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Polymerase chain reaction detects hepatitis B virus DNA in paraffin-embedded liver tissue from patients sero- and histo-negative for active hepatitis B

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Summary. The polymerase chain reaction (PCR) was used to analyse tissues from paraffin blocks of liver needle biopsies retrospectively. Biopsies of 29 patients with proven HBsAg and HBcAg expression in liver tissue and of 8 healthy volunteers served as positive (group 1) and negative tissue controls (group 2), respectively. These were compared with 16 patients with proven HBsAg expression in liver but lack of HBcAg (group 3), with 23 patients with anti-HBc as the only hepatitis B virus (HBV)-related marker (group 4) and with 21 patients with liver disease and without HBV markers in tissue or serum (group 5). PCR detected HBV sequences in all cases of the positive control group and in 94% of group 3, in 65% of group 4, and in 71.4% of group 5, whereas all healthy volunteers were negative. Our data show that PCR is able to detect HBV-DNA sequences in virtually all patients with active viral antigen expression but also in a high proportion of hepatitic patients who are silent for active HB but may or may not show signs of a contact with the HBV. Thus, PCR for HBV-DNA in paraffin sections might become a useful tool for identifying patients carrying HBV-DNA but not expressing HBV antigens.

Key words: Hepatitis B – Polymerase chain reaction – Paraffin sections – Liver biopsy

Introduction

The technique of polymerase chain reaction (PCR) (Saiki et al. 1988) has been successfully adapted to formalin-fixed, paraffin-embedded tissue specimens (Shibata et al. 1988; Lo et al. 1989) and hepatitis B virus (HBV) DNA sequences have been detected in human liver biopsies (Lo et al. 1989; Lampertico et al. 1990; Shindo et al. 1991). Analysis of archival tissues with this highly sensitive technique opens up an exciting opportunity to complement conventional histopathological, serological and immunocytochemical results (Malter and Gerber 1991) in retrospective and prospective investigations. Moreover, it facilitates multicentre studies as material can easily be transferred from centre to centre.

In the present paper we report studies by PCR on human liver biopsies from different groups of patients in our files. We demonstrate HBV-DNA sequences in liver biopsies from patients with anti-HBc as the only marker of a contact with the HBV as well as in patients without any HBV marker in tissue and blood by conventional methods. Proven cases of replicating HBV infection served as positive controls and biopsies from HBV-negative healthy volunteers as negative controls, respectively.

Materials and methods

We studied paraffin-embedded liver needle biopsies from our files (1984–1990) from patients with histologically verified chronic hepatitis according to established criteria (Bianchi et al. 1977) from which a complete immunohistochemical analysis of liver tissue for HBV antigens had been done on unfixed frozen sections using direct and indirect immunofluorescence with primary antibodies against HBsAg, HBcAg and delta antigen as described elsewhere (Stöcklin et al. 1981). HBV markers in serum were determined with currently available radioimmunoassays.

The immunohistological, serological and histological characterization of patients is given in Tables 1 and 2 for the following study groups:

Group 1: positive tissue controls. This group included 29 patients with on-going HBV infection expressing both core (HBcAg) and surface (HBsAg) antigen in liver tissue. A complete serological status for HB was available in 17 patients: 10 were positive for HBeAg, 5 for anti-HBe, 2 negative for both markers of the HBe system, while all 17 had anti-HBc in serum. Out of the 29 patients, 28 had chronic aggressive hepatitis (CAH) and one had chronic persistent hepatitis (CPH).

Group 2: negative tissue controls. This group consisted of 8 clinically healthy volunteer medical students (Rohr et al. 1976) with negative HB serology (HBsAg, anti-HBs, anti-HBc) and normal liver tissue without inflammation.

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Table 1. Immunohistological, serological, histological characterization and polymerase chain reaction (PCR) positivity of patients from the control groups

	Tissue		Serum status					Histology	No.	PCR +
	HBs	HBc	HBs	a-HBs	HBe	a-HBe	a-HBc			
Group 1	+	+	+	−	+	−	+	CAH	10	10
Positive HB tissue control	+	+	+	−	−	+	+	CAH	5	5
	+	+	+	−	−	−	+	CPH	1	1
								CAH	1	1
	+	+			ND			CAH	12	12
Total									29	29
Group 2	−	−	−	−	ND	ND	−	Within normal limits	8	0
Negative HB tissue control										
Total									8	0

HB, Hepatitis B; ND, not determined; HBs, hepatitis B surface antigen; HBc, hepatitis B core antigen; a-HBs, hepatitis B surface antibody; HBe, hepatitis Be antigen; a-HBe, hepatitis Be antibody; a-HBc, hepatitis B core antibody; CAH, chronic aggressive hepatitis; CPH, chronic persistent hepatitis; AH, alcoholic hepatitis

Table 2. Immunohistological, serological, histological characterization and PCR positivity of patients from experimental groups

	Tissue		Serum status					Histology	No.	PCR +
	HBs	HBc	HBs	a-HBs	HBe	a-HBe	a-HBc			
Group 3	+	−	+	−	+	−	+	CAH	1	1
HB with unproven replicative state	+	−	+	−	−	+	+	CPH	1	0
								CAH	4	4
	+	−	+	−	−	−	+	CAH	1	1
	+	−	+	ND	ND	ND	ND	CPH	2	2
								CAH	7	7
Total									16	15
Group 4	−	−	−	−	−	−	+	CAH	13	8
Liver disease with a-HBc only								CPH	3	3
								AH	2	1
								NRH	3	2
								MLD	2	1[a]
Total									23	15
Group 5	−	−	−	−	−	−	−	CAH	12	11
Liver disease with no HB marker								CPH	1	1
								AH	4	2
								NRH	2	1
								MLD	2	0
Total									21	15

NRH, Non-specific reactive hepatitis; MLD, miscellaneous liver diseases; for other abbreviations, see Table 1

[a] Hepatoma

Group 3: HB with unproven replicative state. This group consisted of 16 patients with on-going HB infection in which immunofluorescence failed to detect HBcAg. Serological data were available from 7 of these patients. All 7 had anti-HBc in serum, 5 were positive for anti-HBe but negative for HBeAg, 1 had HBeAg in the absence of anti-HBe, and 1 was negative for both markers of the HBe system. Histology showed CAH in 13 and CPH in 3 of the 16 cases.

Group 4: liver disease with anti-HBc only. This group of 23 patients lacked tissue expression of HBV antigens and other HBV-related

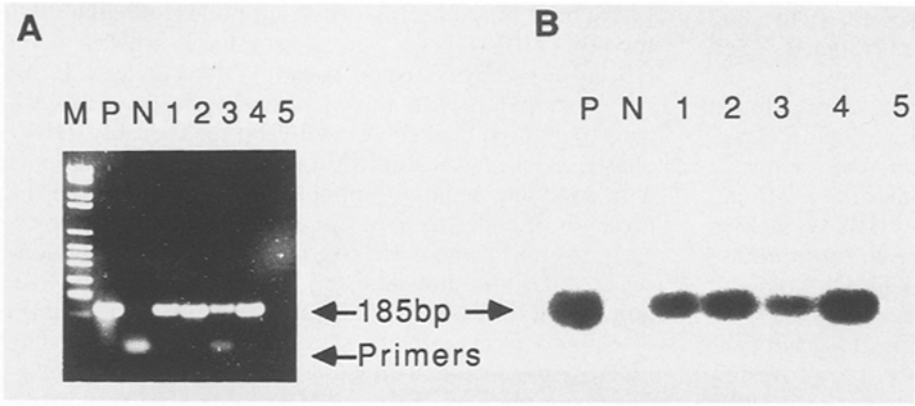

Fig. 1. A A representative ethidium-bromide-stained agarose gel of polymerase chain reaction (PCR) amplification products; 20 µl PCR amplification product was run on a 2.0% agarose gel. *M*, Molecular weight size markers; *P*, pHBV 991 plasmid DNA; *N*, negative control. 1, Hepatocellular carcinoma; 2, $HBsAg^+/HBcAg^+$ case; 3, $HBsAg^-/HBcAg^-/anti\text{-}HBc^+$ case; 4, $HBsAg^-/HBcAg^-$ case; 5, healthy control. **B** A Southern blot analysis of the gel presented in **A**. DNA was hybridized to ^{32}P-radiolabelled HBV 991 plasmid after transferring to nitrocellulose membrane

markers in serum except for anti-HBc. In this group were 13 patients with CAH, 3 with CPH, 2 with alcoholic hepatitis (AH) (both patients were alcohol abusers), 3 with non-specific reactive hepatitis (NRH), and 2 with miscellaneous liver disease (MLD).

Group 5: Liver disease with no HBV markers. This group included 21 patients negative for all HBV-related markers with various forms of inflammation: 12 patients had CAH, 1 had CPH, 4 AH (all were alcohol abusers), 2 NRH and 2 MLD.

Two 5 µm sections were cut from the paraffin blocks under stringent conditions to avoid cross-contamination and placed in a 1.5 ml Eppendorf tube. Subsequent processing was according to Shibata et al. (1988). Briefly, the sections were deparaffinized in 400 µl xylene, vortexed for 1 min and spun for 5 min. The xylene was pipetted off and the residual was removed with 400 µl of 95% ethanol. This was followed by vortexing (1 min) and spinning (5 min). Most of the ethanol was pipetted off and the rest was removed by vacuum desiccation for 10 min. PCR mix (100 µl) was added to the tubes, heated at 95° C for 10 min, followed by 40 cycles of PCR. When DNA was used for amplification, 500 pg plasmid (used only to standardize the method) or 1 µg liver DNA was amplified per reaction.

PCR was carried out essentially as previously described (Saiki et al. 1988). Five units of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin-Elmer Cetus Kuesnacht, Switzerland) were used for each reaction. The reaction buffer and deoxynucleotide triphosphates were obtained from the Perkin-Elmer Cetus Gene Amp DNA amplification reagent kit. Each cycle of PCR consisted of thermal denaturation at 94° C (2 min), primer annealing at 55° C (2 min), and extension at 72° C (3 min), all carried out in an automated thermal cycler (Perkin-Elmer Cetus). During the last cycle, extension time was increased by an additional 7 min. Forty cycles were performed. As an additional control for group 5, PCR was repeated by using 30 and 50 cycles. When DNA was used for amplification, 500 pg plasmid or 1 µg liver DNA was used per reaction. DNA was isolated from liver as previously described (Villari et al. 1989). All the samples were tested twice and in every PCR experiment a positive and a negative control (without DNA) were run simultaneously. To avoid possible contamination by the plasmid, all the necessary precautions were taken and the plasmid was never amplified in the same experiment as the patient samples.

Oligonucleotides were synthesized by using the DNA synthesizer DM (Beckman Nyon, Switzerland). The sequences are written from 5′ to 3′:

PCR 1: GATTGAGATCTTCTGCGACGC
PCR 2: GAGTGTGGATTCGCACTCCTC

For Southern blot analysis 10–20 µl of each PCR sample were electrophorized on 2% agarose gel, stained with ethidium bromide, and photographed under UV light. The separated PCR products were denatured by soaking the gel twice in 0.5 M sodium hydroxide/1.5 M sodium chloride for 20 min each, and neutralized by soaking twice in 0.5 M TRIS-HCl, pH 7.4/1.5 M sodium chloride for 20 min each. The filters were pre-hybridized for 2–4 h at 42° C in hybridization buffer (50% formamide, 5 × SSC, 5 × Denhards, 0.5% SDS, 100 µg/ml denatured salmon sperm DNA) and hybridized overnight with 10^7 cpm/ml of heat-denatured ^{32}P random-primed labelled pHBV 991 plasmid containing the entire HBV sequence (Böttcher et al. 1982). Random priming was performed using the random priming DNA labelling kit (Boehringer, Mannheim, FRG) following the protocol of the supplier. The membranes were washed once at room temperature in a buffer containing 0.1% SDS/1 × SSC for 15 min, once at 37° C in the same buffer for 20 min, and twice at 68° C in a buffer containing 0.5% SDS/0.1 × SSC for 40 min each. After washing the filters were exposed to Kodak XARS autoradiography films at −70° C with intensifying screens.

To standardize the PCR method for HBV sequences we used as target sequence the pHBV991 plasmid which contains the entire 3.2 kb HBV genome. The desired fragment was 185 base pairs long, flanked by primers PCR1 and PCR2 in the region where the core antigen gene overlaps the polymerase gene (Tiollais et al. 1985). This region was chosen because of its conservation amongst different subtypes of HBV (Ono et al. 1983).

As shown in Fig. 1 A the primers amplified the expected 185 bps fragment from the HBV plasmid DNA. Specificity of the amplified sequence for HBV was confirmed with Southern blot hybridization using radioactively labelled pHBV991 plasmid as probe (Fig. 1 B).

Amplification of DNA isolated from a liver with hepatocellular carcinoma: Southern blot analysis confirmed that the DNA contained the HBV genome (data not shown). A band of similar size to that of the amplified HBV plasmid was present in the PCR amplification product of the liver DNA (Fig. 1 A). This DNA fragment hybridized also to HBV-radiolabelled plasmid (Fig. 1 B).

After establishing the conditions for amplifying HBV sequences from plasmid and DNA isolated from hepatocellular carcinoma, we analysed paraffin sections of liver needle biopsies for the presence of HBV-DNA sequences.

Results

The results of the PCR studies on paraffin-embedded liver biopsies are summarized in Tables 1 and 2. A representative ethidium bromide-stained gel and a Southern blot are shown in Fig. 1. Among the group of patients with CAH or CPH positive for HBsAg and HBcAg in liver by immunostaining (group 1), PCR detected HBV sequences in all 29 biopsy samples. Specificity of the amplified sequences was confirmed by hybridization to radioactively labelled HBV plasmid. Group 2 served as a HBV-negative tissue control. It consisted of 8 liver biopsies from healthy medical students in our files (Rohr

et al. 1976) without any markers of HBV in serum and liver and with histologically normal liver. PCR failed to detect HBV-related sequences in all 8 biopsies.

Table 2 summarizes immunohistological expression in liver tissue, serological status of HBV, histological classification of the accompanying liver lesion, and the results of PCR in groups 3–5. In group 3, consisting of 16 patients with CAH or CPH positive for HBsAg in liver and blood but negative for HBc in tissue by immunohistology, PCR detected HBV-DNA in all but 1 patient. The single negative patient had HBe-negative CPH, positive for HBsAg, anti-HBc, and anti-HBe in serum. The common denominator in group 4 is positivity for anti-HBc in serum as the only marker of contact to the HBV. Associated histopathology includes chronic hepatitis and non-hepatitic lesions. PCR detected HBV sequences in 15 of 23 cases. Of 13 CAH patients 8 were positive; among the 5 PCR-negative cases, 2 were drug-related and 1 had serum antibodies against the hepatitis C virus (anti-HCV). All 3 CPH were PCR-positive, as were 1 of 2 cases of AH and 2 of 3 NRH. Among the 2 MLD 1 hepatocellular carcinoma was PCR-positive while 1 case of steatosis was PCR-negative.

In group 5, where conventional methods failed to detect any HBV-related markers, PCR amplified HBV sequences in 15 of 21 patients, even when using the lowest possible number of cycles (30) to minimize contamination. Positive cases included 11 of 12 CAH and the 1 CPH. In the PCR-negative case of CAH a drug aetiology was likely. Two of 4 biopsies of AH and 1 of 2 NRH were positive in PCR, whereas both cases of MLD (both fatty livers) were negative.

Discussion

The role of the expression of different HBV-related antigens in the pathogenesis of HB is still unclear but major importance has been assigned to active replication evidenced by demonstration of HBcAg in liver or presence of HBeAg, DNA-polymerase and/or viral DNA in serum (Bonino 1986; Thomas 1990). The detection of HBV-DNA in patients with chronic hepatitis negative for HBsAg in liver and serum (Brechot et al. 1985) shows that conventional immunological analysis of liver biopsy specimens and serological methods are not sensitive enough to detect small amounts of viral DNA molecules in the liver. PCR, however, is a very sensitive technique which can amplify one single copy of a particular DNA sequence to detectable level in the presence of total DNA from 10^5 cells (Saiki et al. 1988). Such a high sensitivity makes PCR ideally suited for the detection of very low copy numbers of a viral genome but carries also the risk of false-positive and false-negative results (Wright and Wynford-Thomas 1990). To control such artefacts in our study, we included a positive control group (group 1) with proven HBV replication and a negative group (group 2) of biopsies from HBV-negative healthy volunteers. In addition, the preparation of the samples was done under stringent conditions to avoid cross-contamination and positive and negative controls were included in every experiment.

We have tested critical groups of patients for the presence of HBV-DNA. Special emphasis was laid on HBsAg-expression livers without detectable HBcAg when compared with those expressing both viral antigens. The significance of anti-HBc as the only HBV-related marker was studied in comparison with patients with complete negativity of conventional markers in the presence of hepatitic liver disease.

In group 1, where HBsAg and HBcAg were demonstrated by immunostaining, PCR detected HBV sequences in all cases. This is in agreement with findings of Shindo et al. (1991), who detected HBV-DNA by PCR in paraffin-embedded liver tissue in all HBeAg-positive replicative forms of HBV. The HBsAg-positive group of patients without detectable HBcAg in the liver (group 3) is biologically heterogeneous. It includes 1 HBeAg-positive patient who might be considered fully replicative because sampling error for HB antigens in immunohistology is rather high. The critical subgroup, however, is the one with isolated HBsAg synthesis in liver and positivity for anti-HBe in serum (group 3; $n=5$). Four out of five cases with this constellation harboured HBV-DNA sequences but 1 case interestingly was negative in PCR. Such a false-negative result might be related to the presence in paraffin sections of inhibitor(s) of *Taq* polymerase (Lo et al. 1989; Lampertico et al. 1990). This possibility seems to be unlikely, since a fragment of the β-globin gene could be amplified by using a specific set of primers, from DNA isolated from paraffin-embedded material (data not shown). Alternatively, although the presence of viral genomes in numbers below the limit of detection by PCR cannot be ruled out, it might be assumed that this region may be mutated or deleted in the viral genome of the patient (Lo et al. 1989). Since the expected fragment comes from the core-polymerase overlap region, this hypothesis could also explain why HBcAg could not be demonstrated in liver cells in this patient.

This observation also opens up the possibility that PCR can be used to investigate the role of different viral genes in the pathogenesis of disease when used with different sets of primers. Such approaches have already been undertaken to investigate the role of HBV in primary liver cancer (Lampertico et al. 1990; Paterlini et al. 1990). The detection by PCR of HBV sequences in HBcAg-negative patients indicates that analysis of hepatic DNA is more sensitive than the study of viral antigens in detecting HBV infection of the liver. This was also stressed by Villari et al. (1989), who by Southern blot analysis of DNA from liver found DNA replication in 21 of 40 cases, while only 14 of 40 were positive for HBcAg.

In our group 4 (anti-HBc as the only HBV-related marker), PCR detected HBV sequences in 15 of 23 cases. Similarly, Paterlini et al. (1990) found HBV-DNA in the absence of HBV-related serum markers except anti-HBc in a Japanese patient with primary hepatocellular carcinoma in cirrhosis, by PCR. This clearly shows that HBV sequences may be present in liver cells of patients with or without histological signs of chronic hepatitis after HBsAg has become undetectable. One suitable hypothe-

sis to explain this finding would be that the virus detected in the liver is defective as a result of a critical mutation in the viral genome, making the virus relatively inefficient in replication. This assumption is supported by the finding of HBV mutants in patients with detectable HBV-DNA in liver but negative HBeAg in serum (Brunetto et al. 1989; Carman et al. 1989) and by a recent report of Lampertico et al. (1990), who by using PCR and different sets of primers found amplification products shorter than expected, suggesting internal deletions.

Finally, the detection by PCR of HBV sequences in biopsies of various liver diseases where the conventional methods failed to demonstrate any HBV markers (group 5) is astonishing. However, other authors have reported similar experiences. In a study of hepatocellular carcinoma in HBsAg-negative patients Paterlini et al. (1990) identified HBV-DNA sequences in the liver of 17 of 28 patients. Of these 17, 6 were also negative for HBV antibodies. Lampertico et al. (1990) found positive PCR for HBV-DNA in the liver in 3 cases negative for HBV markers with and without chronic liver disease. Moreover, HBV-DNA was detected by PCR in serum in the absence of other HBV markers (Kaneko et al. 1990a). This indicates that in many liver diseases of unknown aetiology HBV may be involved. The PCR technique in such instances may be helpful to investigate further a possible role of HBV in the disease process. So far, however, the number of cases studied is too small to allow firm conclusions on the significance of the HBV in this group of patients.

The fact that the PCR method may be applied to paraffin-embedded tissue samples also offers the opportunity for follow-up of HBV patients and for a closer study of the behaviour of the virus during the evolution of the hepatitic disease. The recent cloning of HCV (Reyes et al. 1990) and the use of the PCR technique to detect HCV infections (Kaneko et al. 1990b; Shindo et al. 1991) in combination with the methods described here may elucidate the analysis of the pathogenetic mechanism, and appropriate treatment of aetiologically specific categories of hepatitis.

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