Role of molecular techniques in the detection of HBV DNA & HCV RNA among renal transplant recipients in India

Sujatha Radhakrishnan, Priya Abraham, Sukanya Raghubaran, G. T. John*, P.P. Thomas*
C.K. Jacob* & G. Sridharan

Departments of Clinical Virology & *Nephrology, Christian Medical College & Hospital, Vellore

Accepted May 4, 2000

In this study we have investigated the occurrence of hepatitis B virus (HBV), hepatitis C virus (HCV) and hepatitis D virus (HDV) infections among 68 renal transplant recipients. Replicative HBV and replicative HCV infections were seen in 12 (17.6%) and 38 (55.9%) patients respectively, the difference was statistically significant (P<0.001). Among the 38 HCV RNA+ individuals, anti-HCV was present only in 23. Anti-HCV in the absence of HCV RNA was detected in one patient. Anti-HDV antibody was seen in 2 (15.4%) of the 13 HBV infected individuals. Nine (13.2%) of the 68 individuals had replicative dual infection with HBV and HCV. Triple infection (HBV DNA+, HCV RNA+, anti-HDV+) was seen in 2 transplant recipients. There was significantly higher demonstration of replicative HCV (P<0.001) in transplant recipients having elevated liver enzymes (n=34) as compared to transplant recipients having normal liver enzyme levels (n=34). Though not significant, a higher detection rate was also seen with replicative HBV infection and replicative dual infection among transplant recipients with elevated liver enzymes. The higher detection of HCV in renal transplant recipients by molecular techniques, emphasizes the need for HCV RNA testing. Further deliberate attempts to change practices to reduce this problem may also improve graft and patient survival in recipients.

Key words HBV DNA - HCV RNA - renal transplant recipients

Patients on haemodialysis (HD) are at high risk of acquiring hepatitis B virus (HBV) and hepatitis C virus (HCV). The risk of acquiring hepatitis D virus (HDV) is low for Indian patients as the virus is widely prevalent in certain geographical regions only. The use of HBV vaccination is expected to decrease the prevalence of HBV among patients on HD and in turn may prevent HBV transmission. However, HCV continues to be a major concern among patients with end stage renal disease (ESRD) requiring HD. HCV prevalence rates vary world-wide in different dialysis centres, from 5-50 per cent based on seroprevalence of anti-HCV antibody (anti-HCV) in these patients. However, studies on HCV by molecular techniques like polymerase chain reaction (PCR) have revealed that anti-HCV has inadequate sensitivity and specificity in detecting acute HCV infection especially in immunosuppressed individuals.

Sero logical markers for the diagnosis of HBV have been well characterised, and most prevalence studies are based on measurement of one or more markers, particularly hepatitis B surface antigen (HBsAg) and anti-Hbc antibody (anti-Hbc). HBV DNA detection by PCR is important in certain situations, for example, in HBsAg negative patients and rarely in anti-Hbc- and anti-HBs- positive carriers where genetic variants of the virus may exist.

There are a few reports on the prevalence of HBV and HCV among renal transplant patients in India indicating prevalence rates of 31-57 per cent and 12-37.5 per cent when measured by HBsAg and anti-HCV.
HCV detection respectively. This is higher than the general population where HBsAg prevalence is reported to be 2-7 per cent and anti-HCV to be 1.5-1.8 per cent. An earlier report from this hospital had shown a low prevalence of HDV among patients on HD and post renal transplant patients. Data from India on HCV infections among HD patients are deficient as they are primarily based on antibody testing alone which is recognised as being inadequate. The main objective of this study was to evaluate the role of molecular testing for HBV and HCV in a cross-sectional group of immunosuppressed renal transplant recipients. Among the patients studied, further analyses were carried out to get some information of the association of HBV and HCV markers under the influence of immunosuppression. In this study, we have in addition to serological testing, investigated the occurrence of replicative HBV, replicative HCV and HDV infections among renal transplant recipients and found that a sensitive molecular technique like nested PCR (nPCR) improves the detection rate, particularly of HCV in the blood.

Material & Methods

Subjects: There is a reported prevalence of 55 per cent for HBV and 25 per cent for HCV as measured by HBsAg positivity and anti-HCV respectively, among Indian renal transplant recipients. In the pilot study reported here, a minimum sample size of 41 was calculated expecting a prevalence of 40 per cent (desired precision 15%) for any one virus, at a confidence level of 95 per cent for assessing the role for molecular techniques in the detection of HBV and HCV.

At this Centre, approximately 100 live related donor renal transplantations are performed annually since 1984. HBsAg testing on patients has been done since then and anti-HCV testing from 1991, at entry to the dialysis and transplant programme. In the post transplant period, HBsAg is tested monthly for the first six months and at subsequent follow up visits. Tests for other serological or vascular markers are done when the liver function tests are deranged or at follow up of patients who have had prior positive viral markers. Samples from 68 such patients referred between January 1997 and June 1998 to the Department of Clinical Virology at this Centre, for molecular detection of HBV DNA and HCV RNA along with appropriate serological markers, were included in the study. There were 55 males (age range 18-57 yr) and 13 females (age range 13-55 yr). All the patients had received blood transfusions (number ranging from 1-33) and had been haemodialysed (range of sessions 3-160). Among these patients, 37 were tested for virological markers in this study within one year of transplantation, while 31 were tested >1 yr after transplantation (range 1-14 yr). All patients were on immunosuppressive therapy. There were 49 patients who had received HBV vaccination while 17 had not and in 2 patients there was no documentation. None of the patients were positive for anti-HIV antibody.

Liver enzyme levels were used to categorise the 68 patients into those with and without hepatitis. Serum aspartate transaminase (AST) and serum alanine transaminase (ALT) levels >40 units/ml were considered elevated. Liver enzyme elevation was not temporally associated with anti-tuberculous therapy. Among the 68 patients, elevated enzyme levels were seen in 34 who were evaluated for virological markers. The remaining 34 patients had normal liver enzyme levels and were referred for molecular testing because of a high degree of suspicion of having infection with HBV or HCV. The equal number of patients with and without liver enzyme elevation was by design but a chance observation.

EDTA blood was collected from renal transplant recipients and plasma separated and stored at -60°C in aliquots. All patients were evaluated by molecular techniques in addition to serology.

HBV PCR: The HBV DNA was extracted using a QIAamp Blood Kit (Qiagen, Germany), according to the manufacturers' instructions from 200 µl volume of plasma. The DNA extract was used immediately for amplification by a nested PCR protocol. Each DNA extract was amplified separately with two sets of primers specific for the surface (outer: S5 and S6; inner: 109 and 585R) and core (outer: 1763 and 2032; inner: 1788E and 2017R-B) genes of the HBV genome. The amplification was carried out in a 50 µl reaction volume containing 10 µl of DNA extract, 1 unit of Taq polymerase, 0.2 mM of dNTP mix containing the four deoxynucleoside triphosphates (dATP, dGTP, dCTP, and dTTP) and 1× PCR buffer. The amplification was performed in a Thermocycler (Perkin-Elmer Cetus, model 2400). The conditions of amplification were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR products were separated on 2% agarose gel and visualised under UV light.

HCV RNA: HCV RNA was extracted from plasma using a QIAamp Viral RNA Kit (Qiagen, Germany) according to the manufacturer's instructions. The RNA was eluted in 30 µl of nuclease-free water. The RNA samples were tested for the presence of HCV RNA using a nested reverse transcriptase-PCR (nRT-PCR) protocol. The primers used for the first RT-PCR reaction were HCO3 for the 5' untranslated region (UTR) of HCV (5'-CAGCCTGGTACCTCGGGGTATTTTGG-3') and the primer 1158F (5'-GACGGTAAACCTCACTTC-3') located in the 3' conserved region of HCV. A second PCR was performed using HCV 1A-Fw (5'-GGATGCTACGTCCTCCTGAA-3') and HCV 1B-Rv (5'-CTGAGAACGAGGCTGGATCG-3') located in the 5'UTR and 3'UTR, respectively. The conditions of amplification were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR products were separated on a 2% agarose gel and visualised under UV light.

The results of the molecular testing were compared with the serological results to determine the sensitivity and specificity of the molecular techniques. The positive predictive value and negative predictive value were calculated for each test.
triphosphates, 50 mM KCl, 10 mM Tris HCl, 3 mM MgCl₂ (Boehringer Mannheim-Roche, Germany) and 20 pmols of outer primers (core or surface gene specific) [Gibco BRL, UK].

The amplification was performed using the GeneAmp 2400 thermal cycler (Perkin-Elmer, USA) at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, for 30 cycles with a final extension at 72°C for 10 min. The annealing temperature was modified from the protocol followed by Kaneko et al. from 42 to 55°C. Second round amplification was carried out using the same conditions with inner primers and 5 μl of the first round product. Standard precautions described to avoid false positives while performing PCR assays were followed.

HCV PCR: The RNA was extracted from 140 μl of plasma using the QIAamp Viral RNA Kit (Qiagen, Germany) and immediately converted to complementary DNA (cDNA). For cDNA synthesis, 9.8 μl reverse transcription mixture containing 1x concentration of reverse transcription (RT) buffer, 1mM each of the four dNTPs, 40 units of RNase inhibitor, 20 units of avian myeloblastosis virus reverse transcriptase and 50 pmols of random hexamer primers (Boehringer Mannheim-Roche, Germany) were taken. To this, 10.2 μl of RNA extract was added to get 20 μl of cDNA after synthesis at 43°C for 60 min, followed by denaturation at 95°C for 5 min. To the above cDNA, 80 μl of PCR reaction mixture containing 1x buffer (1 mM Tris HCl, 50 mM KCl, 1.5 mM MgCl₂), 0.2 mM each of the four dNTPs (Boehringer Mannheim-Roche, Germany), 50 pmols of outer primers from the 5' noncoding region (Gibco BRL, UK) and 2 units Taq polymerase (Boehringer Mannheim-Roche, Germany) were added. The thermal cycling conditions were 94°C for 1.5 min, 50°C for 1.5 min, 72°C for 2 min, for 35 cycles with a final extension at 72°C for 10 min. The second round amplification was done with 5 μl of the first round product in a PCR reaction mixture containing 50 pmols of inner primers.

The amplified products were detected by electrophoresis in a 2.5 per cent agarose gel containing 0.5 μg/ml ethidium bromide (Sigma, USA) and the bands visualised under UV illumination (Mighty Bright, Hoefer, USA). The expected product size for the surface and core coding regions of HBV were 477 and 258 bp respectively. HCV nested product size was 162 bp.

Serology: HBsAg was tested in Hepanostika Uniform II (Organon Teknika, NL-5281 RM Bokrel) or in HBsAg Auzyme EIA or IMx/Axsym (Abbott Laboratories, IL, USA). Other HBV markers were tested by microparticle enzyme immunoassay (IMx, Abbott Laboratories, IL, USA): HBeAg by HBe2, anti-HBe, antibody by Anti-HBe2, anti-HBc antibody by CORE, anti-HBs antibody by AUSAB.

Anti-HCV antibody was tested in UBI HCV EIA 4.0 (United Biomedical Inc., NY, USA) or IMx/Axsym HCV version 3 (Abbott Laboratories, IL, USA). Confirmation of anti-HCV antibody status in samples negative for HCV RNA was carried out with western blot (HCV 3.0, Genelabs Diagnostics, Singapore) or line immunoassay (LiaTek HCV III, Organon Teknika, NL-5281 RM Boktel).

Antibody to delta agent (anti-HDV) was tested in the Anti-delta EIA (Abbott Laboratories, IL, USA).

Statistical analysis: All statistical analysis was carried using the Epi Info 6 (version 6.03) software. Tests of significance for comparisons were carried out with Chi square test and Yates correction applied wherever appropriate. P values <0.05 were considered statistically significant.

Definitions:

Replicative HBV infection - Presence of plasma HBV DNA (positive for any one or both surface/core gene sequences) with or without HBsAg.

Current HBV infection - Presence of HBsAg with or without HBV DNA.

Exposure to HBV - Presence of anti-HBc and/or other HBV serological markers.

Replicative HCV infection - Presence of HCV RNA in plasma with or without anti-HCV.

Exposure to HCV - Presence of HCV RNA and/or anti-HCV.
Results

HBV markers: In 68 patients tested for HBV infection, 11 (16.2%) [8.4 to 27.1, 95% CI] were positive for both HBsAg and HBV DNA, one patient had HBV DNA only while another had HBsAg only. Thus, when measured by both HBV DNA and/or HBsAg, HBV infection was detected in 13 (19.1%) [10.6 to 30.5, 95% CI], while replicative infection was detected in 12 patients. All HBV DNA positive samples showed amplification of both core and surface sequences. HBeAg was not seen in the absence of HBV DNA and it was positive in 8 (66.6%) of the 12 HBV DNA positive individuals. Anti-HBe was positive in 12 of the 68 patients, of these 2 were seen in replicative HBV infection.

Anti-HBc antibody as a marker of exposure to HBV was seen in 35 (51.5%) [39.03 to 63.8, 95% CI] of the 68 patients; 3 of the 35 had anti-HBc as the sole HBV marker. Eight anti-HBc positive individuals were positive for both HBsAg and HBV DNA, one was positive for HBV DNA only while another was positive for HBsAg only. Anti-HBs was seen in 40 (58.8%) of 68 patients; and in 19 as the sole HBV marker. Vaccination history was established in 15 (78.9%) of the 19 individuals positive for anti-HBs alone. Current or replicative HBV infection was seen in 12.2 per cent among the 49 HBV vaccinees and in 41.2 per cent of 17 non-vaccinees (P<0.05; Table I). Total exposure to HBV was significantly higher in the non-vaccinated group (88.2%) than in the vaccinated individuals (48.9%; P<0.001). Anti-HBs was not seen in individuals with replicative HBV infection.

HCV markers: Anti-HCV by EIA was positive in 25 patients (36.8%) and HCV RNA was seen in 38 (55.9%) patients, this difference was statistically significant (P<0.01). Of the 38 HCV RNA positive individuals, only 23 had anti-HCV antibody. Two other patients had anti-HCV in the absence of HCV RNA but western blot confirmed the reactivity in one patient, while the other gave indeterminate reactivity. Exposure to HCV was seen in 39 (57.3%) [44.8 to 69.3, 95% CI].

In 12 (17.6%) of 68 HD patients there was evidence of replicative HBV whereas replicative infection with HCV was seen in 38 (55.9%), this was a highly significant difference (P<0.001).

Dual infection: Replicative dual infection with HBV and HCV was seen in 9 patients (13.2%) as detected by HCV RNA and HBV DNA positivity. Among these 9 patients, 8 were positive for HBsAg, HBV DNA and HCV RNA. HBeAg was seen in 5 of these 8 patients. One patient with dual infection had an unusual HBV serological profile; positive for HBV DNA and anti-HBc, negative for other HBV markers. In addition, there was one HCV RNA positive patient with HBV infection; positive for HBsAg, anti-HBc, anti-HBe but HBV DNA negative.

Anti-delta antibody was present in 2 of 13 (15.4%) patients who had HBV infection (HBsAg+ and/or HBV DNA+). One anti-HDV positive patient was both HBeAg and anti-HBe positive, while the other was HBeAg negative and anti-HBe positive. These two were also concurrently infected with HCV and were HCV RNA positive.

Association of HBV and HCV markers in patients with and without liver enzyme elevation: In those with elevated liver enzymes, there was significantly higher detection of replicative HCV infection (P<0.001; Table II). Higher detection of HBV replicative infection and replicative dual infection were also seen among patients with elevated liver enzymes, the differences were not statistically significant. Of the eight HBeAg positive patients, six were associated with elevated liver enzymes, of these, three patients were also infected with HCV and one with HCV and HDV. Anti-HCV in the presence of HCV RNA was also significantly higher in those with elevated liver enzymes (P<0.001). The two triple infected HBV+, HCV+ patients, who were anti-HDV+, had elevated liver enzymes.
Table II. Association of HBV and HCV markers with normal and elevated liver enzyme levels among renal transplant recipients (n=68)

<table>
<thead>
<tr>
<th>Category</th>
<th>Normal liver enzyme levels (n=34)</th>
<th>Elevated liver enzyme levels (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Replicative HBV infection (HBV DNA positive with or without HBsAg)</td>
<td>3</td>
<td>88</td>
</tr>
<tr>
<td>Replicative HCV infection (HCV RNA with or without anti-HCV)</td>
<td>13</td>
<td>38.2</td>
</tr>
<tr>
<td>HCV RNA positive, anti-HCV negative</td>
<td>8</td>
<td>23.5</td>
</tr>
<tr>
<td>HCV RNA negative, anti-HCV positive</td>
<td>1</td>
<td>2.9</td>
</tr>
<tr>
<td>HCV RNA positive, anti-HCV positive</td>
<td>5</td>
<td>14.7</td>
</tr>
<tr>
<td>Replicative dual infection (HBV DNA &amp; HCV RNA)</td>
<td>2</td>
<td>5.8</td>
</tr>
</tbody>
</table>

* P<0.001 compared to patients with normal liver enzyme levels

Association of HBV and HCV markers in those patients evaluated within a year and more than one year after transplantation: Detection of HBV and HCV in those evaluated within a year of transplantation (n=37) and >1 yr after transplantation (n=31) are given in Table III. The rates of replicative infection of HBV and HCV were not significantly different in the two groups. A higher rate of replicative dual infection was seen, this difference also was not significant. A significantly higher positivity rate for anti-HCV was seen in patients evaluated >1 yr after transplantation (P<0.05). HCV RNA in the absence of anti-HCV was detected at a significantly higher rate in the group of individuals tested within one year of transplantation (P<0.05).

Discussion

In this select group of renal transplant recipients, replicative infection due to HBV and HCV was seen in 17.6 per cent and 55.9 per cent respectively. Replicative dual infection with these viruses was seen in 9 (13.2%). In addition there was one patient who was HBsAg positive in the absence of HBV DNA and was also HCV RNA positive. When measured by HBV DNA and/or HBsAg, HBV infection was seen in 13 (19.1%). If the sample size was estimated with a 10 per cent desired precision for an expected prevalence of 40 per cent, it will be n=93. However, in the study we could recruit only 68. Nevertheless, a relatively narrow 95 per cent CI for the detection of HCV was observed with 57.4 per cent (44.2 to 69.3, 95% CI). There were 13 patients with HBV infection in this group; observed detection of HBV was 19.1 per cent (95% CI, 10.6 to 30.5%).

The exposure to HBV as measured by anti-HBc in the 68 transplant recipients was 51.5 per cent. The HBV exposure rate is significantly higher (P<0.001) than in the control individuals tested in this study in which we have found exposure to be 19.2 per cent as tested by anti-HBc. The controls included 48 patients who underwent endoscopy for non-liver disorders with normal liver function tests, and 1 healthy laboratory personnel. All 57 individuals of the control group were HBsAg negative and anti-HIV negative, and in 50 of these where plasma was available for molecular testing both HBV DNA and HCV RNA were negative, including the anti-HBc positive individuals. A similar anti-HBc prevalence has been reported in blood bank donors from India.
In the present study, the exposure to HBV and HCV was almost equal, with 51.5 per cent positive for anti-HBe and 57.3 per cent for HCV RNA and anti-HCV. However, there was a significantly higher number of patients with replicative HCV infection than replicative HBV infection. This can be explained by the tendency for HCV to persist or to interfere with HBV replication. Suppression of HBV by HCV has been observed when they coexist in an individual.  

We observed HBeAg in 8 (66.6%) of 12 HBV DNA-positive individuals, indicating a higher level of viral replication. Detection of HBV DNA is an extremely sensitive marker and is of particular use in infections involving molecular variants of the virus, for example, in detecting precore mutants incapable of secreting HBeAg⁵⁶. HBV DNA detection by nPCR may not be warranted in individuals positive for HBeAg. There was one HBsAg-negative patient in this study who was found to be positive for HBV DNA and anti-HBe, when tested 3 wk after transplantation. Here, HBV DNA testing has helped to detect a replicative infection, which otherwise would have been regarded as exposure to HBV. Enhanced replication of HBV is known to occur under the influence of certain immunosuppressive drugs, like prednisolone²¹ which may explain the reactivation of HBV in this patient. Follow up of this patient a year later, revealed that he became HBsAg and HBeAg positive (data not shown).  

The antibody response to HCV was absent in 15 (39.5%) of the 38 who tested positive for HCV RNA. This can be attributed to the immunosuppressive therapy. Further evidence to support this is seen in the significantly lower presence of anti-HCV in patients evaluated within a year after transplantation, as immunosuppressive therapy is intense in the immediate six months after transplantation. Tapering of these drugs allows for the immune response against HCV to be mounted. It is of interest to note that a significantly higher occurrence of anti-HCV was seen in individuals with elevated liver enzymes. This suggests that there could be immune damage to the liver even in the presence of immunosuppressive therapy. It has been postulated that damage to the liver is a combination of immune- and viral cytopathogenic effects of HCV²². Likewise, the presence of anti-HBe positivity in replicative HBV infection among individuals with elevated liver enzymes is an indication of the immune system's attempts to control the virus. It is now understood that immune-mediated lysis of hepatocytes plays a major role in the pathogenesis of HBV infection²³.  

A higher detection of replicative HCV was seen in the group with liver enzyme elevation (P<0.001). Though not significant, a similar trend was also seen with replicative HBV infection and with replicative dual infection. This is a relevant observation in our patient population in view of the increased morbidity and mortality associated with HBV and HCV in renal transplant recipients, with an even greater risk with dual infection²⁴-²⁶.  

The documentation of the presence of HBV and more importantly, of HCV, among post transplant recipients emphasizes the need for preventive measures in controlling the transmission of these viruses. Transmission of HBV can be controlled by HBV vaccination of all patients prior to haemodialysis and by screening of blood. In our study group, presence of anti-HBs in 15 patients in the absence of other markers indicates successful response to HBV immunisation that was initiated during the pre-transplant period. Moreover, to improve efficacy of response to HBV vaccine, it was given intradermally to some patients²⁷. There was significantly more exposure to HBV among the non-vaccinates than the vaccinated individuals. However, the inability of some patients on dialysis treatment to respond to HBV vaccination emphasises the need for stringent practices to prevent HBV spread among patients attending dialysis units.  

Mandatory screening for HCV in blood banks will help to reduce the exposure to HCV through transfusions in India. However, studies in dialysis centres have shown that nosocomial transmission of HCV is mostly due to failure of stringent infection control measures rather than being transfusion-related²⁸. Some dialysis centres, including ours, segregate anti-HCV positive patients from those who are negative to prevent transmission. Anti-HCV tests
lack sensitivity and acute HCV infection detectable by HCV RNA, can occur without antibody production in haemodialysis patients. Further, the presence of anti-HCV may indicate past infection and such segregated patients may be vulnerable to reinfection. Hence, there is a need for improved detection methods like PCR for HCV RNA detection to identify anti-HCV negative HCV infected patients and to institute measures to prevent nosocomial transmission of HCV. The high cost and the non-availability of molecular techniques in dialysis centres in India deters from identifying such patients, a problem that needs to be seriously addressed. However, it is the early detection of HCV infection by using PCR that helps in better management of patients in dialysis units and will prove to be cost effective for individual patients. In our study patients the higher detection of current HCV infection relative to HBV may be because of some degree of natural immunity to HBV given the high exposure rate in the general population.

This study reports that the use of molecular techniques increases the detection of parenterally transmitted hepatitis viruses, HBV and especially, HCV. The control of HBV and HCV infections should be a priority in transplant centres, especially in view of reports indicating poor patient and graft survival in individuals with renal transplant with infections by such hepatotropic viruses.

Acknowledgment

This study formed a part of the first author's Ph. D thesis.

References


Reprint requests: Dr Gopalan Sridharan, Professor & Head, Department of Clinical Virology
Christian Medical College & Hospital, Vellore 632004