JHKMTA 1999/2000; 8: 25-31

Quantification of Serum Hepatitis B Virus DNA

Stephen K.N. HO and Tak-mao CHAN

Division of Nephrology, Department of Medicine, The University of Hong Kong,
Queen Mary Hospital, Hong Kong

Abstract

Hepatitis B virus (HBV) infection in an important cause of acute and chronic viral hepatitis. Worldwide there are more than 300 million chronic carriers of HBV, with 100 million carriers in China. In addition to serology markers and liver enzymes, the detection and quantification of HBV DNA in serum appear to be the most reliable methods for monitoring HBV infection and assessing responses to antiviral treatment. This review will discuss the methodology, sensitivity, technical aspects, turn-around time and the clinical applications of the different HBV DNA assays.

Key words: HBV DNA. Quantitation PCR. Real-time PCR.

Introduction

Although the institution of vaccination programmes has decreased the incidence of new HBV infections in many areas, HBV infection remains a significant global health problem with the number of chronic HBV carriers at more than 300 million worldwide. The HBV carrier rate in Hong Kong is about 9 %. Chronic HBV infection may result in an asymptomatic carrier state, reactive fulminant hepatitis, cirrhosis, or hepatocellular carcinoma.

The structure of HBV consists of a partly double-stranded circular DNA virus of the class Hepadnavirus[1,2]. The complete infectious virion (Dane particle) consists of a 42 nm spherical envelope carrying the HBsAg, a 27 nm inner shell of nucleocapsid with the hepatitis B core antigen (HBcAg), and a processing derivative hepatitis B e antigen (HBeAg). The inner nucleocapsid contains the viral genome and its tightly associated DNA polymerase. The partly double-stranded circular DNA genome is composed of approximately 3200 base pairs (Figure 1)[3]. The long DNA strand (L-) is the coding strand carrying four primary translational open reading frames: S, for the surface, pre-S1 and pre-S2 genes; C, for the core and pre-C genes; P, for the polymerase and reverse transcriptase genes; and X, for the X gene that activates viral and cellular promoters. The short DNA strand (S+) is of variable length capable of elongation at its 3'end by the activation of specific DNA polymerase to form a double-stranded molecule with the L- strand[4].

The conventional diagnosis of the status of viral replication after HBV infection is based on the detection of serological markers HBsAg, anti-HBs, anti-HBc, IgM anti-HBc, HBeAg, and anti-HBe, which can indicate the stage of infection, and thus the degree of infectivity, as well as the individual's immune status[5,6]. However, the reliability of these serological tests can be influenced by the host's immune responsiveness. In addition, testing for HBeAg can not detect the presence or absence of viraemia in infections by pre-core or core promoter HBV mutants[7-9]. Rarely is HBV DNA detected in individuals sero-negative for HBsAg[10-12]. Whereas the measurement of liver enzymes reflects hepatocyte lysis, the detection of HBV DNA in serum provides direct evidence of viraemia. Recently, it has also been suggested that the determination of the virus load may provide clinically useful information during the course of infection. Moreover, monitoring of the changes in viral level during therapy of infected patients with interferon alpha and/or nucleoside analogues is of particular importance for the response in terms of virus elimination, viral reactivation, or development of drug resistant viruses[13-17].

DETECTION OF HBV DNA IN SERUM

Direct nucleic acid hybridization or polymerase chain reaction (PCR) assays are commonly used in the detection and quantification of serum HBV DNA. Examples of the former include dot blot hybridization and solution hybridization. Solution hybridization assays can utilize radioactivity (e.g. Genostics HBV

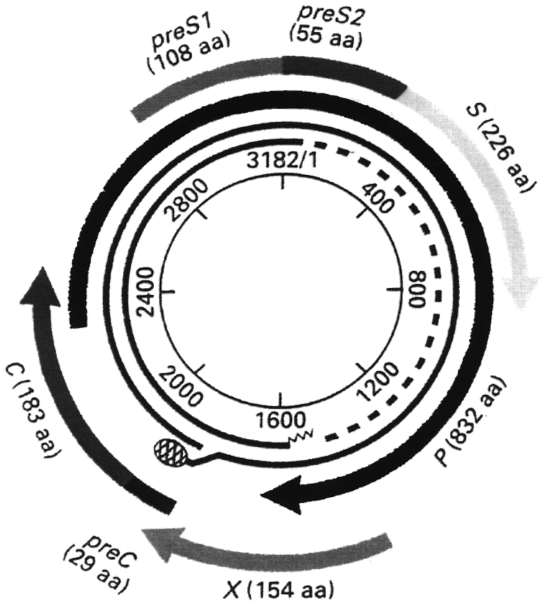

Figure 1. Diagrammatic representation of the hepatitis B virus genome. The viral DNA is partially double-stranded. The long strand of fixed length encodes seven proteins from four overlapping reading frames (surface S, core C, polymerase P, and the X gene X), and three up-steam regions (pre-C, pre-S1, and pre-S2). [aa: amino acids] (Reproduced from reference 3, Lee WM: N Engl J Med 1997; 337(24): 1733-45)

DNA, Abbott Laboratories), antibody capture (e.g. Hybrid Capture HBV DNA Tests, Digene Corp.), branched DNA (bDNA) signal detection systems (e. g. Quantiplex HBV DNA, Chiron Corp.), or photoactive cross-linking (NAXCOR) techniques. In general, assays based on PCR methodology (e.g. Amplicor HBV Monitor Test, Roche Diagnostics) are capable of detecting and quantifying lower levels of HBV DNA. This review will discuss the methodology, the technical aspects, and the clinical applications of the different HBV DNA assays.

Detection of HBV DNA by Direct Nucleic Acid Hybridization

Dot blot hybridization

Dot blot hybridization (DB) has been used to detect HBV DNA in serum since the early 1980's[18,19]. The size and intensity of the hybridized dot on the autoradiogram semi-quantitatively indicates the amount of HBV DNA. The process has a sensitivity of approximately 2.85×10^6 copies/ml or 10 pg/ml, and requires an assay time of 4-7 days. ^{32}P radio-labeled probe was originally used for hybridization. With the use of non-radioactive probes such as alkaline phosphatase, digoxigenin, FITC and biotin, the assay time has been shortened to 2-3 days[20,21]. Imaging densitometry can be applied for more accurate and objective measurement of results[22,23]. However, DB assays have variable sensitivity and low reproducibility. Nevertheless, it can handle a large number of samples concurrently, and is relatively inexpensive.

Solution hybridization

Another method of detection uses solution hybridization with a radio-labeled probe. An example of this is the Genostics HBV DNA assay (Abbott Laboratories, North Chicago, IL, USA). Double-stranded HBV DNA in serum is denatured into single-stranded DNA and then allowed to hybridize with ^{125}I-labeled single-stranded HBV DNA probes in solution for 18 hours at 65°C. ^{125}I-labeled hybridization products are eluted with a sepharose column and radioactivity is measured in a scintillation counter[24]. Although the assay is simple to perform, the use of only one standard has resulted in sub-optimal reliability and reproducibility of results[25,26].

Signal Amplification by bDNA

The Quantiplex HBV DNA assay (bDNA) (Chiron Corp., Emeryville, CA, USA) is a sandwich nucleic acid hybridization assay that utilizes branched DNA (bDNA) for signal amplification (Figure 2)[27]. HBV DNA in serum is first released and denatured by the

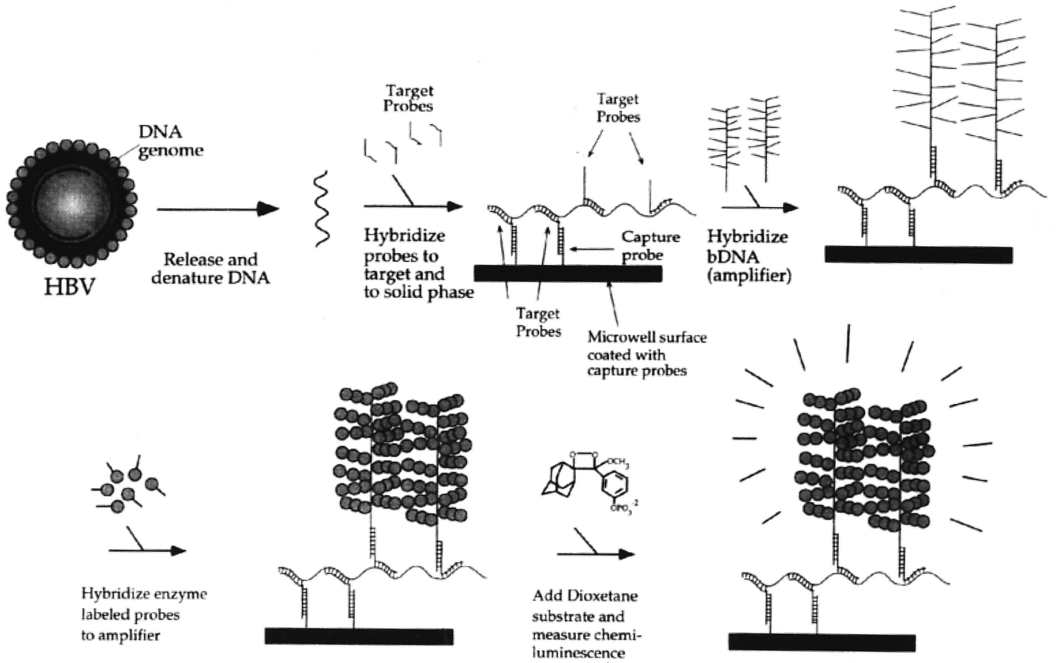

Figure 2. Schematic representation of the branched DNA (bDNA) assay for HBV DNA quantitation. (Reproduced from reference 27, Hendricks DA, et al: Am J Clin Pathol 1995; 104: 537-46)

addition of a lysing agent and heating. It then hybridizes with two different sets of probes complementary to the conserved regions of the surface gene and core gene respectively. One set of probes also binds to microplate wells while the free ends of the other set of probes bind to the synthetic bDNA. Each bDNA molecule has multiple repeat sites for the binding of alkaline phosphatase labeled probes. The amount of HBV DNA captured is indicated by chemiluminescence after the addition of dioxetane substrate. This assay takes approximately 24 hours to perform and the detection range is $7x10^5$ – $5x10^9$ copies/ml (2.5 – 17,700 pg/ml). Apart from the increased sensitivity, the bDNA assay has demonstrated good reproducibility. Intra and inter-assay coefficients of variation have been found to be less than 15%[27-29].

Ultraviolet (UV) cross-linking hybridization

The use of UV results in a highly specific and rapid hybridization process of approximately 30 minutes. An example is the NAXCOR HBV DNA assay (NAXCOR, Menlo Park, California, USA), which can measure HBV DNA concentrations of $5x10^5$ – $5.7x10^8$ copies/ml (2 – 2000 pg/ml) in 6 hours[30,31].

RNA: DNA hybrid capture (HC)

Very specific RNA:DNA hybrids are formed between the HBV DNA in serum and RNA probes with specificity to HBV ad and ay strains. An example of this is the Digene HC HBV DNA test (Digene Diagnostics, Silver Springs, MD, USA). Using an anti-RNA:DNA antibody, the hybrids are captured onto the surface of microplate wells. A second set of alkaline phosphatase conjugated anti-RNA:DNA antibodies then binds to these immuno-captured hybrids. The chemiluminescence after the addition of substrate is measured on a luminometer (Figure 3). The first-generation Digene HC HBV DNA test has a detection range of $1.4x10^6$ – $5.7x10^8$ copies/ml (5 - 2000 pg/ml) and an assay time of 5 hours. The second-generation assay (Digene HC II HBV DNA test) shows improved sensitivity and detection range ($1.4x10^5$ – $1.7x10^9$ copies/ml, or 0.5 – 6000 pg/ml), and a shorter turn around time of 3.5 hours. More recently, the HC II assay also offers an ultra sensitive format which can quantify HBV DNA concentrations down to 5000 copies/ml (17 fg/ml), made possible by an initial concentration step of high speed centrifugation for 2 hours. Apart from the relatively simple technical requirements, the HC II assay also has low variability and high specificity[32-34].

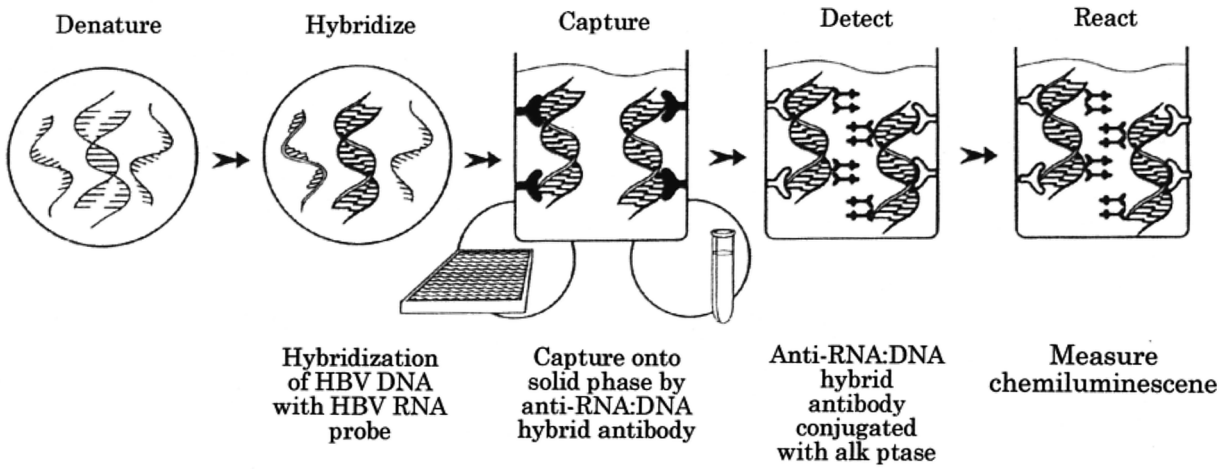

Figure 3. Schematic representation of the RNA:DNA hybrid capture assay. (Reproduced with permission from Digene Diagnostics, Silver Springs, MD, USA)

Detection of HBV DNA by Polymerase Chain Reaction (PCR)

The most sensitive method of HBV DNA detection involves HBV DNA extraction, target sequence amplification by PCR, and detection of the PCR product[35-38]. There have been modifications aiming to improve specificity and reduce contamination. The extraction and denaturation of HBV DNA from serum samples can be achieved by phenol chloroform precipitation[39], alkaline treatment[36], microwave treatment[40,41] or with commercial DNA isolation kit. The number of PCR cycles and the primers which target different regions of the surface can vary with different assays. The amplified DNA product is usually detected by ethidium bromide staining following gel electrophoresis, and the sensitivity is around $3x10^4$ copies/ml (0.1 pg/ml). In theory, improved sensitivity of detection down to 100 copies/ml (0.3 fg/ml) should be achievable when the amplified DNA product is measured by dot blot assay (PCR-DB)[42,43], by DNA enzyme immunoassay (PCR-DEIA)[44], or by nested PCR (nPCR)[45]. However, PCR based methods in general have significant inter-assay and inter-laboratory variability[46]. Qualitative HBV DNA detection by PCR is useful when serological markers yield non-conclusive results. Although a negative result may indicate the absence of HBV in serum, the sensitivity of this assay as well as the possibility of the virus resident in infected cells should be considered. On the other hand, PCR methodology has also been incorporated into quantitative HBV DNA assays.

Quantitative Polymerase Chain Reaction (qPCR)

Competitive PCR is used in these assays for HBV DNA quantification. An example is the Amplicor HBV Monitor Test (Roche Diagnostics, Branchburg, NJ, USA), in which common biotinylated primers allow co-amplification of HBV DNA and a known amount of an internal standard. The primers amplify a 104 base-pair (bp) sequence in the conserved pre-C/C gene region of the HBV genome, and the synthetic internal standard is double-stranded DNA with a 21-mer internal sequence different from the amplified HBV sequence. The PCR products are quantified by hybridization in microplate wells with a dinitrophenyl-labeled HBV specific probe and another dinitrophenyl-labeled probe with specificity for the internal standard, and colorimetric immunoenzymatic detection. Sensitivity of the Amplicor HBV assay is 1000 copies/ml (3.5 fg/ml) and the assay time is 6 hours. An automated assay with the COBAS analyzer can detect HBV DNA at 200 copies/ml (0.7 fg/ml) in 4 hours[47,48].

Real-time PCR

Recent advances in PCR instrumentation and fluorometric detection of PCR products have enabled the detection of PCR product during each amplification cycle using real-time PCR instrumentation. This is possible through the use of specially designed fluorescent probes such as the TaqMan probe, hybridization probes, and molecular beacons. Examples of such systems are the ABI PRISM 7700 Sequence Detection System (PE

Applied Biosystems, Foster City, Calif., USA), the Light Cycler (Roche Diagnostics, Mannheim, Germany) and the iCycler (Bio-Rad Laboratories). Continuous fluorescence measurement and quantification of the amplified products is generated using laser technology or other light detecting systems. Recent studies have shown that these systems can quantify HBV DNA within 5 hours from 200 to $2x10^9$ copies/ml with high reproducibility and specificity[49-50], thus covering the detection range of earlier qPCR assays and the hybridization assays. Besides a wide range of linearity, amplification and signal detection take place in a closed tube system which does not require post-handling of PCR product, thereby reducing the risk of contamination. Furthermore, this methodology may allow for simultaneous detection of more than one virus[50].

CLINICAL APPLICATIONS OF HBV DNA ASSAYS

The PCR method is a sensitive assay to detect low level viraemia, but the clinical usefulness of qualitative PCR may be limited to rare situations when serological markers yield non-conclusive results. Quantification of viraemia is important in the selection of patients for antiviral therapy and to monitor them during treatment. Prior to the availability of quantitative assays for HBV, HBeAg was often used as a surrogate marker of the replicative status. The indirect nature and the presence of pre-core or core promoter mutants present obvious disadvantages of this marker. Furthermore, while disappearance of both HBV DNA and HBeAg from serum correlate with clinical and biochemical improvements[51], HBV DNA is lost earlier than HBeAg and is a more direct index of response to therapy[52]. Although DB hybridization assays are less costly and are able to handle a large set of samples, they do not have sufficient sensitivity and accuracy to corroborate low-grade replication, which is especially important in patients under treatment. The improved sensitivity and reproducibility of nucleic acid hybridization tests with bDNA, UV cross-linking, or hybrid capture confer clinical usefulness, especially in serial monitoring of HBV DNA levels, e.g. during anti-viral treatment or to look for increased viral replication after immunosuppression[53]. Nevertheless, cost, technical complexity, and assay turn-around time remain valid concerns. Based on these considerations, the second generation hybrid capture (HCII) assay appears superior to other non-PCR based assays because of its technical simplicity, rapidity (5.5 hours), and sensitivity (5000 copies/ml, or 17 fg/ml). The cost, technical and hardware requirements of quantitative PCR assays are inherent restrictions on their general clinical use, while the linearity and widened detection range make them valuable research tools.

References

1. Blumberg BS. Australia antigen and the biology of hepatitis B. Science 1977; 197: 17-25.
2. Purcell RH. The Discovery of the hepatitis viruses. Gastroenterol 1993; 104: 955-63.
3. Lee WM. Hepatitis B Virus Infection. N Engl J Med 1997; 337(24): 1733-45.
4. Lau JYN, Wright TL. Molecular virology and pathogenesis of hepatitis B. Lancet 1993; 342: 1335-40
5. Hoofnagle JH, Waggoner JG. Hepatitis A and B virus markers in immune serum globulin. Gastroenterology 1980; 78: 259-63.
6. Miller RH, Kaneko S, Chung CT, Girones R, Purcell RH. Compact organization of the hepatitis B virus genome. Hepatology 1989; 9: 322-27.
7. Blum HE. Hepatitis B virus: Significance of naturally occurring mutants. Intervirology 1993; 35: 40-50.
8. Omata M, Ehata T, Yokosuka O, Hosada K, Ohto M. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med 1991; 324: 1699-1704.
9. Lopez-Alcorocho JM, Moraleda G, Bartolome J, et al. Analysis of hepatitis B precore region in serum and liver of chronic hepatitis B virus carriers. J Hepatol 1994; 21: 353-60.
10. Zhang YY, Hansson BG, Kuo LS, Widell A, Nordenfelt E. Hepatitis B virus DNA in serum and liver is commonly found in Chinese patients with chronic liver disease despite the presence of antibodies to HBsAg. Hepatology 1993; 17: 538-44.
11. Chung HT, LaiCL, Lok AS. Pathogenic role of hepatitis B virus in hepatitis B surface antigen-negative decompensated cirrhosis. Hepatology 1995; 22: 25-9.
12. Gomes SA, Yoshida CF, Niel C. Detection of hepatitis B virus DNA in hepatitis B surface antigen-negative serum by polymerase chain reaction: evaluation of different primer pairs and conditions. Acta Virol 1996; 40: 133-8.

13. Chan TM, Wu PC, Li FK, Lai CL, Cheng IK, Lai KN. Treatment of fibrosing cholestatic hepatitis with lamivudine. Gastroenterology 1998: 115: 177-81
14. Perrillo RP, Schiff ER, Davis GL, et al. A randomized, controlled trial of interferon alfa-2b alone and after prednisone withdrawal for the treatment of chronic hepatitis B. N Engl J Med 1990; 323: 295-301.
15. Lai CL, Chien RN, Leung NW, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia hepatitis lamivudine study group. N Engl J Med 1998; 339: 61-68.
16. Omata M. Treatment of chronic hepatitis B infection. N Engl J Med 1998; 339: 114-15.
17. Malik AH, Lee WM. Chronic hepatitis B virus infection: treatment strategies for the next millennium. Ann Intern Med 2000; 132: 723-31.
18. Bonino F, Hoyer B, Nelson J, Engle R, Verne G, Gerin GL. Hepatitis B virus DNA in the sera of HBsAg carriers: A marker of active hepatitis B virus replication in the liver. Hepatol 1981; 1: 386-91.
19. Scotto J, Hadchouel M, Hery C, Yvart J, Tiollais P, Brechot C. Detection of hepatitis B virus DNA in scrum by a simple spot hybridization technique: comparison wit results for other viral markers. Hepatol 1983; 3: 279-284.
20. Valentine-Thon E, Steinmann J, Arnold W. Detection of hepatitis B virus DNA in serum with nucleic acid probes labelled with 32P, biotin, alkaline phosphatase or sulphone. Mol Cell Probes 1991; 5: 299-305.
21. Akar A, Bournique B, Scholler R. Detection of hepatitis B virus DNA in serum by a nonisotopic hybridization technique. Clin Chem 1992; 38: 1352-5.
22. Weller IV, Fowler MJ, Monjardino J, Thomas HC. The detection of HBV-DNA in serum by molecular hybridisation: a more sensitive method for the detection of complete HBV particles. J Med Virol 1982; 9: 273-80.
23. Lin HJ, Wu PC, Lai CL, Leong S. Molecular hybridization study of plasma hepatitis B virus DNA from different carriers. J Infect Dis 1986; 154: 983-9.
24. Kuhns MC, McNamara AL, Cabal CM, et al. New Assay for the quantitative detection of hepatitis B viral DNA in human serum. Viral Hepatitis and Liver Disease 1988; 258-62.
25. Kapke GF, Watson G, Sheffler S, Hunt D, Frederick C. Comparison of the Chiron Quantiplex branched DNA (bDNA) assay and the abbot Genostics solution hybridization assay for quantification of hepatitis b viral DNA. J Viral Hep 1997; 4: 67-75.
26. Butterworth LA, Prior SL, Buda PJ, Faoagali JL, Cooksley NG. Comparison of four methods for quantitative measurement of hepatitis B viral DNA. J Hepatol 1996; 24: 686-91.
27. Hendricks DA, Stowe BJ, Hoo BS, et al. Quantitation of HBV DNA in human serum using a branched DNA (bDNA) signal amplification assay. Am J Clin Pathol 1995; 104: 537-46.
28. Zaaijer HL, ter Borg F, Cuypers HTM, Hermus MC, Lelie PN. Comparison of methods for detection fo hepatitis B virus DNA. J Clin Microbiol 1994; 32: 2088-91.
29. Lai VCH, Lai CL, Low BG, Lau JYN, Wu PC. Quantitative detection of serum HBV DNA in Chinese patients. J Viral Hep 1997; 4: 359-62.
30. Wood M, Abagli D, Cheng P, Huan B, Van Atta R. Nucleic crosslinking probes for DNA/RNA diagnostics. Clin Chem 1996; 42: S196.
31. Lai VCH, Guan R, Wood ML, Lo SK, Yuen MF, Lai CL. Nucleic acid-based cross-linking assay for detection and quantification of hepatitis B virus DNA. J Clin Microbio 1999; 37: 161-4.
32. Garcia-Meijide M, Bril J, Eddington N, Zhu J, Makar A, Lorincz AT. Performance characteristics of the Digene hybrid capture II hepatitis B DNA test. Hepatology 1998; 28: 486A (Abstract 1295).
33. Garcia-Meijide M, Eddington N, Brill J, et al. Muti-center evaluation of the second-generation digene hybrid capture II HBV test. Hepatology 1998; 28: 579A(Abstract 1666).
34. Ho SKN, Chan TM, Cheng IKP, Lai KN. Comparison of the second generation Digene hybrid capture assay with branched DNA assay for the measurement of hepatitis B virus DNA in serum. J Clin Microbiol 1999; 37: 2461-65.
35. Larzul D, Guigue F, Sninsky JJ, Mack DH, Brechot C, Guesdon JL. Detection of hepatitis B virus sequences in serum by using in vitro enzymatic amplification. J Virol Methods 1988; 20: 227-37.
36. Kaneko S, Miller RH, Feinstone SM, et al. Detection of serum hepatitis B virus DNA in patients with chronic hepatitis using the polymerase chain reaction assay. Proc Natl Acad Sci USA 1989; 86: 312-6.
37. Ulrich PP, Bhat RA, Seto B, Mack D, Sninsky J, Vyas GN. Enzymatic amplification of hepatitis B

virus DNA in serum compared with infectivity testing in chimpanzees. J Infect Dis 1989; 160: 37-43.
38. Liang TJ, Isselbacher KJ, Wands JR. Rapid identification of low level hepatitis B-related viral genome in serum. J Clin Invest 1989 84: 1367-71.
39. Yokosuka O, Omata M, Hosoda K, Tada M, Ehata T, Ohto M. Detection and direct sequencing of hepatitis B virus genome by DNA amplification method. Gastoenterology 1991; 100: 175-81.
40. Cheyrou A, Guyomarc'h C, Jasserand P, Blouin P. Improved detection of HBV DNA by PCR after microwave treatment of serum. Nucleic Acids Res 1991; 19: 4006.
41. Costa J, Rodes J, Jimenez de Anta MT, et al. Microwave treatment of serum facilitates detection of hepatitis B. J Hepatol 1995; 22: 35-42.
42. Theilmann L, Fischer M, Galle PR, Nassal M. Detection of HBV DNA in HbsAg-postive sera after amplification using the polymerase chain reaction. Liver 1989; 9: 322-8.
43. Zeldis JB, Lee JH, Mamish D, et al. Direct method for detecting small quantities of hepatitis B virus DNA in serum and plasma using polymerase chain reaction. J Clin Invest 1989; 84: 1503-8.
44. Garcia F Jr, Garcia F, Bernal MC, Leyva A, Piedrola G, Maroto MC. Evaluation of enzyme immunoassay for hepatitis B virus DNA based on anti-double-standed DNA. J Clin Microbiol 1995; 33: 413-5.
45. Chung HT, Lok ASF, Lai CL. Re-evaluation of alpha-interferon treatment of chronic hepatitis B using polymerase chain reaction. J Hepatol 1993; 17: 208-14.
46. Quint WG, Niesters HG, Gerlich WH, Schirm J, Heijtink RA. Reliability of methods for hepatitis B virus DNA detection. J Clin Microbiol 1995 ; 33: 225-8.
47. Ranki M, Schatzl HM, Zachoval R, Uusi-Oulari M, Lehtovaara P. Quantification of hepatitis B virus DNA over a wide range from serum for studying viral replicative activity in response to treatment and in recurrent infection. Hepatology 1995; 21: 1492-9.
48. Gerken G, Gomes J, Lampertico P, et al. Clinical evaluation and application of the amplicor HBV Monitor test a quantitative HBV DNA PCR assay. J Virol Methods 1998; 74: 155-65.
49. Abe A, Inoue K, Tanaka T, et al. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J Clin Microbiol 1999; 37: 2899-903.
50. Mercier B, Burlot L, Ferec C. Simultaneous screening for HBV DNA and HCV RNA genomes in blood donations using a novel TaqMan PCR assay. J Virol Methods 1999; 77: 1-9.
51. Brook MG, Petrovic L, McDonald JA, Scheuer PJ, Thomas HC. Histological improvement after anti-viral treatment for chronic hepatitis B virus infection. J Hepatol 1989; 8: 218-225.
52. Perrillo R, Mimms L, Schechtman K, Robbins D, Campbell C. Monitoring of antiviral therapy with quantitative evaluation of HBeAg: A comparison with HBV DNA testing. Hepatology 1993; 18: 1306-12.
53. Chan TM, Li FK, Lui SL, Cheng IKP, Lai KN. Treatment of hepatitis B exacerbation in renal allograft recipients with lamivudine. Abstracts of the XVII World Congress of the Transplantation Society 1998, p.68 (abstract 252).

Correspondence to: Mr Stephen K.N. Ho