

POTENTIAL INFECTIVITY OF BLOOD FROM HBsAg ASYMPTOMATIC CARRIERS DUE TO THE PRESENCE OF HBV-DNA AND COMPARISON WITH OTHER MARKERS OF HBV INFECTION

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SUMMARY

Serum samples from 356 HBsAg positive asymptomatic carriers, which were titrated by reverse passive hemagglutination, were analysed for the presence of HBV-DNA, HBsAg and IgM anti-HBc. The samples were divided in three classes, according to the titers of HBsAg and IgM anti-HBc and the distribution of HBV-DNA and HBsAg among these classes was studied. In the high titer class of HBsAg, 65% of samples have one or both markers against only 19% in the low titer class. From the total of 356 samples, 121 gave positive results for IgM anti-HBc (33.9%). From these, 38.9% of HBV-DNA and 47.9% of HBeAg were observed, whereas in samples with absence of IgM anti-HBc, 18.3% and 16.6% were respectively found.

A higher frequency of agreement between all these markers was found in the class of high titers of HBsAg; however, HBV-DNA was detected in the low titer class of HBsAg and little or no IgM anti-HBc, showing potential blood infectivity even in HBsAg positive borderline samples.

KEY WORDS: HBV-DNA; HBsAg; IgM anti-HBc; Asymptomatic carriers.

INTRODUCTION

The Hepatitis B virus (HBV) is one of the viruses that cause hepatitis in man, and the clinical picture is extremely variable, causing acute and chronic forms with different prognosis and degrees of severity, including cirrhosis and a strong epidemiological and biochemical evidence for association with hepatocellular carcinoma.

The infection is endemic in many parts of the world and in our country it poses a significant problem, with high frequencies of the infec-

tion being found in some areas^{2, 12, 29}, mainly in West Amazon where its presence is often associated to the Delta virus^{3, 13}.

The diagnosis of HBV infection is usually achieved by assay of the HBV surface antigen (HBsAg) but this marker does not indicate the presence of infectious particles in the blood. In individuals infected with HBV, HBsAg is found predominantly in the form of 22 nm non-infectious particles and also as part of surface antigen present in complete HBV particles⁶. These com-

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plete particles also have an inner core which contains the Hepatitis B core antigen (HBcAg) and HBeAg in a cryptic form^{1, 24, 31}. The presence of HBeAg in the serum as a soluble protein is associated with the production of complete virion²⁵, and is therefore utilized as a marker of infectivity.

Inside the core, a double-stranded circular DNA and a virus specific DNA polymerase can be found^{15, 16, 20}.

Different techniques are available for the detection of complete virus but they are either of low sensitivity, like electron microscopy, HBcAg detection and DNA polymerase assay or indirect like the detection of HBeAg.

Recently, the use of molecular hybridization analysis allowed the serological detection of HBV-DNA by nucleic acid hybridization, providing a direct and highly sensitive marker of viral replication^{4, 5, 6, 28}.

In this work, we sought to establish the potential infectivity of HBsAg positive sera from asymptomatic carriers as determined by the presence of HBV-DNA. The correlation of HBeAg, IgM anti-HBc and HBsAg levels with HBV-DNA positivity was studied.

MATERIALS AND METHODS

HBsAg positive serum samples from 356 blood donors of different blood banks from Rio de Janeiro were analysed.

Detection of serological HBV markers

HBsAg detection — These samples were confirmed by Elisa — FIOCRUZ⁷ and titrated by R-PHA (Bio-Manguinhos — FIOCRUZ)³⁰.

HBeAg detection — HBsAg positive samples were tested for the presence of HBeAg using Elisa Kits from Organon as well as reagents produced and standardized in our laboratory¹⁹.

IgM anti-HBc detection — All samples were tested with reagents standardized in the Centers for Diseases Control, Atlanta, USA, using HBcAg obtained from cloned *E. coli* Briefly, 125 μ l of goat anti-human IgM (μ -chain specific, Sigma),

diluted in 50 mM carbonate buffer pH 9.6 were coated in polystyrene microplates. After overnight incubation, the plates were washed 4 times in BSA (0.5%) in PBS, and 100 μ l of diluted samples (2×10^{-3} in NHS-PBS) were incubated for 1h/37 C. The plates were washed again, 100 μ l of purified HBcAg (50 ng/ml) diluted in 1% NHS/PBS was added and the reaction was incubated for 1 hour at 37 C. After washing the plates, 100 μ l of rabbit anti-HBc conjugated to peroxidase diluted in 10% NHS/10% NRS/PBS were added and incubation proceeded for 1h/37 C. After washing, 100 μ l of substrate (1.2 ml of 10X-substrate buffer + 10.8 ml distilled water and 0.25 ml of 5 mg TMB/ml DMSO) were added and after 30 minutes at room temperature, the reaction was stopped with 100 μ l of 4N Sulfuric Acid. (The components of 10X substrate buffer are Sodium Acetate 34.0 g, Citric Acid 0.73 g, in 250 ml of distilled water and 0.3 ml H₂O₂ 30%). The reaction is read at 450 nm. The cut-off value is calculated using the mean negative control + 0.1 mean positive control. Negative controls are colourless (extinction around 0.05) and positive controls give a colour that is clearly discernible from the negative control.

Preparation of samples and molecular hybridization

The serum samples were treated as described by SCOTTO et al.²³. To 50 μ l of sample, the same volume of 2M NaCl was added plus two volumes of 1M NaOH. Following a 10 minutes incubation at room temperature, the samples were spotted by filtration through a BRL Hybridot onto nitrocellulose filters prewet with 6X SSC (1X SSC: 0.015 M Na citrate, 0.15 M NaCl, pH 7.0). Neutralization was carried out by addition of 200 μ l of 0.5 M Tris pH 7.4, 3M NaCl, every five samples. The positive and negative controls added to the filters were treated in the same way.

The filters were prehybridized for 1h in a hybridization mix containing 50% deionized formamide, 5X Denhardt solution, 50 mM sodium phosphate buffer pH 7.5, 5X SSC and 100 μ g/ml denatured salmon sperm DNA, and then the radioactively labelled probe was added to the hybridization solution at a concentration of $1-2 \times 10^6$ cpm/ml. Hybridization was carried out at

37°C for 15-18 hs, followed by two 20 minutes washes in 2X SSC, 0.1% SDS at 65°C and two 20 minutes washes in 0.1X SSC, 0.1% SDS at 65°C. the filters were exposed to Sakura X-ray films for 24 hs.

The probe used in this work was the plasmid pCP10 which was a gift from Dr. Pierre Tiollais from the Institut Pasteur. This plasmid consists of the vector pBR 322, containing two copies of the HBV genome. In some experiments the whole plasmid was used as a probe, whereas in others the HBV-DNA was purified by electroelution from agarose gels. The probe was labelled by nick translation²² with ³²P to a specific activity of 1.2×10^7 cpm/ μ g of DNA. The ³²P-alpha-dATP was supplied by Dr. José Carlos Maia, from the University of São Paulo.

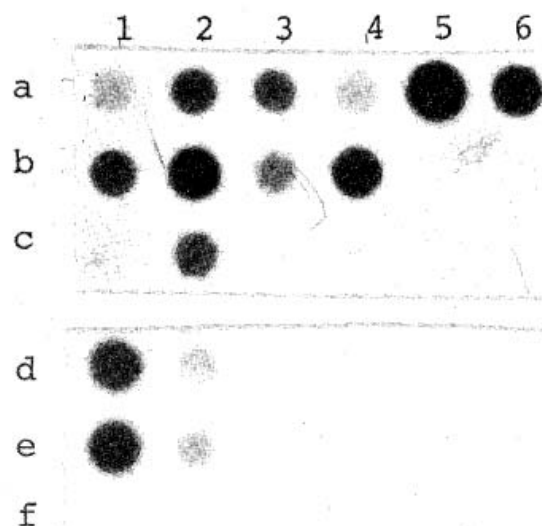
RESULTS

The distribution of 356 samples was analysed according to the hemagglutination titers of HBsAg. The titers varied from 1/16 to greater than 1/4096, of which 28.4% were high titered, with R-PHA titer greater than 1/2048.

The results of the hybridization tests were evaluated by two independent observers and doubtful cases were repeated. 94 out of 356 serum samples were positive when hybridized to pCP10, as exemplified in Figure 1. As it has been reported that false positive cases may arise, possibly as a result of bacterial contamination during storage¹⁰, all positive samples were hybridized to pBR322, the vector in which the HBV-DNA was cloned. Indeed, out of 94, ten samples were found to hybridize also to pBR322 and were therefore hybridized to the purified HBV-DNA insert. To ascertain the purity of the insert, pBR322 DNA was also spotted onto the filter, giving a negative result. Five of the ten pBR322 positive samples were shown to be positive also for the cloned HBV-DNA, being therefore included among the HBV positive samples, which were a total of 90.

The serological markers HBV-DNA, HBeAg and IgM anti-HBc detected in the present samples were correlated with the concentration of HBsAg, semi-quantitatively represented by the R-PHA titer, divided in three groups: 106 samples with low titer (under 1/128), 149 samples with

intermediate titers (1/256 to 1/1024) and 101 samples with high titers (over 1/2048). The percentage of HBV-DNA, HBeAg and IgM anti-HBc increases as the concentration of HBsAg increases (Table I).



a-1, 2, 3, 4, 5; b-1, 2, 3, 4 and c-2: positive samples; b-5, 6 and c-2: negative samples; a-6: positive control (purified Dane particles); d, e and f: HBV cloned DNA (purified from pCP10 plasmid); pCP10 (pBR322 + HBV-DNA) and pBR322 plasmid DNA, respectively. On columns 1 through 6 1000, 100, 10 and 1pg of DNA were spotted respectively.

Fig. 1 — Dot blots of serum of asymptomatic carriers of HBsAg, using pCP10 HBV-DNA insert as a probe.

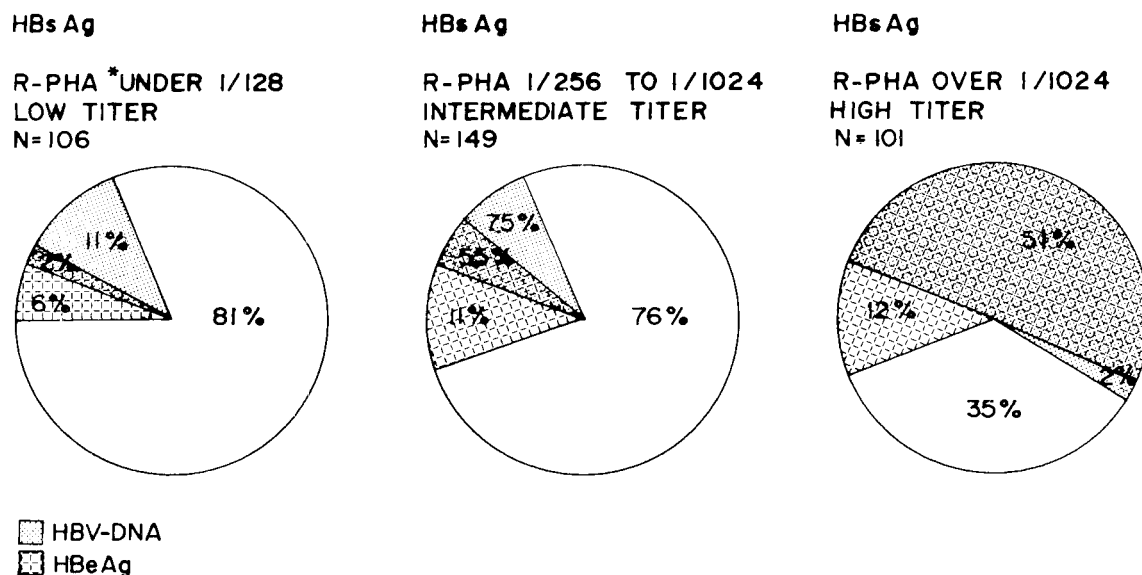
TABLE I
Correlation between HBV serological markers and hemagglutination titer of HBsAg.

R-PHA* titer	number of samples	HBV-DNA (%)	HBeAg (%)	IgM anti-HBc (%)
under 1/128	106	14 (12.3)	10 (9.4)	28 (26.4)
1/256 to 1/1024	149	18 (12.1)	21 (14.1)	52 (34.9)
over 1/2048	101	58 (57.4)	66 (65.3)	41 (40.6)
Total	356	90 (25.0)	97 (27.2)	121 (33.9)

* R-PHA = reverse passive hemagglutination

A clear result can be observed when the percentage of HBV-DNA and/or HBeAg markers is correlated with the titer of HBsAg. In high titer

samples 65% have one or both of these markers against only 19% in low titer samples (Figure 2).



*R- PHA: REVERSE PASSIVE HEMAGGLUTINATION TITER OF HBsAg

Fig. 2 — Percentage of HBV DNA and or HBeAg markers of HBV infection in relation to HBsAg hemagglutination titer.

TABLE 2
Correlation of anti HBc IgM antibodies with HBV-DNA and HBeAg serological markers of HBV infection.

IgM anti HBc	number of samples	HBV DNA (%)	HBeAg (%)
NEG	235	43 (18.3)	39 (16.6)
P/N < 10	90	34 (37.7)	39 (59.2)
P/N 11-50	27	10 (37.1)	16 (59.2)
P/N > 50	4	3 (75.0)	3 (75.0)
	356	90 (25.2)	97 (27.2)

P/N: OD sample / OD negative in Elisa.

Table 2 shows the correlation of IgM anti-HBc antibodies with HBV-DNA and HBeAg. From the total of 356 samples, 121 gave positive results for IgM anti-HBc (33.9%). In samples with P/N less than 10 (weakly positive) and between 11-50, approximately the same presence of HBV-DNA and HBeAg was seen (37% and 59% respectively). It is important to notice that in samples without IgM anti-HBc marker, 18.3% of HBV-DNA and 16.6% of HBeAg were observed, showing

that except for strongly positive samples, the presence of IgM anti-HBc seems to be independent of the presence of either HBV-DNA or HBeAg blood infectivity markers in asymptomatic carriers.

DISCUSSION

Less sensitive methods for the detection of HBsAg, like hemagglutination, are a useful tool to measure the level of antigen present in blood specimens. In sera of asymptomatic carriers of HBsAg, screened from blood donors, a wide variation of titers was found. It was expected that in specimens with a high concentration of HBsAg, other markers of viral replication, specifically HBV particles, would also be present.

The detection of IgM anti-HBc antibodies was also carried out to compare HBV-DNA and HBeAg markers of infectivity, once it could be associated with viral replication.

Indeed, observing Figure 2, it can be seen that the presence of either HBeAg and HBV-

DNA increases in the group with higher titers of HBsAg. In this group (over 1/1024) there is a very high association of HBV-DNA and HBeAg, similar to levels described in the literature. In this class, 96% of HBV-DNA positive sera are also positive for HBeAg, whereas among the total number of HBeAg positive cases, only 81% showed a positive signal for HBV-DNA. This is in agreement with the findings of other authors^{18, 23, 27} and might be explained, according to TAKAHASHI et al.²⁶, by the association of HBeAg with immunoglobulin G causing a lower clearance rate.

This correlation is much decreased among the intermediate titers of HBsAg, although the tendency for higher proportions of HBV-DNA negative/HBeAg positive individuals is maintained. Among the low and intermediate titers of IgM anti-HBc (Table 2), the percentage of individuals carrying HBeAg is also consistently higher than that of HBV-DNA positive cases. However, this is no longer true for the group of individuals with very low titer of HBsAg (Fig. 1), where a relatively high number of cases, 11%, was found which were positive for HBV-DNA while negative for HBeAg and yet some of them showed a very strong hybridization signal. This observation reinforces the current idea that HBeAg is not a very good marker of HBV replication¹⁸. Regarding IgM anti-HBc, only three out of these 11 sera were positive for this marker and they were only weakly positive. The same trend is again observed in the group of individuals negative for IgM anti-HBc: although for a slight margin, carriers of HBV-DNA are more frequent than HBeAg positive ones. The explanation for this kind of situation is not known. Judging from the markers we have, it would appear that there is a considerable level of viral genome replication, coexisting with a low expression of viral antigens. It would be interesting to have also the HBcAg status of these samples.

IgM anti-HBc was detected in 33.9% of the samples but only 25.6% of them represent a considerable titer of antibodies. The great majority showed a low titer, with P/N less than 10. ROGGENDORF et al.²¹ found the same prevalence in healthy carriers, while other authors reported much higher numbers, such as 75%, 81% and 82%^{11, 14, 17}. This apparent discrepancy might be

due to a higher cut-off value employed by us, since inspection of the above mentioned papers shows that a great proportion of the reported titer is low, if compared to those found in acute or chronic hepatitis.

This profile of IgM anti-HBc is commonly found in blood donors, who are routinely screened for clinically inapparent HBV infection, by the presence of HBsAg. Usually, in these cases, the presence of IgM anti-HBc is more likely to represent a residual marker from an earlier infection than an actual marker of replication, as it can be found in acute cases, where it is often observed in very high titer. It is also worth noticing that in these latter cases, the titer of IgM anti-HBc is likely to decrease sharply, while it has been demonstrated that in chronic cases moderate titers are found, which decrease slowly and persist for a very long time^{9, 14}. In our samples, high positivity for IgM anti-HBc was found in only 4 out of 121 cases (13.3%) and 3 of them were also positive for HBV-DNA and HBeAg, indicating a situation similar to an early stage of HBV infection. Thus, it appears that when this antibody is found in very high titers (in this work, the class represented by P/N > 50), it might be regarded as an indicator of viral replication. However, in groups which show intermediate or low positivity for IgM anti-HBc, the frequency of the other markers associated to HBV replication is moderate and very similar. This indicates that in the majority of blood donors, where IgM anti-HBc is present in medium to low titers, it cannot be associated to viral replication and should be considered just an evidence of a past infection.

Regarding our data as a whole, it can be seen that there are many cases in which HBV-DNA is present, even in groups with very low levels of HBsAg and little or no IgM anti-HBc, showing that borderline positivity for HBsAg can mean potential blood infectivity. Therefore, whenever possible, it is better to screen blood donors for markers of Hepatitis B by a more sensitivity assay, like ELISA, which in the case of HBsAg has the ability to detect up to 5 - 10 nanograms of antigen⁷.

RESUMO

Potencial de infectividade em sangue de portadores assintomáticos de HBsAg caracterizado pela presença de HBV-DNA e comparação com outros marcadores da Hepatite B.

Em 356 soros HBsAg positivos de portadores assintomáticos, titulados por hemaglutinação passiva reversa, foi analisada a presença de HBV-DNA, HBeAg e IgM anti-HBc, da seguinte forma: as amostras foram divididas em três classes de acordo com o título de HBsAg e IgM anti-HBc e foi verificada a distribuição de HBV-DNA e HBsAg nestas classes.

Em amostras com títulos elevados de HBsAg, 65% das amostras tiveram um ou ambos os marcadores HBV-DNA e HBsAg e as mesmas foram encontradas em apenas 19% das amostras com baixo título de HBsAg. Do total de 356 amostras, 121 foram positivas para IgM anti-HBc (33.9%). Destas, 38.9% de HBV-DNA e 47.9% de HBeAg foram observadas, ao passo que em amostras com ausência de IgM anti-HBc, 18.3% e 16.6% foram respectivamente encontrados.

A concordância entre os três marcadores foi encontrada em amostras com alto título de HBsAg, entretanto HBV-DNA foi também detectado em grupo de baixo título de HBsAg na ausência de IgM anti-HBc ou em níveis baixos, mostrando o potencial de infectividade mesmo em amostras de HBsAg com valores próximos ao limite da positividade.

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