A HIGHLY SENSITIVE AND SPECIFIC ENZYME-LINKED IMMUNOSORBENT ASSAY OF ANTIBODIES TO HEPATITIS C VIRUS

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Summary. – In this study, a 178 amino acids long portion of the hepatitis C virus (HCV) core gene was cloned, sequenced, expressed in *Escherichia coli*, and purified. The resulting antigen (C178) was tested with human sera enzyme-linked immunosorbent assay (ELISA) in order to assess its ability to diagnose HCV. It was shown by ELISA that 92% of the patients sera, diagnosed previously by a 3rd generation enzyme immunoassay (EIA) as HCV-positive, had antibodies against the C178 antigen. This antigen gave no false positive results when tested with anti-HCV-negative sera.

Key words: hepatitis C virus; core protein gene; cloning; nucleotide sequencing; expression; E. coli; purification; ELISA; RT-PCR

Introduction

HCV is the major causative agent of the non-A and non-B hepatitis. It has been first identified using molecular cloning techniques (Kao *et al.*, 1989; Choo *et al.*, 1989). HCV is a single-stranded positive-sense RNA virus classified within the family *Flaviviridae*. A hundred million individuals are estimated to be chronically infected with HCV and this number increases every year (Murphy *et al.*, 1996). Over 80% of those exposed to HCV become chronically infected, and 20% of them develop cirrhosis, possibly leading to hepatocellular carcinoma. There is no vaccine available to prevent HCV (Urdea *et al.*, 1997). As HCV infection can have such serious consequences and its treatment is rarely efficacious, the tests that can identify HCV-infected patients are crucial for addressing this potentially life-threatening viral disease. The main screening assays for detecting and confirming antibodies to HCV (anti-HCV) are EIA and radio-immunoblot assay. Tests for HCV RNA including PCR-based and branched DNA (bDNA) ones, are used for therapeutic monitoring and prognostics (Younossi and McHutchison, 1996; Urdea *et al.*, 1997). A 1st generation anti-HCV test (EIA-1) uses a single HCV recombinant antigen derived from the non-structural (NS) 4 gene designated C100-3. Only 80% of the patients with clinical and molecular evidence of HCV infection were positive for anti-HCV by this method (Kao *et al.*, 1989; Nagayama *et al.*, 1993). A 2nd generation anti-HCV EIA (EIA-2), developed in 1992, employes the HCV antigens from the core (C22) and NS3 (C33) genes in addition to the NS4-derived (C100) antigen (Mimms *et al.*, 1990). Introduction of new antigens led to a substantial improvement in sensitivity and specificity of this test. It allows the detection of 95% of individuals with molecular evidence of HCV (Kleinman *et al.*, 1992; Aach *et al.*, 1991). A 3rd generation anti-HCV EIA (EIA-3) was designed to further improve sensitivity and specificity of detection by adding more antigens. It uses three recombinant antigens (C22, C100,

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Abbreviations: anti-HCV = antibodies to HCV; bDNA = branched DNA; EIA = enzyme immunoassay; ELISA = enzyme-linked immunosorbent assay; HCV = hepatitis C virus; NS = non-structural; PBS = phosphate-buffered saline; PBST = PBS with 0.05% Tween 20; PCR = polymerase chain reaction; RT-PCR = reverse transcription–PCR

and NS5) derived from four different regions of the HCV genome. It is more sensitive (97%) and slightly more specific than preceding assays (Barrera *et al.*, 1995; Uyttendaele *et al.*, 1994), and the improvement in this assay has been attributed to the reconfigured antigens already present in EIA-2 but not to the NS5 antigen (Gretch, 1997).

Although serological diagnostic accuracy has greatly been improved, there are still a large number of false positive reactions given by EIAs for low risk populations such as blood donors (Gretch, 1997). It has also been reported that in many commercially available EIAs fusion polypeptides and synthetic oligopeptides are used as HCV antigens and these may exhibit non-specific reactions and reduce the sensitivity of the epitopes involved (Seki *et al.*, 1995). To improve the detection rate, identification and generation of new antigens are required. A considerable sequence variation among HCV genotypes and subtypes may contribute to the complicated picture of HCV diagnostics. It should be noted that since the virus core gene is the most conserved genome region, it can be used to detect all HCV subtypes by EIA. The full length core protein is 191 amino acids long (Bukh *et al.*, 1994). Although it is the most conserved region of HCV open reading frame, there are variations among different HCV genotypes. The C terminal end of the core protein is found to be highly hydrophobic and reported to be cleaved (aa 172–191) (Hijikata *et al.*, 1991). For this reason we have amplified and cloned the first 178 amino acids portion of the core gene.

Presently, 1.5% of the general population in Turkey is thought to be infected with HCV which corresponds to about 1 million people (Heintges and Wands, 1997). Very recently, blood donor sera began to be tested by EIAs. The predominant genotype of the virus for Turkey has also recently been identified as 1b (E. Yildiz, unpublished results). From this point of view it was reasonable to develop an EIA using an antigen derived from a Turkish HCV isolate.

In this report we describe cloning, expression, sequencing and production of almost the entire HCV core protein of a Turkish HCV isolate of subtype 1b as a protein tagged with 6 histidines. The recombinant protein C178, when used in ELISA, appeared to contain almost all epitopes of HCV capsid protein and to detect anti-core proteins antibodies specifically.

Materials and Methods

Reagents. All restriction endonucleases and DNA modifying enzymes were purchased from MBI Fermentas. The Ni-NTA resin for purification of C178 was purchased from Qiagen. All other chemicals were from Sigma, Difco, and Carlo-Erba.

Bacterial strains and plasmids. E. coli strains JM109 and M15 were used for construction and propagation of an expression plasmid and production of recombinant proteins. pQE30 expression plasmid (Qiagen) was used for cloning and expression studies.

RNA extraction and reverse transcription–PCR (RT-PCR). Total RNA was extracted from a chronic hepatitis C patient's serum by the single step guanidinium thiocyanate method (Wilson *et al.*, 1995) with slight modifications. Six hundred µl of the serum was mixed with 3 ml of a denaturing solution (4 mol/l guanidinium thiocyanate, 25 mmol/l sodium citrate pH 7.0, 0.1 mol/l 2-mercaptoethanol, and 0.5% N-laurylsarcosine) and vortexed briefly. Then 300 µl of 2 mol/l sodium acetate pH 4.0, 3 ml of water-saturated phenol, and 600 µl of chloroform:isoamylalcohol (49:1) was added and the mixture was vortexed. The mixture was then incubated on ice for 15 mins and centrifuged at 12,000 rpm for 15 mins at 4°C. The aqueous phase was precipitated with one volume of 100% isopropanol at -20°C for 30 mins and centrifuged at 12,000 rpm for 20 mins at 4°C. The pellet was dissolved in 250 µl of the denaturing solution and an equal volume of isopropanol was added. The mixture was incubated at -20°C for 30 mins and centrifuged at 12,000 rpm for 20 mins at 4°C. The pellet was washed with 500 µl of 70% ethanol, centrifuged, air-dried, and resuspended in 10 µl of diethylpyrocarbonate-treated distilled water.

The isolated RNA (10 µl) was reverse transcribed after incubation at 70°C for 5 mins using a RT-PCR kit (Stratagene) according to the producer's instructions.

The obtained cDNA was amplified by PCR in a total volume of 50 µl containing 1U of *Pfu* DNA polymerase (Stratagene), 5 µl of the *Pfu* polymerase buffer, 200 µmol/l dNTPs and 50 pmoles of HCV core gene-specific sense (5′-CGC**GGATCC**ATGAGCACG/AAATCCTAAACC-3′ containing an unique *Bam*HI site (in bold)) and anti-sense (5′-CGC**AAGCTT**GAGGAAGATAGAG/AAAAGAGCAACC-3′ containing an unique *Hind*III site (in bold)) primers. The PCR was performed in 30 cycles, each cycle consisting of a denaturation step at 94°C for 1 min, a primer annealing step at 55°C for 45 secs, and an extension step at 72°C for 1 min. This was followed by a single cycle consisting of a final elongation step at 72°C for 10 mins followed by cooling to 4°C.

Cloning of the HCV core gene. After digestion with *Bam*HI and *Hind*III, the PCR product encoding the HCV core gene was ligated with the corresponding sites in pQE30 vector and the obtained recombinant plasmid (pQECORE) was recovered following transformation of *E. coli* JM109.

DNA sequencing of the core gene in pQE vector was carried out on an ABI Prism 377 sequencer by Dr. B. Cevher of the Bilkent University.

Production of recombinant core antigen in E. coli and its purification. The recombinant plasmid pQECORE was used to transform *E. coli* M15 cells and single colonies were used to induce high-level expression of the core protein. A small-scale induction for initial screening of protein expression was performed by inoculating 5 ml of L broth with 200 µl of an overnight culture that had been inoculated with a single colony. For large-scale induction, 30 ml of medium was inoculated with a single colony, grown overnight at 37°C, and this was then used to inoculate 1 l of L broth at the same temperature. Protein expression was induced by addition of 1 mmol/l IPTG when the cells had reached A_{600} of 0.6–1.0. Incubation was continued for additional 1–5 hrs prior to harvesting the cells by centrifugation. Small-scale cultures were harvested by centrifugation at 12,000 rpm for 2 mins in a benchtop microcentrifuge. Large-scale cultures (100–1000 ml) were

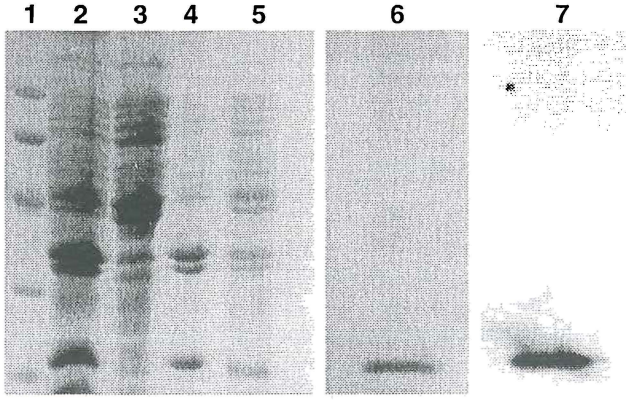

Fig. 1
Results of expression, solubilization, purification and Western blot analysis of C178 protein
Protein size markers (94 K, 66 K, 43 K, 36 K, and 21 K, lane 1), induced insoluble fraction (lane 2), induced soluble fraction (lane 3), the lane 2 sample after solubilization (lane 4), the lane 3 sample after solubilization (lane 5), the affinity chromatography-purified C178 protein (lane 6), the lane 6 sample reacted with human anti-C178.

harvested by centrifugation at 5000 rpm for 10 mins at 4°C in a Beckman JA rotor. Purification of the fraction of induced protein soluble in 8 mol/l urea was carried out essentially as described in the Qiagen's Qiaexpress manual. Concentration of the purified protein was determined according to Bradford (1976).

Western blot analysis. After SDS-PAGE, the gel was blotted onto a polyvinylidene difluoride membrane (Sambrook *et al.*, 1989). The blot was incubated in 50 µl of a blocking solution (10% skimmed milk, and 0.2% Tween 20 in phosphate-buffered saline (PBS) pH 7.4) overnight with continous shaking at 4°C and then washed with PBS containing 0.05% Tween 20 (PBST). It was then incubated in the blocking solution containing the primary antibody (a patient's serum, 1/200 dilution) for 1 hr and washed with PBST for 10 mins. The blot was treated with the secondary antibody (a HRP-conjugated anti-human antibody) for 1 hr in the blocking solution and the bands were visualized using the ECL kit (Amersham) according to the producer's instructions.

ELISA. The sera of the patients were screened by ELISA as described by Sambrook *et al.* (1989) with slight modification. The wells of microtiter plates (96 wells, Nunc) were coated overnight with 100 ng/well of the core antigen in PBS containing 0.02% NaN_3. The coated plates were incubated first with 200 µl/well of a blocking solution (5% skimmed milk and 0.02%NaN_3 in PBS pH 7.4) for 1 hr and then with the blocking solution containing a serum (diluted 1:200) for another hr and washed with PBST 3 times after each of these steps. A blocking solution containing alkaline phosphatase-conjugated antibodies (Sigma) (diluted 1:30,000) against human IgG, M and A was then added (200 µl/well). After 1 hr at room temperature, the unbound conjugate was removed by 3 washings with PBST and 200 µl/well of a substrate solution containing 1 mg/ml PNPP was added. The amount of substrate hydrolyzed was assessed by a Biomek 2000 Automated Laboratory Workstation (Beckman), by reading A_{450}, after incubation in dark for 1–2 hrs at room temperature.

Results

Production of recombinant core antigen (C178) in E. coli and its purification

In order to optimize the expression conditions we examined the influence of growth temperature, induction time and IPTG concentration upon C178 protein expression. We found that optimum IPTG concentration was 1 mmol/l, induction time 3 hrs, and induction temperature 37°C (Fig. 1) It should be noted that inducing of the cells at lower temperatures (30°C or 25°C) dramatically decreased the expression level. At the optimal temperature almost all of the expressed protein was in the insoluble portion. Almost 60% of it was solubilized with 8 mol/l urea. The protein purified by affinity chromatography was more than 90% pure as determined by SDS-PAGE.

C178 protein-based ELISA for anti-HCV

The purified C178 was used in ELISA. The specificity of the C178 protein in ELISA was determined by testing 100 HCV EIA-positive (exclusively 1a and 1b subtypes) and 60 EIA-negative sera as determined by a 3rd generation EIA. For comparison, also RT-PCR was performed to detect HCV RNA in the tested sera. The results are shown in Table 1. A negative control serum was previously tested and found HCV RNA-negative by RT-PCR and anti-HCV-negative by Western blot analysis. ELISA readings at least 2.5 times higher than that of the negative control were regarded as positive, while those at most 1.5 times lower than that of the negative control were regarded as negative. No ELISA readings between 1.5-fold and 2.5-fold of that of the negative control were obtained. Of 100 EIA-positive sera 89 were positive and 11 were negative in ELISA. All the 60 EIA-negative sera were also ELISA-negative. In order to further test the 11 EIA-positive and ELISA-negative sera we performed Western blot analysis using the C178 protein as a probe antigen (data not shown). We could not detect

Table 1. Results of anti-HCV assays by EIA and ELISA and HCV RNA assay by RT-PCR

Sera	No. of sera				
	ELISA			RT-PCR	
	Total	+	–	+	–
Anti-HCV-positive[a]	100	89	11	97	3
Anti-HCV-negative[a]	60	0	60	0	60

[a]Determined by a 3rd generation EIA.
(+), (–) = positive, negative.

any reaction for 3 of these sera. These sera were found HCV RNA-negative in RT-PCR suggesting that they were false EIA-positive. The remaining 8 sera were found Western blot-positive and RT-PCR-positive.

In conclusion, 92% of the patients, which were anti-HCV-positive by EIA, were anti-HCV-positive also by ELISA using the C178 antigen. This antigen showed no false positive results with the negative control sera suggesting that it interacted specifically with anti-HCV.

Discussion

In this study, the 3′-truncated core gene of HCV was cloned and expressed and its product was purified and used in testing of human sera in order to assess its ability to diagnose HCV infection. Since the sequence of this gene varies among genotypes, we have first aligned the available GenBank HCV core gene entries and designed the primer pair to amplify the 178 amino acids long region of the core gene. This region showed variation. Moreover, the C-terminal end of the core protein (aa 172–191) is highly hydrophobic and reported to be cleaved which suggests that its first 171 amino acids contain important domains and possibly all of the immunodominant epitopes (Hijikata *et al.*, 1991). Thus were concluded that the 178 amino acids long core antigen is comparable to the complete one as a probe in detecting antibodies raised by host against the HCV core. The amplified core protein (C178) was cloned into bacterial expression plasmid pQE30, expressed in *E. coli* as an N-terminal his-tagged fusion protein, and purified by Ni ion affinity chromatography under denaturing conditions. The 6-His affinity tag not only enabled to purify the expressed protein by affinity chromatography but also to minimize addition of extra amino acids to the C178 protein. To date, all core region antigens used in detecting HCV infection have either big polypeptides or small synthetic oligopeptides fused at their N-terminus. These fused (attached) polypeptides may exhibit non-specific reactions and reduce the reaction sensitivity of epitope(s). The core protein has been previously expressed in different organisms including insects, yeast, *E. coli* and mammalian cells and found to be useful for early diagnosis of HCV infection (Chiba *et al.*, 1991; Harada *et al.*, 1991; Chien *et al.*, 1992; Yokosuka *et al.*, 1993). However, in these studies, the expressed core antigen was not much purified or did not cover as large as 178 amino acids long portion of the core protein. The largest core protein that was expressed and purified to homogeneity (C115) contained its first 115 amino acids (Seki *et al.*, 1995).

We used the purified His-tagged C178 in establishing an ELISA for detection of antibodies against HCV core protein in human sera. Eighty-nine of the 100 chronic hepatitis patients, that were previously tested by a 3rd generation EIA and found positive, were found positive also in this ELISA, while 60 healthy (control) subjects were negative both in EIA and ELISA. The cut off value for a positive resul was chosen at the 2.5-fold of the average of the negative control readings. The limit for a negative result was chosen at the 1.5-fold of that average. There were no readings between these two limits, however, in the future, samples with such readings should be retested, and if these results would be confirmed, another method such as Western blot analysis should be used. In order to decide whether our 11 ELISA-negative sera were due to insensitivity of our ELISA or lack of antibodies against C178, we subjected them to Western blot analysis and RT-PCR. Eight of these sera were positive in both tests. Three EIA-positive sera were negative in both ELISA and RT-PCR, which suggested that they were false EIA-positive. Therefore it can be concluded that the sensitivity of our ELISA was 92% (89/97) compared to EIA. As C178 was produced in *E. coli*, there might be some impurities in the protein preparation and these could interact with the sera and give false positive results. In order to eliminate this possibility, an *E. coli* lysate was prepared and used as an internal control in ELISA. We found that it did not interact with the sera.

There are 1st, 2nd and 3rd generation EIAs used worldwide for the diagnosis of HCV infection. The sensitivity of these assays ranges from 80% to 97%. When compared to them, the sensitivity of our ELISA is between the 2nd and 3rd generation EIAs. These EIAs use antigens from the core region as well as NS3, NS4 and NS5 regions. The core protein is the putative capsid protein of HCV and antibodies directed to the viral capsid proteins are expected to arise early in infection. Therefore, antibodies against the core protein most probably arise much earlier than those against the non-structural proteins. That is why it is important to include all of the epitopes of the core region into the probe antigen used in an EIA to increase its efficiency. We believe that C178 protein contains all the epitopes of the core region of HCV.

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