

DETECTION OF HEPATITIS B VIRUS DNA BY THE POLYMERASE CHAIN REACTION IN ANTI-HBE POSITIVE CHRONIC HEPATITIS B PATIENTS

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SUMMARY

Detection of HBV-DNA by PCR was compared with other serological markers (HBsAg, HBeAg and anti-HBe) in a series of 49 Chronic Hepatitis B patients, including 12 with a spontaneous clearance of HBsAg. None of these HBsAg negative cases were PCR positive, but 33/37 (89.2%) HBsAg positive cases were PCR positive ($p < 0.0001$). Among HBsAg positive samples, nine cases were HBeAg positive and anti-HBe negative, all of them PCR positive. Other 3 patients were HBeAg and anti-HBe positive and these cases were also found PCR positive. A third group included 21 patients anti-HBe positive and HBeAg negative: 19 of them were PCR positive and 2 were PCR negative. The last 4 cases were HBeAg and anti-HBe negative, two of them were PCR positive.

The detection of anti-HBe viremic cases in the present series suggest that preC variants could occur in our country. In conclusion, the integrated phase of chronic hepatitis B seems to be less frequent than it was assumed, when only HBeAg or dot blot hybridization techniques were used. The new term "low replication phase" might favorably replace the former "integrated phase".

KEYWORDS: HBV; PCR; replication Markers; chronic Hepatitis

INTRODUCTION

The natural history of Hepatitis B has been extensively studied in the last two decades. According to a recent proposed model, two different phases during the evolution of Chronic Hepatitis B (CHB) are observed, i.e. the replicative and the integrated phases²². The latter is characterized by the presence of serum HBsAg derived from integrated defective viral genomes expressing only this protein without active viral replication. The replicative phase is distinguished by detection of serum HBeAg, liver HBcAg and serum HBV - DNA by dot-blot hybridization²².

In spite of a good correlation among these replicative markers, some discrepancies have been observed between serum HBV-DNA and the HBe/anti-HBe system. Thus, serum viral genomes were detected in more than half of the anti-HBe positive patients in Italy², Greece^{15,18} and Taiwan⁸. According to a preliminary paper, based on dot blot hybridization performed in Brazilian patients with CHB, this pattern was not found in our country¹⁹.

Anti-HBe viremic cases were recently elucidated

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by identification of HBV variants with mutations in the pre C region of the genome. These mutations can abolish HBeAg expression from infected cells and are now established as the cause of anti-HBe positive patients with circulating HBV - DNA^{3,4,5}.

The development of Polymerase Chain Reaction (PCR)²⁰ allowed the detection of viral genomes in a larger number of samples, including some HBsAg negative^{14,23,24}. In anti-HBe positive samples, the incidence of HBV-DNA positivity determined by PCR has been reported by several groups ranging from 64 to 100%^{7,9,10,12,13}.

PCR based methodology was utilized by many different groups^{1,6,7,16}. When this technique is used in its most sensitive way (i.e. the final detection step with radioactive Southern blot hybridization), some groups did not find any correlation between the detection of viral DNA with any other immunologically detected viral antigen related to the presence of circulating viral particles (HBeAg, preS1 and preS2 antigens)^{7,10}. In a long term follow-up study, KORENMAN et al.¹⁶, showed that HBV-DNA usually disappeared at or around the time which HBsAg became undetectable. On the other hand, CARMAN et al.⁶, in a shorter follow-up study, found that all patients successfully treated with alpha-IFN have no viral DNA detectable by PCR after 18 months of the end of the therapy, independently of their HBsAg status.

KANEKO et al.^{13,14} developed a simplified non-radioactive nested PCR for the detection of HBV - DNA in serum that would be suitable for clinical application and is as sensitive as the PCR technique followed by the Southern blot hybridization.

Our aim was to compare the detection of serum HBV-DNA by PCR with HBsAg, HBeAg and anti-HBe in patients with CHB and to analyse the impact of the introduction of the PCR in the interpretation of CHB serological profile.

MATERIAL AND METHODS

Samples - Serum samples from 49 patients with CHB attended at an outpatient department of Gastroenterology with a follow-up of 1 to 19 years were collected. Twelve out of these 49 patients had previously been studied²¹ because of a spontaneous clearance of HBsAg. A control group of 11 healthy individuals, including 7 with isolated anti-HBe,

was also analyzed to ensure the specificity of the PCR results.

Serological tests - HBsAg, HBeAg and anti-HBe were detected by ELISA using commercial available reagents (Abbott Laboratories, USA).

Anti-HCV was detected using second generation ELISA kits (Ortho or Diagnostics Pasteur). Positive results were confirmed with the 2nd generation RIBA (Ortho). Anti-HIV was detected using both viral lysate (Genetic Systems) and recombinant (Wellcome) ELISA kits, and results were confirmed with the Western blot test (Dupont/Biotech). Anti-HDV was detected using Organon ELISA kit. Only patients negative for anti-HCV, anti-HDV and anti-HIV were analyzed.

Polymerase Chain Reaction - For the detection of HBV-DNA in serum by PCR, the technique developed by KANEKO et al.¹³ was used with minor modifications. To avoid carry-over between samples, the procedures described by KWOK & HIGUCHI¹⁷ were strictly followed.

Sample preparation - in a 0.5ml GeneAmp reaction tube (Perkin Elmer), serum (10µl) was mixed with 2.5µl NaOH 0.5M (Sigma), overlaid with 100µl of mineral oil and incubated for 1 hour at 37 C. After this time, the solution was neutralized by adding 2.5µl HCl 0.5M (Merck).

OLIGONUCLEOTIDES :

Oligonucleotides covering a well conserved sequence of the C gene were synthesized by Genomics, São Paulo, Brazil, according to the sequences proposed by KANEKO et al.¹³, as shown below.

1763: 5' GCT TTG GGG CAT GGA CAT TGA CCC GTA TAA 3' (30mer)

2032R: 5' CTG ACT ACT AAT TCC CTG GAT GCT GGG TCT 3' (30mer)

1778-E: 5' GAC GAA TTC CAT TGA CCC GTA TAA AGA ATT 3' (30mer)

2017R-B: 5' ATG GGA TCC CTG GAT GCT GGG TCT TCC AAA 3' (30mer)

AMPLIFICATION :

FIRST PCR: using the GeneAmp PCR kit (Perkin Elmer), the volumen as completed to 100µl, containing 2.5U Taq polymerase, 200 µM each deoxynucleotide, 1µM primers 1763 and 2032R, 50mM KCl, 50mM TrisHCl pH 8.3, 1.5mM MgCl₂ and 0.01% gelatin.

The amplification was performed placing the tubes in a DNA Thermal Cycler 480 (Perkin Elmer), programmed to 30 cycles (denaturation 94 C 1.5 min, annealing 42 C 1.5 min, extension 72 C 1.5 min - total duration: 3 hours).

Second PCR: after brief centrifugation, 10µl from the 1st amplification are transferred to another Gene Amp tube, and amplified with primers 1778-E and 2017R-B in the same way as described above.

Analysis of the PCR: after brief centrifugation, 15 µl aliquots from the 1st and 2nd PCR were analysed in an agarosis gel 1% (Sigma) in 0.5xTBE and 0.5µg/ml Ethidium bromide (Sigma).

Samples were considered positive when a 258bp band was visualized after the 2nd PCR and, sometimes, a 270bp band was also visualized after the 1st PCR (Figure 1).

Statistics - The Fisher exact test was utilized for statistical analysis of the results.

RESULTS

Out of 49 patients studied, 37 (75.5%) were HBsAg positive and 12 (24.5%) were HBsAg negative (spontaneous clearance during follow-up). None of the HBsAg negative cases were PCR positive, but 33/37 (89.2%) HBsAg positive cases were PCR positive ($p < 0.0001$) (Figure 2). No patient in the control group was PCR positive.

Another analysis considered only the 37 HBsAg positive patients (Figure 3). Nine of these patients were HBeAg positive and anti-HBe negative, all of them PCR

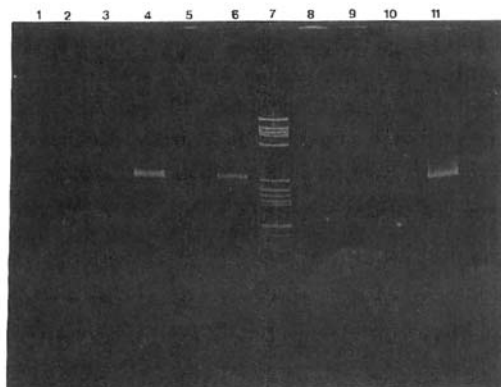


Fig. 1 - Detection of HBV-DNA by PCR. Two negative samples 1 and 2, 8 and 9 (1st and 2nd amplifications, respectively). Three positive samples in lanes 3 and 4, 5 and 6, 10 and 11 (1st and 2nd amplifications, respectively). Molecular weight standard (pBR322 Hae III digested) in lane 7.

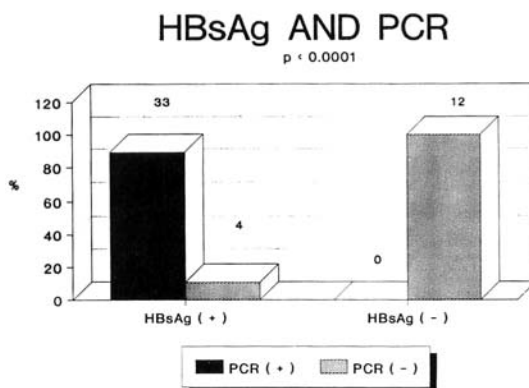


Fig. 2 - Detection of HBV-DNA by PCR according to the presence (+) or absence (-) of serum HBsAg.

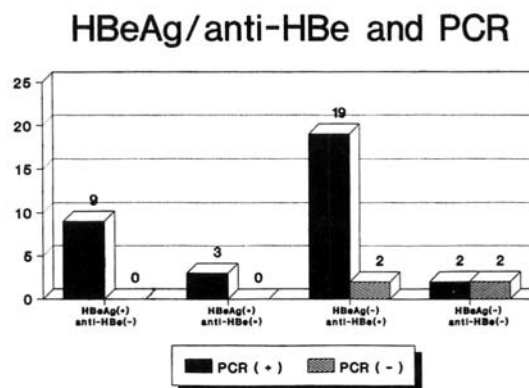


Fig. 3 - Detection of HBV-DNA by PCR in HBsAg (+) samples according to HBeAg/anti-HBe status.

positive. Other 3 patients were HBeAg and anti-HBe positive and these cases were also found PCR positive. A third group included 21 patients anti-HBe positive and HBeAg negative: 19 of them were PCR positive and 2 were PCR negative. The last 4 cases were HBeAg and anti-HBe negative, two of them were PCR positive.

DISCUSSION

In this study, we compared the detection of routine serologic HBV markers (HBsAg, HBeAg and anti-HBe) with the detection of viral DNA by PCR. The utilization of this latter technique substantially modifies the interpretation of serological results. An absolute concordance between the detection of HBeAg and HBV-DNA by PCR was observed in this study. On the other hand, the seroconversion of HBeAg to anti-HBe may not correspond to a complete interruption of viral replication, as

viral DNA in serum was detected in most of our anti-HBe positive cases. The impact of PCR in the interpretation of serological patterns of chronic hepatitis B can be clearly seen if we compare the present data with our previous results: HBV-DNA was detected by dot-blot hybridization in 67% of HBeAg positive and none of anti-HBe positive patients with CHB¹⁹.

HBV-DNA was detected by PCR in most of our HBsAg positive cases, independently of their HBeAg / anti-HBe status, while no viral DNA was detected in the patients with CHB who cleared serum HBsAg. These data reinforce the recent hypothesis that the presence of circulating HBsAg correlates with active viral replication¹¹. The few positive HBsAg and negative PCR cases might be associated with the integration of viral DNA in the genome of host cells, with exclusive HBsAg expression, without active viral replication. In such cases, we cannot rule out the possibility of viral particles circulating at lower levels than the PCR detection threshold.

Two different hypotheses may explain the concomitance of anti-HBe and HBV - DNA in HBsAg positive patients. First, we have to consider the high sensitivity of PCR. This technique detect as less as 3 viral particles¹³, even when the DNA is contained inside viral particles complexed with immunoglobulins. In this way, viral particles could still be present in very low levels, beyond the detection threshold of serological methods. Free DNA in serum derived from lysed hepatocytes or peripheral mononuclear blood cells was also suggested as the source of PCR positivity in some cases¹.

The second hypothesis refers to the presence of viral variants with preC mutations. These variants were initially found in countries where anti-HBe viremic cases were very frequent and a mutation introducing a stop codon in the preC region has been identified in patients with this serological pattern^{3,4,5}. The detection of anti-HBe viremic cases in the present series suggests that these variants could occur in our country; however, further molecular studies are needed to confirm this hypothesis.

The clinical approach for anti-HBe and PCR positive cases ("low replication phase") is a crucial problem arising from these results. The presence of preC variants in the presence of ongoing active disease and active replication, may indicate antiviral treatment. On the other hand, if these results are only due to the

extremely high sensitivity of PCR, a expectant approach would be desirable with careful clinical and serological follow-up of these patients to assess the disease activity. As a matter of fact, it has been observed that PCR positive patients show higher ALT levels than PCR negative ones, indicating the presence of hepatocyte damage¹.

In conclusion, the integrated phase of chronic hepatitis B seems to be less frequent than it was assumed, when only HBeAg or dot blot hybridization techniques were used, as recently suggested by HOOFNAGLE¹¹.

The new term "low replication phase", used by ZOULIM et al.²⁵ might favorably replaces the former "integrated phase", as integration without replication seems to be very rare in Hepatitis B, at least in some geographic areas. Nevertheless, on the clinical standpoint, it is still controversial how important is it to separate the disease in these two news phases. Therefore, the development of sensitive methods to quantify serum HBV-DNA should improve the follow-up of these patients, allowing a direct method to evaluate viral replication and thus providing a better definition of these two different phases.

RESUMO

Deteção do DNA do vírus da Hepatite B pela reação em cadeia da polimerase em pacientes com Hepatite Crônica B soropositivos para o anticorpo Anti-HBe

A detecção do DNA do Vírus da Hepatite B pela Reação em Cadeia da Polimerase (PCR) foi comparada com os outros marcadores sorológicos virais (AgHBs, AgHBe e anti-HBe) numa série de 49 pacientes com hepatite crônica B, incluindo 12 que apresentaram esclarecimento espontâneo do AgHBs. Nenhum caso AgHBs negativo foi PCR positivo, mas 33/37 (89,2%) dos casos AgHBs positivos foram PCR positivos ($p < 0,0001$). Entre as amostras AgHBs positivas, 9 foram AgHBe positivas e anti-HBe negativas, todas elas PCR positivas. Outros 3 pacientes foram AgHBe e anti-HBe positivos, todos também PCR positivos. Um terceiro grupo incluía 21 pacientes anti-HBe positivos e anti-HBe negativos: 19 foram PCR positivos e 2 PCR negativos. Os últimos 4 casos foram AgHBe e anti-HBe negativos, sendo 2 destes PCR positivos. A detecção de casos virêmicos anti-HBe positivos sugere que as va-

riantes preC do VHB podem também estar presentes em nosso país. Em conclusão, a "fase de integração viral" da hepatite crônica B parece ser muito menos frequente do que se imaginava previamente, quando apenas se dispunha das técnicas de detecção de AgHBe e DNA por hibridização. O novo termo "fase de baixa replicação baixa" parece ser melhor que o anterior "fase de integração viral".

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