structural

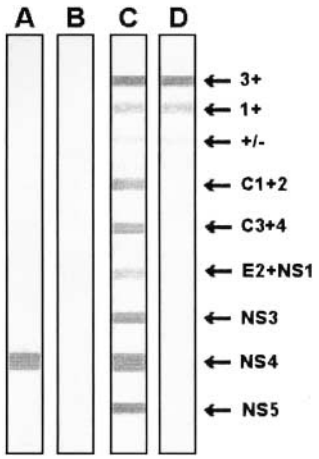

Figure 1. Reactivity of antibody raised in rabbit against recombinant HCV-NS4 antigen using recombinant immunoblotting assay (RIBA). Strip A: the developed rabbit antibody reacted only with NS4 band and showed no reactivity with C1 + 2, C3 + 4, E2/NS1, NS3, and NS5. Strip B: serum from nonimmunized rabbit as a negative control showing no reactivity against all regions. Strip C: serum from HCV infected individual showing reactivity with C1 + 2, C3 + 4, E2/NS1, NS3, and NS5 bands. Strip D: serum from noninfected individual showing no reactivity with C1 + 2, C3 + 4, E2/NS1, NS3, and NS5 bands. The RIBA was done according to the manufacture instruction (strips C and D) to detect anti-HCV antibodies in human sera and was done with slight modification by using anti-rabbit IgG alkaline phosphatase conjugate (strips A and B) to detect reactivity of the developed rabbit anti-sera.

and nonstructural regions of HCV (C1 + 2, C3 + 4, E2/NS1, NS3, NS5) using RIBA. Sera from nonimmunized rabbits showed no reactivity against structural and nonstructural regions (Fig. 1).

Basic Serology and PCR Results

A total of 260 sera were positive for HCV-RNA using a qualitative PCR test (250 were positive for anti-HCV Abs and 10 sera were negative for anti-HCV Abs), and 115 sera were negative for HCV-RNA (65 sera were positive for anti-HCV Abs and 50 were negative for anti-HCV Abs). The HBsAg was detected in 15 out of 115 sera negative for HCV-RNA, but positive for anti-HCV Abs. The reported liver pathology of the 65 individuals showing negative results for HCV-RNA, but positive for

anti-HCV Abs, included: chronic hepatitis with moderate activity and occasional portal–portal bridging fibrosis ($n = 13$), marked bridging fibrosis ($n = 23$), incomplete cirrhosis ($n = 10$), or established cirrhosis ($n = 19$).

Detection of Serum HCV-NS4 Antigen Using ELISA

The well established ELISA technique was adapted to detect the new target marker; HCV-NS4 antigen in sera of infected individuals. We have found 95% agreement between the results of qualitative detection of HCV-RNA using PCR tests and the HCV-NS4 antigen detection using ELISA for a group of 88 HCV infected individuals (their serum samples were positive for anti-HCV Abs and HCV-RNA detection), and 100% agreement between the results for a group of 36 HCV free healthy volunteers (their serum samples were negative for anti-HCV Abs and HCV-RNA detection).

Rapid and Simple Detection of HCV-NS4 Antigen in Serum Using Dot-ELISA

To simplify the detection method, we have developed a dot-ELISA method for simple and rapid qualitative detection of the target HCV-NS4 antigen in serum. However, the produced purple color in the case of serum HCV antigen detection (i.e., positive test) varied in its intensity from weak (1+, 2+) to strong (3+, 4+) according to the antigen level in serum, Fig. 2. To evaluate the efficiency of the dot-ELISA for the detection of serum HCV-NS4 antigen, a total of 260 serum samples from individuals positive for HCV-RNA and 115 sera negative for HCV-RNA were tested blindly (i.e., the PCR results were unknown to the lab worker reading the dot blot results) for HCV-NS4 antigen.

The assay showed 95% sensitivity and 97% specificity, Table 1. All 15 sera positive for HBsAg showed negative results for HCV antigen. Furthermore, the HCV-RNA was quantified in a group of 72 sera positive for qualitative HCV-RNA: 18 with low viremia, 38 with moderate viremia, and 16 samples with high viremia. The dot-ELISA detected HCV-NS4 antigen in 68 out of 72 sera, showing 94% overall sensitivity; 88% sensitivity for samples with low viremia (600 to 100×10^3 IU/mL), 95% sensitivity for samples with moderate, and 100% sensitivity for samples with high viremia. In addition, the

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Figure 2. Rapid and simple detection of the HCV-NS4 antigen in serum samples of HCV infected and noninfected individuals using dot-ELISA. The devices labeled 1 and 2 represent serum samples from noninfected individuals (showing no antigen detection) and the devices labeled 3 and 4 represent serum samples from HCV infected individuals (showing weak and strong positive antigen detection, respectively).

dot-ELISA could detect the target HCV-NS4 antigen in 8 out of the 10 sera negative anti-HCV Abs but positive for HCV-RNA.

DISCUSSION

The HCV antigens have been successfully detected using commercial or noncommercial polyclonal[14] and monoclonal antibodies directed to nonstructure[9,15] and to core proteins.[16] Recently, several immunoassays have been developed for the detection of HCV core antigens in serum and plasma samples.[11,12,16] Tanaka et al.[10,17] reported that the HCV core antigen assay is useful for the diagnosis of acute and chronic hepatitis C, and for predicting and monitoring the effect of IFN-α treatment. The virion structural proteins, C, E1, and E2, have been shown to arise from the viral polyprotein via proteolytic processing by the host signal peptidases. Generation of the virion nonstructural proteins, NS2 to NS5B, relies on the activity of viral proteinases.[18] The 5′ end of the NS-4 protein of different genotypes of hepatitis C virus (HCV) has been shown to be antigenic by epitope mapping, and elicits an antibody in HCV-infected individuals.[19] However, the IgG1

Table 1. Detection of HCV-NS4 antigen using dot-ELISA in comparison with qualitative HCV-RNA detection using PCR test in sera of 375 Egyptian individuals.

HCV-RNA detection using RT-PCR[a]	No.	HCV-NS4 antigen using dot-ELISA[b] Positive	Negative	% Positive
Positive HCV-RNA				
Positive anti-HCV Abs	250	240	10	96
Negative anti-HCV Abs	10	8	2	80
Total	260	248 (TP)	12 (FN)	95
Negative HCV-RNA				
Positive anti-HCV Abs	65	4	61	6
Negative anti-HCV Abs	50	0	50	0
Total	115	4 (FP)	111 (TN)	3

Abbreviations: TP, true positive; FP, false positive; TN, true negative; FN, false negative.
[a]The gold standard for the diagnosis of HCV infection.
[b]Sensitivity (%) = TP/(TP + FN) = 248/(248 + 12) × 100 = 95%.
Specificity (%) = TN/(TN + FP) = 111/(111 + 4) × 100 = 97%.
Positive predictive value (%) = TP/(TP + FP) = 248/(248 + 2) × 100 = 99%.
Negative predictive value (%) = TN/(TN + FN) = 111/(111 + 12) × 100 = 90%.
Efficiency of the test (%) = (TP + TN)/total = (248 + 111)/375 × 100 = 96%.

isotype restriction, relatively low titer, and delayed appearance of antibody responses elicited during HCV infection, suggest that the immunogenicity of HCV proteins is limited in the context of natural infection.[20] These defective humoral immune responses during HCV infection may be attributable to an "immune avoidance" strategy. This may support the detection of nonstructural viral proteins in the blood stream.

In the present study, we have identified a target HCV-NS4 antigen in liver tissues and sera of infected individuals using a specific antibody developed against recombinant HCV-NS4 antigen. Immunohistochemical staining of sections from liver tissue of HCV infected individuals using anti-HCV-NS4 antibody revealed intense granular cytoplasmic staining of hepatocytes. The blocking of anti-HCV-NS4 antibody reactivity using HCV-NS4 antigen resulted in no reaction in liver tissue of HCV infected, as well as liver tissue from noninfected individual used as a negative control (data not shown). The target HCV-NS4 antigen was detected in sera using two enzyme immunoassays; an indirect ELISA and a newly developed dot-ELISA format. The well established ELISA, based on the

produced anti-HCV antibody, showed 95% results agreement with HCV-RNA detection using RT-PCR among HCV infected individuals and 100% results agreement among noninfected individuals. The developed dot-ELISA method allows simple and rapid qualitative detection of the target HCV-NS4 antigen in serum. The assay is easier to perform, is less costly, and has a lower risk of laboratory contamination than assays based on gene amplification technology. All the reaction steps of the developed dot-ELISA were performed at room temperature and takes about 5 min without need of sophisticated equipment.

We evaluated the detection of serum HCV-NS4 antigen using the newly developed dot-ELISA formate in comparison with HCV-RNA detection using RT-PCR as a gold standard. All the assay characteristics e.g., efficiency and predictive values of positive and negative were extremely high. The target HCV antigen was detected in 95% of serum samples positive for HCV-RNA. Moreover, the dot-ELISA allows reliable detection of the HCV-NS4 antigen in 88% of samples with viral loads (600 to 100×10^3 IU/mL) and 80% of sera negative for anti-HCV antibodies, but positive for HCV-RNA. Therefore, the developed antigen detection method may be suitable for use in screening of blood units in order to identify infecting samples that do not contain specific antibodies. The window period in HCV infection during which specific antibodies have not yet been produced is still a major problem in ensuring blood safety.[4] The detection of HCV antigens in the window period could become a useful means of improving the safety of blood and blood products.[21–23] However, a large study population and more definitive and complete data are needed to draw the final conclusion.

In the present study, the developed assay showed 100% specificity among 50 healthy individuals and 94% specificity among 65 patients with liver disease and negative for HCV RNA. The detected false positive results may be attributed to the low level of HCV-RNA viremia in these sera, which may be lower than the detection limit of the RT-PCR or may due to other unknown factors. In addition, the detected anti-HCV Abs and pathological diagnosis of these cases, as chronic hepatitis with moderate activity and liver fibrosis, support the probability of HCV infection. All the HCV-RNA-positive sera used in the evaluation are of Egyptian origin, it is likely that they are predominantly genotype 4.[24] However, the evaluation of assay using sera from patients infected with various HCV genotypes will be performed. Generally, clearance of HCV-RNA after treatment with interferon-α (IFN-α) is associated with disappearance of core protein from serum.[10] Recently, data of Tanaka et al.[17] suggested that antigenemia and viremia of HCV appear and disappear in parallel. Further studies regarding the detection of the

HCV-NS4 target antigen, before and after treatment, will be investigated. In conclusion, all these elements collectively allow the developed dot-ELISA to be used successfully as a simple and rapid screening method of HCV infection.

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