# Nucleic acid amplification technology for hepatitis B virus, and its role in blood donation screening in blood banks

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## ABSTRACT

**الأهداف:** بحث أداء HBV PCR للكشف عن التهاب الكبد الفيروسي Bباستعمال واحدة من الطرق المعروفة حول العالم لفحص التهاب الكبد الفيروسي B في المتبرعين بالدم بينما الاختبارات الأخرى المصلية لها محدودية في الكشف عن الفيروس.

**الطريقة:** تم تصميم امبليكور امبليسكرين في هذه الدراسة لاختبار التهاب الكبد الفيروسي B للكشف عن وجود الحمض النووي لفيروس التهاب الكبد الفيروسي ب في العينات المحضرة بواسطة نظام كوباس امبليبريب بطريقة مطورة لنظام كوباس امبليبريب لجميع الحمض النووي باستخدام طريقة( TNAI ). كل الاختبارات المصلية عملت على عينات المتبرعين للكشف عن مولدات المضاد HBsAgللفيروس والأجسام المضادة لمولد المضاد ( AUSAB )للفيروس والأجسام المضادة ( HBcAg ) للفيروس في فترتين من الدراسة. بدأت الفترة الأولى من فبراير 2005 وبدأت الفترة الثانية أبريل 2007. استمرت الفترتين لمدة 2 شهر بعد البداية في مختبر الباثولوجيا الجزيئية – مستشفى الهدا للقوات المسلحة – الطائف. تم تحليل بيانات 600 متبرع ودراستها.

**النتائج:** وجد 5 عينات بها الحمض النووي لفيروس التهاب الكبد الفيروسي من 600 عينة مختبرة. كانت 3 موجبة للأجسام المضادة لمولد المضاد HBcAb للفيروس، وسالبة للأجسام المضادة لمولد المضاد HBsAg للفيروس. كان 2 اقل من 100 وواحدة كانت أكثر من 100 لقراءة الأجسام المضادة لمولد المضاد أس للفيروس HBsAg.

**خاتمة:** نتائجنا بينت انه يمكن أن يكون هناك إصابة خفية لالتهاب الكبد الفيروسي B في بعض المتبرعين بالدم التي لا يمكن أن تكتشف بواسطة اختبار مولد المضاد HBsAg لفيروس التهاب الكبد الفيروسي ب. إضافة إلى ذلك، أن هذه الدراسة ممكن أن تستخدم في سياسة الرفض لبعض المتبرعين حيث تعتمد على استخدام تقنية الكشف عن الحمض النووي للفيروس.

**Objectives:** To investigate the performance of hepatitis B virus polymerase chain reaction (HBV PCR) using one of the commercial methods used around the world to screen for HBV in some blood donors where other conventional serological assays have limitations to detect the virus.

**Methods:** This study was designed to use Amplicor AmpliScreen for HBV testing to detect the presence of the HBV DNA in the specimens tested by COBAS AmpliPrep™ system using a modified manufacture protocol COBAS AmpliPrep of total nucleic acid isolation (TNAI) kit. All serological tests were carried out on the donors' samples to detect the hepatitis B surface antigen (HBsAg), Australian antibody anti-HBs (AUSAB) and hepatitis B core antigen (HBcAg) in the 2 periods of the study. The first period was started in February 2005 and the second period was started in April 2007. Both periods were continued for 2 months after beginning in the molecular pathology laboratory, Al-Hada Armed Forces Hospital, Taif, Kingdom of Saudi Arabia. The 600 donors' data were then studied and analyzed.

**Results:** Five nucleic acid amplification test (NAT-HBV) positives were found out of 600. There were 3 positive for HBcAb and negative for HBsAg, 2 had reading with <100 mIU/mL anti-HBs (AUSAB), and one had >100 mIU/mL AUSAB readings.

**Conclusion:** Our results show that there is a possibility to have "occult" HBV infection in some donors that cannot be detected by the HBsAg routine serological assays. Moreover, the study can be useful to formulate a new deferral policy based on the implementation of NAT-HBV for blood screening.

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Hepatitis B virus (HBV) infection endemicity is an important health dilemma in many Middle East and Asian countries.[1,2] There are several routes of transmission, but blood transfusion is still high, the risk of transmission is 1/180,000 donations.[3] The transfusion safety has been dramatically improved following the implementation of hepatitis B surface antigen (HBsAg) screening in the early 1970s. However, the studies quoted in this study demonstrate that transmission of HBV by blood components negative for HBsAg can still occur in the acute phase of infection during the seronegative window period, or during the chronic stages of infection for example, "occult" HBV infection (OHB).[4] There are numerous studies reporting the incidence of transmission of HBV by blood or its components that were tested negative for HBsAg during the seronegative window period.[5-9] The incidence might also occur during chronic stages of HBV infection,[10] known as OHB. The OHB is defined as the presence of HBV DNA in blood or liver tissues in patients negative for HBsAg, with or without any HBV antibodies. Additionally, it was observed in different reports that some patients may have HBV DNA circulating in the blood while they continuously tested negative for HBsAg.[11] It has been shown that there is a considerable limitation of practice in the conventional screening assays, which cannot detect HBV infection in those patients with chronic and possible immunologically silent HBV infection that may lead to overlooking such transmission. For a firm blood bank policy development on screening for HBV infection in blood donors, it would be useful to assess the relative contribution of the above 2 sources of transfusion-transmitted HBV infection from HBsAg-negative donations. This would make an important reason to revolutionize screening for HBV infection for blood donors. A new screening policy should be evaluated on the basis of available data or newly designed studies to search for HBV infection with HBsAg-negative donations by the local authority. Initiating and adapting screening protocols based on collecting of data from comparing the conventional and other more sensitive DNA-based technologies might be an option for a feasible solution. While anti-HBc screening can eliminate residual risk of occult HBV transmission by transfusion in low-endemic areas, it would not be practical in many parts of the globe where the studied prevalence of anti-HBc is >10% as too many otherwise healthy donors will be not qualified or permitted to donate blood. In different words, anti-HBc screening does not eliminate residual risk of falsely-tested HBsAg negative donors with OHB, if, in some instance, the prevalence is to high to be ignored from healthy individuals. On the contrary, studies mentioned in this paper indicate that nucleic acid amplification test "NAT-HBV" and/or new HBsAg screening tests of enhanced sensitivity would be effective in the screening of blood donors with OHB in highly endemic countries such as in some parts of the Middle East in general. However, here we want to show that the testing, where nucleic acid is the target for the screening assay, may be considered as an alternative to conventional HBsAg screening testing assays. Moreover, screening by "NAT-HBV" of blood donors would give more enhancements with regards to screening sensitivity of the testing in highly endemic regions. However, the cost-effectiveness of blood screening tests is a major concern in Asia. The cost-effectiveness of blood screening tests using NAT-HBV assays may hinder the target to obtain the safest blood supply but, showing the local regional data of implementing NAT-HBV in correlation to the current testing may be, otherwise, a valid justification. We, therefore, have systemically reviewed the literature on prevalence and infectivity of OHB in Asian countries and the possible role of NAT-HBV for identifying blood donors in the pre-HBsAg window phase or in later stages of OHB, that are usually missed wherever there is no regional data obtained.[10,12-15] There is also consideration and understanding of the low HBV viral load, which reduces the sensitivity of NAT-HBV once mini-pools are used instead of individually tested donor's plasma that will be discussed herein.

**Methods.** After approval of the Research Committee and the Hospital's Ethics Committee, consents from all the donors were obtained. For the collected samples it was decided to use COBAS Amplicor AmpliScreen™ for HBV testing, version 2.0 (Roche, Manheim, Germany) to detect the presence of the HBV DNA in the specimens tested because of the abundance of information with regard to the NAT assay using polymerase chain reaction (PCR) method.[16,17] The specimens of plasma were collected from 600 donors who were already tested for human immunodeficiency virus (HIV-1) and hepatitis C virus (HCV) by similar NAT assay in the same periods of the study, which first started in February, 2005, and the second period started in April, 2007. Both periods continued for 2 months after beginning in the Molecular Pathology Laboratory, Al-Hada Armed Forces Hospital, Al-Taif, Kingdom of Saudi Arabia. Therefore, the samples that were not collected and preformed during this period were excluded from the study. The specimens were stored and archived in -25°C freezers in case further testing was needed. The frozen specimens were thawed and tested for the current NAT-HBV assay. There were 2 periods of times for this study, where all the specimens of the plasma collected from donors were tested qualitatively by the PCR method using COBAS Amplicor - AmpliScreen HBV kit. The plasma specimens were prepared in a 1.5

mL tubes for each donor prior to making mini-pools. The plasma specimens were then thawed on the day of testing to take a 250 µl volume after gentle shaking to consist of 2 donors' samples and a total volume of 500 µl of plasma in each mini-pool. We returned the original donors' plasma samples to the -25°C freezer for storing and archiving, in case there is a need to test an individual plasma sample again. The DNA extraction from plasma samples was performed on a volume of 500 µl of the mini-pools consisting of 2 donors' samples loaded into COBAS AmpliPrep™ system using specialized tubes. The correct volume was checked after loading the tubes on the system against standardized volume tubes of the same type. All mini-pools were then loaded into the COBAS AmpliPrep™ system using a modified protocol of the COBAS AmpliPrep™ of total nucleic acid isolation (TNAI) kit, (Roche, Manheim, Germany). The modification was in the multiprep internal control (MPIC) addition of the COBAS AmpliScreen MultiPrep™ specimen preparation and control kit, (Roche, Manheim, Germany). One vial of MPIC was added to the multi-reagent cassette (TNAI CS3) used in the TNAI kit and approximately a 950 µl of the internal control diluent was added and mixed gently to be ready once the system started to work. The rest of the extraction process of the ribonucleic acid (RNA) for HCV and HIV-1 was according to the manufacturer's protocol as in the insert of the COBAS AmpliPrep™ TNAI kit and the COBAS AmpliPrep™ operation manual. At the end of the RNA extraction procedure steps, a total volume of 70 µl was ready to be taken for the preparation of the HBV-PCR mix for each mini-pool tested. The HBV-PCR assay was carried out as instructed by the COBAS AmpliScreen™ HBV, version 2.0 (Roche, Manheim, Germany) as stated in the insert accompanying the kit. For the hybridization reaction following PCR amplification, the COBAS Amplicor™ Analyzer automatically adds denaturation solution to the reaction tubes to chemically denature the HBV amplicons once created in each test tube of ampliScreen,™ and the HBV internal control amplicons to form single-stranded DNA. Aliquots of denatured amplicons are then transferred to 2 detection cups. A suspension of magnetic particles coated with an oligonucleotide probe specific for HBV amplicons as well as HBV internal control amplicons is added to the individual reaction tube. The biotin-labeled HBV target and HBV internal control amplicons are hybridized to the target-specific oligonucleotide probes bound to the magnetic particles. This hybridization of amplicons to the target-specific probe increases the overall specificity of the AmpliScreen™ assay for amplification and detection of HBV DNA in the plasma of the donors. Following the hybridization reaction, a detection reaction took place; the COBAS Amplicor™ analyzer washes the magnetic particles in the detection cups to remove unbound material, and then adds avidin-horseradish peroxidase conjugate. The avidin-horseradish peroxidase conjugate binds to the hybridized biotin labeled amplicons. The COBAS Amplicor™ Analyzer removes unbound conjugate by washing the magnetic particles and then adds a substrate solution containing hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) to each detection-cup. In the presence of hydrogen peroxide, the particle-bound horseradish peroxidase catalyzes the oxidation of TMB to form a colored complex. The absorbance is measured by the COBAS Amplicor™ Analyzer which uses a wavelength of 660 nm. Therefore, the 2 amplification products generated from HBV and internal control, target DNA were detected calorimetrically after hybridization to HBV-specific and IC-specific oligonucleotide probes bound to magnetic particles in each tube representing one mini-pool. All donation sera enrolled in the study was screened initially as follows: for HBsAg antibody detection, a HBV version 2.0, Abbott AXSYM® system kit (Abbott, Wiesbaden, Germany) was used. The AXSYM® HBsAg (V2) is a third generation microparticle enzyme immunoassay for the qualitative detection of HBsAg (human serum or plasma. The kit was used exactly as instructed in the manufacturer's assay manual. Anti-HBs quantification or HBsAg (recombinant, subtypes ad and ay) on the plasma samples was estimated from a standard curve according to the manufacturer's recommendations. This kit of AXSYM® AUSAB (known as an Australian antibody) or anti-HBs is a microparticle enzyme immunoassay (MEIA) for the quantitative determination of antibodies to HBsAg (anti-HBs) in human serum or plasma. For the third HBV biomarker, that is indicated as an aid in the diagnosis of active and ongoing or previous hepatitis B viral infection known as HBV core antibodies (anti-HBc or HBcAb), an AXSYM® core is used, which is a microparticle enzyme immunoassay for the qualitative detection of antibody to HBV core antigen (anti-HBc) in human serum or plasma. The test for HBV core antigen, when reactive, does not differentiate between acute or chronic hepatitis B infections. However, this was employed and the enzyme immunoassay was processed on a special enzyme-linked immunosorbent assay processor for testing individual samples that were not pooled or diluted. The protocol used followed the manufacturer's instructions. Determination of limit of detection for the NAT-HBV assay was preformed similarly to that described elsewhere,[18] The sensitivity of each assay used here in this study was as follows: for HBsAg was 99.8%, for HBV-NAT was 99.3% and for anti-HBs was 99.8% as mentioned in the manufactures' inserts. The anti-HBc assay has also been indicated to

have a high sensitivity that reaches 98.8%, as per the manufacture's insert.

**Results.** The results obtained in this study show some of the serological tests that are usually used in the laboratory diagnosis of HBV infection, namely, HBsAg, hepatitis B core antigen (HBcAg) and anti-HBsAg with the additional test of nucleic acid amplification technology known as NAT-HBV. The testing has also revealed their 6 possible categorized interpretations as detailed in **Table 2**. The new and old interpretations were based on the data gathered from different publications.[19] Meaning, the references have been selected before and after the implementation of NAT-HBV. We tested negative NAT-HBV individually whenever there is a need to compare between the mini-pools and individual donors' samples, also to emphasize the uncorrelated HBsAg testing. There was no different in our collected data in this study. The NAT-HBV positive cases were initially tested in a mini-pool of 2. **Table 1** indicates the real need for individual NAT-HBV and to encourage the implementation of a policy to repeat positive mini-pools in NAT-HBV testing. The positive NAT-HBV and HBcAb and negative for HBsAg as shown in **Table 2** were tested on individual plasma donors' samples and were confirmed by repeat testing. The rest of the five (2/5) donors that were found positive for NAT-HBV were found to be HBsAg positive, so, they were not OBI or what is known as window period cases.

**Discussion.** In the current study, deferral of a donor was taken into consideration in our laboratory and blood bank services that we provide, despite the low number we found in this study, but, it is relatively high when we compare the number of NAT-HBV screened donors in some studies.[21,22] The 3 donors who were found to be HBsAg negative and positive for anti-HBc and NAT-HBV have triggered our attention for searching and at the same time updating the blood bank guidelines for donors' deferral. As postulated in some

**Table 1 -** Shows the positivity (reactivity) in each test used in the 2 periods of the study and the percentage of the total cases found.

| Period of the study | Number of donors | Nucleic acid amplification technology | | Hepatitis B surface antigen | | Hepatitis B core antibodies | | Anti-HBs (AUSAB) <100 |
|---|---|---|---|---|---|---|---|---|
| | | Negative | Positive | Negative | Positive | Negative | Positive | |
| First | 264 | 261 | 3 | 262 | 2 | 224 | 36 | 15 |
| Second | 336 | 334 | 2 | 336 | 0 | 306 | 33 | 12 |
| **Total (%)** | **600** | **595 (99.16)** | **5 (0.84)** | **598 (99.6)** | **2 (0.33)** | **530 (88.3)** | **69 (11.5)** | **27 (4.5)** |

AUSAB - Australian antibody or it is put also as anti-HBs antibodies to hepatitis B surface antigen (HBsAg)

**Table 2 -** Shows that the principle testing is HBcAb with anti-HBs (AUSAB) quantifications in the presence of nucleic acid amplification technology (NAT)-HBV for donors and possible deferral after implementing HBV-NAT.

| Number of cases found | | Test/results | | | | Old interpretations (before nucleic acid amplification technology) | New interpretations (postulated) |
|---|---|---|---|---|---|---|---|
| First study | Second study | HBsAg | HBcAb | HBV-NAT | Anti-HBs AUSAB | | |
| 261 | 334 | Negative | Negative | Negative | Negative/ Positive | Fit for donation | Fit for donation |
| 34 | 33 | Negative | Positive | Negative | Positive | Check anti-HBs (AUSAB) if positive >35 use the unit, if negative or <35 mIU/mL defer donor | Defer donor |
| 29 | 21 | Negative | Positive | Negative | Positive (≥35 mIU/mL) | Use unit | Defer donor |
| 1 | 0 | Negative | Positive | Positive | Negative | No available interpretation | Check anti-HBs (AUSAB) if >100 mIU/mL use unit, if <100 mIU/mL defer donor, if negative permanent deferral |
| 1 | 1 | Negative | Positive | Positive | Positive (<100 mIU/mL) | No available interpretation | Defer donor |
| 0 | 1 | Negative | Positive | Positive | Positive (>100 mIU/mL) | No available interpretation | Use unit |

Hb - hepatitis B, AUSAB - Australian antibody or it is known also as anti-HBs antibodies to hepatitis B surface antigen.
NAT - nucleic acid amplification technology, HBsAg - hepatitis B surface antigen, HBcAb - hepatitis B core antibodies,
HBV-NAT - Hepatitis B virus nucleic acid amplification technology

studies and opinions gathered elsewhere,[19] anti-HBc and anti-HBs (AUSAB) play an important role in such cases of deferral.

Another important objective for this study was to determine if we could find "window" donations, donations testing positive for NAT-HBV and negative for all other current tests for HBV. It was found there were no indications for window-period donations in all the donors studied in this study, the interpretation of the routine serological markers and the NAT-HBV was assessed to have a formal opinion on the use of these tests with regard to blood donations and donors and for safer blood transfusion. It was very important to address this issue as there is a great deal of confusion with different interpretations that might exist from many blood banking and blood donations centers around the world and also locally. The new interpretations as shown in Transfusion, American Association for Blood Banks (AABB) and other publications,[19] have dramatically influenced us to go back and investigate the old interpretations that were widely used in many local blood bank laboratories. It is also very important to detail the differences to be acknowledged to the concerned professionals.

Data from post transfusion cases indicate that HBsAg is first detected between 50 to 60 days after the incident of transfusion. Studies on the HBV DNA concentrations in serial plasma donations yielded an average 4-day doubling time for HBV DNA during this pre-seroconversion phase. Whereas HCV doubles every few hours and HIV doubles every day, HBV levels in plasma increase relatively slowly.[23] This might be a given reason to see such an outstanding delay in response or OHB cases as found herein (**Table 2**). The HBV remains the last of the major transfusion-transmitted viruses with significant risk of transmission due to long window period and variability of symptoms and expression of serologic markers for HBV tested in many laboratories.[23] Although it is not 100% risk free screening, NAT-HBV does narrow the window period in early-stage infection resulting in an exponential reduction of the virus load that escapes serological screening tests for blood donation designated for blood and blood products transfusions. In the case of NAT-HBV, screening detects HBV DNA in persistently infected individuals with extremely low levels of HBV antigen and antibody often observed in the case of HBV mutants.[1]

The testing of NAT-HBV, HBcAg, HBsAg, and anti-HBs (AUSAB) have all been evaluated to formulate the latest Food and Drug Administration (FDA) deferral guidance for reentry of donors which may still need the collaboration of many blood donation centers to decide what if the donor is falsely tested NAT-HBV positive, would this allow him to reenter for donation again. The vaccination of the donors was not an issue for this current study and may be taken into consideration when a similar study is designed in the future. However, it was suggested that at 6 months, the evaluation should be carried out in the same donation facility with the same screening panel, a continually positive NAT-HBV would permanently defer the donor regardless of the other tests of the same panel. If reentry of a donor is sought out, the donor may wait for a 6 month period to show a NAT-HBV negative result.[24,25] Although in many countries where the prevalence is low there is no implementation of anti-HBc or NAT-HBV screening, we presume that the existing blood bank deferral guidelines needed to include NAT-HBV in addition to the anti-HBc, HBsAg, and anti-HBs (AUSAB). Many of the EU countries have no obligatory role to implement NAT-HBV since they have low endemicity and their valid reason is cost-efficiency. Finally, it is, however, recommended that the use of NAT-HBV is an essential screening assay if the proven HBV infection prevalence is 5-10% of the donors, but the testing should be carried out in individual plasma of donors. Moreover, authorities should not underestimate the cost of HBV infection in a society and a cost-of-illness analysis will justificaty the need for NAT-HBV.[26] Several reports exist of seronegative patients that are immunocompromised and show delayed seroconversion,[27-30] while few reports exist of this occurring in OHB in healthy donors. Postulated mechanisms for this include the inability to detect the antibody response, false-negativity by the used assay, tolerance induced during vertical transmission and the inability to immunologically respond to the virus transmitted in such scenario. Given that our donors' immunocompetence status is not questionable and the test results are true, such important findings would be explained that either he/she does not make antibodies to HBV or basically we cannot detect them by our screening assay. This case highlights the need to test all blood donations by NAT-HBV despite the cost. Although there is a limitation of this study, which is the relatively small sample size, the firm findings here are represented by the fact that all the positive cases that were found in the NAT-HBV mini-pools screening were shown negative when repeated individually. This makes the Roche COBAS AmpliScreen™ HBV, version 2.0 (Roche, Manheim, Germany) not suitable for mini-pool screening even if 2 donors plasma are used in such testing. Concurrently, this is similar to the findings of others, when they write that mini-pool NAT-HBV will not detect most potentially infectious blood units from anti-HBc-positive donors.[31]

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