



Hepatitis B Diagnosis in Blood Bank: Evaluation and Challenges

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ABSTRACT

Hepatitis B virus (HBV) presents a higher residual risk of transmission by transfusion than hepatitis C virus (HCV) or human immunodeficiency virus (HIV). While most infectious blood units are removed by screening for hepatitis B surface antigen (HBsAg), there is clear evidence that transmission by HBsAg-negative components occurs, in part, during the serologically negative window period, but more so during the late stages of infection.

To encourage voluntary blood donation should be the first step of prevention. To reduce the risk of transfusion-associated hepatitis B, test for anti-HBc immunoglobulin M may be included in routine screening of donors' blood, as it has been proved to be an excellent indicator of occult HBV during window period. However, awareness and education of donors regarding the modes of HBV transmission, a stringent one-to-one donor screening and increasing the voluntary donor base should also be implemented to minimize the rate of transfusion-associated hepatitis B.

Keywords: Hepatitis B, HBV, Diagnosis, HBsAg, Nucleic acid testing, Voluntary blood donation.

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INTRODUCTION

Two billion people have been infected with hepatitis B virus (HBV) worldwide and approximately 380 million (6%) are chronic carriers. A total of 4.5 millions new infections are reported annually. Hepatitis B virus kills 6.20 lac people each year. In highly epidemic regions, including Africa, the Amazon Basin, and central South East Asia, the prevalence is 8 to 15% in developing regions. Hepatitis B virus infection mostly occurs early in life with high risk

of chronic liver disease. It can carry through blood and other body fluids, and is transmitted through mother to child, child to child and through sexual or parenteral contact. Vertical transmission is frequent in Asia. The rate of spontaneous HBV clearance is lower in children. Up to 90% of children infected during the first year of life and almost 50% of those infected between the age group 1 to 5 years will develop chronic hepatitis. A total of 25% of adults infected during childhood will die premature from liver cancer or cirrhosis. Hepatic cellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third most common cancer in poor countries. Cirrhosis and HCC cause one million deaths each year mostly 80% in under-privileged regions. These regions face a lack of healthcare workers, poor medical infrastructures, insufficient screening, and poor access to care and treatment. At a time when morbidity and mortality of chronic liver disease has been widely improved in wealthy countries by new innovative strategies and potent antiviral drugs, it is now urgent to face the challenges of better management of chronic hepatitis in resource-poor countries from the perspectives of global health and social justice. And 2.4 million individuals have human immunodeficiency virus (HIV) and HBV coinfection. Hepatic cellular carcinoma is responsible for a quarter of liver-related deaths in HIV patients. Accurate data on prevalence, natural history, and severity of liver injuries in HIV-infected individuals living in resource-limited countries are lacking.

Resource-limited settings maintain the hepatitis epidemic due to many reasons:

Imperfect vaccination coverage—Although most countries vaccinate all children against HBV, it is not covering even 50% in Asia. Poverty and illiteracy, access to treatment for hepatitis B is very limited. The wide spread use of lamivudine rather than tenofovir as first-line treatment of HBV is worrisome due to alarming increase in drug resistance causing uncontrolled viral replication and hence cirrhosis and HCC. The high cost of antiviral therapies, the lack of medical infrastructure and laboratories, shortage of healthcare workers and diagnostic tools. Controlling HBV is very complex. In conclusion, access to screening, with improved diagnostic

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strategies, is of paramount importance for global health and social justice.

It is the responsibility of health policy makers, medical doctors, scientist, and governments to improve screening of HBV markers. The carrying out of cost-effective studies is urgently needed to demonstrate the burden of acute, chronic, and occult infections of HBV.¹

The HBV is an enveloped deoxyribonucleic acid (DNA) virus that belongs to the family Hepadnaviridae and features partially double-stranded relaxed circular DNA. There are eight genotypes of HBV, classified from A to H, and these are distributed across different geographic zones. The entire genome is 3.2 kb in length and replicates by reverse transcription of an intermediate known as pregenomic RNA. The infectious viral particle is spherical, 40 to 45 nm in diameter. The virus consists of an inner nucleocapsid or core, surrounded by a lipid envelope containing virally encoded surface proteins. These protein markers are mainly used to screen the appearance of hepatitis infection with help of serological tests.

All three coat proteins of HBV contains HBsAg, which is highly immunogenic and induces anti-HBs (humoral immunity). Structural viral proteins induce specific T-lymphocytes, capable of eliminating HBV-infected cells (cytotoxic T-cells; cellular immunity). HBsAg is heterogeneous antigenically, with a common antigen designated a, and two pairs of mutually exclusive antigens, d and y, and w (including several subdeterminants) and r, resulting in four major subtypes: adw, ayw, adr, and ayr.

The distribution of subtypes varies geographically. Because of the common determinants, protection against one subtype appears to confer protection to the other subtypes, and no difference in clinical features has been related to subtypes.

In the United States, Northern Europe, Asia, and Oceania, the d determinant is common, but the y determinant is found at lower frequency. The d determinant to the near exclusion of y is found in Japan. The y determinant, and rarely d, is found in Africa and in Australian aborigines. y is also frequently found in India and around the Mediterranean. In Europe, the United States, Africa, India, Australia, and Oceania, the w determinant predominates. In Japan, China, and Southeast Asia, the r determinant predominates. Subtypes adw, ady, and adr are each found in extensive geographic regions of the world. Subtype ayr is rare in the world, but it is commonly found in small populations in Oceania. The c antigen (HBcAg) is present on the surface of core particles. HBcAg and core particles are not present in the blood in a free form but are found only as internal components of virus particles.^{2,21}

The core antigen shares its sequences with the e antigen (HBeAg), identified as a soluble antigen, but no

crossreactivity between the two proteins is observed.² Viral oligopeptides of 8 to 15 amino acids are loaded on host cell MHC-class I molecules and are transported to the surface of the cell. Hepatitis B virus-specific T-lymphocytes can then detect infected cells and destroy them. This cell deletion triggered by inflammation cells may result in acute hepatitis. When the infection is self-limited, immunity results. If HBV is not eliminated, a delicate balance between viral replication and immunodefence prevails, which may lead to chronic hepatitis and liver cirrhosis. In chronically infected cells, the HBV DNA may integrate into the host cell-DNA. As a long-term consequence, integration may lead to hepatocellular carcinoma.

Hepatitis B virus presents a higher residual risk of transmission by transfusion than hepatitis C virus (HCV) or HIV. While most infectious blood units are removed by screening for hepatitis B surface antigen (HBsAg), there is clear evidence that transmission by HBsAg-negative components occurs, in part, during the serologically negative window period, but more so during the late stages of infection. Screening of blood for detection of surface antigen, however, doesn't rule out the risk of transmission of hepatitis B totally, because during serological response of the host to infection, there is a phase during which the HBsAg cannot (avoid contractions in text) be detected in the blood, although hepatitis B infection is present. This phase is called as the 'core window period'. During this window period, detection of the antibodies to the hepatitis B core antigen serves as a useful serological marker for hepatitis B.³ Evaluating the usefulness of anti-HBc screening is critical, particularly for India and other countries that have high hepatitis B endemicity.⁴ It is also a good indicator of occult HBV infection during 'core window' period. Blood collected from the individuals chronically infected with HBV in whom HBsAg is not detectable and from donors with acute hepatitis who are in window period following disappearance of HBsAg and prior to the appearance of anti-HBs can effectively be removed from the inventory. Therefore, some blood banks from India incorporate anti-HBc testing in their donor screening protocol.⁵ Routine anti-HBc screening of individual blood donations and nucleic acid amplification testing by pooling of sera is done in some countries to exclude these donations.⁶

Therefore, it is necessary to introduce the most advance techniques to identify the hepatitis DNA markers in the blood donor screening. The study of two automated nucleic acid amplification systems for blood donor screening shows that the Cobas 201 test was more sensitive than the Prolix Ulterior test.⁷

EVOLUTION IN DIAGNOSIS OF HEPATITIS B

The history of modern research on viral hepatitis began in the year 1963, when Nobel Prize winner Baruch S Blumberg (1925–2011) for the first time publicly reported the discovery of a new antigen named 'Australia antigen' (AuAg).

The earlier method of detection was to see the cytopathic effect of propagating virus in cell culture. Due to limitation of time taken by cytopathic effect, an alternate method was developed by observing viral material in electronic microscope. Another option was to observe hemagglutination reaction. These were the direct antigen detection methods.

The other approach was to detect antibodies produced in patient against HBV. The method most often used was 'compliment fixation reaction' (CFR), which requires four complex biological component mixtures from four different animals. Quantification of the CFR method was only possible by diluting the patient sera.

All attempts to identify the pathogen were unsuccessful for more than 8 years. The problem with viral hepatitis was so big that even human experimentation was done on the prisoners in 1950 in USA. In early days of evolution for blood donor screening, Blumberg used the Agar gel double diffusion, developed by Oculerlony in the discovery of AuAg. The method was sensitive and specific as compared to the biochemical methods and technically simple than CFR.

In 1972, a team including biochemists Lacy Overby, Ghung-Mei Ling, and Richard Decker at Abbott Laboratories (North Chicago) developed a new testing principle for highly-sensitive detection of antigens or antibodies, the solid-phase sandwich radioimmunoassay named—Ausria-125. Antibodies coupled with fluorescent molecules which is measurable under UV light were specially used in viral detection. The fluorescently marked animal antibodies against human antibodies could be used to determine whether a person's serum contain the antibodies of hepatitis B antigen.

The new aspect was to use unlabeled antibodies by simple absorption to a surface (solid phase) and then allow person's serum to react with antibodies bound on special solid surface.

The specifically bonded protein complex could be detected by the antigen-specific antibodies labeled with iodine 125. The process opened new dimensions in detection sensitivity from several microgram/milliliter to nanogram/milliliter. The method was breakthrough for screening of blood donors and diagnosis.

In 1970, David Dane observed under electronic microscope that the Au antigen appears in the form of filamentous and spherical particles which were known

as Dane's particles. Dane's particles on treatment with mild detergent produce core particles which induced the antibody response in human. This strongly suggested that the Dane particles were the actual virus causing hepatitis B. AuAg was obviously the surface antigen of the virus envelope and was named HBsAg (s for surface) thereafter.⁸

The introduction of Austria-125 was the beginning of an impressive development in virus diagnostics; however, the test had one major disadvantage: the radioactivity caused significant difficulties in the normal diagnostic laboratory.

It was, therefore, a big step forward when it became possible to label the antibodies used with enzymes and later with chemiluminescence-generating group. The assay of HBsAg was soon complemented by the detection of antibodies against HBsAg (anti-HBs) and HBcAg (anti-HBc).

Nucleic acid testing (NAT) is a molecular technique for screening blood donations to reduce the risk of transfusion-transmitted infections (TTIs) in the recipients, thus providing an additional layer of blood safety. It was introduced in the developed countries in the late 1990s and early 2000s and presently around 33 countries in the world have implemented NAT for HIV and around 27 countries for HBV. Nucleic acid testing technique is highly sensitive and specific for viral nucleic acids. It is based on amplification of targeted regions of viral ribonucleic acid or DNA and detects them earlier than the other screening methods, thus narrowing the window period of HIV, HBV, and HCV infections. Nucleic acid testing also adds the benefit of resolving false-reactive donations on serological methods which is very important for donor notification and counseling.

Serology assays are performed on individual samples, while NAT is performed on either the individual donation (ID) or on a wide array of minipool (MP)-NAT testing formats. The limit of detection of ID-NAT assays equals the analytical sensitivity and that of pool testing reflects the pool size-dependent decreased sensitivity also known as screening sensitivity. To many end users, it is not obvious that pool testing will have less sensitivity. In this review, mathematically predicted pool size-dependent increase in risk days which is applicable to assays of all technologies is substantiated with published experimental results with NAT standards, clinical NAT only detected yields and detection misses by MP-NAT. In the second half, the blood banking system in India, the donor base, and the variables in serology testing are discussed to explain the wide range of reported NAT yields at 1/300 to 1/17,753. Currently, NAT is not mandated in India and the cost-benefit value of NAT

person is progressing toward resolving their infection. Individuals who have seroconvert from HBsAg to anti-HBs have resolved their infection and are immune to further HBV infection.

The study was conducted by the ICBS to identify high-quality test kits for detection of HBV surface antigen (HBsAg) for the benefit of developing countries. A total of 70 HBsAg test kits from around the world were evaluated comparatively for their clinical sensitivity, analytical sensitivity, sensitivity to HBV genotypes and HBsAg subtypes, and specificity using 394 (146 clinical, 48 analytical, and 200 negative) ICBS master panel members of diverse geographical origin comprising the major HBV genotypes A to F and the HBsAg subtypes adw,²⁴ adr, and ayw 1 to 4. The results of the performance evaluation of 70 HBsAg test kits showed that the diagnostic efficacy of the tests differed significantly. The sensitivity range between the most sensitive HBsAg devices and the least sensitive HBsAg assays was more than 300-fold. Enzyme immunoassays, in general, performed better than rapid assays. However, also within the EIAs there was a significant 200-fold variation in sensitivity; moreover, five EIAs were less sensitive than rapid assays. A relatively high number of assays, including all rapid tests, were of poor sensitivity rendering them unsuitable for HBsAg detection at low concentrations. Therefore, these assays cannot be recommended for use within a public health context, for example, blood screening. Genetic variability in the S gene additionally impaired diagnostic efficacy.¹⁶

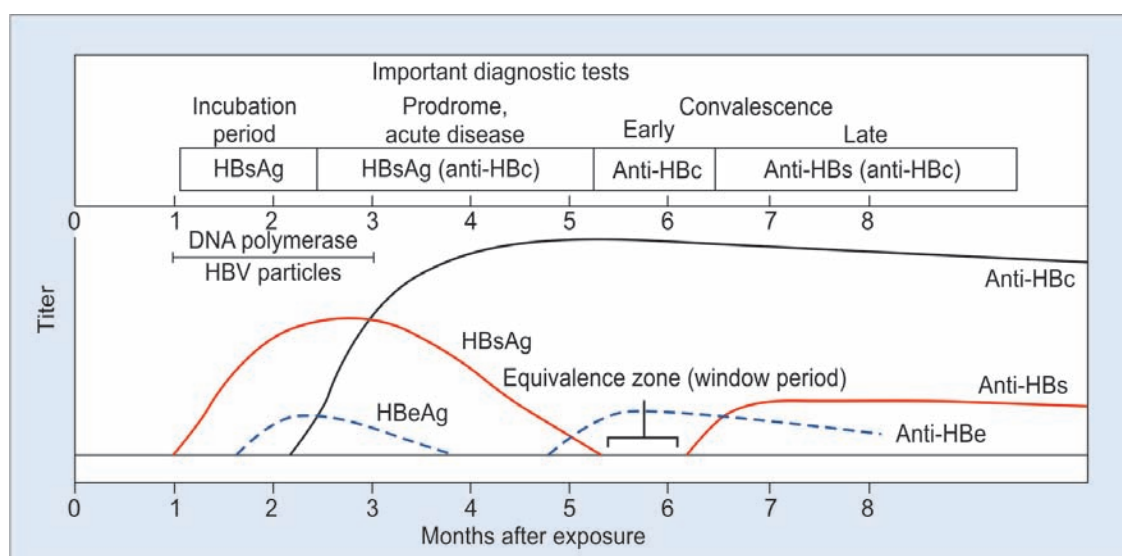
Diagrammatic representation of serology of hepatitis B infection and diagnostic test to be used are shown in Graph 1.

In the following conditions, HBsAg–negative individuals may have undetectable circulating viruses present in the blood:

1. Carriers with HBsAg below the detection level can transmit HBV by blood transfusion.
2. Subjects infected with HBV may show HBsAg negative result owing to point mutation in the precore region of the virus, resulting in inability to synthesize HBsAg. Fulminant hepatitis developed in recipients of HBsAg-negative blood from such donors infected with mutated virus. In all those donors, high levels of anti-HBc were present.
3. In acute infection, there are two periods when HBsAg may be undetectable although the subject can transmit HBV—during early incubation period when both HBsAg and anti-HBc are undetectable, and after clearance of HBsAg but before anti-HBs has become detectable (diagnostic window). In this phase, anti-HBc and anti-HBe can be detected.¹⁹

In India, blood screening for HBV, HIV, and HCV is done by serological tests for HBsAg and antibodies to HIV-1/2 and HCV. The screened seronegative donations are still at risk for TTIs, and thus need for a sensitive screening test arises to decrease this residual risk which has been reduced significantly over the last 2 to 3 decades in western countries, where NAT has been implemented. Nucleic acid testing has been started in few centers in India, but it is not a mandatory screening test for TTIs as per Drug and Cosmetics Act, 1940 and the rules therein.²⁰ Major barriers in implementing routine NAT testing in India are its high cost and lack of technical expertise in most of the blood centers.

Donations negative for HBsAg, but positive for HBV DNA, with or without the presence of HBV antibodies, correspond to 'occult' HBV infection (OBI). The frequency of OBI depends on the relative sensitivity of both HBsAg and HBV DNA assays. It also depends on the prevalence



Graph 1: The serological pattern of hepatitis B virus and helpful diagnosis methods

of HBV infection in the population. Occult HBV infection may follow recovery from infection, displaying antibody to hepatitis B surface antigen (anti-HBs), and persistent low-level viremia, escape mutants undetected by the HBsAg assays, or healthy carriage with antibodies to hepatitis B e antigen (anti-HBe) and to hepatitis B core antigen (anti-HBc). Over time, in the latter situation, anti-HBe and, later, anti-HBc may become undetectable. The critical question is whether or not OBI is infectious by transfusion. All forms have been shown to be infectious in immunocompromised individuals, such as organ- or bone marrow-transplant recipients. In immunocompetent recipients, there is no evidence that anti-HBs-containing components (even at low titer) are infectious. Anti-HBc only, with HBV DNA, can be associated with infectivity, as can rare cases of HBV DNA without any serological HBV marker. If HBV nucleic acid amplification technology is considered, the OBI viral load would usually be <500 IU/ml, making testing of plasma pools unsuitable unless the sensitivity of NAT significantly increases by genome enrichment or test improvement. It is recorded that the healthy blood donors which carry 10^6 IU/ml HBV DNA are positive for HBsAg, anti-HBs, and anti-HBe. But the donors which carry infection with 1000 IU/ml of DNA are surely negative for all serological tests. Therefore, it is suitable to use an assay of highest sensitivity and specificity with detection limit as low as 10 IU/ml and 0.1 ng/ml for HBsAg.²¹

Anti-HBc IgM is a useful marker during the 'core window', a short period in resolving acute HBV infection between the loss of serum HBsAg and the appearance of anti-HBs. Hepatitis B virus DNA is the earliest detectable marker in acute HBV infection. Hepatitis B virus DNA testing is particularly useful in the detection of the early phase of acute HBV infection prior to the appearance of serum HBsAg; for this reason HBV DNA is tested using nucleic acid amplification technology in blood and blood products in resource-rich countries. The appearance of anti-HBe followed by the appearance of anti-HBs is a characteristic of acute resolving HBV infection. The anti-HBs response remains detectable for several years following recovery from acute HBV infection and it indicates protective immunity. Anti-HBc IgG persists for several decades, if not for life, following acute HBV infection. In areas of low HBV endemicity, anti-HBc screening of blood and blood products in addition to HBsAg testing is performed to identify past exposure to HBV.²²

The several molecular mechanisms involved in occult hepatitis B infections include mutation and deletion of HBV genome, treatment-associated mutations, coinfection with other markers, host immune response, epigenetic changes, and genetic integration.²²

Hepatitis B virus precore G1896A mutation is associated with HBeAg seroconversion. This mutation and the adjacent G1899A mutation also appear to be associated with increased risk of hepatocellular carcinoma. Appearance of G1896A or G1899A mutation in the precore region is correlated with increased risk of HCC.^{23,24}

The study among the blood donors of Odisha (Behram, Ganjam) found a stop codon in HBV/D2 at 69th position amino acid which results in a truncated HBsAg gene, lacking the total 'a' determinant region, which might be one reason for HBsAg negativity making the gene non-functional.²⁵ This mutation has also been documented in subgenotype D1.²⁶

CONCLUSION

The use of recombinant multi-epitope protein over expressed in *Escheria coli* can be used in diagnostic kit for hepatitis B, with several advantages: lower cost, facilitated manipulations, and elimination of problems concerning concentration of different peptides in the kits, but still it is not determined.²⁷ To encourage voluntary blood donation should be the first step of prevention. To reduce the risk of transfusion-associated hepatitis B, test for anti-HBc IgM may be included in routine screening of donors' blood as it has been proved to be an excellent indicator of occult HBV during window period. However, awareness and education of donors regarding the modes of HBV transmission, a stringent one-to-one donor screening and increasing the voluntary donor base should also be implemented to minimize the rate of transfusion-associated hepatitis B.¹⁹

Even single-sample HBV NAT may not substitute for anti-HBc screening, as indicated by studies of donors with isolated anti-HBc who have extremely low DNA levels undetectable by standard single-sample NAT and who have been associated with transfusion-transmitted HBV. Moreover, HBsAg testing may still be needed even in the setting of combined anti-HBc and NAT screening. HBsAg-positive units from donors in the chronic stage of infection may contain very low or intermittently detectable DNA levels that single-sample NAT would miss. Although such donors are usually anti-HBc reactive and would be interdicted by anti-HBc screening, some lack anti-HBc. Extensive parallel testing will be needed to determine whether single-sample NAT in combination with anti-HBc might be sufficient to detect all the infectious donors currently interdicted by HBsAg testing. In the future, the current fully automated HBsAg assays may incorporate significant sensitivity improvements and automated single-sample HBV NAT may become a reality. Each country will need to develop its blood screening strategy based on HBV endemicity, yields of

infectious units detected by different serologic/NAT screening methods, and cost-effectiveness of test methods in ensuring blood safety.²⁸

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