Original Articles

Detection of hepatitis B virus DNA in donor blood by the polymerase chain reaction

J. NANDI, K. BANERJEE

ABSTRACT

Background. Blood collected from voluntary donors at local blood banks and blood donation camps was screened for the hepatitis B virus genome using the polymerase chain reaction and for viral markers by standard serological techniques. The sensitivities of the two screening strategies were compared.

Methods. One hundred and twenty-six blood samples were tested for HBV serological markers—HBsAg, anti-Hbs and anti-HBc—by ELISA. The same samples were also subjected to the polymerase chain reaction using primers made by us.

Results. Analysis of the polymerase chain reaction amplified products revealed that 24% of the blood samples which tested negative for HBsAg using the ELISA technique were positive for HBV DNA by the polymerase chain reaction. All the HBsAg positive samples (by ELISA) were also positive by the polymerase chain reaction (which detected additional samples as well). Anti-HBc antibodies showed a much greater concordance with the polymerase chain reaction.

Conclusions. The results emphasize that the screening strategies for donor blood need to be re-evaluated in order to check inadvertent transmission of hepatitis B virus during blood transfusion. The ELISA technique to detect HBsAg as the sole serological marker is inadequate to indicate the actual prevalence of hepatitis B virus in the donor blood. The polymerase chain reaction may be a better screening test. If this is not available, the detection of anti-HBc antibodies appear to be a better means of screening blood than HBsAg.

INTRODUCTION

Our knowledge of the replication and infectivity of the hepatitis B virus rests primarily on the study of the hepatitis B surface antigen (HBsAg), core antigen (HBcAg), e antigen (HBeAg) and their respective antibodies. Although these serological markers are well characterized, they are inadequate indicators of viral infectivity. The absence of HBsAg in the serum does not exclude the presence of hepatitis B virus (HBV) DNA.[1–5] Determining the prevalence of HBV in apparently healthy individuals needs further evaluation. Blood which tests negative for HBsAg but contains HBV DNA is potentially infective and it is important to prevent the inadvertent transmission of HBV from donor blood during transfusion. The use of the polymerase chain reaction (PCR) may be a more sensitive screening method for donor blood.[6,7] This study investigated the role of PCR to ascertain the actual prevalence of HBV DNA in donor blood and compare the results with those obtained by the ELISA technique.[8,9]

SUBJECTS AND METHODS

Blood samples were collected from 126 voluntary donors at local blood banks and blood donation camps. Tests for HBsAg, anti-HBs and anti-HBc were carried out by ELISA using kits from the National Institute of Virology, Pune, India, Hepanostika (Holland) and Abbott Laboratories (North Carolina, USA). For the PCR assay, DNA was extracted from sera by either the standard phenol/chloroform extraction[10] or the sodium hydroxide (NaOH) extraction method of Kaneko *et al.*[11] The latter is quicker and gives results comparable to phenol/chloroform extraction.

Briefly, 100 to 200 ml of serum was incubated with an equal volume of 0.1M NaOH for 1 hour and centrifuged at 20 000 rpm. The supernatant was neutralized with hydrochloric acid and used as the PCR template. Oligonucleotide primers were synthesized in an automated DNA synthesizer (Gene Assembler Plus, Pharmacia, Sweden) by phosphoramidite chemistry. The primers were purified on FPLC after deprotection. The primer sequences were chosen from a conserved region of the S and S–P overlap regions of the HBV genome. This has been reported to be highly specific for the HBV genome.[12]

Primer 1: (30 mer)

5' – GCGAAGCTTTTAGGGTTTAAATGTATACCT – 3'

Primer 2: (24 mer)

5' – GCG CTG CAG CTA TGC CTC ATC TTA – 3'

The PCR was carried out in a 100 µl assay volume with 2.5 units of Taq polymerase (Perkin Elmer Cetus, USA or Boehringer Mannheim, Germany), 200 µM each of dNTPs (Boehringer Mannheim, Germany) and 100 pM of each

National Institute of Virology, 20-A Dr Ambedkar Road, Post Box No. 11, Pune 411001, India
J. NANDI, K. BANERJEE
Correspondence to J. NANDI

primer (in later runs the concentration of primers was reduced to 20 pM after the necessary titration), 1.5 mM $MgCl_2$, 50 mM KCl and 50 mM Tris-HCl (pH 8.3). A master mix was made, aliquoted appropriately and stored at -20 °C. The reaction was started by adding 10 µL of the template DNA. Negative reagent controls with no primer or no DNA were routinely included in the PCR runs. The positive control was either DNA from HBsAg positive sera or a suitably diluted plasmid containing the entire HBV genome (gifted by Dr Robert Purcell, National Institutes of Health, USA). For PCR denaturation a-temperature of 94 °C for 1 minute, annealing temperature of 55 °C for 1 minute, an extension temperature of 72 °C for 2 minutes and a cycle number of 35 were found suitable. The PCR run was carried out in a programmable thermal cycler (Perkin Elmer Cetus, USA). Analysis of the PCR product was carried out either by agarose gel (3%) or polyacrylamide gel (10%) electrophoresis (PAGE). The DNA bands were visualized by ethidium bromide (EtBr) staining followed by ultraviolet transillumination or silver staining respectively. The PAGE silver staining (Fig. 1) was found to be more sensitive than agarose gel electrophoresis. Appropriate DNA markers were run simultaneously in the gel to ascertain the fragment DNA size of the amplified product (450 bases). The specificities of the amplified products from different sera were compared with that of the positive control, namely plasmid pHH_2 containing an insert of the HBV genome. Serial dilutions of the EcoR1 digest of the plasmid were used to determine the lowest detection limit in the PCR assay. The samples were scored positive by semi-quantitatively estimating the intensity of the amplified band. Absence of a signal in the gel electrophoresis was scored as PCR negative. Sometimes the PCR product showed a doublet pattern (Fig. 2; possibly related to the unusual partially double stranded DNA genome of HBV[13]).Occasionally reannealing events may occur between the PCR target and product resulting in double band amplification.

RESULTS

One hundred and twenty six voluntary donor blood samples were screened by ELISA for the presence of HBsAg, anti-HBs and anti-HBc, and PCR was done to confirm the presence of the HBV genome in the serum. Seven samples were positive for HBsAg while 119 were negative. Of the 126 samples, 3 were positive for anti-HBs and 35 for anti-HBc (Table I). A comparison of the HBsAg status and the results of the PCR is shown in Table II. All the HBsAg positive samples were positive on PCR while 89 of the HBsAg negative samples were negative by PCR. However, 30 HBsAg

Table I. Serological markers of hepatitis B virus in voluntary donor blood samples

Serological markers	Status	*n*	(%)
HBsAg	negative	119	(94)
HBsAg	positive	7	(6)
anti-HBs	positive	3	(2)
anti-HBc	positive	35	(28)

Table II. Comparison of the results of HBsAg and PCR

Serological markers	PCR status	*n*	(%)
HBsAg positive	positive	7	(5.6)
HBsAg negative	negative	89	(70.6)
HBsAg negative	positive	30	(23.8)

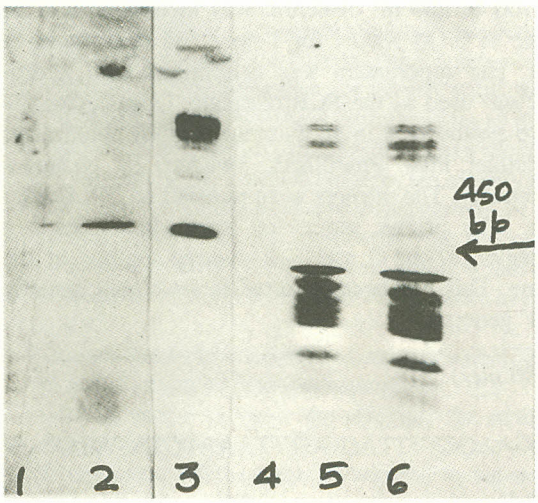

Fig. 1. PCR with primer pair 1 and 2 from conserved S and S-P overlap region (35 cycles). Phast gel continuous gradient PAGE (10% to 20%) with silver staining.

Lane 1 HBV seronegative and PCR negative
2 & 3 HBV seronegative and PCR positive
4 reagent negative control
5 & 6 DNA marker (PBR 322 Hind III + $EcoR_1$ cut)

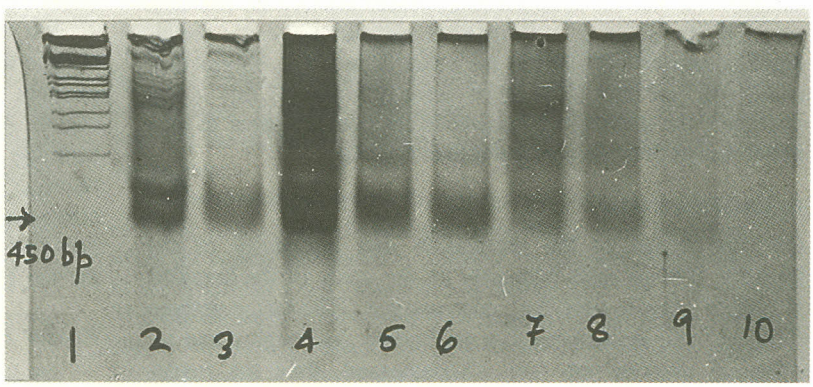

Fig. 2. PAGE (10%) analysis of PCR products of serum sample from donor blood with primer pairs 1 and 2 (35 cycles). Expected fragment size 450 bp.

Lane 1 DNA marker (DNA Hind III and $EcoR_1$ cut)
2 to 6 HBV seropositive and PCR positive
7 & 9 HBV seronegative but PCR positive
8 positive control plasmid pHH_2 with HBV genome DNA
10 reagent negative control

negative samples were positive on PCR. Anti-HBc antibodies detected a larger number of positive samples than HBsAg and correlated well with the PCR results (Table III).

Table III. Comparison of the results of anti-HBc and PCR

Serological markers	PCR status	*n*	(%)
anti-HBc positive	positive	35	(27.8)
anti-HBc negative	negative	89	(70.6)
anti-HBc positive	negative	2	(1.6)

DISCUSSION

In the donor blood samples, anti-HBc was positive in a higher proportion than HBsAg. Our PCR data clearly indicates the higher sensitivity of the test. This has obvious implications since anti-HBc is considered to be a more sensitive serological marker than HBsAg for detecting HBV.[14] Out of the 126 blood samples analysed, 94% were HBsAg negative. The proportion of samples negative for HBsAg but positive for HBV DNA by PCR assay was 24%. This percentage is comparable with that reported in a similar study from Taiwan. The prevalence of HBV DNA in healthy blood donors has been previously shown by slot blot hybridization. A higher percentage of HBV DNA positivity was reported in HBsAg negative samples by using PCR. This is logical considering the greater sensitivity of the technique. In our study we have found many instances in which HBV DNA was found in HBsAg negative donor blood samples. This has important clinical implications. Acute hepatitis has been induced by inoculation of serum negative for HBV serological markers but positive for HBV DNA detected by PCR.[15] The reason for HBsAg negative blood samples to score positive on PCR may be due to the lower detectability of HBsAg by ELISA. Another possibility may be the formation of immune complexes between HBsAg and anti-HBs resulting in the non-availability of HBsAg for detection by ELISA. The formation of immune complexes has been implicated in the pathogenesis of hepatitis.

Even though PCR is not a very cost effective technique, its sensitivity justifies its consideration as a new strategy for screening donor blood. Since our study demonstrates a high correlation between PCR and anti-HBc detection, the latter may be adopted where PCR cannot be carried out due to lack of facilities or cost restraints. In major laboratories of the country, where facilities exist for PCR, the technique should be used for validating screening procedures based on serological techniques.

ACKNOWLEDGEMENTS

The authors are grateful to the Janakalyan and Red Cross Blood Banks, Pune, for donor blood samples. The interaction of Dr K. N. Ganesh and Dr A. Natu of the Organic Chemistry Division, National Chemical Laboratory, Pune, is acknowledged with gratitude. Thanks are due to Ms Varsha Bhavalkar for excellent technical assistance.

REFERENCES

1 Brechot C, Degos F, Lugassy C, *et al*. Hepatitis B virus DNA in patients with chronic liver disease and negative tests for hepatitis B surface antigen. *N Engl J Med* 1985;**312:**270–6.
2 Sun CF, Pao CC, Wu SY, Liaw YF. Screening for hepatitis B virus in healthy blood donors by molecular DNA hybridization analysis. *J Clin Microbiol* 1988;**26:**1848–52.
3 Shafritz DA, Lieberman HM, Isselbacher KJ, Wands JR. Monoclonal radioimmunoassays for hepatitis B surface antigen. Demonstration of hepatitis B virus DNA or related sequences in serum and viral epitopes in immune complexes. *Proc Natl Acad Sci USA* 1982;**79:**5675–9.
4 Thiers V, Nakajima E, Kremsdorf D, *et al*. Transmission of hepatitis B from hepatitis B seronegative subjects. *Lancet* 1988;**2:**1273–6.
5 Lai ME, Farci P, Figus A, Balestrieri A, Arnone M, Vyas GN. Hepatitis B virus DNA in the serum of Sardinian blood donors negative for the hepatitis B surface antigen. *Blood* 1989;**73:**17–19.
6 Wang J-T, Wang T-H, Sheu J-C, *et al*. Detection of hepatitis B virus DNA by polymerase chain reaction in plasma of volunteer blood donors negative for hepatitis B surface antigen. *J Infect Dis* 1988;**163:**397–9.
7 Larzul D, Guigue F, Srinsky JJ, Mack DH, Brechot C, Guesdon JL. Detection of hepatitis B virus sequence in serum by *in vitro* enzymatic amplification. *J Virol Methods* 1988;**20:**227–37.
8 Kaneko S. State of HBV DNA in liver of patients with HBV surface antigen positive liver disease its variation and its correlation with HBV serological markers. *Acta Hepatol Jpn* 1985;**26:**846–55.
9 Chemin I, Baginski I, Petit I, *et al*. Correlation between HBV DNA by polymerase chain reaction and pre-S1 antigenemia in symptomatic and asymptomatic hepatitis B virus infections. *J Med Virol* 1991;**33:**51–7.
10 Maniatis TF, Fristsch EF, Sambrook J. In: *Molecular cloning: A laboratory manual*. New York:Cold Spring Harbor Laboratory, 1985:458.
11 Kaneko S, Feinstone SM, Miller RH. Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. *J Clin Microbiol* 1989;**9:**1930–3.
12 Larzul D, Chevrier D, Thiers V, Guesdon JL. An automatic modified polymerase chain reaction procedure for hepatitis B virus DNA detection. *J Virol Methods* 1990;**27:**49–60.
13 Malter JS, Gerber MA. Polymerase chain reaction for hepatitis B virus DNA. *Hepatology* 1991;**13:**188–90.
14 Kaneko S, Miller RH, Bisceglie AMD, Feinstone SM, Hoofnagle JH, Purcell RH. Detection of hepatitis B virus DNA in serum by polymerase chain reaction. *Gastroenterology* 1990;**99:**799–804.
15 Kaneko S, Miller RH, Feinstone SM, *et al*. Detection of serum hepatitis B virus DNA in patients with chronic hepatitis using the polymerase chain reactions assay. *Proc Natl Acad Sci USA* 1989;**86:**312–16.