Serum hepatitis B virus DNA detection with S- and C-region-directed probes

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Summary. Developments in molecular biology have offered a wide range of nucleic acid probes to detect the genome of hepatitis B virus (HBV). We have tested the ability of two enzyme-linked (alkaline phosphatase) probes to detect HBV-DNA. These hybridise with the S and C regions of the genome of HBV and are used to determine the clinical significance of detecting the two regions. A total of 66 serum samples from patients at different stages of HBV infection was examined. HBV-DNA was detected with at least one of the probes in 17 (85%) patients with HBeAg-positive chronic hepatitis, five (26.3%) with anti-HBe-positive chronic hepatitis and six (66.6%) with acute hepatitis. Although both probes were able to detect as little as 10 pg/ml (2.86×10^6 g.E./ml) of a full length HBV-DNA standard, the C-region-directed probe did not react in one patient with acute hepatitis, two with HBeAg-positive and three with anti-HBe-positive chronic hepatitis. When C-region-directed probes are used for diagnostic purposes, results should always be accompanied by hybridisation with probes directed against other regions showing less variability (e.g. S region).

Introduction

Nucleic acid hybridisation assays with DNA probes play a major role in the identification of hepatitis B virus (HBV) and have proved essential for monitoring the course of disease and evaluating treatment. The level of viral replication is an essential marker of disease status and among the markers of serum HBV replication, HBV-DNA is the most sensitive.^{1,2} Data accumulated from many studies show that viral DNA is a more specific and sensitive determinant of infectivity than HBeAg. Serum HBV-DNA detection shows a strong correlation with active liver disease and the detection of liver HBcAg. HBV-DNA is not only a better marker of infectivity, it is extremely helpful in the interpretation of the progressive seropathological state.³

The HBV genome has four open reading frames (ORF) which divide it into four constant regions named S, C, X and P.⁴ The use of synthetic oligonucleotide probes (SOP), and their advantages for the detection of HBV-DNA over genomic probes, have been documented previously.^{5–7} SOPs for HBV-DNA detection allow the selection of specific genome fragments. In the present study, we have evaluated the significance of the detection of specific HBV genome fragments from the S and C regions and their value in the routine detection of HBV-DNA in the serum of patients at different stages of HBV infection.

Patients and methods

Patients

Sixty-six patients were included in the study: 18 healthy asymptomatic carriers, 20 patients with HBeAg-positive and 19 with anti-HBe-positive chronic hepatitis (CH), and nine with acute hepatitis.

Serological assays

HBV markers (HBsAg, anti-HBs, anti-HBc, anti-HBc IgM, HBeAg and anti-HBe) were determined by commercially available enzyme immunoassays (EIAs; Abbott Laboratories, Chicago, IL, USA).

For serum HBV-DNA detection, a spot hybridisation technique with two commercially available enzyme-linked oligonucleotide probes (Dupont Laboratories), one annealing with the S region and the other with the C region of the genome of HBV, was performed. The probes were 26 and 25 bp long (S and C probes respectively), complementary to nucleotides 209–234, and nucleotides 1983–2007 of the *ayw* L(–) strand;⁸ oligonucleotide sequences were selected to avoid mismatches > 20% with *ayw*, *adw* and *adr* subtypes⁹ of HBV. The enzyme alkaline phosphatase was covalently linked to the C-5 position of a thymidine base through a spacer arm. Twenty-five μ l of each sample were incubated with 50 μ l 1 N NaOH for 10 min at room temperature and transferred by means of a filtration apparatus on to a nylon filter soaked in $5 \times$ SSC. Neutralisation was performed by adding

Table I. Serum HBV-DNA detection: S and C probes

Patient group	Number of samples tested	Number (%) that were HBV-DNA-positive with probes for	
		S region	C region
Acute hepatitis	9	6 (66.66)	5 (55.55)
Asymptomatic carriers	18	0 (0)	0 (0)
HBeAg-positive chronic hepatitis	20	17 (85)	15 (75)
Anti-HBe-positive chronic hepatitis	19	5 (26.31)	2 (10.52)

Table II. Summary of probe correlation

Patient group	Number of samples tested	Number (%) for which results were	
		Concordant	Discordant
Acute hepatitis	9	8 (88.88)	1 (11.2)
Asymptomatic carriers	18	18 (100)	0 (0)
HBeAg-positive chronic hepatitis	20	18 (90)	2 (10)
Anti-HBe-positive chronic hepatitis	19	16 (73.60)	3 (26.30)

100 µl of 2 M ammonium acetate to each well. After sample immobilisation, filters were washed several times in 5 × SSC and air-dried at room temperature. Pre-hybridisation was performed for 15 min at 55°C (S region probe) and 50°C (C region probe) in sealed plastic bags containing 10 ml of pre-hybridisation solution (20 × SSC 25%, BSA 0.5%, PVP 40000 0.5%, SDS 0.5%). Hybridisation was performed in sealed plastic bags containing 2.4 ml of pre-hybridisation solution for 15 min at 55°C (S region probe) and 50°C (C region probe). Filters were washed twice in 1 × SSC plus SDS 1% for 5 min at 50°C, twice in 1 × SSC plus Triton X100 1% for 5 min at 50°C, twice in 1 × SSC plus Triton X100 1% for 5 min with shaking at room temperature, and finally twice in 1 × SSC for 5 min with shaking at room temperature. Colour was developed by incubating the filter in sealed plastic bags with 7.5 ml of substrate buffer, 50 µl of nitro blue tetrazolium (NBT) solution and 38 µl of 5-bromo-4-chloro-3-indolylphosphate (BCIP) solution (Dupont Laboratory) for 6 h at room temperature and 9 h at 37°C. The reaction was stopped by washing the filters with de-ionised water.

For HBV-DNA assays, all sera were tested twice; only concordant results were considered for analysis. Serum specimens from seronegative healthy individuals were used as negative controls; for positive controls, and to assess the sensitivity of the assay, serial dilutions of an HBV-DNA standard ranging from 10⁶ to 1 pg/ml (2.86 × 10¹¹–2.86 × 10⁵ genomic equivalents/ml – g.E./ml – [1]) were used.

Results

Specificity and sensitivity of the assays

The specificity for both probes was 100%: HBV-DNA was not detected either in the negative controls

or in the healthy asymptomatic carriers of the virus. Both probes were able to detect as little as 10 pg/ml (2.86 × 10⁶ g.E./ml) of the HBV-DNA standard.

Overall HBV-DNA detection

HBV-DNA was detected with the S-region-directed probe in 17 (85%) patients with HBeAg-positive CH, five (26.3%) with anti-HBe-positive CH and in six (66.6%) patients with acute infection. With the C-region-directed probe, hybridisation was detected in 15 (75%) patients with HBeAg-positive CH, two (10.5%) with anti-HBe-positive CH and five (55.5%) with acute infection. These results are summarised in table I.

Comparison of detection with S and C region HBV-DNA probes

Concordant results with both S and C region probes were found in 60 cases (90.9%). A summary of the probe correlation is shown in table II. When discordant results were found, the specimens were always scored negative with the C-region-directed probe and positive with the S-region-directed probe.

Discussion

Following the introduction of HBV-DNA testing, it became evident that HBeAg and anti-HBe were not entirely reliable as parameters of viraemia.¹⁰ Currently, it is generally accepted that HBV-DNA is the most direct marker of viral replication. First attempts to detect HBV-DNA in the serum of infected patients were made with radioactively labelled full length genomic probes. These full genomic probes were substituted by synthetic oligonucleotide probes and,

subsequently, non-radioactively labelled probes were developed. These probes proved to be as sensitive as radioactively labelled probes for the routine detection of HBV-DNA.^{6,7} Several probes have been described for HBV-DNA detection. In this study, the differences found in the detection of specific fragments from the S and the C regions of the genome of HBV were evaluated.

The oligonucleotide probes showed a high specificity, as evidenced by the lack of positive results when samples from healthy individuals and from asymptomatic healthy HBsAg carriers were tested. Both probes achieved sufficient sensitivity (up to 10 pg/ml; 2.86×10^6 g.E./ml). These probes were able to detect HBV-DNA even in the group showing the lowest levels (anti-HBe-positive chronic active liver disease) of HBV-DNA (mean values *c.* 30 pg/ml).¹¹ An overall evaluation of the results (table I) shows the detection of HBV-DNA with at least one of the probes in 85% of the chronically infected patients with HBeAg and in 26.3% of those with anti-HBe-positive chronic infection. For acute hepatitis, HBV-DNA was detected in 66.6% of the samples tested. When these results are compared with those reported with radioactive genomic probes¹²⁻¹⁵ or with synthetic oligonucleotide probes,^{6,7} a high correlation is observed. These results support the premise that enzymically labelled oligonucleotide probes have technical advantages in the routine detection of HBV-DNA.

However, when the results for the specific detection of the selected S and C sequences are compared and evaluated (table II), some discrepancies arise: the S-region-directed probe could detect HBV-DNA in a greater number of cases than the C-region-directed probe. In the present study, one sample from a patient with acute hepatitis (11.2% discordance), two from HBeAg-positive CH patients (10% discordance) and three samples from anti-HBe-positive CH cases (26.3% discordance) were only positive when hybridisation was carried out with the S-region-directed probe. The study conducted by Manzin *et al.*⁶ in α -IFN-treated patients reported that positivity against C-region-directed probes was lost before reactivity to the S probe. The authors also found differences between the two probes when samples from patients with acute hepatitis were tested. Since an HBV variant characterised by a mutation in the pre-core region was described,^{11,16} newer mutations in the HBV genome have been reported. These mutations have been described not only for anti-HBe-positive but also for HBeAg-positive patients.^{17,18} Therefore, it seems that the differences found in our study could be explained by the higher sequence variation in the region recognised by the C-region oligonucleotide, compared to that recognised by the S-region probe. We believe that for diagnostic purposes, C-region-directed oligonucleotide probes should be carefully evaluated, even if stringent hybridisation conditions are used.

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