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COMPARISON OF SERUM HEPATITIS B VIRUS REPLICATION MARKERS IN PATIENTS WITH CHRONIC HEPATITIS B: STUDIES ON HBeAg/ANTI-HBe SYSTEM, VIRAL DNA POLYMERASE AND HBV-DNA

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SUMMARY

The detection of HBV-DNA in serum by molecular hybridization is the most sensitive and specific marker of replication and infectivity of hepatitis B virus and currently is proposed as a routine diagnostic technique in the follow up of HBV — related diseases. Comparing different techniques already described, we found that direct spotting of serum samples on nitrocellulose membranes under vacuum filtration, followed by denaturing and neutralizing washes is more practical, simple, sensible and reproducible.

DNA polymerase assay using phosphonoformic acid as specific viral inhibitor has shown 86.8% of concordance with HBV DNA detection, and so, it is an useful alternative in the follow up of hepatitis B chronic patients.

We found 19.2% HBeAg positive samples with no other markers of viral replication and no anti-HBe positive sample had detectable HBV DNA. Discordance between the 2 systems have been extensively described, and we confirm this for the first time in our country. Molecular biological techniques are essential to determine the replication status of chronic hepatitis B patients.

KEY WORDS: Hepatitis B; Molecular hybridization; DNA polymerase; HBV-DNA; HBV replication markers; Chronic hepatitis B.

INTRODUCTION

Hepatitis B is a worldwide public health problem involving more than 200 million people as Hepatitis B Virus (HBV) carriers, with high risk of developing severe chronic liver disease, cirrhosis and hepatocellular carcinoma[27]. Its aetiological agent has been extensively studied and characterized and is now classified in the new group of Hepadnaviridae, which includes other animal partial double — strand DNA hepatotropes virus that share many other common features with the human virus[14].

During the natural history of this disease, there is a initial period with active viral replication, corresponding to the acute phase. In most of the cases, the evolution is benign, and the

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disease is aborted at this stage. In some cases, however, the host immune system is not capable to neutralize viral replication, and the disease progresses with continued viral replication for more than 6 months, characterizing the chronic forms of hepatitis B. In other cases, the viral DNA becomes integrated in the host cellular genome, and the viral replication ceases or decreases to undetectable levels[12].

The assessment of HBV replication status in following these patients is very important for prognosis, infectivity monitoring, and, furthermore, for indication and follow up of antiviral or corticosteroid therapy[6].

Many serum markers have been developed to characterize viral replication. Initially, it was shown that the detection of HBeAg in sera was related in many cases with other evidences of viral replication[23]. HBeAg, detected by standard immunoassays, is a soluble antigen, and its synthesis occurs in the liver generally when the virus is actively replicating[13]. However, some direct methods to detect Dane particles constituents have shown discordance between the presence of viral replication and the detection of HBeAg[8].

KAPLAN et al., 1973[16] developed a technique to detect the HBV DNA polymerase activity in serum after ultracentrifugation. This technique is very cumbersome and not suitable for routine screening. LIN et al., 1984[19] avoided the ultracentrifugation step using phosphonoformic acid as a specific viral polymerase inhibitor, creating a simpler and more sensitive method suitable for routine screening.

Detection of HBV-DNA in serum by molecular hybridization was developed by some groups in the early 80's[1, 3, 18, 30] and is currently proposed as a routine diagnostic assay in the follow-up of HBV related diseases. The superiority of this technique has led to the detection of a more sensitive and specific marker of replication and infectivity of HBV[9]. The use of this technique allowed the detection of HBV homologous sequences in HBsAg negative chronic hepatitis cases[4, 10]. Since then discrepances between the HBeAg/anti-HBe system and direct HBV-DNA detection have been described.

Hybridization techniques described so far vary in many crucial features: direct immobilization of samples onto nitrocellulose membranes[18], pre-treatment of samples with proteinase K[22], previous denaturation of samples with Sodium hydroxide[26], utilization of Dextran-Sulphate in washes[21]. WALTER et al., 1986[28] compared various techniques and proposed pre treatment of samples with formalin-sarkosyl as the most sensitive one, with no other treatment. Other groups have also shown that proteases digestion has adverse effects in sensitivity[31].

Comparisons of the "e" system with others parameters of viral replication has led to variable results. Some groups found 67%[22] and 68%[6] HBeAg positive cases also HBV-DNA positive. For other groups, about 90 to 100% HBeAg positive cases have detectable DNA[2, 18, 26, 30]. Considering anti-HBe cases, less concordance is found, with very high geographic variability[17]: in Northern Europe and USA anti-HBe positive patients were rarely HBV-DNA positive[15, 28], but in Italy[2], Greece[18] and Taiwan[7] more than half anti-HBe positive patients have detectable serum viral genomes.

Considering the data discussed above, the aims of our study are: first, compare the hybridization technique proposed by WALTER et al. with Scotto's technique proposed by ZELDIS et al. and the technique utilized by us; second, compare the DNA polymerase assay developed by LIN et al. with the detection of HBV-DNA; and, finally, compare the detection of HBeAg, anti-HBe, viral DNA polymerase and HBV-DNA in 38 serum samples from chronic hepatitis B patients.

MATERIAL AND METHODS

REAGENTS — Restriction endonucleases for isolating HBV-DNA from the vector DNA were supplied by Pharmacia and Sigma. Radioactive probes were labeled to specific activities between 10^7 and 10^8 cpm/ug with DNase I and DNA polymerase I from Sigma using alpha — ^{32}P — dATP manufactured at Instituto de Química da USP (a gentle gift of Dr. J.C.C. Maia and Dr. M.H. Juliani) (800 mCi/mmol). Nitrocellulose membranes BA85 were supplied from Schleicher & Schuell. Samples were concentrated using a

manifold apparatus manufactured at Tecnica Permatron (São Paulo, SP). Hybridizations were performed in heat sealed plastic bags. Autoradiographies were performed with Kodak QH S film and intensifying screens. ^{3}H — dTTP was supplied from Amersham. Other reagents were mainly supplied by Sigma or Merck.

PREPARATION OF HBV-DNA PROBES — Cloned HBV-DNA was prepared in Escherichia coli HB101 transformed by p211 (a gentle gift of Dr. Francis Galibert, Laboratoire D'Hematologie Experimentale, Centre Hayem, Hopital St. Louis, Paris, France) according to MANIATIS et al, 1982[20]. This plasmid contains the entire HBV genome cloned in the EcoRI site of pBR322. Probes were labelled by nick translation as described by RIGBY et al. (1976)[24]. Standards were prepared by serial dilutions of purified HBV DNA in water in a way that each 25 µl of solution contains always 100 µg of sheared herring sperm DNA and HBV-DNA quantities cited in the text. Serum samples standard were made by diluting a strong positive sample with a negative sample to the concentrations cited in the figures.

PREPARATION OF SAMPLES FOR HYBRIDIZATION:

TECHNIQUE A — Routine laboratory technique is a modified Liebermann's technique. Samples (25 µl) were directly applied onto a nitrocellulose membrane (previously soaked in water) using a manifold system. The filters where then submitted to a denaturing treatment with NaOH 0.5 M, NaCl 1M for 20 minutes, neutralized with Tris HCl 0.1 M pH 7.4, NaCl 0.5 M for 5 minutes twice and then rinsed with 2 x SSC 5 minutes twice.

TECHNIQUE B — A modified Scotto's technique. Samples (25 µl) were incubated previously with 25 µl 2 M NaCl and 50 µl 1 M NaOH for 10 minutes in microtitration plaques wells and then filtered through a nitrocellulose membrane (previously soaked in 6 x SSC) using a manifold system. Filters were then neutralized as described above.

TECHNIQUE C — Walter's technique — samples (25 µl) were denatured in 10 x SSC, 2 N NaOH, 15% formaldehyde and 0.5% Sarkosyl at 70°C for 10 minutes and then filtered through a nitrocellulose membrane using the manifold system. Filters were then neutralized as described above.

Whatever was the technique used, filters were air dried and then baked for 2 hours at 80°C in vacuum oven. Procedures were performed at room temperature, unless other temperature is cited.

PRÉ-HYBRIDIZATION AND HYBRIDIZATION

PRE-HYBRIDIZATION was performed submerging the filter in 20 ml of 5 x SSC, 1 x Denhardt, 0.1 M Sodium Phosphate pH 7.5, 1 mM EDTA, 0.1 mg/ml freshly heat denatured sheared herring sperm DNA at 65°C for at least 2 hours.

HYBRIDIZATION was performed with 10 ml of 4 x SSC, 2.5 x Denhardt, 0.1 M Sodium phosphate pH 7.5, 1 mM EDTA, 0.2 mg/ml freshly denatured sheared herring sperm DNA, freshly heat denatured nick translated probe (10^{6} 10^{7} cpm/ml) gentle shaking at 65°C overnight.

REMOVAL OF NON-SPECIFIC HYBRIDIZATION was made by filter treatment twice in 2 x SSC, 0.1% SDS, 65°C, 10 minutes each, 1 x SSC, 0.1% SDS, 65°C, 10 minutes, and 0.1 x SSC, 0.1% SDS, 65°C, 10 minutes.

AUTORADIOGRAPHY — Filters were air dried and exposed for 2 and 7 days to film and intensifying screens cited above.

DETERMINATION OF VIRAL POLYMERASE ACTIVITY — Lin's technique modified — in two 1.5 ml plastic tubes each serum sample (25 µl) is incubated at 37°C for 16 hours in 1 M Tris pH 7.4; KCl 0.15 M; MgCl2 45 mM; NH_4Cl 55 mM; 2 — mercaptoetanol 0.15% ; Nonidet P-40 0.8%, 20 nM each non-radiolabelled nucleotide and 1 µCi 3 H-dTTP. In one of the 2 tubes was added 10 mM phosphonoformic acid as specific viral polymerase inhibitor. The reaction was

then stopped adding Pronase 1 mg/ml, that breaks viral capsid. Samples were then deposed on 2.5 cm diameter GF/C filters and non-incorporated nucleotides were removed by three washings in 5% tricloroacetic acid, 50 mM Sodium Pirophosphate. After drying, filters were counted in scintillation solution in a beta-counter. Samples were considered positive when there is a significant difference between the 2 tubes, compared to negative controls.

DETECTION OF HBeAg AND ANTI HBe was performed with Abbott diagnostic Kits.

SAMPLES — 38 HBsAg positive serum samples from chronic hepatitis B patients were assayed by the 3 methods described above.

RESULTS

Comparative results between the three samples preparation procedures are shown in figure 1. In terms of detection of purified cloned HBV DNA, all the techniques could detect as little as 100 pg of DNA diluted as described above. No hybridization signal was detected with the negative control sera. When comparing the detection of our standard serum sample dilutions, techniques A and B gave signals till the 1/16 dilution, but technique C shown no signal, even with the half-diluted sample.

Operationally the most simple technique is by far technique A that needs no separated treatment of individual serum samples and allows a rapid and efficient filtration of samples. Technique B, besides a longer operation time, elicits in some samples the appearance of protein clots that block total filtration of samples in commercial manifold systems. Considering these results, the other hybridizations were performed according to technique A. Longer autoradiographic exposures (7 days) allow the detection of as little as 10 pg of purified DNA, as shown in figure 2, without compromising specificity.

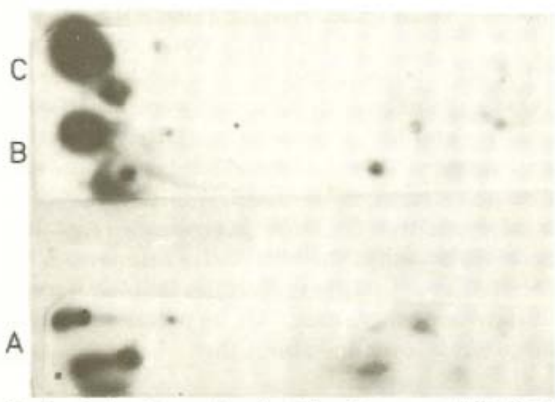

Fig. 1 — Autoradiographies after 1 day of exposure of hibridized nitrocellulose filters submitted to spotting techniques A, B and C as described in text. From left to right: purified cloned DNA: 10ng, 1ng, 100pg, 50pg, 25pg, 10pg, positive serum sample: 1/2, 1/4, 1/8, 1/16; negative serum sample.

The results of the analysis of 38 serum samples by the four serologic methods described above, are shown in table 1. Hibridization signals with varied intensity over a clear background from some of these samples were shown in figure 3 even with some spillage and no round spot caused by the manifold apparatus available.

Fig. 2 — Autoradiography of hybridized nitrocellulose filter after 7 days of exposition performed according to technique A. Serial dilutions of HBV DNA as described in text. From left to right 10ng, 1ng, 100pg, 50pg, 25 pg, 10 pg, 1 pg. Positive serum sample dilutions 1/2, 1/4, 1/8, 1/16; negative serum sample.

TABLE 1

		DNA and Pol. +	DNA + Pol. −	DNA − Pol. +	DNA and Pol. −	Total
I	HBeAg + Anti HBe −	17 65.5%	3 11.5%	1 3.9%	5 19.7%	26
II	HBeAg + Anti HBe +	0	0	0	1	1
III	HBeAg − Anti HBe +	0	0	1 11.1%	8 88.9%	9
IV	HBeAg − Anti HBe −	0	0	0	2	2
Total		17	3	2	16	38

Comparative results of hybridization (DNA) and DNA polymerase (POL.) assays in 4 different groups according to HBeAg anti HBe status.

DISCUSSION

Development of molecular biology allowed the standardization of very specific and sensitive

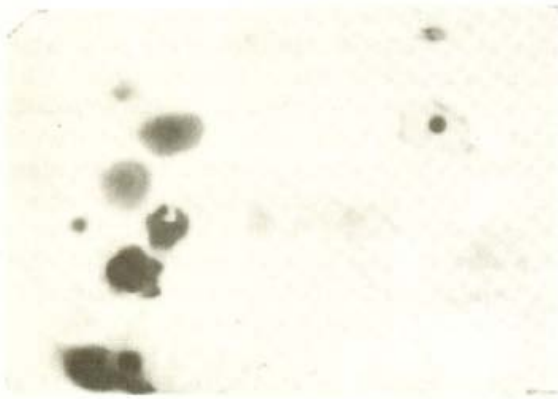

Fig. 3 — Autoradiography of a nitrocellulose filter containing spotted serum samples and hybridized as described in the text. The filter contains negative samples and positive samples of varying intensity spotted in alternate wells. Results were expressed from + to + + + + according to the signal intensity.

methods driven to the direct detection of complete HBV particles components, such as DNA polymerase and viral DNA. Initially restrained to research laboratories, these techniques have been so simplified that, nowadays, the detection of HBV-DNA and DNA polymerase are considered essential diagnostic tools for determining the replication status of chronic hepatitis B patients, necessary for indication, follow-up and prognosis of antiviral or corticosteroids therapy[6].

Since its first application in the beginning of this decade, many groups proposed several different procedures for serum HBV DNA detection. Recently, WALTER et al., 1987[29] and ZELDIS et al., 1986[31] compared some different techniques, and each proposed one different technique as the best one. We compared the Scotto's technique proposed by ZELDIS et al. and Walter's tecnique with our rotine technique, which is a modification of Liebermann's technique. We got disappointing results with Walter's technique, and, comparable results using Scotto's procedures.

In our hands, previous denaturation of serum samples could elicit appearance of protein clots that will block filtration in commercial manifold apparatus. Other group using this technique utilise a self-planned equipment with larger wells, that will not be blocked by these clots[26]. Most of the groups proposing direct spotting of serum samples do not perform vacuum filtration to concentrate them, as the porus diameter of nitrocellulose membranes (220 or 450 nm) is larger than the diameter of Dane particle (42 nm), and some virus are not retained on nitrocellulose. WALTER et al., 1987[29] have even showed positive hybridization signals in four overloaded membranes submitted to vacuum filtration. However, these groups had to work with smaller quantities of serum (5 μl) than us (25 μl), because samples diffuse rapidly through nitrocellulose (5 μl sample directly spotted corresponds to about 1 cm diameter spot). So, even with the lost of some virus through nitrocellulose membrane porus, we can assay a quantity of serum 5-fold higher than simple direct spotting, allowing greater sensitivity. Unlike other groups[26, 29], we did not identify any serum inhibitor of DNA binding to nitrocellulose, as our sensitivity was increased when performing direct spotting. ZELDIS et al., 1986[31] suggest that the 5'binding protein could work as a bridge to the membrane. Perhaps interactions between viral envelope and core proteins with the nitrocellulose, involving or not polymerised serum albumin, enhance the retention of viral particles.

Other method proposed to HBV replication monitoring is the detection of serum viral DNA polymerase activity. LIN et al., 1984[19] developed a simplified technique, slightly modified by us. To our knowledge, Lin's method was never compared with HBV-DNA detection. This assay has shown a good correlation with hybridization (33/38 - 86.8%) and HBeAg positivity (29/40 - 72.5%). We found 3 sera HBV-DNA positive and DNA polymerase negative, and 2 other with inverse parameters. The former were all HBeAg positive and the latter were one HBeAg positive and one anti-HBe positive. This last serum was also anti-delta positive, and, it might represent a false positive for DNA polymerase. Another possibility is that the virus delta interferes with the results of this assay. Studies involving more anti-delta positive cases will be performed to enlarge our knowledge about this.

In conclusion, assaying viral DNA polymerase using the technique developed by LIN et al. is a good marker for HBV replication, although false positive samples could rarely ap-

pear. Comparing to hybridization, it has the advantage of involving tritium labelled nucleotides, that are less hazardous, easier to handle, and have longer half-life. Careful storage of sera is very important because the enzyme is very labile[13]. We are now trying to simplify the precipitation and washing steps, optimize positive ne gative ratio and reprodutibility, which is a common problem involving enzymatic reactions.

For the first time in our country, the comparison between HBeAg/anti-HBe system and molecular biological replication markers in chronic hepatitis B patients was performed. This is very important due to high geographic variability of this correlation. Our results show that among HBeAg positive/anti-HBe negative patients, 19.2% do not show other serum replication marker and 80.8% show one of them at least. On the other hand, 1 9 anti-HBe positive patient had detectable DNA polymerase but no HBV-DNA. This sample was already discussed above. None of our anti-HBe positive sample shown HBV DNA.

These results are intermediate to those from other groups, where HBV replication markers were found in most (67 - 100%) HBeAg positive samples and in few to more than half anti-HBe positive samples. Many reasons may explain our results. First, we studied chronic hepatitis B patients, some of them evolving by spontaneous or induced HBeAg/anti-HBe seroconversion, that could explain the HBeAg positive/DNA negative sera. Second, some patients in advanced stages of disease could maintain this serological pattern[6, 17]. Third, São Paulo is a region were the HBV prevalence is about 2%[5], corresponding to medium endemicity region, with a paucity of studies through the world at this point. This could explain the intermediate characteristic of our population, with more HBeAg/DNA negative patients than Northern Europe and USA, and with fewer anti-HBe/DNA positive patients than found in highly endemic areas. Studies with larger populations, from different ethnic and regional origins will be performed in a larger study.

Anyway, these data clearly demonstrate some disagreement between HBeAg and HBV-DNA or DNA polymerase detection in chronic hepatitis B patients and reinforce the necessity of monitoring them in the follow up of these patients. Comparison between sorologic markers and detection of tissual viral antigens and in situ hybridization[25] are under investigation in our laboratories.

RESUMO

Comparação dos marcadores sorológicos da replicação do vírus da hepatite B: estudos sobre o sistema AgHBe/anti-HBe, DNA polimerase viral e HBV-DNA.

A detecção do genoma do HBV no soro por hibridização molecular é o mais sensível e específico marcador da replicação e infectividade do HBV, sendo sua utilização proposta como técnica rotineira no acompanhamento de doenças relacionadas a este vírus. Comparando diferentes técnicas descritas anteriormente, escolhemos a deposição direta das amostras séricas sobre a membrana de nitrocelulose sob filtração a vácuo, seguida de banhos desnaturantes e neutralisantes como mais prática e simples, com sensibilidade equivalente.

O ensaio da DNA polimerase usando ácido fosfonofórmico como inibidor viral específico mostrou 86.8% de concordância com a detecção direta do DNA viral, sendo, portanto, uma alternativa viável no acompanhamento de pacientes com hepatite crônica B.

Encontramos 19,2% das amostras AgHBe positivos sem outros marcadores de replicação viral. Por outro lado, nenhuma amostra anti-HBe positiva teve HBV-DNA detectável. Discordância entre estes dois sistemas foi extensamente descrita, e confirmamos pela primeira vez este fato em pacientes com hepatite crônica B em nosso país. Técnicas de biologia molecular são, portanto, fundamentais na determinação da replicação viral em cada paciente.

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ABREVIATIONS

HBV — Hepatitis B Virus

1 x SSC — 0.15 M NaCl, 0.015 M Sodium Citrate

1 x Denhardt — 0.02% Ficoll, 0.02% Polivynilpirrolidine, 0.02% BSA

BSA — Bovine sorine albumin.

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