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Original Article

Study of Antibody Recognition Pattern of HCV by Western Blot Method

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Abstract
HCV is a positive strand RNA virus, and is a member of the Family Flaviviridie. HCV show substantial nucleotide sequence variability distributed throughout the viral genome, particularly the HVR1 region that is suggested to be immunogenic in humans. HVR1 is the most heterogeneous region within the E2 genome and peptide corresponding to this region, can be detected by homologous sera. To study the serum reactivity to peptides obtained from HVR1 region of the E2 region of the HCV genome, western blot can be performed in place of Enzyme - Linked Immuno Sorbent Assay (ELISA), if the expressed recombinant protein is insoluble & cannot be extracted or purified from the host cell.

Key words: - HCV, serum reactivity, western blot

Towards the end of 1980s a significant number of cases of parentally transmitted viral hepatitis could not be ascribed to any of the then known hepatic viruses (HAV, HBV or hepatitis delta virus) and the associated diseases was designated as non A non B (NANB hepatitis). In 1989, Choo et al cloned a part of the non-A non-B hepatitis virus and found it to be a positive, single stranded RNA molecule of approximately 9.5 kb in length [1, 2]. Today, this virus is commonly known as the hepatitis C virus. Later, an assay for the circulating antibodies in HCV infected patients was developed using viral antigen derived from recombinant yeast [3]. The persistent infection with HCV commonly results in the chronic active hepatitis that may lead to liver cirrhosis and hepatocellular carcinoma [4, 5].

HCV genotypes show diversities of around 30% in the nucleotide sequences of their whole genome and comprehensive analysis of these sequences has revealed the existence of at least 6 genotypes and more than 30 subtypes throughout the world [6,7].

The pathogenesis of HCV infection is poorly understood, despite our knowledge of its epidemiology and molecular virology; because of the lack of animal or cell culture systems. Within a single infected patient, HCV also exist as a population of closely related genome known as quasi sp.[6, 8, 9, 10]. Increased HCV quasi species heterogeneity has been linked to longer duration of HCV infection, age level of viremia and HCV type1 infection. HCV quasi species heterogeneity has been found to affect interferon therapy [9].

E2 represents the most variable region of the HCV genome [11, 12] and the variation is assumed to be caused by error prone nature of the RNA dependent RNA transcriptase random mutation and selection of mutants capable of escaping cellular and humoral immune response of the host [13, 14, 15, 16]. Antibodies against E2 correlate with the protection from HCV challenge in chimpanzees and assays are now available to directly assay for neutralizing antibodies using cell binding of structural proteins [17].

HVR1 is the most heterogeneous region within the E2 genome and peptide corresponding to this region, can be detected by homologous sera [18, 19, 20]. This can be done by cloning the genome to express the protein of interest for immunological study by western blot or by other methods like the neutralization assays or ELISA. Here we discuss the antibody recognition of HCV protein by western blot protocol.

Materials and Methods

To enable the expression and capture of the 150 bp long HVR1 peptides, previously amplified first round single molecule amplimers (verified from the sequence analysis) were re-amplified in second round reactions using new sense and anti-sense primers. Each primer pair was designed taking into account, the HVR1 variability of each patient's quasi species within the primer site, to amplify a fragment of DNA encompassing only the HVR1 region.

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The primers also contained restriction enzyme sites to allow directional in- frame cloning of the HVR1 PCR product. PCR products were cloned into pre-cut GST tagged pET- 42a, as per protocol described in pET system manual (Novagen)[21]. The clones were established in non - expression host JM 109 competent cells (Novagen) and re-transformed in BL21DE3 expression hosts (Novagen).

A single recombinant colony or a loopful of the glycerol stock of the single recombinant colony was sub-cultured in 10 ml of LB broth with kanamycin and incubated at 37°C for 3 to 4 hours in presence of isopropyl thioβ D galactoside (IPTG) for induction of recombinant protein.

As per objective of the project, in order to measure the antibody responses to the recombinant HVR1 peptide, the protein extract was prepared and the protein was purified using GST bind kit (Novagen). The eluate was collected in four fractions and western blot was performed to check the purification and elution efficiency.

For better resolution of protein separation, a bigger gel was used and was run overnight at 7 m amp.
The result was such that the recombinant protein (size 39 kDa) was not captured.

The protein was present in the crude cell suspension but it was not present in the cell extract or in any of the purified protein fraction. A low concentration was seen in the Bug Buster extract. (See fig. 1)

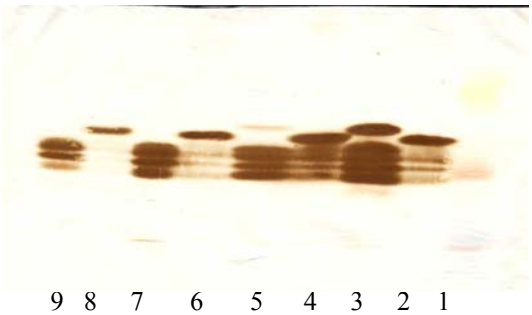


Fig 1. Western blot for GST purified protein fraction. [Lane 1- low weight protein molecular marker; Lanes 2, 4, 6 & 8 - induced cells, bug buster extract, GST purified peptide and fraction unbound to GST column respectively of the negative control i. e. pET-42a plasmid without insert

Lanes 3, 5, 7 & 9 are induced cell, bug buster extract, GST purified peptide and fraction unbound to the GST column respectively of the sample]

GST tag in the vector is the soluble fraction of the vector peptide; we had assumed that the 150-bp long insert would also be soluble. But the recombinant protein encompassing the HVR1 region was found to be insoluble and retained in the host *E. coli* cells as inclusion body. So, instead of extracting and purifying the protein itself, the inclusion body was extracted from the cell pellet using the Bug buster cell extract reagent & was lysed using lysozyme. When it was run on gel and probed in western blot the 39-kDa proteins was present in test sample whereas it was not there in the negative control.

Because of time constraints, the protein from the inclusion body was not purified but it was decided that instead of ELISA, the peptides would be probed with sera in Western blot.

To perform western blot probed with patient sera, following procedure was performed
First of all the concentration of total protein in each induced sample was determined spectrophotometrically. Before probing the protein sample with patient sera, concentration of all the 18 samples were optimised. For this, one of the peptide samples and a negative control (pET-42a vector without insert) was selected; and a series of dilutions of the samples was prepared in SDS-PAGE loading buffer. To determine the amount of protein to be loaded, this serially diluted sample was probed with anti his antibody and the patient sera.
It was found that when probed with anti-his antibody, 1: 20 dilution gave neat band and with patient sera 1: 5 dilution gave neat and readable bands in the western blot.
Hence on the basis of this result, two sets, i.e. 1: 5 and 1: 20 dilutions of all the recombinant peptide samples were prepared to probe with anti-his and patient serum respectively.

Analysis of expressed protein on SDS -PAGE Gel (Sodium Doedecile Sulphonate - Polyacrylamide Gel Electrophoresis)
Reagents for SDS PAGE were prepared as follows:

1× TBE (Tris base): 0.2g KCl, 3.7g Tris, 8g NaCl, 500ml distilled water, pH 7.6

1×SDS gel loading buffer: 50 mM Tris.Cl (pH 6.8), 100mM dithiothereitol, 2 % SDS, 0.1 % bromophenol blue, 10 % glycerol. 1×SDS gel loading buffer lacking dithiothereitol can be stored at room

temperature. Dithiothereitol was added just before use.

Tris- glycine electrophoresis buffer: 25 mM Tris, 250 mM glycine (pH 8.3), 0.1% SDS. A 5× stock was made by dissolving 15.1g of Tris base and 94g of glycine in 900 ml of de-ionised H_2O. Then, 50ml of a 10% (w/v) stock solution of electrophoresis grade SDS is added and the volume was adjusted to 1000ml with water.

Solution for 20ml of 10% resolving gel for Tris glycine SDS - PAGE was prepared by mixing: H_2O - 7.9ml, 30 % acrylamide mix - 6.7ml, 1.5 M Tris (pH 8.8) – 5ml, 10 % SDS- 0.2ml, 10%ammonium persulphate -0.2ml, TEMED - 0.008ml.

Solution for 20ml of 15% resolving gel for Tris glycine SDS -PAGE was prepared by mixing: H_2O - 4.6ml, 30 % acrylamide mix – 10ml, 1.5 M Tris (pH 8.8) - 5ml, 10 % SDS - 0.2ml, 10%ammonium persulphate - 0.2ml, TEMED - 0.008ml.

For the stacking gel, all the materials were the same except that the 1 M Tris (pH 6.8 is used instead of 1.5 M Tris)

The ammonium per sulphate and N, N, N', N'-tetramethylenediamine (TEMED) were added just prior to pouring the gel, as these reagents promote and catalyse the polymerisation of acrylamide. The resolving gel mix was poured into assembled gel plates, leaving sufficient space at the top for the stacking gel to be added later. The gel was overlaid with some water and was allowed to polymerise for 15-30 min. After polymerisation, the water overlay was removed and the surface of the resolving gel was rinsed gently with water to remove any un-polymerized acrylamide. The remaining space was filled with stacking gel solution and the comb was inserted immediately. After the stacking gel had polymerized, the combs were removed and the wells were rinsed with water to remove un-polymerized acrylamide. It was made sure that at least 1 cm of the stacking gel was present between the bottom of the loading wells and the resolving gel. The gel plates were placed in the electrophoresis tank and the tank was filled with 1× tris glycine electrophoresis buffer.

10 µl of the heat denatured samples and low range protein molecular markers (2,500 kDa - 45,000 kDa, Amersham Pharmacia Biotech) diluted 1:1 were loaded into the wells and the gel was run at 200V for 30 min (or until the dye front reached the lower brim of the gel plate).

The gel was removed carefully from the plates and was placed immediately in Coomassie blue solution and was stained for an hour at room temperature with gentle agitation. Coomassie brilliant blue solution was prepared by dissolving 0.25g Coomassie brilliant blue in 90 ml of methanol /H_2O (1: 1)(45ml: 45 ml), & then 10 ml glacial acetic acid was added. After staining, the Coomassie solution was poured off and de-staining solution was added. De-staining was carried out for approximately an hour with gentle agitation or until the background was clear. The gel was kept in water until ready for drying.
The Coomassie stained gel was dried for 2 hours at 80^0 C on the gel dryer.

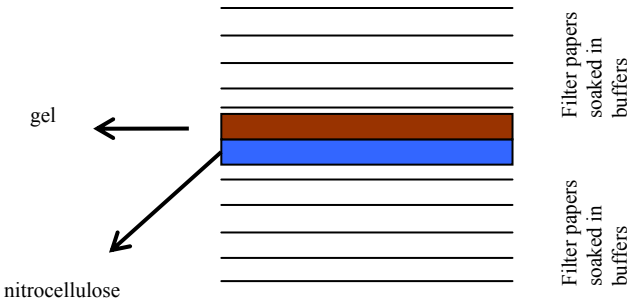
Detection of the recombinant protein on western blot

[*For this, the reagents prepared were as follows:* Wash buffer was prepared by mixing 1× TBS with 0.1 % tween 20. The blocking buffer was prepared by mixing 0.3g milk powder in 10ml of wash buffer. 1000ml western blot buffer (transfer buffer) was prepared by mixing: 48mM Tris base -5.8g, 39 mM glycine - 2.9, 0.037% (vol/vol) SDS- 0.37 g, 20 % methanol - 200 & distilled water to make the volume 1000ml]

The western blot was performed as follows:

10 pieces of Whatman no 1 filter paper, and 1 piece of nitrocellulose was cut out as the same size of the gel. The filter papers were soaked one by one in transfer buffer and were aligned on the bottom (anode) of the western blot apparatus. The gel, nitrocellulose & 5 layers of filter paper were assembled as shown in fig.2.

Fig. 2 Western blot arrangement of filter papers, gel and membrane



The sandwich arrangement was carefully checked for trapped air bubbles and was removed by rolling a pipette over it after each layer was placed. The nitrocellulose paper was first thoroughly soaked in methanol as it is highly hydrophobic. It was allowed to dry and then soaked thoroughly in transfer buffer. To avoid any short circuit, any buffer that leaked in the surrounding area of the gel paper sandwich was dried. The blot was carried out at 25 V for at least an hour. The membrane was removed carefully and then washed in 1 ×TBS tween 20 washing buffer. The membrane was then incubated in blocking buffer for an hour with gentle agitation. The blocking buffer was tipped off and incubated at least for 2 hours at room temperature or overnight at 4^0C in blocking buffer containing primary antibody (1: 3,000 dilution of anti–his mouse antibody). The membrane was washed in washing buffer 3 times for 5 min. Then the membrane was incubated at least 2 hours at room temperature in blocking buffer containing secondary antibody. (1:1000 dilution of peroxidase conjugated anti - mouse antibody). The membrane was washed thrice for 5 min in washing buffer. The color signal was developed by adding 5 ml DAB (diamino benzidine) substrate solution. Once the signal was strong enough, the membrane was washed thrice in water and dried by placing it between filter papers and stored in dark.

Each sample lane was cut into strips of about 3mm. For each peptide, one strip each was probed with HCV negative serum and patient serum of each time point at a final concentration of 1:100. The western blot strips with 1: 20 dilution of the samples were probed with anti-his antibody at a concentration of 1: 3000.

Result

When probed with anti-his antibody, about 35 kDa band in the negative control & 39 kDa band was detected in the sample (fig. 3). The size of the recombinant peptide was calculated as follows: - (Promega protocols and application guide), 1Kb of DNA = 333 amino acids of coding capacity = 37 KDa protein.

Number of amino acids without insert DNA (GST tag only) = 319 amino acids = 35.44 kDa

Number of amino acids with 150 bp insert DNA = 351 = 39 KDa

1 2 3

Fig3. Western blot of the expressed protein (lane 1 low molecular weight protein marker, lane 2 negative control (35.4 kDa), lane 3 patient sample (size 39 kDa)

Discussion

Current approaches to the prevention of Hepatitis C Virus (HCV) infection are less than satisfactory, despite considerable progress during the past several years, in the understanding of this heterogeneous virus and virus-host interactions. Neutralizing protective antibodies can be detected in a small proportion of infected individuals and these seem to be isolate-specific and relatively ineffective in vivo presumably as a consequence of frequent mutation in genome encoding the envelope proteins. Although experimental studies are promising, vaccine development remains in an early stage. The presence of circulating neutralizing antibodies directed to the immune dominant epitopes of HCV should serve to protect the susceptible hepatocyte or other target cells from HCV infection after exposure to the virus. Neutralizing antibodies may be acquired passively by the administration of immune globulin preparations containing these antibodies or actively by the administration of a vaccine that induces formation of antibodies in the recipient. As there is to date, no efficient in vitro replication system to grow the virus and develop neutralisation assays, alternative assays to assess the biological function of anti – E1 E2 antibodies have been searched for. Neutralisation of binding (NOB) assay developed by Rosa et al, exploiting the specific binding of a highly purified E2 protein onto susceptible target cells has allowed the quantitative evaluation of NOB antibodies that are capable of neutralising the binding of E2 onto such cells. Chimpanzees immunised with E1 and E2 proteins developed high anti-NOB titres & were protected against challenged infection, suggesting that NOB activity could be an indication for in vivo neutralisation of viral infection [17]. The HVR1 region is immunogenic in humans [22, 23]. Ray et al hypothesized that the persistence of infection may be

directly related to sequence variability in the envelope genes [15].

In this study we described western blot protocol to study the serum reactivity to peptides derived from HVR1 region of the E2 region of HCV genome.

With pET-42a vector, we were able to express the recombinant peptide from the PCR product. This was detected by probing with anti-his antibody. The protein cell lysate was diluted 1: 10 to capture a neat clean band. An approximate band of 39 kDa was detected. A slightly smaller band (approximately 35 kDa) was detected in the negative control (fig. 3). Once the cloned plasmid was established, an attempt was made to extract the GST-fusion peptide using bug buster protein extraction kit and GST bind kit. The protein was found to be insoluble as it retained in the cell (see fig. 1). The extraction of fusion protein from the inclusion bodies is a very tedious and time-consuming process. Hence the GST fusion peptide was not purified; instead, the whole rude cell lysate was used to probe in the western blot.

SDS- PAGE

The relative electrophoretic mobility of proteins in the presence of sodium dodecyle sulphate (SDS) is related to their relative molecular mass. The method is dependent on the fact that the binding occurs predominantly to hydrophobic regions and that proteins differ in their hydro-mobility. SDS- PAGE separates SDS-protein complexes on the basis of their relative molecular mass provided, that the charge: mass ratios and the shape of the complexes are the same. The strongly anionic detergent SDS is used in combination with a reducing agent and heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind SDS and become negatively charged. Because the amount of SDS bound is almost always proportional to the molecular weight of the polypeptide and is independent of its sequence, SDS polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide. By using markers of known molecular weight, it is therefore possible to estimate the molecular weight of the polypeptide chain(s).

SDS-PAGE is carried out with a discontinuous buffer system in which the buffer in the reservoirs is of a different pH and ionic strength from the buffer used to cast the gel. The SDS –polypeptide complexes in the sample that is applied to the gel are swept along by a moving boundary created when an electric current passed between the electrodes. After migrating through a stacking gel (pH 6.8) of high porosity, the complexes are deposited in a very thin zone (or stack) on the surface of the resolving gel. The ability of discontinuous buffer systems to concentrate all of the complexes in the sample into a very small volume greatly increases the resolution of SDS –polyacrylamide gel.

The sieving properties of the gel are determined by the size of the pores, which is a function of the absolute concentration of acrylamide and bisacrylamide used to cast the gel. The size of the pores decreases as the biscacrylamide: acrylamide ratio increases reaching a minimum when the ratio is approximately 1:20. Most SDS-polyacrylamide gels are cast with a molar ratio of bisacrylamide: acrylamide of 1:29,which has been shown empirically to be capable of resolving polypeptides that differ in size by as little as 3%.

Immunological detection of immobilized proteins (Western Blotting)

SDS- PAGE achieves fractionation of protein mixture during the electrophoresis process. It is possible to use this fractionation to examine individual separated proteins. The first step is to transfer or blot the pattern of separated proteins from the gel on to a sheet of nitrocellulose paper and probe with reagents (in case of proteins, antibodies that react specifically with antigenic epitopes displayed by the target protein attached to the solid support) that are specific for particular sequences of amino acids. The transfer of proteins from the gel to nitrocellulose can be achieved by electro blotting.

The sensitivity of western blotting depends on reducing the background of non-specific binding by blocking potential binding sites. Protein solutions e.g. 10% w/v bovine serum or 5% (w/v non-fat dried milk); which will block all remaining hydrophobic binding sites on the nitrocellulose sheet can be used. The blot is then incubated in dilution of primary antibody directed against the protein of interest. This IgG molecule will bind to the blot if it detects its antigen, thus identifying the protein of interest. In order to visualize the interaction, the blot is incubated further in a solution of secondary antibody directed against the IgG that provide the primary. The second antibody is labeled appropriately with a substrate so that the interaction of the second antibody with the primary antibody can be visualized on the blot by, incubating it with appropriate enzyme (peroxidase, alkaline phosphatase) that converts the substrate into an insoluble colored product i.e. precipitated on to the nitrocellulose. The presence of the colored band indicates the position of the protein of interest and by comparing it with a Coomassie stained gel of the same sample; the target protein can be identified.

The enzyme used in enzyme- linked antibodies is usually either, alkaline phosphatase, which converts

colorless 5-bromo-4-chloro-indolyl phosphate (BCIP) substrate into a blue product or horse- radish peroxidase, which with H_2O_2 as a substrate oxidises either 3-amino-9 ethylcarbozyle into an insoluble brown product [24, 25].

Conclusion

In case the recombinant protein is insoluble or cannot be purified, serum reactivity to HVR1 region of the E2 genome of HCV can be studied by western blot instead of ELISA.

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