STABLE EXPRESSION OF ENVELOPE GLYCOPROTEINS OF HCV GENOTYPE 3A IN HUH-7 CELL LINE.

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**ABSTRACT:** Characterization of antibodies targeting the attachment and entry of the Hepatitis C virus (HCV) particles into host cells is important for studying antibody mediated neutralization. Antibodies against envelope proteins have neutralizing capacity and may prevent HCV infections. The aim of the present study was the development of a system for screening of HCV anti envelope neutralizing antibodies in the serum of HCV patients during acute and chronic HCV infections. We stably transfected Huh-7 cell line with envelope glycoproteins coding expression vector. Selection of positive cell clones was done with antibiotic G418. We observed the cell lines for up to six months for the sustained expression of the envelope proteins. The expressed E2 protein has a molecular mass of ~68kDa and was detected both in cytoplasm and on the plasma membrane. The developed stable cell lines were utilized to study a panel of serum samples from HCV infected patients for anti envelope antibodies through calorimetric analysis. The measurement of absorbance was directly related to the antibody titer in the host. Inhibition of viruses at the stage of viral entry provides a route for therapeutic intervention. The developed system has the potential to detect and characterize anti envelope neutralizing antibodies from HCV infected patients and can also be used for the screening of antiviral molecules that can block the entry of the virus into host cell.

**Key words:** Hepatitis C virus, glycoprotein, attachment, entry, vaccine, mammalian cell lines

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INTRODUCTION

Approximately 3.5% of world population is infected with HCV (Lindenbach, et.al., 2001, 2005, 2006, 2007). In Pakistan HCV disease prevalence in local population is ≥ 10% (Idrees 2008, Syed, et al., 2009). Chronic HCV infection is frequently associated with an elevated risk for development of hepatocellular carcinoma (Karmochkine, et.al, 2006, Liang, et.al., 2000). The standard treatment for chronic hepatitis C is pegylated interferon alpha (IFN-α) plus ribavirin which leads to a sustained virological response in ≥50% of treated patients and is associated with serious side effects [Saito, et.al., 1999, Deutsch, et.al., 2008].

Antibodies against the envelope glycoproteins may have neutralizing capacity [Chen, et.al., 2007, Op De Beeck, et.al., 2004]. Studies showed antibodies specific for E2 block the binding of HCV from infected serum to human cell lines (Rosa, et.al., 1996). The chimpanzees immunized with the recombinant envelope glycoprotein (E1E2) were protected against experimental challenge with homologous virus (Zibert, et.al., 1995). Co-injection of HCV and an antiserum against E2 also protected chimpanzees from infection suggest that antibodies against E1 and E2 have the ability to neutralize HCV infections [Dash, et.al., 2001, Cho, et.al., 1994]. In addition, protection was associated directly with the titer of anti-E1E2 antibodies, suggesting a likely role for antibodies in protection. A role for antibodies in protection has also been suggested from rare cases of spontaneous resolution of chronic infection in patients [Bartenschlager, et.al., 2008, Bigger, et.al., 2000 & 2004].

Inhibition of viruses at the stage of viral entry provides a route for therapeutic intervention (Matthews, et.al., 2004). In this study we collected serum samples from HCV patients who were not undergoing interferon therapies at the time of enrollment and without any evidence of liver failure. RNA was isolated from serum; cDNA was synthesized using gene specific anti sense primer and amplified after addition of sense primer. Amplified product was cloned in mammalian expression vector and stable expression was studied in continuous Huh-7 cell lines. We utilize the stable cell lines to detect anti-HCV envelope antibodies in the serum of HCV infected patients. The developed system has the potential to detect and characterize anti envelope neutralizing antibodies from HCV infected patients and can also be used for the screening of antiviral molecules that can block the entry of the virus into host cell.
Materials and Methods

HCV E1E2 genes Complimentary DNA (cDNA) Synthesis and PCR amplification: Hepatitis C virus RNA of local 3a genotype was isolated from the serum sample of a chronic HCV carrier using RNA isolation kit (Gentra, Purescript, USA) according to the procedure given in the kit protocol. Patients 18 years of age and older who are infected with HCV alone were eligible for this study. All serum samples were negative for hepatitis B virus surface antigen (HBsAg; DRG Germany) but positive for anti-hepatitis C virus antibody (anti-HCV ELISA, DRG Germany). The cDNA was synthesized using gene specific anti sense primer using M-MLV (Invitrogen USA) reverse transcriptase enzyme according to the kit protocol. PCR amplification of the envelope gene was carried out using sense and anti-sense primers (Table-1). The PCR products were run on 1.5% agarose gel and photographed. The 1632-bp product was excised from the gel and purified using DNA purification kit (Fermentas Inc. Germany), digested with EcoR1 and HindIII and was then gel purified using QIAQUICK gel extraction kit (Qiagen, Hilden).

Table-1: Primer sequences used for the generation of cDNA and PCR amplification of the E1 and E2 regions of HCV genomes

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’- 3’)</th>
<th>Product size bp</th>
<th>Gene</th>
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<tbody>
<tr>
<td>PE1E2FA</td>
<td>GCAAGCTTGCCATGGTTTCGCAACAGGGAACTTG</td>
<td>1632</td>
<td>E1E2</td>
</tr>
<tr>
<td>PE1E2RA</td>
<td>GCGATATCTACGAGAAAAACGAGGATGACGA</td>
<td>1632</td>
<td>E1E2</td>
</tr>
<tr>
<td>PE1E2FB</td>
<td>GCAAGCTTGCCATGGTTTCATCCAGCAAGCCGCTCT</td>
<td>1632</td>
<td>E1E2</td>
</tr>
<tr>
<td>PE1E2RB</td>
<td>GCGATATCTACGTTCAGCGTGACAGCTCT</td>
<td>1632</td>
<td>E1E2</td>
</tr>
<tr>
<td>P3E1FA</td>
<td>GCAAGCTTGCCATGGAATGATGTCATTGCCACAC</td>
<td>575</td>
<td>E1</td>
</tr>
<tr>
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<td>GCGATATCTAC GGCTATGTAAAAGACCAG</td>
<td>575</td>
<td>E1</td>
</tr>
<tr>
<td>P3E1FB</td>
<td>GCAAGCTTGCCATGGTGGCCTATTACTCCATGC</td>
<td>1032</td>
<td>E2</td>
</tr>
<tr>
<td>P3E1FB</td>
<td>GCGATATCAT ACCTACCAGCCGATACCATGT</td>
<td>1032</td>
<td>E2</td>
</tr>
<tr>
<td>P3E2FB</td>
<td>GCAAGCTTGCCATGTTTCATGTTATGTCCTAAGG</td>
<td>1032</td>
<td>E2</td>
</tr>
<tr>
<td>P3E2FB</td>
<td>GCGATATCAT ACCAGCCGATACCATGT</td>
<td>1032</td>
<td>E2</td>
</tr>
</tbody>
</table>

Cloning of envelope genes in mammalian expression vector pcDNA3.1: The amplified sequence encoding E1E2 gene was cloned into plasmid pcDNA 3.1 (Invitrogen Tech USA) between hindIII and EcoRI sites downstream of CMV promoter. Translation of E1/E2 initiates from the ATG artificially introduce as start codon at the 5’ end of the proposed signal peptide of E1 to permits expression of the genes in mammalian cell culture.
Transformation: Adding the entire ligation reaction to a 100 µl aliquot of TOP10F’ cells and incubated on ice for 20 min. The cells were then heated to 42°C for 1 min and returned to ice. A 500 ml of SOC medium was added to the cells and incubated at 37°C for 1 hour. Competent cells that have taken up plasmid were selected by spreading the culture onto a Luria-Bertani (LB) agar plate containing 100 µg/ml ampicillin and 12.4 µg/ml of tetracyclin. Colonies were selected by incubating the plate overnight at 37°C.

Colony PCR: To identify bacteria harboring cloned E1E2 genes, individual colonies were used to directly inoculate PCR reactions. The PCR reactions were prepared with 5 pM each of vector-specific primers T7 (5'-TAATACGACTCACTATAGGG-3’) and BGH (5'-TAGAAGGCACAGTCGAGG-3’). Each reaction was prepared to a final volume of 20 ml following a single round of amplification; products were isolated on a 1.5%, ethidium bromide-stained agarose gel, a successful cloning reaction being visualized as a product at approximately 2 kb. Colonies identified as possessing a desired clone were then used to inoculate a 3 ml LB culture containing 100 mg/ml ampicillin, shaking at 225 rpm overnight at 37°C. The plasmids were isolated through plasmid isolation kit (Fermentas Inc. Germany). Quantification of the plasmid prep was performed on a spectrophotometer.

Sequence analysis: The DNA strands from both PCR products as well as from plasmids was sequenced in both directions with automated sequencer (Applied Biosystems USA) BigDye Termination method using specific sense and anti sense primers. Approximately 350 ng of plasmid was used for each sequencing reaction, containing 5 pmol of forward or reverse primer, 2 µl of BigDye, 2 µl of dilution buffer (Applied Biosystems) in a total volume of 10 µl. Sequencing reaction was done in a thermal cycler (ABI 2700) for 25 cycles with the parameters 94°C for 20 s, 58°C for 20 s 60°C for 4 min, transferred to a 0.5 ml microcentrifuge tube, labeled DNA was then precipitated with 50 µl of 100% molecular grade ethanol. The DNA was incubated at room temperature for at least 20 min and then centrifuged at 14000 x g for 30 min. The supernatant was discarded and the pellet was washed twice with 250 ml of 70% ethanol, centrifuged at 14000 x g for 15 min each time. The pellet was air dried and re-hydrated in 15 ml formamide and loaded into sequencer.

Cell culture: Huh-7 human liver hepatoma cell line was obtained from American type cell culture (ATCC CCL-185) and were grown in Dulbeccos modified Eagles medium (DMEM) (ICN, USA) supplemented with 100 mg/ml of streptomycin and 100 U/ml of penicillin and 10%FBS (GibcoBRL, Life Technologies Inc. USA).
**Transfection:** To establish a stable cell line expressing the HCV E1E2 glycoprotein of local 3a genotype, Huh-7 cells were plated in a six well plate 24 hours prior to transfection in DMEM supplemented with 10% FBS (Life Technologies Inc. USA), 100 mg/ml of streptomycin (Sigma USA) and 100U/ml of penicillin (Sigma, USA). These Huh 7 cells were transfected at 75% confluency. A 10 µg of linearized E1E2 construct was diluted in 500 µl of transfection media in 1.5 ml centrifuge tube and in the second tube 10 ml of lipofectamine was diluted in the 500 µl transfection media. Both the tubes were allowed to stand for 10 minutes at room temperature and the contents of both the tubes were combined and incubated at room temperature for 30 minutes. Prior to transfection the media of the cells was replaced with no FBS and antibiotics media. The resulting DNA/lipid complex was vortex and poured drop wise onto the plated cells. The plate was placed at 37º C for 12 hours after that the media having DNA/ lipid complex was replaced with 10% FBS DMEM supplemented with 100 µg/ml of streptomycin and 100U/ml of penicillin. At 24 hours post transfection, cells were split in 60 mm culture dishes at various cell densities. The positive cell colonies were selected by incubation with DMEM containing 10% FBS and 1mg/ml of G418 (Life Technologies Inc. USA) for approximately 5 weeks. Stable cell lines were picked up and amplified. The expression of the E1E2 genes was checked by RT-PCR, western blot analysis and immunofluorescence assay by using anti-E1 and anti-E2 antibodies.

**Reverse transcriptase-polymerase chain reaction (RT-PCR):** To characterize the E1E2 producing cell, the expression profiles of cell line were screened by RT-PCR. The cell were grown in DMEM supplemented with 10% FBS, 100U Penecillin, 100 µg Streptomycin and 500ug/ml G418. Total RNA was isolated from G418Tag cells using an RNeasy Midi kit (Qiagen Inc., Valencia USA) the residual genomic DNA was removed by RNase-Free DNase (Qiagen Inc. Valencia USA) treatment. Total RNA (about 1 µg) was reverse transcribed using Superscript-IITM reverse transcriptase kit (Invitrogen, USA) according to the manufacturer's instructions given in the kit protocol. PCR was performed using sense and antisense primers to produce gene specific fragments. The PCR cycling profiling was: 94°C for 5 min (one cycle), followed by 35 cycles with 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, and final extention was given at 72°C for 10 min. PCR products were analyzed on 1.5% agarose gel electrophoresis containing ethidium bromide and photographed.

**Western blot analysis:** The amount of proteins produced by the stable cell line was determined using a protein assay reagent (Biorad Dye). Briefly, 60 µg of the total protein from cell extracts was electrophoresed in 10% SDS polyacrylamide gel and then transferred on to nitrocellulose membrane.
The proteins on the membrane were blocked by incubation with 5% skim milk. The level of HCV E1E2 gene expression was determined using monoclonal antibodies (Santa Cruz Biotechnologies Inc. USA) specific for HCV E1E2 proteins.

The proteins were subsequently visualized by using horse reddish peroxidase conjugated goat anti-mouse immunoglobulin G (Chemicon International Inc., USA) and staining with chemiluminescence’s substrate (Amersham Biosciences, Buckinghamshire). GAPDH was used as an internal control and was detected by using monoclonal anti actin antibody (Chemicon International Inc., USA).

**Immunofluoresence assay (IFA):** Stable cell line expressing HCV envelope glycoprotein was grown overnight on a cover slip in a 6 well plate. Cells were washed with 1X PBS, fixed with 30% acetone at -20º C. Subsequently, fixed cells were blocked with 1% donkey serum in 1X PBS. HCV E1E2 envelope glycoprotein produced by the cells were detected by incubation with E1E2 specific antibodies (1:100) for 2 hours at 37º C and visualized with secondary donkey anti mouse IgG antibody conjugated with FITC (1:100) (Chemicon International Inc., USA). Cover slips were mounted onto the slides and HCV envelope proteins and analyzed on the flow cytometer within 1 hour.

**Calorimetric analysis:**

Huh-7 cells stably expressing the HCV envelope glycoproteins were plated in micro wells of 96 well plates. Patients 10 years of age and older infected with HCV alone, with no evidence of liver failure and not undergoing HCV therapy at the time of enrollment were eligible for this study. Samples were diluted in 1:100 with 1xPBS in the disposable tubes and mixed well. 100 µl of negative, positive control and diluted samples were added separately in the corresponding micro wells. Mixed gently and incubated at 37ºC for 60 minutes. Wells were washed gently with 500 µl of 1xPBS for 5 times. 100 µl HRP conjugate anti-human secondary antibody was added into each well, the plate was incubated at 37ºC for 60 minutes. Wells were again washed with 1xPBS for 5 times. 100 µl chromogen/ substrate were added into each well, and the micro plate was kept at room temperature for 20 minutes. Absorbance was measured at 450nm.
RESULTS

Cloning of envelope genes in mammalian expression vector

HCV cDNA Synthesis and PCR amplification of envelope genes

Hepatitis C virus RNA of local 3a genotype was isolated from the serum sample of a chronic HCV carrier. The serum samples used for isolation of envelope genes were negative for hepatitis B virus surface antigen (HBsAg) but positive for anti-hepatitis C virus antibody (HCV IgG). The primers used for the amplification of E1 and E2 were designed based on knowledge of existing sequence data for these genes (Table-1). Frequent mutations in the Hypervariable regions of envelope genes the recovery of these genes from patient samples is difficult (Legrand et al., 2007 & Herring et al., 2005). Different sets of primers were designed, out of which one primer set worked and result in the amplification of the whole envelope genes. The primers artificially introduce a start codon at the 5’ end of the proposed signal peptide of E1. This permits expression of the genes in mammalian cell culture.

Cloning of HCV envelope genes in mammalian expression vector pcDNA3.1

In order to characterize the role of envelope genes in disease progression we cloned the amplified PCR product in mammalian expression vector pc DNA 3.1. This vector has a CMV promoter which represents an effective mean to transduce eukaryotic cells for transient and stable expression studies. The cloned genes were sequenced in both direction and the consensus sequence was matched to HCV genotype 3a sequence when blast was made with other HCV sequences in GenBank data base. The resulting sequence was submitted to NCBI GeneBank database. The Accession number assigned to this sequence is EU399722.

Generation of stable cell lines: The expression vector was linearized and transfected into Huh7 cells by lipofectamine. Twenty-four hours post transfection, selection was applied to the transfected cells by growing them in the presence of 1mg of G418/ml. The majority of the cells (75%-85%) did not develop resistance to the selecting agent, but in the long run it was possible to identify G418-resistant cell clones, which were picked after four weeks of culture and grown as individual cell lines. Once the clones had been isolated and individually grown as cell lines the concentration of neomycin was decreased to 500 µg/ml. The individual cell lines showed some variability in growth rate.
Detection and quantification of E1E2 RNA in Huh7 cells transfected with envelope protein coding vector: To check the presence of RNA in transfected cells, total RNA was extracted from cells and Non-quantitative RT-PCR was performed with gene specific set of primers. PCR products were obtained only when a reverse transcription step was included, indicating that amplification was exclusively RNA dependent and not due to the presence of residual DNA in the RNA preparation (Figure -1).

Fig-1: RT-PCR of cells transfected with expression vector pcDNAEPk. Lane 1: 1kb ladder, Lane: 2, 3 exp, Lane: 4-6 -ve control.

Detection of envelope proteins.

Western blot analysis: To check expression of HCV envelope proteins produced from replicon clones, we performed Western blotting analyses with protein extracts of transfected Huh-7 cells using mouse monoclonal sera against E1 and E2 (Santa Cruz Biotechnologies Inc. USA). The Western blot analysis identified specific bands of the expected electrophoretic mobility for E2 having molecular weight of 70 kDa respectively (Figure-2).

Figure 2: A-Western blot with anti-E2
B- Western blots with anti-GAPDH antibodies (Diluted 1: 500).
Immunofloresence analysis.

Immunostaining was done to investigate the presence of a protein and its sub-cellular localisation. Envelope proteins expression was detected in cytoplasm and on the plasma membrane. No changes in the morphology of the cells were observed (Figure-3).

![Figure-3: Immunostaining of stable cell lines 1,2 and 3 with anti envelope antibodies. Lane A merge. Lane B; staining with FITC, Lane C; counter staining with DAPI.](image)

Detection of anti envelope antibodies in serum samples of HCV patients:

We used stable cell lines expressing the HCV envelope glycoproteins to check anti envelope antibodies in chronic HCV patients. Cell lines grown in micro well plate were incubated with serum samples from HCV patients for 1 hr and afterward incubated with HRP conjugated anti-human secondary antibody. Plate was developed with diamino benzedine and read on the microplate reader at 450nm (Table-2). The measurement of absorbance was directly related to the antibody titer in the host. The absorbance for negative control was subtracted to find the cutoff values and plotted against age of patients. A linear correlation between ages of patients and Anti-HCV was observed (Figure-4). Pearson's correlation was applied and found that correlation was significant at the 0.05 level (Table-3).
Figure-4: Comparison between AGE and Anti-HCV. The slope of the trend line shows that Anti-HCV and age have positive relation.

Table-2: Measurement of absorbance at 450nm for detection of anti envelope antibodies in the serum of HCV infected individuals.

<table>
<thead>
<tr>
<th>Sr No</th>
<th>AGE</th>
<th>GENOTYPE</th>
<th>Absorbance Λ=450nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>3a</td>
<td>0.003</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>3a</td>
<td>0.243</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>3a</td>
<td>0.008</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>3a</td>
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</tr>
<tr>
<td>5</td>
<td>22</td>
<td>3a</td>
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</tr>
<tr>
<td>6</td>
<td>26</td>
<td>3a</td>
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</tr>
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<td>9</td>
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<tr>
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</tr>
<tr>
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<tr>
<td>15</td>
<td>60</td>
<td>3a</td>
<td>0.18</td>
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Table-3: Pearsons correlation depicting that Correlation is significant at the 0.05 level (1-tailed)

<table>
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<th></th>
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<th>VAR2: Ab titer</th>
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<tr>
<td>Pearson Correlation</td>
<td>1</td>
<td>.575*</td>
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<tr>
<td>Sig. (1-tailed)</td>
<td>.032</td>
<td></td>
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<td>11</td>
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Discussion

HCV infections are increasing at an alarming rate in Pakistan (Idrees 2008, Syed, et al., 2009). Inhibition of viruses at the stage of viral entry provides a route for therapeutic intervention, as evidenced by the recent development of inhibitors of HIV entry (Matthews, et.al., 2004). Because of difficulties in propagating HCV in cell culture, entry inhibitors have not yet been reported for HCV. The unstoppable spread of HCV can only be contained by a vaccine with high protective efficacy, low cost, and simple immunization schedules that elicit the broadest and most robust immune responses against HCV (Deutsch, et.al., 2008, Chen, et.al., 2007, Rosa, et.al., 1996, Zibert, et.al., 1995). HCV genome codes two envelope glycoproteins E1 and E2, which play an important role in the attachment and entry of virus particles into the host cell (Op De Beeck, et.al., 2001 & 2004). These are supposed to be the first viral component that comes in contact with the host cell surface receptor and are obvious candidates for vaccine development [Cooper, et.al., 1999, Strickland, et.al., 2008, Deutsch, et.al., 2008).

In this study we collected serum samples from HCV patients who were not undergoing interferon therapies at the time of enrollment and without any evidence of liver failure. cDNA was synthesized using gene specific anti sense primer and amplified after addition of sense primer. Amplified product was cloned in mammalian expression vector and stable expression was studied in continuous cell lines. Cloned envelope genes were sequenced and sequence is available on NCBI database with accession no eu399722. We used this expression construct to direct the expression of the HCV envelope glycoproteins in continuous cell lines to characterize expressed viral antigens.
Based on earlier observation that liver cells show the maximum tropism for the virus, we stably transfected Huh-7 cell line with envelope glycoprotein coding expression vector pcDNAEPk (Harada, et al., 1995, Hu, et al., 2005, Pietschmann, et al., 2001 & 2003, Gosert, et al., 2003). Selection of positive cell clones was done with antibiotic G418 at a concentration of 500µg/ml. We have seen that majority of the cells did not develop resistance to the selecting agent, but in the long run it was possible to identify G418-resistant cell clones, which were picked after one month of culture and grown as individual cell lines. We have observed the cell lines for up to six months for the sustained expression of the envelope proteins. The doubling times of cells were similar to that of the parental cells. The protein expression was checked by western blot analysis and immunostaining. Western blot showed that the expressed E2 protein has a molecular mass of ~70kDa (Figure 4). Envelope proteins expression was detected in cytoplasm and on the plasma membrane (Fig 5). The results are in accordance with the studies in which this characteristic of protein expression at the surface of plasma membrane has been exploited to generate HCV pseudotype viruses containing the E1/E2 proteins (Bartosch, et al., 2009) Harada. The E1/E2 proteins are also contained in HCV-like particles (HCV-LPs), which are produced in either insect or human cell lines infected with a recombinant baculovirus (Daniel, et al., 2004, Rouille, et al., 2006). The identification and characterization of antibodies targeting distinct steps of viral entry is an important strategy for the understanding of the molecular mechanisms of antibody-mediated neutralization (Hu, et al., 2005, Pietschmann, et al., 2001 & 2003, Gosert, et al., 2003). It is important to mention here that the developed system provides easy detection of anti envelope antibodies in serum of HCV patients. We utilized this system to study a panel of serum samples from HCV infected patients, to detect anti envelope antibodies through calorimetric analysis (Op De Beeck, et al., 2001). Here we relate the rate of absorbance to the titer of anti envelope antibodies. According to the results obtained (Table-2) all serum samples were positive for anti envelope antibodies. However; the neutralization of infection in patients was completely absent and do not meet the rapidly evolving glycoproteins. Possible mutations in the binding epitope of envelope glycoprotein may be responsible for the failure of these antibodies to resolve infection. Interestingly when the absorbance values were plotted against the ages of patients a linear correlation was observed. Studies have proved that treatment of HCV infection with interferon alone is not sufficient for viral clearance (Saracco, et al., 2001) therefore new therapeutic approaches are required on urgent basis. The developed system has the potential to detect and characterize anti-HCV envelope neutralizing antibodies from HCV infected patients and can also be used for the identification of antiviral molecules able to bind to HCV envelope glycoprotein.

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REFERENCES


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