Polymerase chain reaction for the diagnosis of viral hepatitis B and C

C Bréchot

Abstract
Polymerase chain reaction is a highly sensitive technique for the detection of hepatitis B virus-DNA and hepatitis C virus-RNA in serum, liver tissue, and peripheral mononuclear blood cells. In chronic hepatitis B, it is particularly useful for identification of infectious subjects who are hepatitis B surface antigen positive and anti-hepatitis B e antigen antibody-positive, and for follow up of hepatitis B virus infections in liver transplantation programmes. Polymerase chain reaction detection of hepatitis C virus-RNA in serum may be the only means of confirming acute hepatitis C infection and also of identifying viraemia in the chronic disease, particularly in at-risk hepatitis C virus antibody-negative individuals. It can also be used for direct evaluation of mother to child hepatitis C virus transmission. As in hepatitis B, polymerase chain reaction can be used for monitoring reinfection with hepatitis C virus after liver transplant, and has proved invaluable in identification of different hepatitis C virus genotypes. The efficacy of antiviral treatment can also be monitored using polymerase chain reaction. Polymerase chain reaction has thus shown numerous advantages for disease detection and monitoring despite the limitations imposed, for example, by possible contamination problems and semiquantitative evaluations.

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Polymerase chain reaction (PCR) is certain to modify the approach to the diagnosis of viral hepatitis. While it provides a direct and highly sensitive identification of viral genomes, however, it has three important limitations: (a) the risk of false positive results because of contamination; (b) difficulties in developing quantitative tests; (c) the need for automation and use of non-radioactive probes for wider availability of the technique. This review will discuss different clinical and practical uses of PCR for the diagnosis of viral hepatitis, focusing on the hepatitis B and C viruses.

PCR for the appraisal of viral multiplication

HBV (HEPATITIS B VIRUS)

Conventional hybridisation procedures have now been used widely for the detection of HBV-DNA in serum, tissues, and mononuclear blood cells.1 2 HBV-DNA is the most direct and sensitive test for viral multiplication and semiquantitative tests have been developed, based on liquid hybridisation.3 4 The limit of sensitivity, however, is 105–106 particles/ml of serum or plasma.

There is a need for more sensitive tests to identify the HBV-DNA sequences, based on the following: (a) hepatitis B surface antigen (HBsAg) positive blood donors or mothers have been shown to transmit HBV infection despite being positive for antibodies to hepatitis B e antigen (anti-HBe) and HBV-DNA negative in serum3 5; (b) some patients with HBsAg positive/anti-HBe positive chronic active hepatitis have active liver disease despite serum HBV-DNA negativity and absence of hepatitis delta virus or hepatitis C virus (HCV) coinfections, autoimmune liver disease, or other causes of liver disease; (c) it is important to have a precise follow up of patients under antiviral treatment, to evaluate partial or complete responses and the subsequent risk of reactivation; (d) PCR is necessary for subsequent sequencing of the amplified products (either direct sequencing or sequencing after cloning).5 It has provided information on the genetic variability of HBV and the potential implication of mutations in the pre-C, C, and pre-S/S viral sequences in the persistence of HBV infection, severity of liver disease, and response to treatment.6 7; (e) HBsAg negative patients with acute and chronic liver diseases have been shown to contain HBV-DNA sequences in the blood, liver, or mononuclear cells. HBV-DNA has also been shown in blood donors with no HBV serological marker.8 9 10

The reliable detection and characterisation of these sequences is facilitated by PCR.

HBsAg-positive patients
Several studies have highlighted the potential use of PCR for HBV-DNA detection.27–40 Representative results obtained in our laboratory are shown in the Figure,36 showing serum HBV-DNA in 50% of HBsAg positive/anti-HBe positive asymptomatic carriers who were HBV-DNA negative in a regular spot test.

In contrast, HBV-DNA was shown in almost all HBsAg positive subjects with chronic active hepatitis, but not in cases of resolved acute hepatitis or negative controls.

Two main conclusions can be drawn from these findings: firstly, PCR is a highly sensitive test, detecting as few as 10 HBV particles/ml; secondly, the PCR test is not positive for all HBV carriers and thus may have prognostic implications.
It is clear, however, that quantitative tests will be necessary to evaluate the real significance of the test in chronic HBV infection. For example, it would not yet be wise to start antiviral treatment systematically in patients who are HBV-DNA positive on PCR but negative in spot test assays. Some of our recent results indicate that at least a semiquantitative estimation of the HBV-DNA sequences will be obtained, allowing precise follow up of antiviral treatment.

HBsAg negative patients with acute or chronic liver diseases

PCR is the most useful test for the appraisal of HBV infection in seronegative patients with liver disease. It has allowed confirmation of previous findings made with the spot test or Southern blot, and has provided access to the nucleotide sequence analysis of these viral mutant forms.

In addition, the detection of both HBV-DNA and HCV-RNA in non-A, non-B infections allows us to determine the respective importance of these viral infections. An appraisal of HBV infections in liver transplantation is given later in this paper.

Conclusions

The use of PCR for the detection of HBV-DNA is still limited in routine clinical use to three areas: detection of HBV multiplication in HBsAg positive/anti-HBe positive patients with active liver disease despite a negative spot test for HBV-DNA; appraisal of HBsAg negative liver diseases; follow up of HBV infections in liver transplantation programmes. Future potential uses will include follow up of antiviral treatment (dependent on quantification) and detection of HBV-DNA in blood donors (dependent on automatisation).

Hepatitis C virus (HCV)

The recent development of 'second and third generation' tests for anti-HCV antibody detection has improved considerably the sensitivity and specificity of serological tests. There are still several limitations, however, including delayed seroconversion in acute infection (about six to eight weeks on average), apparent HCV infection in seronegative individuals, difficulties in interpreting some 'indeterminate' results, and the absence of a test for detecting HCV antigens and, thus, of serological tests for HCV multiplication.

Detection of HCV-RNA

PCR can be used for the detection of HCV-RNA in serum, liver, and mononuclear cells. The efficiency of detection is dependent upon conservation of samples, the amount of material available (100 to 200 microlitres are sufficient), and the specific primers used. Primers located on the 5' non-coding part of HCV are the most efficient; negative results may be confirmed by using primers on other parts of HCV genome (capsid, NS5, for example). There is also the question of nested single step PCR. Nested PCR is highly sensitive but also highly susceptible to contamination. To minimise these problems, we use either nested PCR with the two steps performed in a single tube, or single step PCR with three primers (one for cDNA and two different primers for the PCR).

Determination of HCV viraemia

PCR allows for a direct determination of HCV viraemia; in acute infection it can identify HCV viraemia one week after contamination. Furthermore, it is now clear that most anti-HCV positive patients with chronic hepatitis have HCV-RNA detectable in the serum (Table I). A few anti-HCV positive individuals are HCV-RNA negative, however, and it is not yet clear whether they have intermittent viraemia. In contrast, recent evidence from our laboratory indicates that only a subset of blood donors with repeatedly normal activities of alanine aminotransferase has
detectable serum HCV-RNA; this suggests that PCR might allow the identification of individuals for further investigation and, possibly, treatment. It has also been found that anti-HCV negative subjects can be HCV-RNA positive. This has been shown both in patients with chronic hepatitis and in blood donors implicated in post-transfusional hepatitis.\(^{53,54}\) As for hepatitis B, HCV-RNA detection might be used in the future for the follow up of hepatitis C patients receiving treatment with interferon. Semiquantitative evaluation of HCV-RNA has been performed but is not really applicable at present. This will be a major challenge in the next few years.

**Evaluation of the role of HBV or HCV in some liver diseases: the value of negative PCR tests**

A negative result with PCR is of significance because of the extreme sensitivity of the test.

**HCV AND FULMINANT HEPATITIS**

We have evaluated the potential role of PCR in fulminant hepatitis (Table II).\(^{43}\) In this study, HCV-RNA was not detected in any patients with fulminant hepatitis of unknown cause. In contrast, HCV-RNA was detected in a significant proportion of patients with HBSAg positive fulminant hepatitis, suggesting co-infection or superinfection by HCV as a potential risk factor.

**FROZEN TISSUES**

PCR can be used to detect HBV-DNA, HBV-RNA, and HCV-RNA in frozen liver biopsy specimens. PCR has also been used for HBV-DNA detection in paraffin embedded liver sections\(^{57-61}\) despite a reduction in sensitivity of around tenfold in our experience. HCV-RNA has yet to be detected in paraffin embedded liver samples. It has been possible to confirm the presence of HBV-DNA and HCV-RNA in liver samples of patients with HBSAg negative chronic liver diseases, including hepatocellular carcinoma.\(^{52}\) The HBV-DNA sequences identified in tissue tumours can be transcriptionally active as HBV-RNA has also been identified. More recently, HCV-RNA positive and negative strands have been shown in these tumours indicating ongoing viral multiplication in the tumour cells. Finally, HCV-RNA can be identified in some liver biopsy specimens despite a negative serum test.

**PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)**

HBV-DNA has been identified in PBMC using classic hybridisation procedures.\(^{62}\) PCR has recently proved the presence of HBV-RNA in these cells.\(^{63}\) HCV-RNA positive and negative strands can also be shown in PBMC, indicating that, as for flavi and pestiviruses, HCV infects mononuclear cells.\(^{64}\)

There are limitations associated with the interpretation of PCR tests for the detection of viral genes in tissues or PBMC. For example, how can contamination from serum or non-tumorous liver cells (in studies on hepatocellular carcinoma) or from serum particles absorbed on PBMC (in studies on mononuclear cells) be ruled out, given that

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Anti-HCV and HCV-RNA in chronic NANB hepatitis (From: Porchon et al. J Hepatol)(^{50})</th>
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<tbody>
<tr>
<td>Patients</td>
<td>Anti-HCV</td>
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<tr>
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<tr>
<td>18</td>
<td>+</td>
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<td>4</td>
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<td>5</td>
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<td>1</td>
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<td>-</td>
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<td>36</td>
<td>27/36</td>
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<thead>
<tr>
<th>TABLE II</th>
<th>PCR results in patients with HBSAg positive or indeterminate fulminant hepatitis (From: Fésay et al. J Clin Invest 1992; 99: 1361–5)(^{41})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HCV</td>
<td>Anti-HBV</td>
</tr>
<tr>
<td>----------</td>
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</tr>
<tr>
<td>Serum</td>
<td>Liver</td>
</tr>
<tr>
<td>Group 1</td>
<td>(n=17)</td>
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<tr>
<td>Group 2</td>
<td>(n=23)</td>
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<tr>
<td>(Indeterminate)</td>
<td>1</td>
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<table>
<thead>
<tr>
<th>TABLE III</th>
<th>Nucleotide sequence homology in the E1/NS, hypervariable domain (From: Fésay et al. J Clin Invest 1992; 99: 1361–5)(^{43})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>After liver transplantation</td>
</tr>
<tr>
<td>Patient 2</td>
<td>After liver transplantation</td>
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<tr>
<td>Patient 3</td>
<td>Before liver transplantation</td>
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</table>

**HBV AND HCV REINFECTION AFTER LIVER TRANSPLANTATION**

Immunotherapy with anti-HBs specific immunoglobulin can prevent HBV reinfection in HBSAg positive patients who were HBV-DNA and HBeAg negative at the time of liver transplantation. Testing by PCR for HBV-DNA in mononuclear cells and liver samples in this group of HBSAg negative subjects after transplantation has shown negative results in liver biopsy samples but positive results in peripheral blood mononuclear cells. This indicates that PCR can be useful for the evaluation of HBV infection in liver transplantation. Also, the presence of HBV-DNA in peripheral blood mononuclear cells probably accounts for the reappearance of HBV infection in the liver graft in up to 30% of these subjects.\(^{59}\) We have recently validated PCR as the most reliable test to identify HCV infection after liver transplantation. It has also been possible to show direct recurrence of HCV infection in the liver graft by the original HCV strain\(^{53}\) (Table III).

**PCR for the detection of HCV-RNA and HBV-DNA in tissues and peripheral blood mononuclear cells**

For example, how can contamination from serum or non-tumorous liver cells (in studies on hepatocellular carcinoma) or from serum particles absorbed on PBMC (in studies on mononuclear cells) be ruled out, given that...
PCR is such a sensitive procedure? We have adopted a number of different techniques. For HBV-DNA and hepatocellular carcinoma we have used different primers and shown different patterns of positivity in serum and non-tumorous cells as compared with the tumour – that is, presence of defective viral genomes only in the tumour.61 For HCV-RNA in PBMNC, we have shown that mitogens (such as PHA/PMA) will enhance HCV multiplication in short term (24–76 h) cultures of PBMNC.44

It is also possible to take advantage of the viral genetic variability. For example, sequences of the E2/NS1 hypervariable region show significant mutations in the same strain when comparing HCV-RNA extracted from tumorous and non-tumorous liver tissue from patients with hepatocellular carcinoma. This means that the positivity of HCV-RNA in the tumour is not due to minute contamination from non-tumorous cells. It also underlines a different rate of replication and mutation in tumorous and non-tumorous liver cells.61 65 66

PCR for the analysis of HBV and HCV genetic variability: clinical implications

HCV
The comparison of nucleotide sequences from the published HCV genomes in Japan, USA, and Europe highlights the existence of distinct subtypes of HCV. The 5′ non-coding and, to a lesser extent, the core and NS3-NS4 regions of the viral genome are well conserved among different isolates. In contrast, the open reading frames encoding the E1 and E2/NS1 envelope proteins are much less conserved. In the 5′ part of the E2/NS1 sequence is located a 'hypervariable' domain (Table III).65 66

It is possible to take advantage of this 'hypervariable' domain to identify precisely an HCV strain as well as its mutations during follow up. This will be achieved by amplification of the 'hypervariable' domain using primers located on both sides of the E2/NS1 sequence, followed by nucleotide sequence analysis of the hypervariable domain.

Mother to child HBV and HCV transmission

HBV
Mother to child HBV transmission can be identified by serological tests. PCR may only be of interest in clinical investigations on the transmission of some HBV mutants.

HCV
HCV-RNA is currently the only direct marker used to investigate mother to child HCV transmission. Several studies have indicated that, in most cases, anti-HCV from the mother infected with the virus will persist in newborns until about 12 months after birth. In some rare instances, HCV-RNA has been detected repeatedly in the neonates, indicating HCV transmission. This seems to be a rare event, except for mothers infected by both HCV and HIV (when the level of HCV viraemia is usually higher).67–72 Prospective studies are now required to evaluate the long term consequence of these infections.

Conclusions
Despite the numerous advantages listed in this review, there are still limitations to the routine use of PCR, both in the procedure itself as well as in the interpretation of the results. The Tables IV and V summarise main applications, advantages, and limitations of PCR.

MAIN CLINICAL INDICATIONS FOR PCR IN HBV AND HCV DIAGNOSIS

HBV
PCR can be used for the identification of low level HBV viraemia in HBsAg positive subjects with active liver disease without HBV-DNA positivity in standard hybridisation techniques or evidence of other causes of liver disease. It is also useful for the appraisal of HBV infection in liver transplantation, evaluation of antiviral treatment (although the question of quantification remains), and for the diagnosis of HBsAg negative acute or chronic liver disease. The implications of genetic variability of HBV have yet to be determined.

HCV
PCR can be used for the diagnosis of acute HCV infection and the anti-HCV negative patient with chronic hepatitis, evaluation of HCV viraemia in asymptomatic blood donors with normal alanine aminotransferase activity,

### Table IV

<table>
<thead>
<tr>
<th>Advantages associated with the use of PCR in viral hepatitis</th>
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<tr>
<td>Sensitive estimation of viral multiplication (too sensitive?)</td>
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<tr>
<td>= HBV, HCV, HDV</td>
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<tr>
<td>– evaluation of degree of infection?</td>
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<tr>
<td>-&gt; Quantification?</td>
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<tr>
<td>– follow up of antiviral treatment</td>
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<tr>
<td>Detection of HBV-DNA and HCV-RNA in seronegative patients</td>
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<tr>
<td>– delayed seroconversion in acute HCV infection</td>
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<tr>
<td>– seronegative chronic carriers (HBV and HCV)</td>
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<tr>
<td>Direct evaluation of mother to child HCV transmission</td>
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<tr>
<td>Analysis of genetic variability and its implications (HBV, HCV)</td>
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### Table V

<table>
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<tr>
<th>Limitations of the PCR assay</th>
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<tbody>
<tr>
<td>1 Technical</td>
</tr>
<tr>
<td>False positives (contamination) and false negatives</td>
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<tr>
<td>(quality of DNA and RNA)</td>
</tr>
<tr>
<td>– PCR with HLA (DNA) or cyclic (RNA) primers</td>
</tr>
<tr>
<td>– Amount of serum or plasma used. Conservation.</td>
</tr>
<tr>
<td>– Design of primers (prediction of sequence analysis?)</td>
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<tr>
<td>– Single step (three primers) or nested PCR (one tube assay)?</td>
</tr>
<tr>
<td>– Quantification?</td>
</tr>
<tr>
<td>2 Interpretation</td>
</tr>
<tr>
<td>– Intact or defective genomes?</td>
</tr>
<tr>
<td>– 'Capture' with anti-HBs (HCV) before PCR?</td>
</tr>
<tr>
<td>– Comparison with infectious doses</td>
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<tr>
<td>– Contamination of PBMNC or tissue samples by serum particles?</td>
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<tr>
<td>– In situ hybridisation</td>
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<tr>
<td>– PCR profile with different primers</td>
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<tr>
<td>– Sequence comparison</td>
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diagnosis of anti-HCV positive chronic hepatitis with anti-LKM1 autoantibodies, and the decision of whether or not to treat these patients with interferon. It is also useful in the evaluation of HCV infections after liver transplantation, investigation of mother to child HCV transmission, and follow up of the effects of antiviral treatment (although quantification is still questionable).


