Quantitative DNA analysis of very low-level hepatitis B viremic patients reporting to the gastroenterology clinic

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ABSTRACT

الأهداف: التحقق من بيانات المرضى الذين أظهرت فحوصهم مستويات متدنية من فيروس التهاب الكبد ب، واختبار الطرق المناسبة من أجل جعل الفحوص التحليلية أكثر دقة، ومن أجل التحكم بهذا المرض، والتقليل من نسبة انتشار المرض والوفيات.

الطريقة: أُجريت هذه الدراسة في عيادة الجهاز الهضمي في مستشفى القوات المسلحة بالهدا، الطائف، المملكة العربية السعودية، وشملت جميع سجلات المرضى ونتائج المسح المصلي عن مولدات الأجسام المضادة لفيروس التهاب الكبد والمعروفة باسم HBsAg منذ العام 2007م. لقد كان مجموع عدد المرضى الذين تضمنتهم الدراسة 104 مريضاً ممن أظهرت فحوصهم مستويات متدنية من فيروس التهاب الكبد ب، حيث كانت نتيجة قراءة التفاعل التسلسلي المبلمر ذو التوقيت الفعلي أقل من 12 وحدة دولية/مليلتر. ولقد قمنا أيضاً بتقييم نتائج تحليل أنزيمات الكبد، وناقلة أمين الألانين، وناقلة أمين الأسبارتات في بعض الحالات.

النتائج: أظهرت نتائج الدراسة أنه بعد تحليل بيانات المرضى الذين بلغ عددهم 1,178 مريضاً فقد تبين أن 104 مريضاً (8.83%) كانت تنطبق عليهم شروط الدراسة بما فيها قراءة التفاعل التسلسلي المبلمر التي تقل عن 12 وحدة دولية/مليلتر. لقد قمنا بتقسيم المرضى إلى 6 مجموعات وذلك اعتماداً على تفاعل مولدات الأجسام المضادة HBsAg، وتدني ظهور الفيروس في الدم، أو ارتفاعه، أو عدم ظهوره على الإطلاق، ولقد وجدنا 4 حالات مصابة بالتهاب الكبد الخفي.

خاتمة: أثبتت الدراسة مدى تأثير المستويات المتدنية من الحمض النووي على تشخيص وعلاج التهاب الكبد ب، كما أنها قد تعطي تلميحات للطبيب المعالج أثناء متابعة مثل هؤلاء المرضى. ويجب استخدام تقنية التفاعل التسلسلي المبلمر ذات التوقيت الفعلي من أجل مراقبة هذا المرض والكشف عنه.

Objectives: To examine data on very low-level viremic hepatitis B virus (HBV) infections in patients reporting to a gastroenterology clinic, and to investigate methods to improve analysis to avoid missing follow-up data and improve the management of HBV infection, and minimize morbidity and mortality outcomes.

Methods: A total of 104 patients with very low-level viremic HBV whom reported to the gastroenterology clinic at Al-Hada Armed Forces Hospital, Taif, Saudi Arabia and had a reading of <12 IU/mL on the real time (RT) polymerase chain reaction (PCR) detection system were enrolled in this study. For serological testing (for example, hepatitis B surface antigen [HBsAg]), we examined patients' results recorded in the laboratory information system since early 2007. Liver enzymes, alanine aminotransferase, and aspartate aminotransferase were assessed in some cases.

Results: After analyzing the data collected from 1,178 patients, we found 104 (8.83%) cases that fit the criteria for our study, including a reading of <12 IU/mL. We formed 6 groups of participants based on HBsAg reactivity and very low, elevated, or no viremia, and found 4 cases of continuous occult hepatitis B infection.

Conclusion: The very low levels of DNA found had a diagnostic impact on the management of HBI and yielded several suggestions for clinicians regarding follow-up with patients. It is important to use a sensitive RT PCR to monitor the course of HBV infection.

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Hepatitis B virus (HBV) is a major cause of chronic liver disease.[1] Around 2 billion people worldwide are infected with HBV, and 350 million are in the chronic stage of the infection.[2] The HBV carriers have a high risk of developing long-term sequelae of hepatitis

B, such as liver cirrhosis, and hepatocellular carcinoma.[3] Countries with a moderate prevalence of HBV infection, such as some European countries, account for nearly 25% of indications for liver transplantation in reference centers.[1] Several assays based on polymerase chain reaction (PCR) are commercially available for HBV DNA detection, as well as mutation detection. The widely known real-time (RT) PCR technique[4] can direct investigators to the quantification of viral load. The RT PCR technique is preferable to other conventional endpoint PCR techniques due to its high sensitivity and wide dynamic range.[1,5-7] However, in some cases of HBV infection, absence of HBV DNA or presence of low viremia[8] may confuse clinicians as to the appropriate interpretation of these serological markers.[9] Even with a commercially available, well-known, fully automated system for RT PCR, such as the COBAS AmpliPrep-COBAS TaqMan HBV test (CAP-CTM, Roche Molecular Systems, Inc., Branchburg, NJ, USA), problems of detection may persist. Regardless of the presence of serological markers, some patients will show very low-level viremia.[1,10-16] Toyoda et al[17] studied the effect of circulating low-level HBV, defined as a state of occult HBV infection (OHBI). The correlation with the development of hepatocellular carcinoma (HCC) in HBV surface antigen-negative (HBsAg–) patients has been described as controversial. It was suggested that OHBI prevalence strongly depends on the sensitivity of the HBV detection method employed.[17] Some researchers suggested that coinfection with HIV could be an important risk factor in OHBI.[18] Defining OHBI was an objective of an international consensus conference,[16] and there have been many attempts to define OHBI through the absence of HBsAg response and/or anti-HBc in the presence of HBV DNA in the serum or plasma of a patient or donor.[6,15,19,20] Very low-level viremia may be the cause of undetectable HBV biomarkers, such as HBsAg, but in some instances, HBsAg may be detected even though continuous low-level viremia persists.[16] In the current study, all patients were serologically investigated to determine their reactivity to HBsAg and anti-hepatitis B core antigen (anti-HBcAg). In this study the TaqMan chemistry PCR amplified the HBV DNA for the virus in patients' sera from the patients who were evaluated on suspicion of contracting HBV by the gastroenterologist at the clinic. The studied 1178 patients data were analyzed after they had visited the clinic and attended follow-up visits. We also retrospectively studied cases of very low level viremia and serological markers for those patients.

Methods. A total of 104 patients with very low-level viremic hepatitis B infection (HBI) reported to the gastroenterology clinic at Al-Hada Armed Forces Hospital, Taif, Saudi Arabia and meet the criterion of having a reading of <12 IU/mL on the RT PCR system, Cobas TaqMan 48 (Roche, Mannheim, Germany). These patients were enrolled in the study for further investigation. Data were collected over 14 months, from January 2008 to April 2009, to gain RT PCR results. For serological testing, we used patients' results from early 2007, recorded in the laboratory information system (LIS). Regardless of the stage of chronic HBI, the serum HBV DNA concentration was determined using the RT PCR assay mentioned earlier. We searched for 3 to 4 readings, and if more RT PCR testing reading was needed, we searched for more results in 1-9 month intervals.

Using a combination of routine serological and RT PCR assays, we investigated the occurrence of very low-level viremia in patients with HBI. The CAP-CTM COBAS® AmpliPrep/COBAS® TaqMan® HBV test (Roche, Mannheim, Germany) is an in vitro nucleic acid amplification test for the quantification of HBV DNA in human plasma using the COBAS® AmpliPrep instrument (Roche, Mannheim, Germany) for automated specimen processing, and the COBAS® TaqMan® CTM analyzer (Roche, Mannheim, Germany) for automated amplification, detection, and quantification of viral load. For the sample preparation step, we transferred all plasma samples to 1.5 mL screw tubes and stored them at -20°C before preparation. Afterward, we placed 1.05 mL of each plasma sample into an S-input tube provided by COBAS® AmpliPrep (Roche, Mannheim, Germany).

The HBV DNA extraction from plasma samples was performed by COBAS® AmpliPrep (Roche, Mannheim, Germany). All samples were loaded into the COBAS AmpliPrep™ system and using the kit (Roche, Mannheim, Germany). The extraction of HBV DNA was performed according to the manufacturer's protocol, as stated in the insert of the COBAS AmpliPrep/COBAS® and TaqMan® CAP-CTM HBV kit (Roche, Mannheim, Germany) and the COBAS AmpliPrep™ operation manual. No modification to the procedure was needed. A RT PCR (amplification and detection) step was then followed. Processed specimens were added to the amplification mixture in amplification tubes. The PCR amplification occurred using the thermal cycler in the CTM analyzer.

Serological markers for HBV were also studied. The HBsAg was tested in all patients' samples by using the ARCHITECT HBsAg kit (Abbott on ARCHITECT System™, Wiesbaden, Germany) according to the instructions of the manufacturer. Anti-HBc also was studied. The assay was performed on all samples using the ARCHITECT Anti-HBc II kit (Abbott on ARCHITECT System™, Wiesbaden, Germany). The liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also assessed in some cases using the ARCHITECT System™ kits (Abbott, Illinois, USA), especially in cases in which

Table 1 - Groups of patients in this study with very low-level viremia.

Group	Cases of very low-level viremia (n=104)	Description	HBV RT-PCR quantitative readings				HBsAg testing
			1st	2nd	3rd	4th	
1	70	Continuous very low-level viremia – HBsAg (+)	VL	VL	VL	VL	Positive
2	21	Very low-level viremia, elevated viral load detected – HBsAg (+)	VL	EL	EL	EL	Positive
3	7	Very low-level viremia, no viral load detected – HBsAg (+)	VL	VL	ND	ND	Positive
4	1	2 readings of very low-level viremia, then elevated viral load – HBsAg (+)	VL	VL	EL	EL	Positive
5	1	Very low-level viremia, elevated viral load, then very low-level viremia – HBsAg (+)	VL	EL	EL	VL	Positive
6	4 (OHBI)	Very low-level viremia, continuous HBsAg nonreactivity – HBsAg (–)	VL	VL	VL	VL	Negative

HBsAg (+) - positive for HbsAg, HBsAg (–) - negative for HbsAg, VL - very low level of HBV DNA (<12 IU/mL), EL - elevated level of HBV DNA (>12 IU/mL), ND - no HBV DNA detected. HBV - Hepatitis B virus, RT-PCR - real-time polymerase chain reaction, OHBI - occult HBV infection, HBsAg - HBV surface antigen-negative. Readings were obtained in this study using RT PCR and ranged from 22 to 110,000,000 IU/Ml.

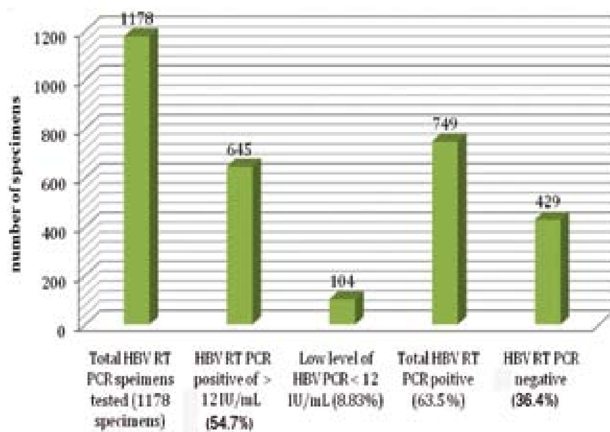

Figure 1 - Results for HBV RT-PCR testing of patients' specimens showing the total number of specimens tested, the number of positive RT-PCR results for HBV DNA, and the number of cases of very low-level viremia. HBV - Hepatitis B virus, RT-PCR - real-time polymerase chain reaction.

there was doubt regarding the serological markers of HBV infection. However, such testing was limited to a few cases. A specialized kit for testing for ALT and AST was used as instructed by the manufacturer's assay manual. Basic statistical analyses were used throughout the study, such as calculating the percentage and total for each group. The study was finalized after approval of the research committee and the hospital's ethics committee. All participants in the study signed consent forms.

Results. Retrospective data collection took place in the laboratory of the Molecular Pathology Department at Al-Hada Armed Forces Hospital. We used data available from the LIS to check patients' serological tests for HBV. Based on the availability of the test results, results of a quantitative RT PCR and previous consecutive testing were collected and studied. At least 3-4 RT PCR readings for HBV were gathered from the LIS. Additionally, we used the serological testing results from RT PCR to create 6 groups from the 104 patients with readings of <12 IU/mL. Participants were chosen from 1,178 HBV patients tested for HBV DNA during the time of the study (**Table 1**). All patients who tested positive for HBV with a RT PCR result of ≥12 IU/mL were not included in study. Of the 1178 patients we found 104 patients with very low levels of HBV DNA, 645 (54.7%) tested positive and 429 (36.4%) tested negative (**Figure 1**). We used the first available RT PCR reading of <12 IU/mL and the next available reading, whether it showed very low, elevated, or no level of HBV DNA (**Table 1**). The RT PCR assay showed the presence of HBV DNA with a reading of <12 IU/mL in 104 (8.8%) out of 1178 patients, confirming very low-level viremic HBI. This determination was made regardless of HBsAg-positive sera results, which were obtained separately at a different location in the laboratory. All of the readings of the RT PCR assay were <12 IU/mL in the first instance of testing, and we did not enroll patients with readings above this level in the initial 6 groups (**Table 1**). We found that ALT and AST levels were within normal range, according to the assay protocols, in 4 of 4 patients in the OHBI group, which had very low-level viremia and an undetectable level of anti-HBsAg. However, no abnormality in these liver enzymes was observed in the 104 patients. Thus, the results indicated that there is a need to recommend a defined protocol for all physicians with regard to the logical ordering of HBV laboratory assays. The quantitative reading used for very low levels of DNA was not given in copies/mL or copies/microliter; only IU/mL was used. Conversion of the readings of different assays is found in a paper by Ronsin et al,[21] which fully explains how different assays might be used by converting from one assay to another.

Discussion. There was uncertainty in some studies as to why HBsAg was undetectable in some patients with very low-level viremia. One interesting assumption was that the cause is a rapid noncytolytic HBsAg-specific T-Cell response, which leads to low-level expression

of HBsAg.[22] Nonetheless, it remains unknown how very low-level HBV viremia might affect the safety of health workers in endemic areas. Hepatitis B virus viremia among health care workers would restrict them from working in an endemic area known to have many patients reporting to clinics and undergoing surgical procedures. There is potential to use data gathered from health care workers with very low-level viremia to lift restrictions, similar to that proposed in the European Union and modified by others.[23]

The sequencing, followed by detection, of a single amino acid deletion may not provide a solid explanation of HBsAg detection failures, because the targeted epitopes are variable in most of the current detection assays.[16] As an alternative, some highly sensitive and commercially available PCR assays could play a major role in mitigating the failures of serological assays that cannot detect HBsAg. In our groups of patients with very low-level viremia (**Table 1**), the majority of patients identified with very low levels of HBV DNA through the RT PCR assay had continuously very low-level viremia accompanied by the presence of HBsAg. In Group 2, the follow-up yielded very low-level viremia then an elevated viral load with continued HBsAg positivity. This could explain the slow replication process at the beginning of the infection. Later in the course of infection, there were indications of a slightly higher level of HBV DNA, in some instances 22 to 250 IU/mL.

One limitation of this study was that we were unable to access other serological markers of HBV infection. This was unavoidable because the physicians who ordered the tests did not order tests of these markers. This underscores the need to instruct specialized physicians to follow up regarding HBI with a systematic testing of HBV markers in general, and in the case of very low-level viremia (≤12 IU/mL). It is highly recommended to circulate a clinical laboratory diagnostic guide of the tests to clinicians who follow up on patients with chronic HBI (**Table 2**). A similar recommendation was made by Kao et al,[24] who stated the natural history of chronic HBI could be divided into 4 dynamic phases for HBV carriers who acquire the virus early in life. In addition, Kao et al[24] indicated that serological and virological markers related to HBsAg are the hallmarks of HBV infection because they are the first serological markers to appear in acute HBI. Moreover, the persistence of HBsAg for more than 6 months suggests CHBI, whereas HB envelope Ag (HBeAg) usually indicates active HBV replication and risk of transmission of infection. This is especially important for regulating the donation of blood. Unfortunately, we cannot provide complete information through the present study of HBeAg testing of 104 patients with very low-level viremia.

The third group, mentioned in **Table 1**, exhibited a very unusual pattern, or phase, of CHBI. The inability to detect viral DNA by using the sensitive RT PCR assay employed could have indicated no replication at the time of sampling and clinical follow-up. Thus, efforts should be made to study this phenomenon in the future. The phenomenon may be described as disappearance of detectable HBV DNA indicated by RT PCR, even when HBsAg can be detected. In many recent studies,[10,19,20,25-38] OHBI is defined as the absence of detectable HBsAg in individuals whose serum or tissue tests positive for HBV DNA, irrespective of other HBV serological markers. At present, the most important assay to monitor serum or plasma HBV DNA level is RT PCR, which is considered as an invaluable laboratory test for assessing liver disease activity and infection activity in HBV carriers. Predicting the risk of HCC development[35,39,40] or liver-related mortality is another use of the assay.[24]

In group 4, which consisted of one patient, another crucial finding was revealed. Two readings of less than 12 IU/mL, as given by the RT PCR assay, were obtained, separated by 3 months. Later, during follow-up, elevation of HBV DNA was documented, along with continued HBsAg positivity. The patient showed an HBV DNA reading of 14,501,493 IU/mL approximately 8 months after the <12 IU/mL reading. The HBsAg detection was also the same, marking a continuous presence of this important HBV serological marker. It is essential to study more of these cases in

Table 2 - Recommendations for systemic follow-up ordering of serological and virological marker tests in cases of very low-level viremia (≤12 IU/mL).

Clinical visit and follow-up result	Serological and virological markers ordered						Comments
	HBsAg	Anti-HBcAg	RT PCR	HBeAg	Anti-HBs	Anti-HBe	
1. Patient suspected of having HBI at first visit	Y	Y	Y	N	N	N	If any test result is positive, arrange for a second visit.
2. Patient tested positive for any tests ordered	Y	Y	Y	Y	Y	Y	If HBsAg and anti-HBcAg are negative, order tests again, providing a very low level of HBV DNA was detected in initial testing.
3. Patient tested for HBV viral load	N	N	Y	N	N	N	In all subsequent visits, there is no need to order any other serological markers.

N - no need to order, Y - recommended to order, HBsAg - hepatitis B surface antigen, Anti-HBcAg - hepatitis B core antigen, HBeAg - hepatitis B envelope antigen, the antigenic determinant closely associated with the nucleocapsid of HBV that circulates as a soluble protein in serum, Anti-HBs - antibodies to HbsAg, Anti-HBe - antibodies to HbeAg, HBV - hepatitis B virus, RT-PCR - real-time polymerase chain reaction, sensitive to the lowest possible level of hepatitis B virus DNA, HBI - hepatitis B infection.

detail by enrolling more patients with good analytical capacity provided by the LIS and defined follow-ups. There have been tremendous improvements in lower-limit detection with the current RT PCR, with reports of a detection limit of 3.8 IU/mL.[41]

The fifth group (**Table 1**) consisted of one patient who showed a reading of <12 IU/mL and during follow-up, approximately 5 months later, tested positive with a reading of 182 IU/mL. Seven months later, the same patient returned to a reading of less than 12 IU/mL. There is no explanation why the reading would slightly rise and then decrease, expect that replication might have been suppressed in a way yet to be investigated. None of the patient's other serological markers had been examined in the laboratory. The patient continued to have HBsAg positivity after the first order of laboratory testing. We could not find any indication of antiviral treatment for the patient, suggesting the need for careful follow-up and investigation when the patient returned to the clinic.

Finally, in group 6, a very low level of viremia continued to appear in the patients' samples (n = 4) and remarkable results of HBsAg negativity also were observed. This was similar to findings by others,[42,43] in which resemblances to the findings of OHBI cases were described. It is not possible to further investigate the samples to find the HBV DNA sequences in these OHBI-like cases. This underscores the need for a reference national laboratory in which all similar cases could be collected and studied, as this would enhance knowledge and understanding of HBV infections in cases with HBsAg negativity and very low levels of HBV DNA. At present, there is no national program that could assist in the follow-up of such cases in which there might be a silent HBV infection with no serological markers. Generally, chronic occult infection is asymptomatic and associated with low levels of viral replication (namely, low DNA levels).[44] It is important to carefully differentiate between OHBI and the deficiency of some immunoassays available on the market. The HBsAg sequence may altered and, thus, may not be recognized by the assay.[45]

In conclusion, it is principally important for physicians to screen for HBI in HBV-endemic areas, and to monitor liver disease progression in HBV carriers by using both serological and virological markers. In this way, effective treatment could be initiated early, before the development of advanced liver disease and, possibly, death.

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