Comparison of ultracentrifugation and polyethylene glycol precipitation for concentration of hepatitis B virus (HBV) DNA for molecular hybridisation tests and the relationship of HBV–DNA to HBe antigen and anti-HBe status

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Summary. A ^{32}P -labelled DNA probe was used to examine 50 hepatitis B surface antigen (HBsAg)-positive sera for the presence of hepatitis B virus (HBV) DNA. HBV–DNA was detected in all 21 HBeAg-positive samples, in one out of 21 anti-HBe-positive samples and in three out of eight HBeAg- and anti-HBe-negative samples. The results of this DNA hybridisation test correlated well with HBeAg status and could be used to determine infectivity in HBeAg- and anti-HBe-negative samples. Ultracentrifugation was marginally superior to polyethylene glycol precipitation for concentrating HBV–DNA from serum.

Introduction

The laboratory diagnosis of acute hepatitis B and detection of the chronic carrier state of hepatitis B virus (HBV) depends upon serological tests. Hepatitis B surface antigen (HBsAg) is the first serological marker to appear in clinical infection¹ and is followed by HBeAg, a core-associated antigen that correlates closely with the number of virus particles, the presence of free HBV–DNA in the serum, and relative infectivity.² Antibody to HBe (anti-HBe) is not protective but is associated with lower infectivity.

The presence of HBV–DNA in serum can be detected with a ^{32}P -labelled DNA probe. This was first done in 1980 with human and woodchuck hepatitis agents.³ In previous studies in which molecular hybridisation methods were used to detect HBV–DNA in serum,^{4–6} close correlation was found between the presence of either HBeAg or the DNA polymerase enzyme, as measured by radio-immunoassay; DNA hybridisation was a more sensitive means of detecting complete virus particles as well as for monitoring antiviral therapy.⁷

Several techniques have been used for harvesting HBV–DNA from serum including pelleting the Dane particles by ultracentrifugation and precipitation with polyethylene glycol. Ultracentrifugation has proved to be superior to other methods of DNA extraction such as gel electrophoresis or direct application of serum samples to a nitrocellulose membrane because it gives a better autoradiography signal and minimises interference by serum components.⁸

The purpose of this study was to examine the correlation between the presence of HBV–DNA and HBeAg and anti-HBe status in 50 HBsAg-positive serum samples, and to compare ultracentrifugation and polyethylene glycol precipitation methods of concentrating HBV–DNA in serum samples.

Materials and methods

Serum samples

Fifty HBsAg-positive serum samples were selected for examination; HBsAg was detected by enzyme-linked immunosorbent assay (ELISA) (Wellcome Diagnostics). Some were from healthy blood donors and some from patients with acute or chronic hepatitis. All were tested for HBeAg and anti-HBe by ELISA with reagents from the Division of Microbiological Reagents and Quality Control (Central Public Health Laboratory, Colindale Avenue, London). Twenty-one samples gave positive results for HBeAg only, 21 for anti-HBe only and eight samples gave negative results for both. In addition, of the original 50 sera, 10 were paired sera from five patients who were initially HBeAg-positive and then became anti-HBe-positive.

DNA extraction and hybridisation

HBV–DNA was extracted from virus particles obtained from the serum samples by ultracentrifugation or polyethylene glycol (PEG) precipitation.

Ultracentrifugation. The Dane (virus) particles were pelleted by centrifugation at 160 000 *g* for 4 h at 4°C. The pellet was resuspended in buffer for DNA extraction.

Polyethylene glycol precipitation. To 100 µl of serum, 50 µl of polyethylene glycol 6000 (BDH, Poole) 30% was added and left overnight at 4°C. The sample was then centrifuged for 30 min at 1000 *g* and the pellet was resuspended in buffer for DNA extraction.

The HBV-DNA was extracted and detected by dot-blotting on a nitrocellulose membrane soaked in buffered salt solution (BSS; 3 M NaCl, 0.3 M sodium citrate). Double stranded DNA in the sample was denatured by adding 200 µl of 1 M NaOH to 100 µl of virus suspension. The samples were transferred to the nitrocellulose membrane and neutralised by adding 200 µl of 0.5 M HCl to each well of the dot-blot apparatus (BioRad, Hemel Hempstead). As a positive control, dilutions of an HBV-DNA standard (obtained from a pool of laboratory positive samples) were made at concentrations of 100 pg, 10 pg, 5 pg, 1 pg and 0.5 pg and applied to the nitrocellulose membrane as above. The membrane was then baked at 80°C for 2 h after which it was treated to prevent non-specific binding by adding 15 ml of skimmed milk (0.25% v/v) in BSS and incubating overnight at 65°C.

The DNA probe (kindly donated by Dr J. Monjardino, Royal Free Hospital, London) was labelled by the random priming method of Klenow.⁹ The specific activity of the labelled probe was measured in a scintillation counter and gave a value of 1.2×10^9 cpm/pg of DNA. The probe was added to the nitrocellulose membrane with 8 ml of skimmed milk 0.25% in BSS and left to hybridise overnight at 65°C. After one low and one high stringency wash in BSS, the nitrocellulose membrane was overlaid with photographic film (Fuji RXG) and held at -70°C for 24 h before developing. The cut-off for positivity was a dot intensity greater than that of the 0.5-pg standard.

Results

A positive dot-blot result for HBV-DNA was obtained with 25 specimens after ultracentrifugation preparation and from 23 specimens after PEG precipitation. All 21 HBeAg-positive specimens gave positive dot-blot results for HBV-DNA after preparation by ultracentrifugation and 20 of them gave

positive results after PEG precipitation. Only one of 21 anti-HBe-positive but HBeAg-negative samples gave a positive dot-blot for HBV-DNA; this was from a sample prepared by ultracentrifugation. There was no noticeable difference in the intensity of autoradiography signals between dot-blot preparations from specimens harvested by ultracentrifugation and by PEG precipitation.

In the 10 paired serum samples from five patients who were initially HBeAg-positive and then became anti-HBe-positive, the initial HBeAg-positive samples gave positive dot-blot results for HBV-DNA and the anti-HBe-positive samples gave negative results for HBV-DNA in each case.

Discussion

Our results show a strong positive correlation between the presence of HBV-DNA and HBeAg, confirming previous studies.^{5,6,10} HBV-DNA was detected in samples prepared by ultracentrifugation from 21 HBeAg-positive sera. It would be reasonable to assume that the presence of HBV-DNA is responsible for the greater infectivity of HBeAg-positive sera. There was also a strong negative correlation between the presence of anti-HBe and HBV-DNA. In only one sample was a small amount of HBV-DNA detected and this specimen had previously given only a low positive result in the anti-HBe ELISA.

Our results are consistent with the evidence that viral DNA disappears from the serum and becomes integrated into hepatocytes in anti-HBe-positive carriage¹¹ and that active virus replication does not occur in such circumstances. However, they differ from the results of previous studies which showed that a substantial proportion of patients with anti-HBe may have free HBV-DNA in their serum.^{6,10} It is possible that more anti-HBe-positive samples may have given an autoradiograph signal in the dot-blot analysis (albeit weakly) if the exposure time of the film had been prolonged to (e.g.) 48 h. Furthermore, the number of specimens in our study was small. However, the overall correlation was well demonstrated in the

Table. Distribution of HBV-DNA-positive specimens prepared by ultracentrifugation or PEG precipitation in relation to HBeAg status

HBeAg status	Number of samples tested	Number of samples that were HBV-DNA positive after preparation by	
		UCF	PEG
HBe Ag + ve	21	21	20
Anti-HBe + ve	21	1	0
HBeAg-ve, anti-HBe-ve	8	3	3
Total	50	25	23

UCF, ultracentrifugation; PEG, polyethylene glycol precipitation.

paired sera from five patients in which disappearance of HBeAg correlated with disappearance of HBV-DNA and with the appearance of anti-HBe.

The HBV-DNA probe was of particular use in determining the presence of viral DNA (and hence infectivity) in those patients whose sera gave negative results for both HBeAg and anti-HBe in ELISA. Some of these samples gave strong dot-blot reactions and might, therefore, be of high infectivity. Some patients may remain HBeAg-negative and anti-HBe-negative for some time and molecular hybridisation could be useful in separating them into potentially infectious and non-infectious groups.

Non-radioactive biotinylated DNA probes have been evaluated with a streptavidin-alkaline phosphatase conjugate^{12, 13} but they are less sensitive than the ³²P-labelled probe,¹⁴ although alkaline phosphatase may be as sensitive as the ³²P-labelled probe.¹⁵ Non-

radioactive probes have the advantage of greater safety and a longer shelf-life.

Preparation of samples by ultracentrifugation gave marginally better results than preparation by PEG precipitation. However, the difference in intensity of reactions between dot-blots prepared by the two methods was not sufficiently large to be of general practical significance in routine testing. Nevertheless, all HBeAg-positive samples were HBV-DNA positive after ultracentrifugation, but one was HBV-DNA-negative after PEG precipitation. Furthermore, in the only sample that was anti-HBe-positive and had detectable HBV-DNA, the HBV-DNA was detected in a sample prepared by ultracentrifugation; the sample prepared by PEG precipitation gave a negative dot-blot. However, PEG precipitation is cheaper and more convenient to perform, especially if a large number of samples are to be processed.

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