Detection of hepatitis B virus DNA in serum using synthetic non-radioactive oligonucleotides

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SUMMARY A rapid and simplified technique for detecting hepatitis B virus (HBV) DNA by spot hybridisation in the sera of patients with different clinical forms of HBV infection was investigated using enzyme conjugated synthetic oligodeoxyribonucleotides as probes. These are able to hybridise to the S and C regions of the HBV L(-) DNA strand. When compared with a complete ³²P-labelled HBV DNA probe, the synthetic oligonucleotides provided a sensitive and quick method for the routine survey of HBV infection. Moreover, the DNA extraction procedure used allowed the spot hybridisation technique to be applied and read easily and the results obtained within a few hours.

It is concluded that synthetic cold probes can be used in hybridisation assays for HBV DNA detection as part of current clinical laboratory procedures.

Hepatitis B virus (HBV) infection is currently a problem of worldwide relevance.¹ Its control at present largely depends on both laboratory diagnosis and evaluation of infectivity,² and molecular hybridisation techniques have provided a sensitive and specific approach to the detection of HBV DNA sequences in serum samples and tissues.³ Moreover, when a large number of samples needs to be tested for routine purposes, these methods represent a quick and useful tool for the widespread survey of the infection.⁴⁻⁶ The availability of radiolabelled DNA probes for routine diagnosis, however, is limited to a few laboratories because of the need for propagating, isolating, purifying and labelling procedures. Moreover, as autoradiography requires from 48 hours to two weeks for the results to be read, the use of such probes does not allow a rapid diagnosis to be made. On the other hand, production of biotinylated⁷ HBV DNA probes has only partially overcome this problem. In fact, hybridisation by cloned, complete biotinylated probes, detected by complexing with streptavidin conjugated to an enzyme, has been shown to be relatively insensitive in some circumstances,⁸ compared with the ³²P-labelled probes or different cold probes.

As the importance of and the interest in using sensitive non-radioactive HBV DNA probes for routine purposes has been underlined recently⁹ and the

complete sequence of several cloned HBV serotypes is known,^{10,11} a potential approach lies in the use of synthetic, enzyme conjugated oligodeoxyribonucleotides as probes which can hybridise to specific sites on the HBV L(-) strand.

To investigate this possibility, synthetic oligodeoxyribonucleotides, which are specifically recognised by fragments of the S and C open reading frames of the HBV genome, were used to test serum samples from different groups of patients infected with HBV.

Material and methods

Serum samples from 213 HBsAg chronic carriers and 25 patients with acute hepatitis B were tested for the presence of HBV DNA sequences. Serological markers of HBV infection (HBsAg, anti-HBs, HBeAg, anti-HBe, total anti-HBc and IgM anti-HBc) were detected using a commercially available enzyme immunoassay (EIA) (Connaught Laboratories, Ontario, Canada). Laboratory diagnosis of acute hepatitis B was performed using the methods mentioned above, excluding hepatitis A virus, cytomegalovirus, and Epstein-Barr virus infections, which were assayed using routine EIA methods for specific IgM antibodies.

SYNTHETIC OLIGONUCLEOTIDE PROBES

Sequence data for *ayw*,¹⁰ *adw*, and *adr* subtypes¹¹ were studied comparatively, and oligonucleotide sequences

in the S and C regions of HBV DNA were chosen to avoid mismatches greater than 20%. The oligonucleotide sequences for the S and C regions of the HBV genome were synthesised and purified using reverse phase high pressure liquid chromatography by Molecular Biosystems (San Diego, California, USA), consisting, respectively, of synthetic 26 and 25 bases complementary to nucleotides from 209 to 234, and from nucleotides 1983 to 2007 of the *ayw* L(-) strand.¹⁰ The probes were covalently linked to alkaline phosphatase at the C-5 position of a thymidine through a 12-atom spacer arm and lyophilised. Finally, the lyophilised probes were resuspended in deionised, sterilised water which resulted in a 10 µg/100 µl stock of each probe.

³²P-LABELLED PROBE

The probe used was the purified HBV DNA (subtype *ayw*) inserted in tandem into the EcoRI restriction site of a pBR322 plasmid¹⁰ and labelled with ³²P d-TTP by the nick translation method¹²; the specific activity was 4–6 × 10⁸ dpm/µg.

NUCLEIC ACID EXTRACTION PROCEDURE

Serum (500 µl) in a final volume of 1.5 ml was incubated at 50°C for one hour in 150 mM NaCl, 10 mM EDTA (pH 8.0), 10 mM TRIS-HCl (pH 8.0), 2% sodium dodecyl sulphate (SDS), 2.0 µg/ml salmon sperm DNA and 250 µg/ml proteinase K. Afterwards phenol:chloroform:isoamyl alcohol (25:24:1) extraction was performed. Extracted DNA was then precipitated with cold absolute ethanol, dried, and redissolved in 200 µl of 10 mM TRIS-HCl (pH 7.5) 1 mM EDTA.

DNA:DNA HYBRIDISATION

For the hybridisation assay using the cold, synthetic probes, redissolved DNA (50 µl) was applied to nylon filters (Bio-Rad, Richmond, California, USA) using vacuum filtration apparatus (Bio-Dot Apparatus, Bio-Rad, Richmond, California, USA), and then denatured in situ in 0.5 M NaOH, 1.5 M NaCl for 10 minutes, neutralised in 1.5 M NaCl, 0.5 Tris-HCl (pH 8.0) for 10 minutes, and then dried for one hour in a vacuum oven at 80°C.

The filters were pre-hybridised for 15 minutes in pre-warmed hybridisation buffer at 50°C. The hybridisation solution was 5x SSC (1x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% bovine serum albumin (BSA), 0.5% polyvinylpyrrolidone (PVP) and 1% SDS. The hybridisation was performed at 50°C for 30 minutes in the same buffer containing 5 µl/ml of the stock of probes. The filters were washed twice with 50 ml of 1x SSC, 1% SDS at room temperature, followed by two washes with 1x SSC, 1% Triton X-100 at the hybridisation temperature, and

finally two washes with 1x SSC at room temperature.

Hybridisation with the purified, ³²P-labelled complete probe was carried out according to Weller *et al*¹³ with minor modifications. The filters were hybridised at 42°C for 50–60 hours in 50 ml of a solution containing 50% formamide, 5x SSC, 1x Denhardt's solution (Ficoll 400, PVP 360, BSA 0.2 mg/ml each), 0.02 M NaPi (pH 7.0), 0.05% SDS, 50 µg/ml denatured salmon sperm DNA, and 10⁸ dpm of ³²P-labelled nick-translated HBV DNA probe.

The unincorporated radioactivity was removed by washing out with the following solutions: (i) 5x SSC, 0.1% SDS, 50% formamide at 42°C for 30 minutes; (ii) 2x SSC, 0.1% SDS at 65°C for 30 minutes.

HYBRIDISATION DETECTION

Alkaline phosphatase was detected in 7.5 ml of a substrate buffer (100 mM TRIS-HCl pH 8.5, 100 mM NaCl, 100mM ZnCP₂, 0.02% sodium azide) containing 33 µl of nitro-blue tetrazolium (NBT) and 25 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 70% of dimethylformamide. The colour (blue) development was checked after four and 24 hours. Autoradiography was carried out for 48 hours and for one week at -70°C in a cassette containing Kodak X-Omat G film and Dupont intensifying screens.

Results

PRESENCE OF HBV DNA IN SERA OF HBsAg CHRONIC CARRIERS

Out of 213 HBsAg chronic carriers studied, 39 were HBeAg positive, 165 anti-HBe positive, and nine were negative for both HBeAg and anti-HBe. Thirty three of 39 (84%) HBeAg positive chronic carriers were positive for serum HBV DNA; 21 out of 165 (13%) chronic carriers positive for anti-HBe antibodies had detectable concentrations of HBV DNA in their sera (table 1). Serum HBV DNA was not found in HBeAg/anti-HBe negative carriers, nor in different groups of subjects negative for serological HBV markers, nor in subjects with anti-HBc or anti-HBs antibodies, or a combination. HBV DNA negative samples were not strongly positive when tested using the ³²P-labelled HBV DNA, but two HBeAg and one anti-HBe positive/HBsAg carrier, negative using oligonucleotides, were borderline positive for HBV DNA after one week's exposure to the radioactive probe (table 1).

Furthermore, a sensitivity test was performed using a plasmid control DNA (100 ng/ml) containing 10⁸ genomic copies/ng, thus finding a 10-fold and a 20-fold loss of sensitivity when comparing, respectively, the S and C probes with the complete, ³²P-labelled probe (data not shown).

Table 1 *HBV DNA detection by synthetic oligodeoxyribonucleotides in sera of HBsAg chronic carriers and patients with acute hepatitis B*

Group	Sera	HBV DNA detection				
		Probe -S-	-C-	Positive (%)	Probe 32-P	Positive (%)
Chronic HBsAg:						
HBsAg positive	39	33	33	(84.6)*	35	(89.7)†
Anti-HBe positive	165	21	21	(12.7)	22	(13.3)
HBsAg/anti-HBe negative	9	0	0	(—)	0	(—)
Total	213	54	54	(25.3)	57	(26.8)
Acute hepatitis B:						
HBsAg positive	10	8	7	(80.0)	8	(80.0)
Anti-HBe positive	10	2	1	(20.0)	2	(20.0)
HBsAg/anti-HBe negative	5	0	0	(—)	0	(—)
Total	25	10	8	(40.0)	10	(40.0)

-S- = synthetic oligodeoxyribonucleotides recognised by part of the S region of the HBV genome

-C- = synthetic oligodeoxyribonucleotides recognised by part of the C region of the HBV genome

32-P = complete, radiolabelled HBV DNA probe

*Per cent of samples positive for at least one oligodeoxyribonucleotide probe

†Per cent of samples positive for the complete, 32P-labelled HBV DNA probe

PRESENCE OF HBV DNA IN ACUTE HEPATITIS B

Serum samples from 25 patients with acute hepatitis B were collected on their first day of admission to the infectious diseases department of the University of Ancona. Ten (eight HBeAg positive and two anti-HBe-positive) out of 25 (40%) samples from these patients showed positive hybridisation signals, while 15 (60%) were negative when hybridised using both the complete and the synthetic oligonucleotide probes (table 1).

HBV DNA DETECTION IN SERA OF HBsAg CHRONIC CARRIERS TREATED WITH INTERFERON

HBV DNA was tested in six HBsAg chronic carriers with different degrees of evolving chronic hepatic damage and treated with recombinant α -interferon (3 million units/daily for three days/week for three months) (table 2). In a follow up survey during treatment progressive decrease in terms of positive signals was observed in five out of six patients; in particular, in three cases the C probe was no longer able to detect HBV DNA after 60 days: on the other hand, the S probe still gave positive results. In these

cases this pattern was predictive of HBeAg/anti-HBe seroconversion (data not shown) and complete clearance of detectable HBV DNA.

Discussion

The detection of HBV replication is of major importance in clinical laboratories. In fact, serum HBeAg positivity has been shown to be associated with HBV replication, serum infectivity, and presence of serum HBV DNA in almost all chronic carriers tested.^{13,14} Conversely, HBV DNA positivity in anti-HBe positive/HBsAg carriers may indicate that persistent replication is developing in spite of HBe/anti-HBe seroconversion, and that the patients risk severe, chronic liver disease.^{15,16}

In this study we investigated the use of non-radioactive oligodeoxyribonucleotides for detecting HBV DNA in the sera of patients with acute or persistent HBV infection. In our opinion, several general aspects may particularly lead to the use of synthetic oligonucleotides as probes; firstly, recent reports clearly discuss the possibility of spurious hybridisation when

Table 2 *Serum HBV DNA detected by S and C probes of HBV genome in HBsAg chronic carriers treated with interferon*

Case No	Serum HBV DNA during treatment (days)							
	0		30		60		90	
	-S-	-C-	-S-	-C-	-S-	-C-	-S-	-C-
01	+	+	-	-	-	-	-	-
02	+	+	-	-	-	-	-	-
03	+	+	+	+	+	-	-	-
04	+	+	+	+	+	+	+	+
05	+	+	+	+	+	-	-	-
06	+	+	+	-	+	-	-	-

-S- = synthetic oligodeoxyribonucleotides recognised by part of the S region of the HBV genome

-C- = synthetic oligodeoxyribonucleotides recognised by part of the C region of the HBV genome

Use of synthetic non-radioactive oligonucleotides to detect HBV

HBV DNA probes contain or are contaminated with plasmid sequences.¹⁷⁻¹⁹ In this case hybridisation with some episomal DNA present in human tissue may occur, thus giving false positive results. This possibility has mainly been related to DNA extracted from human tissues, but it may also involve serum DNA. Accurate purification of inserted HBV DNA is needed when cloned genomes are used as probes, whereas in our assay the specificity of the synthetic probes was satisfactorily guaranteed. The specificity of the oligodeoxyribonucleotide probes was assayed with several samples of viral and non-viral DNA (cytomegalovirus, Epstein Barr virus, plasmid pBR322, herring and salmon sperm, human placental, *Escherichia coli* DNA). Secondly, a routine method calls for the wide distribution of stable materials: both enzyme labelled and unlabelled synthetic oligonucleotides are stable for months at low temperature. Recently, a 21 nucleotide sequence probe, end-labelled with ³²P, was tested for HBV DNA detection in serum by spot hybridisation.²⁰ Use of oligonucleotides, it was emphasised, makes the assay simpler and faster, but even in that case all the problems due to the use of radioactive material clearly remained.

A third aspect concerns the sensitivity of the test. Generally, molecular hybridisation methods have exhibited high levels of sensitivity in detecting specific DNA sequences when ³²P-labelled probes are used. Sensitivity of non-radioactive methods is generally lower and problems of interpreting results may be present when borderline samples are tested. Additionally, spotting serum samples on to nylon or nitrocellulose filters directly may make a radioactive method easier,⁴ but it may also interfere with the reading of low levels of enzymatic reaction. These are the main reasons why we adopted the DNA extraction procedure reported above, which allows results to be read more easily.

A method of amplifying short DNA fragments that enhances the sensitivity of hybridisation procedures has recently been described,²¹ and preliminary results on HBV DNA detection using a short (128 nucleotides), ³²P-labelled DNA fragment complementary to part of the S gene have been satisfactory.²² Clearly, the application of this assay to HBV DNA detection using a non-radioactive method could be of great interest.

Our results indicate that, although a low concentration of HBV DNA has been detected using the complete ³²P-labelled probe in three samples giving negative results when using the oligonucleotide probes, the sensitivity of the two cold probes used was acceptable for diagnostic purposes. The results obtained in terms of prevalence of HBV DNA positive/HBsAg carriers by using the synthetic probes are in line with previous data obtained using a complete ³²P-labelled probe.¹⁶

Furthermore, because one of the main purposes of HBV DNA detection in human sera is to have a handy tool to test sequential samples from treated chronic carriers, we examined the sera of patients treated with α -interferon. The loss of positivity using the oligonucleotide probe for the C region of the HBV genome was faster during treatment than for the S probe. This probably reflects the lower detection rate observed with the second oligonucleotide, and the different sensitivities of the two probes probably reflect the higher sequence variation in the region homologous to the C probe, compared with the region homologous to the S probe, among different subtypes. It was also predictive of the decrease in HBV DNA and, in three cases, of HBe/anti-HBe seroconversion, and of the complete clearance of HBV replication.

In conclusion, the results point to the usefulness of synthetic oligodeoxyribonucleotides as cold probes for the detection of HBV DNA in sera by spot hybridisation, thus highlighting this technology as an important tool in the survey of HBV infection in clinical laboratories. In our opinion, this alternative approach using non-radioactive probes is clearly of value, and further development and improvement may well generate diagnostic assays for general use. One possible approach would be to use several oligonucleotides from regions of the genome which show little sequence variation between viral subtypes, because if appropriately chosen, there would be no steric hindrance and a stronger signal.

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