Genotype analysis and assessment of antigenic sensitivity for recombinant HCV proteins by indigenous SIBA for detection of Hepatitis C Virus infection: A comparison with 3rd EIA and RT-PCR

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The first serological testing for the detection of anti-HCV antibodies using recombinant antigens was introduced in 1991. Since then many developments have taken place and at present third generation ELISA kits are being used most widely and globally. Detection of anti-HCV does not distinguish past from present infections and in diagnostic virology particularly ELISA’s, a positive HCV test result may be non-specific and therefore has to be crosschecked by another test of different principle for which Immunoblots were initially developed. Patients with liver disease attending the inpatient and outpatient wards of the CLRD (Center for Liver Research and Diagnostics) between Aug 2004 and Feb 2007 were screened for HCV by using 3rd generation ELISA, HCV blot, and RT-PCR. Genotyping was done for all the positive samples. Out of 531 samples tested, 211 samples showed identical results as reactive by ELISA, HCV blot and RT-PCR. Out of the 214 genotype samples, genotype 1a was found to be prevalent by 52.33% (n=112), followed by others. RNA based detection by RT-PCR remains the reliable method of HCV diagnosis, however, where there are no facilities for the PCR to be performed particularly in the small to medium laboratory and diagnostic centers, HCV blot could be done as a supplemental assay.

Keywords: Genotyping, RT-PCR, HCV blot, 3rd generation ELISA

Introduction

Discovery of hepatitis C virus (HCV) by Choo et al1 is a major breakthrough in the field of infectious diseases, and since then many developments in diagnosis, pathogenesis and treatment have taken place. The HCV is associated with the majority of cases of post transfusion hepatitis distributed worldwide. About half of the infected individuals develop chronic hepatitis of which 10-20% progress to liver cirrhosis with an associated increased risk of developing hepatocellular carcinoma (HCC)2,3. HCV is a single positive stranded RNA virus belonging to the family flaviviridae and genus hepacivirus. HCV infection is estimated to affect more than 170 millions all over the world4, and this infection in particular is increasing day by day in the developing countries. It is estimated that in India approximately 1.8-2.5% of the population is presently infected by HCV5-7 and it is estimated that about 20 million people are already having HCV infection8.

Ever since the screening of blood and blood products for transfusion has been made mandatory, the severity of the infection has reduced drastically. Although USA made the screening mandatory from July 1992, in India, however, the HCV screening in blood banks was made mandatory only from June 20019. Even then infection by blood and blood products has not come to an end, as many blood banks are unable to screen for HCV antibodies on a regular basis owing to high cost involved in the purchase of kits. Globally, the ELISA technique has been widely used for the routine screening of anti-HCV Ab.

The first serological testing for the detection of anti-HCV antibodies using recombinant antigens was introduced in 199110. Since then many developments have taken place and at present 3rd generation ELISA kits are used most widely and globally. Using this new 3rd generation ELISA kits the average period of HCV sero-conversion after blood transfusion has been reduced to 7-8 wk resulting in improved sensitivity of assay in comparison to the earlier generation kits of EIA-1 and EIA-211 but the question still remains unanswered for specificity. Detection of anti-HCV does not distinguish past from present infections and in
diagnostic virology, particularly ELISA’s, a positive HCV test may be non-specific i.e. false positive and therefore has to be crosschecked by another test of different principle for which immunoblots were initially developed. The immunoblotting assays present the same recombinant proteins of enzyme immuno assays as bands immobilized on solid phases.

The detection of HCV RNA by PCR has been widely used and is currently the most sensitive procedure for the diagnosis of HCV infection. HCV RNA detection is a valid diagnostic alternative in anti-HCV negative infections and in cases of early acute hepatitis. There are 6 genotypes numbered from 1 to 6 and more than 120 subtypes of HCV have been reported with distinct geographical distribution\textsuperscript{12-16}. Genotype 1 is seen as 1a common in the USA and Northern Europe, 1b as the most common genotype found world wide, genotype 2 seen as 2a and 2b is also world wide in distribution and common in North Italy and Japan\textsuperscript{17-22}, genotype 3 is common in the Indian subcontinent, genotype 4 in Africa and Middle East, genotype 5 in South Africa and genotype 6 in South East Asia and Hong Kong\textsuperscript{23}.

The aim of the present study was to evaluate the efficiency of the first indigenously developed HCV blot assay by J Mitra and Co, New Delhi. For this, we have used the most widely used method of screening technique i.e. $3^{rd}$ generation ELISA and a reverse transcriptase RT-PCR to detect the presence of HCV RNA. The prevalence of HCV RNA positive individuals was compared with both $3^{rd}$ generation ELISA and HCV blot in order to assess the usefulness of both the tests for screening and supplementary assay, respectively. Genotyping was done in order to assess the capability of HCV blot to pick up different genotype samples prevailing in Andhra Pradesh, South India.

Materials and Methods

Patients

The patient population consisted of 531 patients; this patient pool consisted of dialysis, liver transplanted cases (immune suppressed state), chronic cases, cirrhosis and non-viral hepatitis. Among these 159 were females and 372 were males who attended Centre for Liver Research and Diagnostics, Hyderabad during Aug 2004 to Feb 2007.

Serology Testing

ELISA Test System

Anti-HCV antibody was tested by using $3^{rd}$ generation ELISA kit (Ortho 3.0 Ortho clinical diagnostics, Raritan, New Jeresy). Ortho HCV 3.0 ELISA test system is a qualitative, enzyme linked immunosorbent assay, each microwell coated with a combination of recombinant hepatitis C virus (r HCV) antigen c22-3, c200 and NS5. The amino acid sequence of the three HCV recombinant proteins were c22-3 AA # 2-120, c200 AA # 1192-1931, NS5 AA # 2045-2995. Assay was carried out as per the standard test procedure mentioned by the manufacturer.

HCV Blot

The HCV blot is an in vitro qualitative immunoblot assay and is based upon traditional western and dot blotting techniques. It has been developed and designed using HCV antigen representing the immuno dominant regions of HCV genome. HCV antigens from Core, NS3, NS4 and NS5 regions of the virus were immobilized onto the membrane along with two different concentrations of internal quality control bands. Assay was carried out as per the standard test procedure mentioned by the manufacturer.

Nucleic Acid Extraction and Detection

HCV RNA was isolated from the serum by guanidinium isothiocyanate (GITC)-acid-phenol method\textsuperscript{24}. Briefly, 200 $\mu$L of the serum was mixed with 500 $\mu$L lysis solution (4 M GITC, 0.75 M sodium acetate, 0.5% Sarkosyl and 0.1 M 2-mercaptoethanol), 500 $\mu$L water-saturated phenol and 200 $\mu$L chloroform:isoamyl alcohol (24:1). RNA was precipitated from the aqueous phase sequentially with isopropanol and resuspended in 25 $\mu$L of diethyl pyro carbonate (DEPC)-treated water. Prior to RT-PCR, the RNA was denatured by heating at 95°C for 2 min, followed by rapid chilling. Amplification by PCR was done essentially by the method of Das et al\textsuperscript{25}. Briefly, amplification of the 50 non-coding region (NCR) was carried with 25 pico mole each of the primers, 10X Taq buffer (Mg2+ free), 2.5 mM MgCl2, 200 mM dNTPs, 25 units of ribonuclease inhibitor (RNAsin), 4 units of AMV reverse transcriptase (Promega, Madison, USA), 1 unit of Taq DNA polymerase and RNA template was made up to a volume of 50 $\mu$L. RT-PCR step was done carried out in single tube using a programmable thermocycler (MJ Research, MA, USA) at 42°C for 1 h, followed by 95°C for 2 min, 35 cycles at 94°C for 30 sec, 50°C for 45 sec, 72°C for 1 min and a final extension at 72°C for 5 min. First round PCR was done using the forward primer (5’-ACTGTCTTTCACGCAAGACGTTGCTAGCCAT-3’) and reverse primer (5’-CGAGACCTCCCCGGG
CACTCGCAAGCACCC-3'); 10 µL of the first round PCR product was reamplified with internal primers (forward primer 5'-ACGCAAGACGCTTAGCCATGGCCTTAGT-3' and reverse primer 5'-TCCCGGGGCACTCGCA-AGCACCCTATCAGG-3') for another 35 cycles under same conditions. A negative control, a positive control and water blank was tested during RNA extraction, reverse transcription and amplification for quality control and to exclude false positive results in the PCR due to cross contamination. PCR products were analyzed on 2% agarose gels followed by staining with ethidium bromide and visualized under a UV trans-illuminator. A 100 bp ladder (Promega, Madison, WI) was used as a size marker. Detection of a 256 bp PCR product indicated that the sample was positive for HCV (Fig. 1).

**RFLP Analysis of the 5’ UTR Amplified Product**

The amplified product obtained by RT-PCR was subjected to RFLP analysis by restriction digestion with *Hae III*, *Hinf I* and *Bst NI* (New England Biolabs, Beverly, MA, USA). The size of the undigested amplicon was 256 bp, which was cleaved separately with these three enzymes. After treatment, the restriction fragments were separated by agarose gel electrophoresis and visualized in gel doc. The restriction patterns of the samples were compared with the predicted patterns to determine the genotype. For generating the above patterns, representative full-length sequences of various genotypes [HCV-1 (1a); HCV-J (1b); HCV-G9 (1c); HC-J6 (2a); HC-J8 (2b); Bebel (2c); NZIL (3a); and TR (3b)] were selected and the electrophoretypes predicted using RESTRI and DIGEST programs of PCGENE software package. *Hae III* and *Mva I* enzymes were originally used for typing of the HCV isolates from India 25,26.

**NS5 Typing**

As expected, size of DNA fragments for each genotype was as follows: 1a, 3356 bp; 1b, 143 bp; 2a, 240bp; 2b, 309 bp; 3a, 143 bp; and 3b, 201 bp. Primer sequences used in the study were:

1a: 5'-GAGTCCTGAGGAGGCACTCCGTACG-3'
1b: 5'-AGGCCACCTGCAGCCTATCGAGCTGG
AA-3'
2a: 5'-TATGTGTCAACAGCAAGCCAGG-3'
2b: 5'-GGCTGTGTCCCTGCTCAAGAGGCA-3'
3a: 5'-CTCGGACCTGGACTTTTGCT-3'
3b: 5'-CCGCGCTTAGCGCGCTTTGCT-3'
CA: 5'-CTCGGTCACATCGGCTCCGTGAA-3' (Anti-sense primer for all genotypes).

**Results**

In the present study, a total of 531 samples were screened for HCV using 3rd generation ELISA HCV blot (J Mitra & Co, New Delhi) and RT-PCR. Of which, by ELISA 213 samples were reactive and 318 samples were non-reactive, by HCV blot 211 were reactive and 317 were non-reactive, 3 were indeterminate and by RT-PCR, 214 samples were positive and 317 samples were negative (Fig. 1; Table 1).

Out of the 531 samples tested, 211 showed identical result as reactive by ELISA, HCV blot and RT-PCR. Whereas 3 samples were found non-reactive by ELISA but indeterminate by HCV blot and positive by RT-PCR. Another 2 samples were reactive by ELISA but non-reactive by HCV blot and negative by RT-PCR. Remaining 315 samples were negative with Ortho 3.0 ELISA, HCV blot and RT PCR (Table 2).

Out of the 214 positive RT-PCR samples, 211 were reactive by HCV blot and 3 were indeterminate. The 3 HCV blot indeterminate samples reacted only with

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**Table 1—Result of each test against total number of samples**

<table>
<thead>
<tr>
<th>Result</th>
<th>ELISA</th>
<th>HCV blot</th>
<th>RT PCR</th>
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<tbody>
<tr>
<td>Reactive</td>
<td>213</td>
<td>211</td>
<td>214</td>
</tr>
<tr>
<td>Non Reactive</td>
<td>318</td>
<td>317</td>
<td>317</td>
</tr>
<tr>
<td>Indeterminate</td>
<td>NA</td>
<td>003</td>
<td>NA</td>
</tr>
<tr>
<td>Total</td>
<td>531</td>
<td>531</td>
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core antigen. The percentage of specific antibody band reactivity differed in HCV blot for all the 214 samples. Among them, NS3 antibodies represented in large numbers with 98.59% (n=211) followed by Core antibodies 85.98% (n=184), NS4 antibodies 73.83% (n=158), and NS5 antibodies 62.61% (n=134) (Table 3; Fig. 2).

Genotyping was carried out for 214 positive samples; genotype 1 was the common and observed in 142 (66.35%) samples while genotype 3 was observed in 72 (33.64%) samples. The sub-typing analysis by NS5 typing showed that the hepatitis group having genotype 1, 112 (52.33%) samples belonged to 1b genotype, while 30 (14.01%) belonged to 1a genotype. The hepatitis group having genotype 3, 54 (25.23%) samples belonged to genotype 3a and 18 (8.41%) belonged to genotype 3b (Table 4; Fig. 3).

**Discussion**

Diagnostic kits are usually developed for their applications to human sera or plasma by different manufacturers, however, their suitability for application must be verified. Prior to their
Introduction into the market different commercial kits that are developed, need a thorough evaluation, before they are released for wider use. PCR technology is very expensive and requires high technical expertise and not easily accessible to many laboratories, particularly in the developing world. Hence, there is a necessity to evaluate various sero-diagnostic methods and to compare them with RT-PCR to determine their efficacy.

For improving the reliability of HCV diagnosis, supplementary tests such as HCV blot and RT-PCR has been applied to 3rd generation ELISA screened samples. In our present study, the commercial HCV 3rd generation ELISA and supplementary test assay HCV blot have shown concordance comparison; a correlation was observed between HCV 3rd generation ELISA, HCV blot and RT-PCR in 531 samples with minor discrepancy (Table 2). Out of the 214 RT-PCR positive samples, in 211 (98.59%) samples HCV blot with two or more bands were observed, and in 211 (98.59%) samples 3rd generation ELISA as reactive. The comparison of HCV blot and ELISA differed by 5 (2.33%) samples.

In 3 (1.40%) samples, HCV blot was indeterminate with core band, but 3rd generation ELISA was non-reactive, although both the kits are for antibody detection and share the same HCV protein coatings but RT-PCR was positive, indicating these samples were potentially infectious for HCV infection in follow up studies.

In 2 (0.93%) samples, 3rd generation HCV ELISA was reactive, but HCV blot and RT-PCR were negative. The results indicated the false positivity by 3rd generation ELISA might be since the patients were undergoing interferon therapy, and serum HCV RNA disappear from the serum of most responders while the great majority of these patients maintained anti-HCV in the serum for a long time (3 to 4 years).

ELISA tests are not specific sometimes because antibodies are found in patients with undetectable HCV RNA that may represent past infection or intermittent viremia. But a recent study by Vicente et al has shown a positive test for anti-HCV antibodies in the absence of serum HCV RNA; its negativity cannot be ignored, as HCV RNA can be present in liver or PBMC (peripheral blood mononuclear cells) indicating an ongoing infection. HCV RNA was detected in 50% of the PBMC samples obtained from anti-HCV antibody positive, serum HCV RNA negative patients who had normal ALT levels. Similar observation was also made by Radkowski et al who found HCV RNA in uncultured PBMC samples obtained from 7 of the 11 patients who also had normal ALT values and anti-HCV antibodies and who did not have detectable RNA in serum. In our study, HCV RNA showed a better correlation with PCR than 3rd generation ELISA, which is in agreement with other studies. In our study, the results demonstrated that NS3 the HCV antibody response to antigen was better than that to Core, NS4 and NS5.

The influence of genetic variability upon antibody detection represents a challenge for serological diagnosis of HCV infection. We felt the need to do genotyping as the regional distribution of HCV genotypes can have an impact on the configuration of diagnostic assays and vaccine design. To check whether the genotypes prevailing in South India can be picked up by the present kit, as the assays available at present are all based on antigens derived from HCV genotype 1. According to the classification of Simmonds et al sera from genotype 3-6 show a decreased reactivity with proteins of the NS3 region.

There is a paucity of information on HCV genotypes in India. HCV genotypes 1a, 1b, 2a, 3a, 3b, and 3g have been reported from northern and western India. But genotype 1 is more predominant in South India than genotype 3. The HCV genome sequences from various geographical regions of the world show substantial heterogeneity of nucleotide sequences within several regions of viral genome. On the basis of these genomic differences HCV has been classified into various genotypes. In large population studies as it is economical 5' UTR and NS 5 typing techniques have been used for the genotyping of HCV and for subtyping. In our study, genotype 1 is predominant with 66.35% of the total number of samples tested. The subtyping analysis showed that 1b (52.33%) was more prevalent than 1a (14.01%). Our data correlate with other published data from South India, which shows the prevalence of genotype 1 over genotype 3 and 1b prevalent over 1a. Subtyping analysis of the patients belonging

<table>
<thead>
<tr>
<th>Table 4—Genotype-subtype with number of samples</th>
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<tbody>
<tr>
<td>Genotype</td>
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</tr>
<tr>
<td>Genotype 1 (n=142)</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Genotype 3 (n=72)</td>
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Number in parenthesis for * indicates percentage.
to genotype 3 showed that the genotype 3a is more prevalent than genotype 3b. In our study, out of the three indeterminate samples, two samples were found with genotype 1 subtype 1a and the other with genotype 3 subtype 3b. Our study also showed that no individual was with 2, 4, 5 and 6 genotypes, as well as with more than one genotype. Another noteworthy observation in our study was the presence of genotypes 3b, and 3a, indicating that multiple HCV genotypes were prevalent in South India.

Though the improved 3rd generation ELISA shows better sensitivity as compared to the first and second, it still shows some non-specific results. So there is a necessity to re-test such samples with immunoblot. But another disadvantage of all immunoblots is relatively large number of indeterminate results but our study showed a drastic reduction of the indeterminate stage, limiting it to 3 (0.53%) samples, which correlates with other results thereby suggesting HCV blot as best supplementary testing for HCV infection. However, RT-PCR is preferred by many clinicians, because it gives information on current infection. Also the immunoblot may give false negative results in immune suppressed individuals and in early infection. It is also not useful to diagnose vertical transmission in an infant.

In conclusion, HCV blot showed good co-relation with RT-PCR than 3rd generation ELISA. The accuracy indices for the above three tests are high with RT-PCR (100%) followed by ELISA (99.5%) and HCV blot (98.5%). However, the antibody assays have evolved into a 3rd generation EIA, but still requires supplementary tests, like HCV blot. Based on these results we recommend HCV blots could be done as a supplemental assay in the small to medium laboratory and diagnostic centers. HCV blot has also picked up the genotypes 1a, 1b, 3a, and 3b that are more prevalent in South India. The serology does not always mirror the RT-PCR results because the former is marker of exposure (past or present infection) whereas the later is a marker of active or current infection. The immunoblot is a supplemental antibody assay, confirming the presence of antibody by ELISA. But the detection of RNA by RT-PCR remains the method of choice for the diagnosis of current HCV infection, because a reliable assay for HCV antigen detection is still unavailable.

References
19. Chowdhary A, Santra A, Chaudhuri S, Dhal G K, Chaudhary


