CHAPTER 7

Antigenic studies of recombinant HCV Core, NS3, and NS5.
INTRODUCTION

Detection of antibodies in serum against hepatitis C viral infection has become the principle method for the diagnosis of HCV infection in individuals with chronic hepatitis and for the screening of blood donors. The original assay based upon the c100-3 recombinant proteins derived from NS4 showed nonspecific (false positive) as well as insensitive (10 - 20%) results, however, the later developed assays used the recombinant proteins from the Core (c22-3) and NS3(c33c) regions of the HCV genome along with NS4 (Second -generation assays). But this assay is able to detect the infection only after 12 weeks and more over indeterminate results may occur. Currently developed assays include four HCV proteins i.e. NS4, core, NS3 and NS5 (Third -generation assays) and are proved to be more effective for the screening of patients and blood donors. The use of third -generation assays has led to substantial reduction in the incidence of post transfusion hepatitis. In prospective studies, the incidence of HCV transmission among recipients of blood screened by first generation assay was 1.5% in Spain (Gonzalez et al., 1995), 3.7% in Japan (Takano et al., 1996), and 11% in Taiwan (Chang et al., 1996). Screening by second-generation assays reduced the incidence to 0.9% in Japan (Takano et al., 1996), 1% in Spain (Gonzalez et al., 1995), and 2.5% in Taiwan (Chang et al., 1996).

Even the third generation assays fail to detect antibody to HCV in donated blood collected from an individual with acute infection before seroconversion for antibody. This period between exposure to virus and development of antibodies is called “window” period which is long for HCV compared to other viruses with means of 66 to 88 days (Courouce et al., 1994). By measuring the incidence of HCV infection in blood donors, it has been estimated that the current residual risk of HCV transmission through collection of “window” samples is 1 per 100,000 donations in the United States (Schreiber et al., 1996). This frequency of infection is similar to the residual risk of infection from blood screened by third-generation assays from French blood donors.

Some samples false negative for antibody have been reported for a small portion of immunocompetent individuals persistently infected with HCV (Kao et al., 1996; Cerino et al., 1997). Even in anti-HCV positive individuals, there is considerable variability in the frequency of reactivity to the individual HCV proteins used in supplementary assays like
RIBA (3rd generation recombinant immuno blot assay of Ortho). Among 90 anti-HCV-positive blood donors screened by Ortho, antibody reactivity in the third-generation assay varied over a range of 5,000-fold, with some serum samples having antibody levels only just above the cutoff value (Dhaliwal et al., 1996).

HCV can be classified into at least six major genotypes, whose nucleotide and amino acid sequences over the whole genome differ by approximately 30%. This degree of amino acid sequence variability is similar to that observed between variants of other RNA viruses (e.g., dengue virus), in which significant antigenic differences have been documented and which form the basis of their classification into serotypes. We have previously measured the serologic reactivity's of individuals infected with different HCV genotypes to antigens used in two third-generation anti-HCV assays (3rd generation anti-HCV EIA from Ortho and Abbot Murex). Samples from HCV genotype-1 infected individuals showed an approximately five times greater reactivity than those infected with HCV genotype-2 or -3, and the reactivity was independent of other factors that may have influenced the antibody response, such as the degree of viremia, donor age, and severity of hepatitis as assessed by liver function tests.

The main purpose of the current study is to make comparison between the performance of the recombinant antigens expressed by us with the kit antigens developed from HCV strains of US and Europe. HCV antigens expressed in the present work are exclusively from Indian isolates of HCV i.e. genotype 1 and 3 where as the kit antigens are developed from genotype 1 only. An important observation made by us during the work is failure of kit antigens to detect HCV infections which are other wise confirmed by PCR method. As a further development, in the current study we have successfully cloned and expressed the HCV antigenic proteins core, NS3 and NS5 from different genotypes in E.coli expression system, purified the proteins by different methods, confirmed by western blotting and evaluated by ELISA.
MATERIAL AND METHODS

HCV RNA isolation: The RNA was extracted from 200 μl of serum according to the protocol of Chomczynski and Saachi (1987) as described in chapter 2.

PCR amplification: The PCR amplification protocol was described in chapter 2.

<table>
<thead>
<tr>
<th>No. of Cycle</th>
<th>Steps</th>
<th>Temperature</th>
<th>Time/Core</th>
<th>Time/NS3 and NS5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>42℃</td>
<td>60 min</td>
<td>60 min</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>95℃</td>
<td>3 min</td>
<td>3 min</td>
</tr>
<tr>
<td>36</td>
<td>3</td>
<td>94℃</td>
<td>30 sec</td>
<td>60 sec</td>
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<td></td>
<td>4</td>
<td>54℃</td>
<td>45 sec</td>
<td>90 sec</td>
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<td>120 sec</td>
</tr>
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<td>5 min</td>
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<tr>
<td>1</td>
<td>7</td>
<td>4℃</td>
<td>Infinite</td>
<td>Infinite</td>
</tr>
</tbody>
</table>

Purification of PCR product: The protocol for purification of PCR product is already discussed in chapter 2.

Preparation of Plasmid DNA: 5 μl PET 21 a and b cultures were inoculated onto 5 ml LB medium with ampicillin. Over night grown cultures have been used for plasmid DNA isolation. Isolation of plasmid DNA was described in chapter 2.

RFLP Pattern:

<table>
<thead>
<tr>
<th>Genes</th>
<th>Restriction enzymes used for vector digestion</th>
<th>Restriction enzymes used for orientation check</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core</td>
<td>EcoRI</td>
<td>KpnI and XbaI</td>
</tr>
<tr>
<td>NS3</td>
<td>EcoRI and HindIII</td>
<td>Sall</td>
</tr>
<tr>
<td>NS5</td>
<td>NdeI and Bam HI</td>
<td>Hind III</td>
</tr>
</tbody>
</table>
**Linearization and dephosphorylation of Plasmid DNA**

1. 10 µg of closed circular plasmid DNA is digested with 5-10 units of the desired restriction enzymes for 2-3 hours at 37°C.

2. When digestion is completed, the sample was extracted once with Phenol: chloroform and DNA was precipitated in ethanol. The solution was mixed and stored at -20°C overnight.

3. The DNA prep was recovered by centrifugation at 10,000g for 10 minutes at 4°C and the pelleted DNA was dissolved in 110 µl of 10 mM Tris Cl (pH - 8.3).

4. 90 µl of the linearised plasmid DNA was added to 10 µl of 10 X CIP buffer with 5-10 units of CIP enzyme and incubated for 1 hour at 37°C.

5. To inactivate CIP activity at the end of the incubation period, the reaction mixture was incubated for 15 minutes at 65°C.

6. The reaction mixture was cooled to the room temperature and extracted once with Phenol and once with Chloroform mixture.

7. Precipitation the DNA in ethanol. Mix the solution again and store it in -20°C overnight.

8. The DNA was recovered by centrifugation at 10,000 rpm for 10 minutes at 4°C. The pellet was washed with 70% ethanol at 4°C and centrifuged again at 10,000 rpm for 10 minutes.

9. The tubes are kept opened and incubated at 65°C for 10 min. to evaporate the residual ethanol in the preparation.

10. The precipitated DNA was dissolved in TE (pH-8.0) to a concentration of 100 µg/ml and stored as aliquots at -20°C.

**Ligation**

1. In a microfuge tube, the ligation mixture was constituted as follows:

   - 25 µg/ml amplified target DNA: 1 µl
   - Enzyme digested plasmid: 20 ng
   - 10X ligation buffer: 1 µl
   - Bacteriophage T4 DNA ligase: 3 Units
   - H2O: to 10 µl
2. The ligation mixture was incubated for 4 hours at 14°C.

3. After ligation, the mixture is stored at 40°C until transformation of competent *E. coli*.

**Transformation:** Transformation of plasmid DNA was carried out according to the protocol described in chapter 2.

**Screening of recombinants:** For screening over night incubated transformed LB agar plates individual colonies were picked and inoculated in 30 ml screw cap tubes containing 5 ml autoclaved LB medium containing ampicillin. The tubes are incubated at 37°C for over night. For all the plasmid DNA was isolated and digest with an enzyme, all the samples confirmed by electrophoresis.

**Expression screening:** For expression screening, individual colonies with insert are inoculated in 30 ml screw cap tubes containing 5 ml autoclaved LB medium containing ampicillin. The tubes are incubated at 37°C for 2 – 3 hours until cell growth reaches to $\text{OD}_{600} = 0.8 – 1.0$. After reaching this OD, 1μl IPTG (100mM stock) is added to each tube for induction and then tubes are kept for shaking at 37°C for 5 – 6 hours. Cell samples are taken before induction and after the termination of culture.

The samples before and after induction are adjusted to 1 OD/ml and lysed in presence of 2x PAGE gel loading buffer by boiling for 5-6 minutes. The boiled samples in 2x buffer are loaded on to the wells of PAGE gel (20μl/ well) along with protein marker. After PAGE the gel is stained by commassie blue and then destained. The lanes of uninduced and induced samples are compared to find a new protein band in induced sample. If a new protein observed in induced sample further analysis carried out by western blotting.

**Shake flask culture:** The shake flask culture was used for accumulation of sufficient cell pellet to undertake downstream processing. For shake flask culture 2.0 litres LB medium is prepared and dispensed into 4 X 2 lit flasks with 500 ml medium in each flask. The medium is autoclaved at 121°C/15 lb pressure. After cooling to room temperature the flasks are placed in laminar air flow. Before placing inoculum, to each flask 500μl ampicillin (1mg/ml stock) is added and then inoculum of 1% is added. Then the flasks are
kept in orbital shaker at 37°C for 2 – 3 hours until cell growth reaches to OD$_{600}$ = 0.8 – 1.0. After reaching this OD, 20µl IPTG is added to each flask for induction and then flasks are kept for shaking at 37°C for 5 – 6 hours. Cell samples are taken before induction and after the termination of shake flask culture.

Then fermentative production of HCV - CORE, NS3 and NS5 proteins was done on tabletop fermentor (BIOFLOW 2000 of New Brunswick) of 3 litres capacity. The cells with induced protein are harvested by centrifugation at 6000g and the cell pellets are stored at -70°C until further use. Again the induction of NS5 was checked by PAGE and expressed protein is confirmed by western blot assay.

**Downstream processing and purification of recombinant HCV antigens:** The most important characteristic of many recombinant diagnostic proteins is their purity. They are used for coating onto ELISA strips or rapid strips for the detection of antibodies in the serum of infected patients. If the antigenic protein is not pure or co purified with host proteins, diagnostic assay may show false positive signal. Many recombinant proteins expressed in *E.coli* are produced as inclusion bodies i.e. insoluble protein aggregates which can be solubilized with chaotropic agents like urea or guanidine -HCl. In the present investigation we have processed the HCV recombinant antigens expressed in *E.coli* by different methods and eliminated the host proteins gradually from cell lysis to protein purification.

In the present work HCV – CORE, NS3, and NS5 coding sequences have been cloned in PET expression vectors and introduced into BL21 strain of *E.coli* by salt induced transformation. Later screening of recombinants was done and promising clones are used for cell culture and large scale expression of HCV proteins. The purification procedure include, Cell lysis, isolation of inclusion bodies, solubilization of protein from inclusion bodies and purification of protein from host protein impurities.

**Buffers and Reagents**

The following instruments, reagents and buffers are used for the cell lysis and solubilization of protein from inclusion bodies.
1. **TES Buffer** (pH 8.0)
   - 20 mM Tris
   - 5 mM EDTA
   - 20% Sucrose
   - 1 mM PMSF

2. **TED Buffer (pH 8.0)**
   - 20 mM Tris
   - 5 mM EDTA
   - 5 mM DTT

3. **Lysozyme:**
   - 100 mg/ml (stock)

4. **NaCl :**
   - 5 M stock

5. **Triton-X 100 (non ionic detergent)**

6. **Solubilization Buffer - 1 (pH 10.5)**
   - Carbonate buffer containing
   - 0.1% SLS (Sodium Laryl Sarcosine)

7. **Solubilization Buffer - 2 (pH 8.0)**
   - Tris – EDTA buffer containing
   - 8 M urea

**Instruments required**

- Cell homogenizer
- Magnetic stirrer
- High-speed centrifuge
- Ultra sound sonicator
- PH meter
Cell Lysis

_E. coli_ recombinant strain BL21 cells expressed HCV CORE, NS3 and NS5 proteins in fermentor, harvested by centrifugation and stored at \(-70^\circ\text{C}\). Before processing, the pellet is brought to room temperature for thawing.

1.0 g cell pellet is suspended in 10 ml TES Buffer and made into homogenous suspension with cell homogenizer. To the homogenous cell suspension, 100 μl of lysozyme is added and the suspension is stirred for 30 minutes at 4°C to allow digestion of cell wall and partial cell lysis. The suspension became sticky after the cell lysis.

The suspension is sonicated for by 20 seconds X 5 pulses. A time pause of 2 minutes given between successive pulses to cool the cell suspension. After sonication the viscous cell suspension became free solution.

To the sonicated suspension, 1 ml of 5 M NaCl (0.5 M final concentration) and 100 μl of triton-X 100 are added and left for stirring at 4°C. NaCl solublize some host proteins and triton-X 100 removes membrane proteins. Then the suspension was centrifuged at 21000g for 20 minutes at 4°C. Supernatant-I was saved for analysis. Pellet-I contains insoluble bulk HCV protein.

Pellet-I was resuspended in 10ml of TED buffer. The suspension is sonicated again briefly (15 seconds X 3 pulses) and spun at 21000 g for 20 minutes at 4°C. Supernatant –II was preserved for further analysis. Pellet – II contains insoluble HCV protein as inclusion bodies to a purity of 80 – 90%.

Pellet-II of HCV – Core is suspended in 10 ml carbonate buffer (pH 10.5) containing 0.1 % Sodium Lauryl Sulphate. The suspension is incubated at 65°C for 1 ½ hrs for heat shock. The combination of heat shock and non-ionic detergent treatment solublize the protein. After heat shock, the suspension is spun at 21,000 g for 20 min at 4°C. Final supt. contains solubilized protein. The pellet is saved for analysis.
Where as the Pellet-II of HCV – NS3 or NS5 is suspended in 10 ml TE buffer (pH 8.0) containing 8M urea and left for stirring at room temp for ½ hour to solubilize the protein. The solubilized protein is stored in −70°C until further processing. Small aliquots of all samples are taken for analysis by SDS-PAGE

All supernatants and final pellet are checked by SDS-PAGE to know which sample is containing the HCV proteins.

Purification of HCV – proteins from solubilized protein samples

HCV - CORE by Electroelution

There are different methods used for the purification of proteins. Most frequently used is chromatography which depends on parameters like size, charge, pl, hydrophobic nature of a protein. Often a combination of ion exchange chromatography followed by gel filtration or reverse phase chromatography followed by gel filtration are usually employed. After purification the protein is refolded and activated. Since HCV – CORE is a diagnostic protein it is need not be an active form or refolded form. Even the denatured form can work very well for diagnostic procedures.

The method which can yield good quantity of pure protein without resorting to chromatography is electroelution i.e. extraction of proteins separated on polycarlamide gels with the help of externally applied current.

Method

Before the start of purification, the concentration of solubilized protein increased to 2mg to 5mg/ml by placing protein sample in dialysis bag and keeping the dialysis bag in sucrose.

After concentration, the protein is loaded along with 2x loading dye in two wide wells of a big gel 24cm x 12 cm. Along with sample, pre-stained marker is loaded in a narrow well for reference.

PAGE is carried out at 80 m Amp / 250 V with cooling system at 10°C. After PAGE run, the gel portions corresponding to the separated HCV – CORE are excised.
Excised gel slices are placed in dialysis membrane bag with cut off value 10 kda (treated with BSA for blocking) with 1x gel PAGE running buffer containing 1mM PMSF but without SDS.

The dialysis membrane bag is placed in horizontal electrophoresis chamber filled with 1x PAGE running buffer. The proteins are eluted from excised gel slices into dialysis membrane at 240mAmperes/70V current for 2 hours at 4°C.

After electro elution, the protein elute is collected from dialysis bag and checked by SDS-PAGE along with known concentration of BSA.

After confirmation on gel, the elute is filtered through 0.2 μ filter then subjected to concentration using molecular weight cut of membrane of 10kda pore size. The concentration is done by placing the concentrators in SS34 rotor of Sorval centrifuge and spun at 3000rpm for 5 minutes. The concentrated protein sample in the concentrator is collected.

The concentrated HCV – CORE protein is again checked by PAGE to know the concentration.

**Purification HCV – NS3 and NS5 by Ion exchange column chromatography**

Unlike HCV – CORE, for the purification HCV – NS3 and NS5, column chromatography is employed. Initially gel filtration technique was used but it is not efficiently separated the protein. Later using DEAE sepharose chromatography was done, but the method is not giving purified protein as the protein is eluting at different concentrations of NaCl used. Finally CM sepharose matrix binding studies were carried out at very small volume level followed by column elution.

**NS3 and NS5 protein binding studies with CM-Sepharose**

- CM-sepharose slurry of 0.5 ml was taken into 1ml eppendorf tube and spun at 13000g on table top centrifuge for five minutes to remove 20% ethanol
- To the pellet of CM sepharose, TsoEs - 8Murea buffer is added and mixed thoroughly. The eppendorf tube was spun at 13000g for 5 minutes.
The above step was repeated for 3–4 times until equilibration of the matrix.

NS3 and NS5 solubilized proteins (OD_{280nm} = 2.0 ABS) of 1ml in T_{50E5} - 8Murea is added to equilibrated CM-Sepharose and mixed well. The sample was spun at 13000g for 5 minutes. Supernatant-1 was scanned at 200λ to 400λ.

To the pellet, 1ml of T_{50E5} buffer with 1mM DTT is added, mixed thoroughly, and spun at 13000g for 5 minutes. Supenatant-2 was separated and scanned at 200λ to 400λ.

To the above pellet, 1ml of T_{50E5} buffer with 1mM DTT and 0.5 M NaCl is added and mixed well. After five minutes the suspension was spun at 13000g for 5 minutes. Supenatant-3 was separated and scanned at 200λ to 400λ.

To the above pellet, 1ml of T_{50E5} buffer with 1mM DTT and 1.0 M NaCl is added and mixed well. After five minutes the suspension was spun at 13000g for 5 minutes. Supenatant-4 was separated and scanned at 200λ to 400λ.

Fractions showing high OD_{280nm} are checked by PAGE to know the presence of NS5 protein. PAGE revealed that pooled protein between fractions 45mL to 75 ml is having bulk of NS3 and NS5 protein without any host proteins. This protein solution is lyophilized. Lyophilized powder solubilized in 5 ml of T_{50E5} buffer with 8M Urea and 1 mM DTT. Protein in this fraction was estimated with Bio-Rad micro-assay.

<table>
<thead>
<tr>
<th>Sample value @ 280λ</th>
<th>NS3</th>
<th>NS5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubilized protein</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Supt-1 Flow through</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Sup-2 Wash</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>Sup-3 in 0.5 M NaCl</td>
<td>0.88</td>
<td>0.88</td>
</tr>
<tr>
<td>Sup-2 in 1.0 M NaCl</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

All supernatants are concentrated using centrifon MWC membranes and loaded on PAGE gels to know the presence of NS3 and NS5 in which fraction. The binding studies
revealed that HCV NS5 is binding to the CM sepharose matrix and is getting eluted at 0.5 M NaCl.

**Scale up with CM-Sepharose chromatography**

- 100 ml volume Pharmacia column thoroughly washed and fixed to the stand. Prepared CM-Sepharose matrix slowly poured onto the column and is allowed to settle under gravity.
- After sedimentation, the column is washed with 5 bed volumes of autoclaved deionized water.
- Then column is equilibrated with 500 ml of T_{50}E_{5} buffer pH: 7.6 with 8M Urea and 1mM DTT.
- Solubilized NS3 and NS5 protein of 10ml (10.0 OD/ml) was loaded on to the column. Then the column is connected to the gradient mixer having 300 ml of T_{50}E_{5} 8M Urea in one cylinder (starting gradient) and 300 ml of T_{50}E_{5} 8M Urea with 0.5M Nacl (end gradient) in other cylinder. The outlet of the column is connected to Pharmacia fraction collector. Each fraction of volume 5.0 ml is collected till end of the run.
- All fractions after the end of the chromatography are scanned at A_{280nm} for their spectral properties. The OD values of all fractions are recorded and plotted as an excel graph.

**CHARACTERIZATION**

*Preparation of SDS-PAGE gels*

**Gel stocks**

- 30% Acrylamide mix (29% (w/v) Acrylamide and 1% (w/v) N,N'-Methylenebisacrylamide)
- 1.5 M Tris-HCl, pH 8.8
- 1 M Tris-HCl, pH 6.8
- 10% (w/v) Sodium dodecyl sulfate (SDS)
- 10% (w/v) Ammonium per sulfate
- TEMED (N,N,N',N'-Tetramethylethylenediamine)
Tris-Glycine electrophoresis running buffer

25 mM Tris base,
250 mM Glycine (pH 8.3),
0.1% SDS.

2X SDS gel loading buffer

50 mM Tris.Cl (pH6.8)
100mM dithiothreitol
2% SDS(electrophersis grade)
0.1%bromophenol blue
10%glycerol

<table>
<thead>
<tr>
<th>Component</th>
<th>12% gel</th>
<th>15% gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>1.6 ml</td>
<td>1.1 ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>2.0 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>1.5M Tris (PH 8.8)</td>
<td>1.3 ml</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>TEMED</td>
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<td>0.002 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>5.002 ml</td>
<td>5.002 ml</td>
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</table>

<table>
<thead>
<tr>
<th>Component</th>
<th>5% gel</th>
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<tbody>
<tr>
<td>H₂O</td>
<td>3.40 ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>0.83 ml</td>
</tr>
<tr>
<td>1.0M Tris (PH 6.8)</td>
<td>0.63 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.05 ml</td>
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<td>TEMED</td>
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</tr>
<tr>
<td>TEMED</td>
<td>0.005 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>5.002 ml</td>
</tr>
</tbody>
</table>
Gel staining solution
0.25 g of coomassie brilliant blue R250
90 ml of methanol
10 ml of glacial acetic acid

Destaining solution
30 ml of methanol
10 ml of glacial acetic acid
60 ml water

Western blot transfer buffer (pH 8.3)
39 mM glycine
48 mM Tris base
0.037% SDS (Electrophoresis grade)
20% methanol

Blocking Solution
5% (w/v) non-fat dried milk
0.05% Tween 20 in phosphate-buffered saline

Western blot Wash buffer
PBS + TWEEN 20

Buffer and substrates for western blot development
Alkaline phosphate Buffer
100 mM NaCl
5 mM MgCl₂
100 mM Tris.Cl (pH 9.5)

NBT (Nitro Blue Tetrazolium)
Dissolve 0.5 g of NBT, in 10 ml of 70% dimethylformamide.
**BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate)**

Dissolve 0.5 g of BCIP disodium salt, in 10 ml of 100% dimethylformamide.

**Equipment**

1. Slab gel apparatus: Midjet (LKB) or equivalent
2. 0.75 mm spacers and 15-well comb
3. Power supply with constant current capability
4. Boiling water bath
5. 15 ml Hamilton syringe
6. Pipettes
7. LKB laser densitometer or equivalent

**Pouring SDS-PAGE gels**

1. Assemble the glass plates according to the manufacturers instructions.
2. Determine the volume of the gel mold in an Erlenmeyer flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel, mix the components in the order shown. Polymerization will begin as soon as the TEMED has been added. Without delay, swirl the mixture rapidly and proceed to the next step.
3. Pour the acrylamide solution into the gap between the glass plates. Leave sufficient space for the stacking gel. Using a Pasteur pipette, carefully overlay the acrylamide solution with 0.1% SDS (for gels containing >10%acrylamide).place the gel in a vertical position at room temperature.
4. After polymerization is complete (30 minutes), pour off the overlay and wash the top of the gels several times with deionized water to remove any unpolymerized acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining water with the edge of a paper towel.
5. Prepare the stocking gel as follows: In a disposable plastic tube, prepare the appropriate volume of solution containing the desired concentration of acrylamide, mix the components in the order shown. Polymerization will begin as soon as the TEMED has been added. Without delay, swirl the mixture rapidly and proceed to the next step.
6. Pour the stocking gel solution directly onto the surface of the polymerized resolving gel. Immediately insert a clean Teflon comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Place the gel in a vertical position at room temperature.

7. While the stocking gel is polymerizing, prepare the samples by heating them to 100°C for 3 minutes in 1x SDS gel-loading buffer to denature the proteins.

8. After polymerization is complete (30 minutes), remove the Teflon comb carefully. Using a squirt bottle, wash the wells immediately with deionized water to remove any un polymerized acrylamide. If necessary, straighten the teeth of the stocking gel with a blunt hypodermic needle attached to a syringe. Mount the gel in the electrophoresis apparatus. Add Tris-glycine electrophoresis buffer to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe.

9. Load up to 20 μl of each of the samples in a predetermined order into the bottom of the wells.

10. Attach the electrophoresis apparatus to an electric power supply. Apply a voltage of 8 V/cm to the gel. After the dye front has moved into the resolving gel, increase the voltage to 15V/cm and run the gel until the bromophenol blue reaches the bottom of the resolving gel (about 120 min). Then turn off the power supply.

11. Remove the glass plates from the electrophoresis apparatus and place them on a paper towel. Using a spatula, pry the plates apart. Mark the orientation of the gel by cutting a corner from the bottom of the gel that is closest to the leftmost well.

12. The gel can now be fixed stained with coomassie brilliant blue or silver salts flurographed or autodiographed or used to establish a western blot.

Staining SDS-PAGE gels with coomassie blue

1. Immerse the gel in at least 5 volumes of coomassie blue staining solution and place on a slowly rotating platform for a minimum of 2 hrs at room temperature.
2. Remove the stain. Destain the gel by soaking it in the methanol/acetic acid destaining solution with out the dye on a slowly rocking platform 2-4 hrs, changing the solution three or four times.

3. The more thoroughly the gel is destained, the smaller the amount of protein that can be detected by staining with coomassie brilliant blue.

4. After destaining, gels may be stored indefinitely in water in a sealed plastic bag without any diminution in the intensity of staining. Store fixed polyacrylamide gels in water containing 20% glycerol.

5. To make a permanent record, either photograph the stained gel or dry the gel.

Western Blot

Transfer of proteins from SDS-polyacrylamide gels to solid supports

1. When the SDS-polyacrylamide gel is approaching the end of its run, rinse the graphite plates with distilled water and wipe off any beads of liquid that adhere to them with Kimwipes or other non absorbent tissues.

2. Wearing gloves, cut six pieces of whatmann 3MM paper of nitrocellulose filter (Millipore HAWP or equivalent) to the exact size of the SDS-polyacrylamide gel. If the paper or filter is larger than the gel, there is a good chance that the overhanging edges of the paper and the filter will touch, causing a short circuit that will prevent the transfer of protein from the gel. Mark one corner of the filter with a soft-lead pencil.

3. Float the nitrocellulose filter on the surface of a tray of deionized water and allow it to wet from beneath by capillary action. Then, submerge the filter in the water for at least 5 minutes to displace trapped air bubbles.

4. Soak the six pieces of 3MM paper in a shallow tray containing a small amount of transfer buffer.

5. Wearing gloves, set up the transfer apparatus as follows:
   a) Lay the bottom electrode (which will become the anode) flat on the bench, graphite side up.
   b) Place on the electrode three sheets of 3MM paper that have been soaked in transfer buffer. Stack the sheets one on top of the other so that they are
exactly aligned. Using a glass pipette as a roller, squeeze out any air bubbles.

c) Place the nitrocellulose filter on the stack of 3MM paper. Make sure that the filter is exactly aligned and that no air bubbles are trapped between it and the 3MM Paper.

d) Remove the glass plates holding the SDS-polyacrylamide gel from the electrophoresis tank. Transfer the gel briefly to a tray of deionized water, and then place it exactly on top of the nitrocellulose paper. Orient the gel so that the mark on the filter corresponds to the bottom with a gloved hand.

e) Place the final three sheets of 3 MM paper on the gel, again making sure they are exactly aligned and that no air bubbles are trapped.

f) Place the upper electrode (which will become the cathode) on top of the stack, graphite side down. Connect the electrical leads (positive or red lead to the bottom graphite electrode) Apply a current of 0.65mA/sq.cm.of gel for a period of 1.5-2hrs

6. Turn off the electric current and disconnect the leads. Disassemble the transfer apparatus from the top downward, peeling off each layer in turn. Transfer the gel to a tray containing coomassie brilliant blue, and stain it. This will allow you to check whether electrophoretic transfer is complete.

7. Remove the nitrocellulose filter from the sandwich and transfer it to a clean piece of 3MM Paper. Allow the filter to dry for 30-60 minutes at room temperature.

8. Cut off the bottom left-hand corner of the filter. This serves as insurance against obliteration of the pencil mark. Stain the filter with ponceau S or India ink. India ink should be used if the western blot is to be probed with a radio labeled antibody or probe.

Blocking binding sites for Immunoglobulins on the Nitrocellulose Filter

1. Place the nitrocellulose filter in a heat-sealable plastic bag and add 0.1ml of blocking solution per square cm of filter. Seal the bag, leaving as few air bubbles as possible, and incubate the filter for 1-2 hours at room temperature with gentle agitation on a platform shaker.
Kc1 - 0.2gms
Na2HP4 - 1.44gms
KH2PO4 - 0.24gms

Dissolve the contents in 900 ml of double distilled water. Adjust the pH to 7.4 and make the final volume to 1 liter.

**ELISA wash buffer**

1000ml PBS & 0.5ml TWEEN-20 mix thoroughly.

**Blocking Solution: (Per 1 liter)**

1000ml PBS
0.5ml TWEEN-20
40 grams of skimmed milk powder

Mix the contents thoroughly and filter sterilize through Whatman no.3 filter paper.

**Citrate Phosphate buffer (pH 5.0)**

0.1 M Citric acid
0.1 M Sodium phosphate

Mix equal volumes of sodium phosphate and citric acid solution and adjust the pH 5.0.

**Substrate buffer**

5 ml Citrate Phosphate buffer (pH 5.0)
2μl 30% H2O2
3,3', 5,5'-tetramethylbenzidine (TMB).
Make up the buffer to 10 ml.

**ELISA coating**

1. To optimize plate coating, purified antigens each 100ng/ml were diluted with phosphate buffer saline (PBS) of pH 7.4 and load 100μl in each well. After loading incubate the plate at 37°C for 90 minutes.

2. After incubation wash the plate for 8-10 times with a soak time of 60 sec for each wash.

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3. After washing dry the plate and fill the wells with blocking solution and incubate for another 60 minutes at 37°C.

4. Repeat the wash procedure for 6 times with soak time of 60 sec after each wash.

5. Dry the plate in laminar airflow and wrapped with Para film and store the plate at 2 – 8°C until next use.

**ELISA development**

1. Dilute the sera (primary antibody) in 1:9 (10μl sera + 90μl PBS) ratio and load 100μl of diluted sera and incubate at 37°C for 60min.

2. After incubation, the plate was washed for 6 times with wash buffer. For every wash the soaking time is about 1 minute.

3. Add 100 μl of enzyme conjugate (secondary antibody) to each well with a final concentration of 1:30000 (1μl Ab + 30μl PBS) and incubate it for 1 hour at 37°C.

4. After incubation, repeat the wash procedure for 6 times.

5. Add 100μl of substrate in citrate buffer (pH 5.0) to each well and keep it in dark for 20 min for development.

6. The color development reaction was stopped by the addition of 50μl of 1N H₂SO₄ and optical densities (ODs) were measured at 450nm.

**Cut off calculation**

\[ NCx (\text{Negative cut off value}) = \bar{X} + 3\sigma \]

Whereas \( \bar{X} \) = mean of all negative values

\( \sigma \) = Standard deviation of mean

**Estimation of Protein**

The protein purified after electro elution is quantitatively estimated by spectrophotometer using Bio-Rad reagent. Before estimating the concentration of HCV-CORE, a standard curve or calibration curve is prepared by macro assay using known linear concentrations of Bovine Serum Albumin (BSA) protein. The absorbance or optical density values of BSA are plotted to get a calibration curve. Then HCV CORE protein is also taken and its absorbance is measured after treatment with Bio-Rad reagent at 595nm.
The OD\textsubscript{95} of HCV-CORE was compared with standard curve. Final concentration close to 1 mg/ml, is stored in −70°C freezer until next use. The purified HCV − CORE protein is tested by ELISA before transfer to −70°C freezer.

**CORE:** Each gram pellet of induced cells yielding 1.5 mg pure protein

The HCV recombinant antigenic proteins expressed in *E.coli* expression system are purified and characterized by the following protocols.

The cell pellets are suspended in lysis buffer and treated with lysozyme. Then the cell suspensions are sonicated briefly to break the cell walls and to get out the host proteins including recombinant foreign protein. The recombinant proteins generally exist as inclusion bodies. After sonication the suspension is centrifuged to remove host proteins. Then the foreign protein in inclusion bodies is solubilized by treatment with detergents like SLS/SDS and Urea. Then the solubilized recombinant protein is fractionated by Ammonium sulphate precipitation. Then further purification is done by ion exchange chromatography and molecular exclusion chromatography. The purity of the protein is checked by qualitative and quantitative methods. The qualitative methods include western blotting and ELISA and the quantitative methods include SDS- PAGE and protein estimation by Biorad reagent using spectrophotometry.

**Protein Estimation by Biorad Assay**

In the present investigation, the concentrations of purified recombinant HCV proteins namely CORE, NS3, and NS5 are carried out by Bio-Rad assay.

The Bio-Rad protein Assay, based on the method of Bradford, is a simple and accurate procedure for determining concentration of solubilized protein. It involves the addition of an acidic dye to protein solution, and subsequent measurement at 595nm with spectrophotometer. Comparison with standard curve provides a relative measurement of protein concentration.

**Principle**

The Bio-Rad protein assay is a dye binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. The dye binds
primarily basic and aromatic amino acid residues especially arginine. Spector (1978) found that the extinction coefficient of a dye-albumin complex solution was constant over a 10 fold concentration range. Thus Beer's law may be applied for accurate quantitation of protein by selecting an appropriate ratio of dye volume to sample concentration.

**Materials**

Protein Assay dye reagent concentrate from Bio-Rad  
Lyophilized Bovine Serum Albumin standard from Bio-Rad  
Spectrophotometer set to wave length 595nm  
Quatx cuvettes  
Vertex mixer  
Pipettes  
Whatman # 1 filter papers  
5ml test tubes

**Reconstitution of standard:** To the lyophilized BSA powder, milliQ water is added for reconstitution. The final protein concentration prepared as 1mg/ml. This protein is used for preparation of standard curve or calibration curve. Micro assay procedure is adapted for the preparation of standard curve.

**Micro Assay Procedure**

**For standard curve**

1. Five dilutions of BSA protein standard is prepared with linear range of 1.2 to 10.0 µg/ml. along with a blank containing no protein. In the present work, protein standard solutions are assayed in duplicate.  
2. 800 µl of each standard is dispensed into clean, dry test tube. 200 µl of dye reagent concentrate is added to each tube and vertexed gently.  
3. The tubes are incubated at room temperature for 5 min. and then the absorbance of samples are measured at 595nm by spectrophotometer.  
4. The absorbance values of standard BSA linear dilutions are plotted on graph to get a standard curve.
Standard Procedure

1. Bio-Rad reagent was prepared by diluting 1 part Dye Reagent Concentrate with 4 parts distilled, deionized (DDI) water. Diluted reagent was filtered through Whatman #1 filter (or equivalent) to remove particulates.

2. The protein standard BSA was prepared to five dilutions. The linear range of the assay for BSA is 0.2 to 0.9 mg/ml. The linear range of dilutions are maintained as duplicate.

3. From each standard dilution, 100 μl is pipetted into a clean, dry test tube.

4. To each tube Add 5.0 ml of diluted dye reagent to each tube and vortex.

5. Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour.

6. Measure absorbance at 595 nm.

For HCV - CORE, NS3 and NS5 protein samples

5. The purified proteins of CORE, NS3 and NS5 are solubilized in Tris-Urea buffer. The purity of the proteins is checked by HPLC and PAGE.

6. These protein samples are diluted to threshold range of Bio-Rad assay and 800 μl of each sample is dispensed into clean, dry test tube. 200 μl of dye reagent concentrate is added to each tube and vertxed gently.

7. The tubes are incubated at room temp. for 5 min. and then the absorbance of samples are measured at 595nm by spectro photo meter.

8. The absorbance values of samples are compared with standard curve and the concentrations are derived.
RESULTS

1. Marker specific amino acid variations among different genotypes

From the sera of the HCV infected patients, RNAs of genotypes 1a, 1b and 3b were isolated and amplified with specific primers for regions of Core (380 bp), NS3, and NS5. The amplified DNAs were cloned in *E. coli* expression vectors and transformed into competent BL21 cell line. From the cloned sequences, the amino acid sequences have been derived using Bio edit software. The cloned sequences with respect to amino acid sequences were compared with other known published sequences (Fig. 7.1a, 7.1b and 7.1c). Variations are observed among different genotypes with regard to specific marker. The core protein is highly conserved among all the hepatitis C viral subtypes and is localized to nucleotides 342 to 722. The corresponding protein has 120 amino acids and with a molecular weight of about 18 kDa (Fig. 7.1a). With respect to HCV Core protein, genotype 1a differing from genotype 1b by 6 amino acids (5% divergence) and from genotype 3b by 19 amino acids (15% divergence). Genotypes 1b and 3b are differed by 16 amino acids (13% divergence).

HCV NS3 region selected from nucleotides 3925 to 4919. The corresponding protein has 374 amino acids and with a molecular weight of about 37 Kda (Fig. 7.1b). The NS3 protein of genotype 1a differing from genotype 1b by 5 of 338 amino acids (1.5% divergence).

The NS5A region selected from nucleotides 6281 to 7403. The corresponding protein has 374 amino acids and with a molecular weight of about 45 kDa (Fig. 7.1c). The NS5 protein of genotype 1a differing from genotype 1b by 15 of 374 amino acids (4.0% divergence).

2. Cloning and expression

HCV Core, NS3 and NS5 plasmid DNA were introduced into competent cells by standard transformation protocol. All the colonies were screened for detection of positive clones using restriction digestion of their DNA followed by AGE (Fig. 7.2a, 7.2b and 7.2c). In positive clones, insert orientation was checked by RFLP analysis (Fig. 7.3a, 7.3b and 7.3c). All positive clones with correct orientation of the insert were involved for
expression screening. The induction was done at shake flask culture as well as at fermentation and expression was screened by PAGE (Fig. 7.4a, 7.4b and 7.4c). Further confirmation of expression was done by western blotting (Fig. 5).

In case of HCV- Core clones, for each genotype nearly 25 colonies were screened. 10 clones have been identified with insert and only three of these clones had correct orientation of the insert. These clones are from three genotypes 1a, 1b and 3b. These clones were showing good expression after induction with IPTG at shake flask level.

For HCV - NS3 clone, only 15 colonies were screened for each genotype after transformation. After screening, only 4 of them showed insert. In all these four, there was correct orientation of the insert. These four clones include two from genotype 1a and other two from genotype 1b. All clones showed expression after induction.

Similarly for HCV - NS5 clone, 18 transformed colonies were screened for each of genotype 1b and genotype 3b. Only one clone was identified as positive for presence of insert and right orientation of the insert in case of genotype 1b, in genotype 3b, two clones were identified with correct insert orientation. These 3 clones were checked for expression at shake flask level. All clones showed expression.

Cell lysis and purification of HCV - core, NS3 and NS5

Cells were grown after shake flask culture was harvested and processed using standard lysis protocols and purification. In the present work, HCV-Core, NS3, NS5 recombinant proteins processed to the stage of inclusion bodies (insoluble protein aggregates). These inclusion bodies are solubilized in carbonate buffer with 0.1% SLS for Core (Fig 7.6a) and tris buffer with 8 M urea for NS3 and NS5 (7.6b and 7.6c). The solubilized proteins still containing few host protein contaminants.

The HCV- Core inclusion bodies were further purified by gel elution method (10a) NS3 and NS5 proteins have been purified by ion exchange column chromatography i.e. CM sepharose cation exchange chromatography (Fig. 7.7a and 7.7b). The profiles of chromatography fractions and their absorbance values at 280 nm for NS3 and NS5 are furnished as excel graphs (Fig. 7.8a and 7.8b). The purified protein was lyophilized,
expression screening. The induction was done at shake flask culture as well as at fermentation and expression was screened by PAGE (Fig. 7.4a, 7.4b and 7.4c). Further confirmation of expression was done by western blotting (Fig. 5).

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suspended in Tris-8M urea and stored at -70°C until further use. Final purified proteins were loaded on PAGE on specification gels and stained by comassie blue dye. The pictures are furnished as 7.9a, b and c.

**Estimation of purified HCV – Core, NS3 and NS5 proteins**

All three proteins after purification were quantitatively estimated by Bio-Rad assay. Before estimation of samples, a standard curve was prepared from Bovine serum albumin (BSA) of 1mg/ml stock. The standard BSA protein after reconstitution, scanned at $\lambda 280$ by UV spectrophotometer. The absorbance of the protein at 280 nm was 0.63 abs. From this stock linear concentrations of 2µg to 10µg are prepared, treated with dye and OD values were measured at 595 nm and from the OD values a standard curve was plotted. The absorbance values of Core, NS3 and NS5 proteins with unknown concentrations was also measured and the values was compared to standard curve to derive the exact concentration. The absorbance values of BSA, Core, NS3 and NS5 proteins are furnished in the table 7.1. According to the Bio-Rad assay, the concentration of the three proteins is estimated as follows.

- Core – 1.2 mg/ml (Graph 7.1)
- NS3 – 1.5 mg/ml (Graph 7.2)
- NS5 – 0.8 mg/ml (Graph 7.3)

**CHARACTERIZATION**

**ELISA**

A sample size of 115 individuals was tested by ELISA using commercially available kits and indigenous recombinant antigens. The individuals patients include 50 negative and 65 positive for HCV. The positive individuals were already confirmed by RT-PCR and genotyping analysis was also carried out. These positive samples were used to check with ELISA include genotype 1a (20), 1b(20), 3a(10) and 3b(15). These genotypes exhibited variation in the reactivity to indigenous and commercially available antigens of Core, NS3, and NS5. For Core, frequency of reactivity against type 1a, 1b, 3a, and 3b antisera to homologous core protein ranged from 93.3 to 95%, where as the reactivity frequency was between 86.6% and 95% with heterologous combinations. For NS3 the frequency of reactivity between homologous antigens and antibodies ranged from 80 to
85%, compared to a range of 70 and 85% between heterologous combinations. Similarly, the frequency of reactivity of NS5 antigen with homologous combinations was 60 - 65% and heterologous combinations was 50 to 55% With respect to Core region, high frequencies of reactivity was observed for both homologous as well as heterologous combinations which indicated greater proportion of shared epitopes between many genotypes for Core protein (Table 7.2).

A significant observation in this study is identification of 4 positive samples (genotype 1a (2), 3a (1) and 3b(1) ) confirmed by RT-PCR but not picked up by standard Ortho kit. The same samples were picked up in ELISA with indigenous antigens.

Figure 7.1: Comparison of the inferred amino acid sequences of antigens expressed from recombinant clones of core, NS3, and NS5 of genotypes 1a, 1b, and 3b with representative of each of the major genotypes in the world.
### Fig. 7.1a

**Clustal W multiple alignment of core gene with various countries strains**

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### Fig. 7.1b

**Clustal W multiple alignment of NS3 gene with various countries strains**

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Fig. 7.1c

Clustal W multiple alignment of NS5 gene with various countries strains.
Fig. 7.2a
HCV – Core screening of the positive clones (with insert)

1) Plasmid DNA without insert; 2) Plasmid DNA with insert; 3) Plasmid DNA without insert; 4) Plasmid DNA with insert; 5) Plasmid DNA without insert; 6) 100 bp DNA ladder
(from bottom to top 100, 200, 300, 400, 500, 600, 700….1500bp)

Fig. 7.2b
HCV NS3 screening of the positive clones (with insert)

1) Plasmid DNA without insert; 2) Plasmid DNA with insert; 3) Plasmid DNA without insert; 4) Plasmid DNA with insert; 5) Plasmid DNA with insert; 6) 100 bp DNA ladder
(from bottom to top 100, 200, 300, 400, 500, 600, 700….1500bp)
Fig. 7.2c

HCV NS5 screening of the positive clones (with insert)

1) Plasmid DNA with insert; 2) Plasmid DNA without insert; 3) Plasmid DNA without insert; 4) Plasmid DNA with insert; 5) 100 bp DNA ladder (from bottom to top 100, 200, 300, 400, 500, 600, 700…1500bp)

Fig. 7.3a

HCV core DNA insert orientation check

1) Insert with right orientation; 2) Insert with wrong orientation; 3) Insert with right orientation; 4) Insert with wrong orientation; 5) 100 bp DNA ladder (from bottom to top 100, 200, 300, 400, 500, 600, 700…1500bp)
**Fig. 7.3b**

HCV NS3 DNA insert orientation check

1) 100 bp DNA ladder (from bottom to top: 100, 200, 300, 400, 500, 600, 700,...,1500bp)
2) Plasmid DNA without insert; 3) Insert with right orientation; 4) Insert with wrong orientation;
5) Insert with wrong orientation

**Fig. 7.3c**

HCV NS5 DNA insert orientation check

1) Insert with right orientation; 2) Insert with right orientation;
3) 100 bp DNA ladder (from bottom to top: 100, 200, 300, 400, 500, 600, 700,...,1500bp)
Fig. 7.4a

HCV recombinant CORE protein expression at shake flask culture and fermentation. The cell lysates before and after induction are loaded along with protein marker.

1) Cell lysate before induction (10 µl); 2) Protein marker (116, 66, 45, 35, 25, 18.4 & 14.4 kDa)
Fig. 7.4b

HCV recombinant NS3 protein expression at shake flask culture and fermentation. The cell lysates before and after induction are loaded along with protein marker.

1) Protein marker (116, 66, 45, 35, 25, 18.4 & 14.4 kda); 2) Cell lysate before induction (10 μl)
Fig. 7.4c

HCV recombinant NS5 protein expression at shake flask culture and fermentation. The cell lysates before and after induction are loaded along with protein marker.

(a) 1) Protein marker (116, 66, 45, 35, 25, 18.4 & 14.4 kda); 2) Cell lysate before induction (10 μl); 3) Cell lysate after induction (10 μl).

(b) 1) Cell lysate before induction (10 μl); 2) Protein marker (116, 66, 45, 35, 25, 18.4 & 14.4 kda); 3) Cell lysate after induction (10 μl).
Fig. 7.5
Western Blot image of purified HCV proteins

1) HCV Core (1μg); 2) HCV NS3 (1μg); 3) Prestained marker
(from top to bottom 116, 85, 47, 36, 26 and 20kDa)

Fig. 7.6a
HCV CORE 10 gram cell pellet lysis and processing for the preparation of soluble protein

1) uninduced; 2) induced; 3) Marker 116, 66, 2, 45, 35, 18.4, 14.4; 4) Sup1 (10μl) in SLS-carb; 5) BSA 4μgl; 6) Sup2 (10μl) in TES buffer; 7) Sup 1 (10 μl) in TES buffer

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Fig. 7.6b
NS3 cell pellet lysis and processing

1) Supt. 1 in TES; 2) Supt. 2 in TE; 3) Protein marker 116, 66, 45, 35, 25, 18.4 & 14.4 kDa;
4) Supt.3 in TE + 8M Urea

Fig. 7.6c
NS5 cell pellet lysis and processing

1) Protein marker 116, 66, 45, 35, 25, 18.4 & 14.4 kDa; 2) Supt. 1 in TES; 3) Supt. 2 in TE; 4) Supt.3 in TE + 8M Urea
Fig. 7.8a
HCV NS3 CM sepharose Ion Exchange chromatography
Fig. 7.8b
NS5 CM sepharose Ion Exchange chromatography
Fig. 7.9a

HCV Core purified protein with uninduced and induced culture

1) Whole cell pellet without induced protein; 2) Whole cell pellet with induced protein; 3) Protein Marker (from top to bottom 116, 66.2, 45, 35, 25, 18.4 and 14.4 kDa); 4) Purified CORE Protein
Fig. 7.9b
HCV NS3 purified protein with uninduced and induced culture

1) Whole cell pellet without induced protein; 2) Whole cell pellet with induced protein; 3) Protein Marker (from top to bottom 116, 66.2, 45, 35, 25 and 18.4 kDa); 4) Purified NS3 Protein

Fig. 7.9c
HCV NS5 purified protein with uninduced and induced culture

1) Whole cell pellet without induced protein; 2) Whole cell pellet with induced protein; 3) Protein Marker (from top to bottom 116, 66.2, 45, 35, 25, 18.4 and 14.4 kDa); 4) Purified NS5 Protein
Graph 7.1

Estimation of core protein along with the BSA standard.
Graph 7.2
Estimation of NS3 protein along with the BSA standard.
Graph 7.3

Estimation of NS5 protein along with the BSA standard.
### Table 7.1

Bio-Rad Assay of BSA (standard) and unknown HCV - Core, NS3 and NS5

<table>
<thead>
<tr>
<th></th>
<th>BSA (Vol/conc.)</th>
<th>Bio-Rad dye concentrate</th>
<th>Water</th>
<th>OD@595 nm Triplicate 1</th>
<th>OD@595 nm Triplicate 2</th>
<th>OD@595 nm Triplicate 3</th>
<th>OD@595 nm Average</th>
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<tbody>
<tr>
<td>01</td>
<td>20 µl (2µg)</td>
<td>200 µl</td>
<td>780 µl</td>
<td>0.098</td>
<td>0.101</td>
<td>0.097</td>
<td>0.098</td>
</tr>
<tr>
<td>02</td>
<td>40 µl (4µg)</td>
<td>200 µl</td>
<td>760 µl</td>
<td>0.202</td>
<td>0.199</td>
<td>0.198</td>
<td>0.199</td>
</tr>
<tr>
<td>03</td>
<td>60 µl (6µg)</td>
<td>200 µl</td>
<td>740 µl</td>
<td>0.294</td>
<td>0.304</td>
<td>0.298</td>
<td>0.298</td>
</tr>
<tr>
<td>04</td>
<td>80 µl (8µg)</td>
<td>200 µl</td>
<td>720 µl</td>
<td>0.397</td>
<td>0.394</td>
<td>0.407</td>
<td>0.399</td>
</tr>
<tr>
<td>05</td>
<td>100 µl (10µg)</td>
<td>200 µl</td>
<td>700 µl</td>
<td>0.499</td>
<td>0.502</td>
<td>0.495</td>
<td>0.498</td>
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**HCV - CORE**

<table>
<thead>
<tr>
<th></th>
<th>20 µl</th>
<th>200 µl</th>
<th>780 µl</th>
<th>0.125</th>
<th>0.135</th>
<th>0.120</th>
<th>0.126</th>
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<tbody>
<tr>
<td>02</td>
<td>40 µl</td>
<td>200 µl</td>
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<tr>
<td>03</td>
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<td>0.361</td>
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<td>0.365</td>
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<tr>
<td>04</td>
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<td>0.470</td>
<td>0.485</td>
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<td>0.479</td>
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**HCV - NS3**

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<th>200 µl</th>
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<th>0.158</th>
<th>0.152</th>
<th>0.157</th>
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</thead>
<tbody>
<tr>
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<td>0.316</td>
<td>0.313</td>
<td>0.302</td>
<td>0.310</td>
</tr>
<tr>
<td>03</td>
<td>60 µl</td>
<td>200 µl</td>
<td>740 µl</td>
<td>0.450</td>
<td>0.456</td>
<td>0.442</td>
<td>0.449</td>
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<tr>
<td>04</td>
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<td>0.591</td>
<td>0.601</td>
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**HCV - NS5**

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<th>0.084</th>
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<td>03</td>
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<td>0.243</td>
<td>0.246</td>
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<tr>
<td>04</td>
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<td>200 µl</td>
<td>720 µl</td>
<td>0.316</td>
<td>0.322</td>
<td>0.320</td>
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Table 7.2
Recombinant antigens Core, NS3 and NS5 reacted with genotype specific antibodies.

<table>
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<th>Anti serum</th>
<th>Core</th>
<th>NS3</th>
<th>NS5</th>
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<tbody>
<tr>
<td></td>
<td>1a</td>
<td>1b</td>
<td>3b</td>
</tr>
<tr>
<td>Type 1a</td>
<td>19/20 (95)</td>
<td>18/20 (90)</td>
<td>18/20 (90)</td>
</tr>
<tr>
<td>Type 1b</td>
<td>19/20 (95)</td>
<td>19/20 (95)</td>
<td>18/20 (90)</td>
</tr>
<tr>
<td>Type 3a</td>
<td>09/10 (90)</td>
<td>09/10 (90)</td>
<td>09/10 (90)</td>
</tr>
<tr>
<td>Type 3b</td>
<td>13/15 (86.6)</td>
<td>13/15 (86.6)</td>
<td>14/15 (93.3)</td>
</tr>
</tbody>
</table>
DISCUSSION

Since 1989, the sequence analysis of different HCV genotypes and subtypes lead to the development of immuno assays based on synthetic peptides and recombinant HCV antigens for detection of anti-HCV in various blood samples. The first generation assays, which are based on HCV nonstructural NS4 antigen, suffered a major set back as they could fail to detect many positive samples. A second generation assay was subsequently developed, which included a structural Core protein (c22-3) and a nonstructural NS3 region (c33c) in addition to NS4 protein. As sequences of many HCV genotypes were revealed, the third generation assays have evolved to increase the fidelity of the test. In these assays, a recombinant NS5 antigen has been added in addition to other antigens. As a result, 3rd generation EIA have a great degree of sensitivity and increased specificity. (Soffredini et al., 1996; Jeffrey et al., 2000) However, in 3rd generation EIA also there are few drawbacks like indeterminate results and it has not eliminated the need for the direct detection of HCV RNA in all individuals.

In the present study, we have tested the antigenicity of more prevalent Indian strains of HCV (1a, 1b, and 3b) by including recombinant indigenous antigens Core, NS3, and NS5 in comparison with commercially available 3rd generation EIAs in which the antigens are based on prevalent HCV strains (specifically genotype 1b) outside India. The more reliable results with the indigenous antigens in comparison with those of commercial antibody detection tests may be attributed to the fact that we have used local HCV strains to generate the recombinant antigenic proteins and also the serological response tested was from local specimens. The frequency of detection of antibodies with indigenous recombinant proteins of core and NS3 region plays an important role in HCV diagnosis as the results of ELISA are more consistent and accurate (Claeys et al., 1995). Recent studies have identified several epitopes in the N-terminal portion of the nucleocapsid protein which are predominantly recognized by HCV infected patients (Buratti et al., 1997). We have detected antibodies against core region in 95% serum samples. Antibodies against this region are regarded to be reliable markers of virus replication since their presence was found to be closely associated with the presence of specific mRNA.

NS3 region codes for a protease and a helicase and we have detected antibodies against the NS3 region in 85% of the serum samples (Houghton et al., 1991; Bradly et al., 1993; Matsuura et al., 1993). We have observed two samples which were negative for 3rd
generation EIA but strongly reactive to NS3 indigenous proteins. NS5 region codes for RNA-dependent RNA polymerase and we have noticed that NS5 has relatively less reactivity because only 65% of the samples are positive for this antigen (Miller et al., 1990). Moreover this antigen showed cross reactivity with the antibodies evoked by other viruses like HIV as HIV also has the enzyme RNA-dependent RNA polymerase.

In the present study, we have also investigated the degree of type-specific serological reactivity to antigens used in the current assays. The use of recombinant antigens expressed from different genotypes in the enzyme immunoassay allowed the reciprocal measurements of homologous and heterologous reactivity, and these provided a more rigorous assessment of the type-specific components of reactivity to the core, NS3, and NS5 regions. This study revealed weaker reactivity of sera from individuals infected with non-type1 genotypes with respect to ELISA screening as well as to individual antigens in the confirmatory recombinant immunoblot assay. In the current study we have noticed a consistent and stronger reactivity of sera to antigens of a homologous type than to antigens of heterologous type. Furthermore, each of the three antigens investigated is likely to contain a range of linear and conformational epitopes, and these vary to certain degree of cross reactivity. Recognition of different epitopes in the antigens may be one explanation for the wide range of reactivity to homologous and heterologous antigens observed between sera of the same genotype. In the extreme case, the Core protein which is specific in reactivity with one genotype may be poorly reactive or non reactive with other genotype (Chan et al., 1991; McOmish et al., 1993; Dow et al., 1996; Powlotsky et al., 1996).

Conversely, the high degree of cross-reactivity observed between certain sera and NS3 or NS5 protein of heterologus genotypes may have resulted from their recognition of shared epitopes. The finding of significant antigenic variability is used for serologic screening which form the basis for a number of future investigations. Now antigens from other genotypes have been produced, it may be possible to carry out large scale screening of populations infected with non-type1 genotypes (for example, type 3 antigens prevalent in North India, Bangladesh, Nepal, Pakistan etc) (Zein et al., 1995). This may reveal the frequency with which anti-HCV samples are being missed by conventional assays and may ultimatey help reduce the frequency of posttransfusion hepatitis further, particularly when population with high frequency of acute infection are screened.