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Assessment of Former and Newly Developed HBV Assays in a Third World Setting

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Newly available HBV serological assays have not been established routinely in most underdeveloped countries. Utilizing enzyme-immune assays to determine the presence of pre-S1 antigen and anti-pre-S2, and using two conventional hybridization techniques and the PCR assay to detect HBV-DNA, we studied 30 HBsAg chronic carriers and as a reference group 10 subjects whose only HBV routine marker was anti-HBc. Seventy-nine percent of the HBeAg positive carriers showed detectable HBV-DNA by a non-radioactive slot-blotting technique. The PCR assay was more sensitive than the slot-blotting technique, detecting HBV-DNA in anti-HBe positive patients with moderate or normal ALT activity. Pre-S1 antigen was mostly related to the presence of HBsAg and anti-pre-S2 was associated with active viremic state, increased ALT activity (ranges 51 to 640 IU/L), and with self-limited HBV infection. The presence of HBV-DNA in the group with anti-HBc only was detectable solely by the PCR assay. For an underdeveloped country the addition of a PCR assay or pre-S/anti-pre-S protein tests to the current assessment procedures of HBV chronic infection should be used only in selective cases. HBeAg/anti-HBe serological evaluation and HBV-DNA detection by a non-isotopic conventional hybridization technique still remain as useful tools to screen initially for the presence of viremia in chronic HBsAg carriers. The presence of HBV-DNA in individuals with anti-HBc only suggests that anti-HBc screening should be maintained and expanded to all the blood banks of less industrialized countries where the rate of HBV infection in apparently healthy people tends to be high.

KEY WORDS: Hepatitis B virus, DNA hybridization, pre-S proteins, anticore

INTRODUCTION

Recently developed assays have permitted the detection of different related proteins encoded by the hepatitis B virus (HBV) envelope genes [Heermann et al., 1984; Kuijpers et al., 1988]. Pre-S1 and pre-S2 antigens have been reported in sera and liver tissue of patients with HBV infection at different clinical stages, showing a particular correlation with markers of viral replication such as HBeAg and HVB-DNA [Heermann et al., 1984; Kuijpers et al., 1988, 1989; Petit et al., 1990; Dienes et al., 1990]. Several reports also suggest that antibodies to pre-S2 epitopes (anti-pre-S2) may precede the immune response to the major viral protein implying that these antibodies may contribute to neutralization and possible elimination of the virus [Neurath et al., 1986; Itoh et al., 1986]. However, the presence of anti-pre-S has been described in cases of chronic hepatitis B, suggesting that they are probably not responsible for viral clearance [Hoofnagle and Di Besceglie, 1991]. To date, the clinical significance of the pre-S encoded proteins and their specific antibodies remains controversial. In the last 2 years, the detection of serum HBV-DNA has been greatly enhanced by the use of the polymerase chain reaction technique (PCR) [Kaneko et al., 1989; Sumasaki et al., 1989], suggesting its optimal application as a monitoring tool for HBV infection [Chemin et al., 1991; Gerken et al., 1991]. This report attempts to define the implications of these newly available HBV diagnostic procedures in the clinical approach currently established for HBV infection in an underdeveloped country.

PATIENTS AND METHODS

Study Groups

Group A included 11 HBsAg chronic carriers with liver disease (3 chronic persistent hepatitis, 5 chronic active hepatitis and 3 active cirrhosis), with a mean age of 37 years. All attended the out-patient care unit of the Instituto de Inmunologia, Caracas, Venezuela. Alanine-aminotransferase (ALT) was measured by an UV-Kinetic method (a-gent® Abbott Laboratories, North

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Chicago, IL, USA; upper normal limit 37 IU/L). At the time of the study, ten patients had a mean ALT activity 4.7-fold above the normal upper limit (mean ALT 175 IU/L) and only one patient had a normal ALT value. Furthermore, 90% presented increased circulating immune-complexes (mean 5.4 μg/ml of human aggregated gammaglobulin control values 2.1 ± 1 μg/ml) as detected by a solid phase Clq microassay as previously described [Orozco et al., 1983] and 70% showed increased cryoprecipitate protein content (mean 0.608 mg/ml, control values 0.254 ± 0.110) after a 7 day period following the method described by Contreras et al. [1982]. Patients with HBsAg had high titers of anti-HBc. None had anti-HBs and only one had anti-Delta during the follow-up period. Group B consisted of 19 asymptomatic HBsAg chronic carriers, also anti-HBc positive and anti-HBs negative, with a mean age of 35 years. In 11 patients the mean ALT activity was 2.7-fold above the normal upper limit (mean ALT 103 IU/L), while 8 maintained normal ALT values, with 83% with increased circulating immune-complexes (mean 5.3 μg/ml) and 33% elevated cryoprecipitate protein content (mean 0.464 μg/ml). The last group (Group C) consisted of 10 subjects with persistent high titers of anti-HBc only during at least 6 months of follow-up. The group was represented by 3 polytransfused patients with high risk to HBV infection, 3 health care workers (intermediate risk), and 4 subjects without risk factors for hepatitis B (low-risk). None had abnormal ALT activity (mean 21 ± 8 IU/L) and all were repeatedly negative for HBsAg, anti-HBs, IgM-anti-HBc, and anti-hepatitis C virus (HCV) antibodies during 6 months of follow-up prior to entering the study.

Assays for Different HBV Markers

HBsAg, anti-HBs, anti-HBc, IgM-anti-HBc, HBeAg, anti-HBe, and anti-Delta antibodies were measured by commercial enzyme-immune assays (Hepanostika, Organon-Teknika, Turnhout, Belgium). Anti-HCV antibodies were detected by a second generation enzyme-immune assay (Abbott Laboratories, North Chicago, IL). Detection of pre-S1 protein and anti-pre-S2 was carried out following the microELISA technique developed by Organon-Teknika, the Netherlands [Kuijpers et al. 1988]. All serum samples were re-tested for both pre-S1 and anti-pre-S2 to minimize non-repeatable false positive reactions. Levels of anti-HBc and anti-pre-S2 were quantitated by calculating the percentage inhibition utilizing the formula described by Parkinson et al. [1990]: % inhibition = 100 − (O.D. sample reading/O.D. negative reading) × 100.

HBV-DNA

HBV-DNA was first detected by the slot-blot technique [Scotto et al., 1983]. The DNA probe (recombinant plasmid pAM kindly provided by Dr. Robert Purcell, NIH, USA) was labeled by the oligonucleotide random-priming method with digoxigenin-dUTp according to the instructions of the manufacturer (Boehringer-Mannheim, USA). HBV-DNA content in positive samples was quantitated by a solution-phase hybridization assay (Genostics, Abbott Laboratories, North Chicago, IL [Kuhns et al., 1988]. The presence of HBV DNA was further assessed by the PCR using one set of oligonucleotides specific for the pre-core-core region of the HBV genome. Serum samples were processed according to Kaneko et al. [1989]. Amplification was carried out in a final volume of 100 μl containing 10 μl of serum DNA sample, 2.5 units of Taq polermase (Perkin-Elmer Cetus, USA), 200 μM of each dNTP, 0.5 μM of each primer, 50 μM KCl, 10 mM Tris-HCl (ph 8.3), and 1.5 mM $MgCl_2$. After overlayering with 100 μl of mineral oil, the reaction was performed in an automatic DNA thermal cycler (Perkin-Elmer Cetus, USA) using an amplification program of 35 cycles. Each cycle consisted of a) denaturation step (94°C for 1 min), b) annealing step (42°C for 2 min), and c) polymerization step (72°C for 2 min). A final polymerization step was carried out for 10 min. After the last cycle, PCR products were analysed by 2% agarose gel electrophoresis, observing the DNA by UV fluorescence after staining with ethidium bromide.

RESULTS

Group A

Irrespective of the liver histology and the HBeAg/anti-HBe serological status, serum pre-S1 protein was found in each patient (Table I).The HBeAg positive subgroup had HBV-DNA by slot-blotting, while only one anti-HBe positive patient proved HBV-DNA positive by this assay. The quantity of HBV-DNA content ranged from 4.19 to 358 pg/ml. The three patients without HBV-DNA by slot-blotting or by the solution-phase hybridization assay had detectable HBV-DNA by PCR, two of whom had mild or normal ALT activity. Eight out of 11 patients (73%) were repeatedly reactive for anti-pre-S2 (% inhibition ≥ 75%), 6 patients wilth a 6.7-fold increase in ALT activity (mean 249 IU/L). The HBsAg patient positive for anti-Delta during the follow-up maintained anti-pre-S2 reactivity and elevated ALT activity with a viremia detectable only by PCR.

Group B

Only one patient (No. 19) was negative for pre-S1 without HBeAg/anti-HBe, and without anti-pre-S2, had HBV-DNA detectable only by PCR with a moderate ALT activity (Table II). As in group A, the presence of pre-S1 was found in the majority of the carriers independently of the HBeAg/anti-HBe status. The conventional hybridization techniques for HBV-DNA detection were less sensitive for identifying the viremia in this group since only 5 carriers demonstrated (4 HBeAg positive carriers and 1 HBeAg/anti-HBe negative patient) HBV-DNA by slot-blotting with a concentration which ranged from 11 to 503 pg/ml. The PCR proved to be more sensitive than the slot-blotting technology for detecting HBV-DNA in the remaining four HBeAg positive patients, three of whom had normal ALT values.

TABLE I. Pre-S1, Anti-Pre-S2 and HBV-DNA in Patients With Chronic HBV Infection†

Liver Histology	HBeAg	Anti-HBe	Pre-S1	Anti-pre-S2	HBV-DNA slot-blotting	HBV-DNA PCR	ALT (IU/L)
CPH	+	−	+	+	+	*	117
CPH	+	−	+	+	+	*	80
CPH	−	+	+	+	−	+	60
CAH	+	−	+	+	+	*	117
CAH	+	−	+	−	+	*	51
CAH**	−	+	+	+	−	+	640
CAH	−	+	+	−	−	+	40
CAH	−	+	+	+	+	*	118
Active cirrhosis	+	−	+	−	+	*	67
Active cirrhosis	+	−	+	+	+	*	299
Active cirrhosis	+	−	+	+	+	*	204

†CPH = chronic persistent hepatitis. CAH = chronic active hepatitis. *HBV-DNA positive by slot blotting. **Positive anti-delta.

TABLE II. Pre-S1, Anti-Pre-S2, and HBV-DNA in Asymptomatic HBsAg/Anti-HBc Chronic Carriers

Asymtomatic HBV carriers	HBeAg	Anti-HBe	Pre-S1	Anti-pre-S2	HBV-DNA slot-blotting	HBV-DNA PCR	ALT (IU/L)
1	+	−	+	−	+	*	55
2	+	−	+	−	−	+	38
3	+	−	+	+	+	*	66
4	+	−	+	−	−	+	17
5	+	−	+	+	+	*	237
6	+	−	+	+	−	+	91
7	+	−	+	−	−	+	38
8	+	−	+	+	+	*	105
9	−	+	+	−	−	+	19
10	−	+	+	−	−	+	33
11	−	+	+	−	−	−	32
12	−	+	+	−	−	+	106
13	−	+	+	+	−	+	66
14	−	+	+	−	−	+	108
15	−	+	+	−	−	+	110
16	−	−	+	+	−	+	35
17	−	−	+	+	+	*	118
18	−	−	+	+	−	−	39
19	−	−	−	−	−	+	71

*HBV-DNA positive by slot-blotting.

Moreover, the PCR also detected the present of HBV-DNA in 7 out of 9 (78%) patients (6 anti-HBe positive carriers and 1 HBeAg/anti-HBe negative patient). The two carriers without HBV-DNA by PCR had normal ALT values (patients numbers 11 and 18). Eight subjects (42%) had anti-pre-S2 (% inhibition $\geq$ 76%), 7 with HBV-DNA detected by slot-blotting or by PCR, and 6 associated with moderate or high ALT values (mean 114 IU/L).

Group C

Table III shows the serological HBV pattern of individuals who were positive for anti-HBc only. The percentage inhibition of the anti-HBc test ranged from 89.6% to 96%. Such anti-HBc levels have previously been considered as high [Parkinson et al., 1990]. An association with anti-HBe and anti-pre-S2 was observed in 4 and 3 individuals respectively. None were positive for pre-S1 protein or HBeAg. HBV-DNA was detectable only by PCR in nine individuals (90%), including those with low exposure to HBV. Figure 1d shows one of these HBV-DNA positive samples as determined by PCR.

DISCUSSION

Several clinical serological patterns have been described in association with the natural history of HBV infection [Robinson, 1990]. The clinical and diagnostic benefit of newly available HBV assays still remains to be fully established [Kuijpers et al., 1989; Sumasaki et al., 1989; Petit et al., 1990]. The possibility that the pre-S determinants might represent active viral replication, and the notion that the appearance of anti-pre-S could indicate impending resolution of the infection has

TABLE III. Presence of HBV-DNA by PCR in Individuals Who Were Positive for Anti-HBc Only*

Risk	Anti-HBc	HBeAg	Anti-HBe	Pre-S1	Anti-Pre-S2	HBV-DNA slot-blotting	HBV-DNA PCR
High-risk[a]	+	–	–	–	–	–	+
High-risk	+	–	+	–	–	–	+
High-risk	+	–	+	–	–	–	+
Intermediate risk[b]	+	–	–	–	–	–	+
Intermediate risk	+	–	+	–	+	–	+
Intermediate risk	+	–	–	–	–	–	–
Low-risk	+	–	–	–	–	–	+
Low-risk	+	–	–	–	–	–	+
Low-risk	+	–	–	–	+	–	+
Low-risk	+	–	+	–	+	–	+

*Mean ALT = 21 ± 8 IU/L.
[a]High-risk = polytransfused patients.
[b]Intermediate-risk = health-care workers.

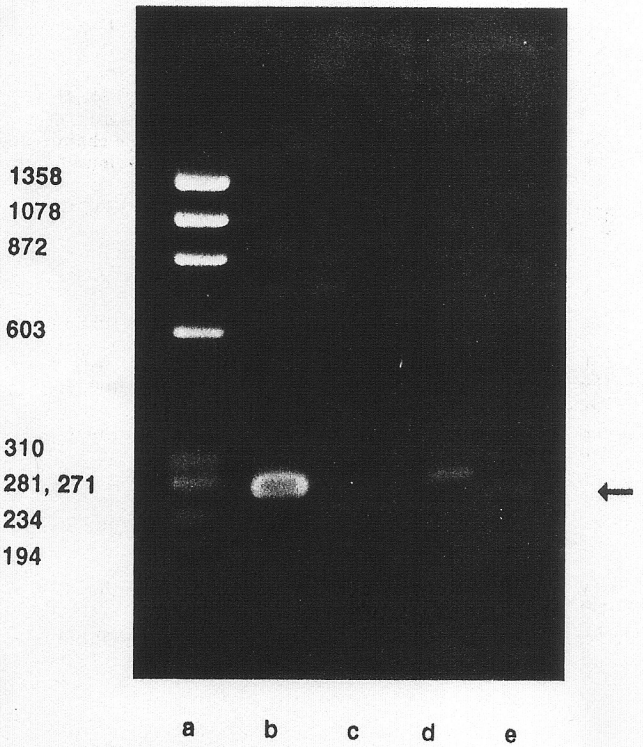

Fig. 1. Agarose gel electrophoresis of the PCR products specific for HBV. The gel was stained with ethidium bromide and visualized under UV light. **a:** Molecular weight markers. Φ X174 DNA digest with Hae III. **b:** Positive control plasmid pAM. **c:** Negative control. Serum sample negative for HBV markers. **d:** Serum from an HBV subject whose only routine viral marker was anti-HBc. **e:** Negative control. PCR mix without template DNA. The arrow shows the PCR product (270 base pairs).

not been fully accepted [Hoofnagle and Di Bisceglie, 1991]. Furthermore, the need to identify the viremic status of the HBV chronic carriers is necessary for monitoring antiviral therapy [Gerken et al., 1991]. In less industrialized countries the diagnosis of viral hepatitis is too costly due to the need to import reagents and diagnostic kits [Machado et al., 1985]. For example, in Venezuela the average cost of medical care for a patient with chronic active hepatitis has been estimated at US $8,540, employing solely the traditional HBV serological markers [Fernández et al., 1991]. To assess the implications of incorporating a broader diagnostic approach to the several stages of HBV infection in underdeveloped countries, we studied a group of HBsAg chronic carriers with different degrees of liver inflammation. We included the detection of the pre-S1 protein, anti-pre-S2, and circulating HBV-DNA, the latter investigated by two conventional hybridization techniques and by PCR. It was found that pre-S1 antigen determination cannot substitute the information provided by HBeAg/anti-HBe for clinical assessment. Pre-S1 protein was found either in patients with established chronic liver disease or in asymptomatic HBsAg carriers with a lesser degree of liver inflammation reflected by a moderate elevation of ALT activity. In our reference group, represented by individuals who were positive for anti-HBc only, pre-S1 protein was consistently absent. As reported by others, these results suggest that the presence of pre-S1 antigenemia is related mainly to the titer of HBsAg and to an excess of subviral particles [Budkowska et al., 1988; Yuki et al., 1990]. Therefore, for preliminary clinical evaluation of HBV chronic carriers, HBeAg/anti-HBe determination is still useful to indicate the replicative viral status. It has been claimed that PCR is more accurate than other serological assays for identifying viral replication [Gerken et al., 1991]. We found that the subgroup of viremic patients already identified by the presence of HBeAg had detectable levels of HBV-DNA by a non-isotopic slot-blotting technique. The PCR assay was more sensitive for identifying the low replicative viral activity of HBsAg patients who were firstly classified as non-viremic carriers due to the presence of anti-HBe and the absence of HBV-DNA as detected by conventional hybridization techniques. All those carriers were within the group of asymptomatic patients with moderate or normal ALT activity. These patients need not necessarily fulfill the criteria for entering antiviral clinical trials [Hoofnagle, 1990]. Consequently, assess-

ment of the HBV replicative status by HBeAg serological determination or by conventional hybridization techniques seems to be reliable. The PCR assay, which requires a rigorous technical performance, probably has a place in studying the potential viremic status of selective individuals. For instance, our group with anti-HBc only showed circulating HBV DNA only when the sera were tested by PCR. The presence of free viral DNA in the serum of anti-HBc positive carriers has been reported recently [Sumasaki et al., 1989; Gerken et al., 1991]. These findings have several implications in countries where the prevalence of HBV infection is high in apparently healthy people [Machado et al., 1988, 1991]. Moreover, in contrast with most of the developed countries, the majority of the underdeveloped countries, including Venezuela, have only introduced recently anti-HBc testing in blood bank guidelines.

Finally, it was found that anti-pre-S2 could be associated with active viremia. The coexistence of anti-pre-S2 and HBV-DNA has been reported rarely in patients with HBV liver damage [Hellstrom et al., 1986]. We found anti-pre-S2 more frequently in patients with active inflammatory liver disease confirmed either histologically, by an increased ALT activity, or by both procedures. This suggests that the production of serum anti-pre-S2 may be related to the necroinflammatory liver process. Furthermore, three patients with anti-HBc only also had anti-pre-S2, supporting the notion of a self-limited HBV infection.

In conclusion, for underdeveloped countries the addition of PCR as well as pre-S determinants to the current assessment of HBV chronic infection seems to have a place in selected cases. A clinical approach which includes routinely HBeAg/anti-HBe tests and HBV-DNA detection by conventional non-isotopic hybridization techniques remains helpful and easy to interpret.

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