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REVIEW

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Biomarkers of transfusion transmitted occult hepatitis B virus infection: Where are we and what next?

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Abstract

Blood transfusion is a vital procedure, where transfusion-transmitted infection of hepatitis B virus (HBV) remains an important issue, especially from blood donors with occult hepatitis B virus infection (OBI). Occult hepatitis B virus infection is a complex entity to detect using surrogate blood biomarkers for intrahepatic viral transcriptional activity, requiring a continually refined battery of tests utilised for screening. This review aims to critically evaluate the latest advances in the current blood biomarkers to guide the identification of OBI donors and discuss novel HBV markers that could be introduced in future diagnostic practice. Challenges in detecting low HBV surface antigen levels, mutants, and complexes necessitate ultrasensitive multivalent dissociation assays, whilst HBV DNA testing requires improved sensitivity but worsens inaccessibility. Anti-core antibody assays defer almost all potentially infectious donations but have low specificity, and titres of anti-surface antibodies that prevent infectivity are poorly defined with suboptimal sensitivity. The challenges associated with these traditional blood HBV markers create an urgent need for alternative biomarkers that would help us better understand the OBI. Emerging viral biomarkers, such as pre-genomic RNA and HBV core-related antigen, immunological HBV biomarkers of T-cell reactivity and cytokine levels, and host biomarkers of microRNA and human leucocyte antigen molecules, present potential advances to gauge intrahepatic activity more accurately. Further studies on these markers may uncover an optimal diagnostic algorithm for OBI using quantification of various novel and traditional blood HBV markers. Addressing critical knowledge gaps identified in this review would decrease the residual risk of transfusion-transmitted HBV infection without compromising the sustainability of blood supplies.

Abbreviations: Anti-HBc, Antibodies against the hepatitis B virus core antigen; Anti-HBe, Antibodies against the hepatitis B virus e antigen; Anti-HBs, Antibodies against the hepatitis B virus surface antigen; cccDNA, covalently closed circular DNA; CHB, Chronic hepatitis B virus infection; ER, endoplasmic reticulum; FFP, fresh frozen plasma; HBcAg, Hepatitis B virus core antigen; HBcrAg, Hepatitis B virus core-related antigen; HBeAg, Hepatitis B virus e antigen; HBsAg, Hepatitis B virus surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HLA, human leucocyte antigen; HSPG, heparan sulphate proteoglycan; ID-NAT, individual donation nucleic acid test; IFN-γ, interferon-gamma; IL, Interleukin; IU/mL, international units per millilitre; LLOD, lower limit of detection; LMIC, Low-or middle-income country; LOD, limit of detection; miRNA/miR, microRNA; MHR, major hydrophilic region; MP-NAT, mini-pool nucleic acid test; NAT, nucleic acid test; NTCP, sodium taurocholate co-transporting peptide; OBI, occult hepatitis B virus infection; ORF, open reading frame; PCR, Polymerase chain reaction; PD-1, programed cell death protein 1; pgRNA, pre-genomic RNA; RBC, red blood cell; rcDNA, relaxed circular DNA; SNPs, single nucleotide polymorphisms; SVPs, sub-viral particles; Th1, type 1 T helper cell; TTI, transfusion-transmitted infection.

KEYWORDS
blood safety, diagnostic, HBV, serum markers

1 | INTRODUCTION

Blood transfusions remain integral to managing many conditions, surgeries, and traumas, so preventing transfusion-transmitted infections (TTIs) remains a priority. Hepatitis B virus (HBV) is one of the most common TTIs, with an approximate 50% mortality rate within 6–12 months post-transfusion.[1] Chronic hepatitis B virus infection (CHB), with detectable HBV surface antigen (HBsAg) for ≥ 6 months, is associated with high HBV DNA levels and often elevated HBV e antigen (HBeAg) levels[2] (Figure 1). Following decades of chronic infection or the resolution of acute HBV infection, HBV DNA may persist under incomplete immune control despite HBsAg seroclearance, an entity termed occult hepatitis B virus infection (OBI).[3] OBI constitutes the presence of replication-competent HBV DNA in the liver without detectable HBsAg, where the episomal covalently closed circular (cccDNA) mini-chromosome maintains low-level intrahepatic replication of infectious HBV virions.[3] It is hypothesised that more robust immune-mediated suppression of viral replication and highly restricted gene expression differentiates OBI from HBsAg-positive infection.[4] In blood donor screening, OBI is diagnosed by detectable circulating HBV DNA as a surrogate cccDNA biomarker, combined with detectable HBV core antibody (anti-HBc) in low-endemic countries.

These infectious HBsAg-negative but DNA-positive blood donations account for most of the residual risk of HBV TTIs in developed countries,[5] transmitting HBV to approximately 8%–29% of recipients.[6,7] These transfusions may culminate in fatal complications, especially in recipients with existing immunological disturbances.[8] The prevalence of OBI in blood donors worldwide mirrors patterns of HBV endemicity. Occult hepatitis B virus infection is detected at significant frequencies from 0.06% in low-endemicity countries to 12% in high-risk groups in high-endemicity countries.[9] The real-world prevalence may be even higher than estimated, as the current assays for HBV DNA utilised to detect OBI have suboptimal sensitivities.[3] Blood establishments could, therefore, benefit from potentially alternative biomarkers to identify these OBI donors with typically low viral loads and HBsAg-negativity to prevent HBV transmission and improve blood safety. However, to our knowledge, the blood markers modulating the transmission of OBI have not been critically reviewed. We summarise these markers currently utilised to identify OBI and propose novel markers for future utilisation. We searched MEDLINE and Embase databases to identify relevant articles using Covidence (Veritas Health Innovation, Australia) and snowballing.

1.1 | Current virological markers that enable the diagnosis of occult hepatitis B virus infection

1.1.1 | Hepatitis B virus surface antigen

The undetectability or absence of HBsAg in OBI could be due to various factors. Low cccDNA levels in hepatocyte nuclei limit the expression of HBV transcripts and subsequent HBsAg expression, resulting in its undetectability.[10] Therefore, the first-line diagnosis of OBI highly depends on the sensitivity of the HBsAg assay. More sensitive assays may uncover greater frequencies of positivity and change a diagnosis from OBI to overt HBV infection. For example, while commercial HBsAg assays used in donor screening may have lower limits of detection (LLOD) of 50 mIU/mL,[3] re-testing samples using assays with an LLOD of 5 mIU/mL detected HBsAg in often substantial proportions (1%–48%) of samples that were HBsAg-negative in standard screening assays.[11–13] Development of an immune complex transfer chemiluminescence enzyme assay showed even greater sensitivity for HBsAg of 0.5 mIU/mL with high specificity, equivalent to sensitivities of current molecular assays.[14] However, these assays were developed to monitor HBV reactivation and are yet to be evaluated with blood donors. Incorporating ultrasensitive HBsAg assays into diagnostic practice may detect minute levels of HBsAg in a substantial proportion of donors previously diagnosed with OBI. The presence of HBsAg may reflect the production of HBsAg from transcriptionally active integrated HBV DNA as previously described in CHB[15]; their presence may indeed potentially contribute to the observed elevated risk of hepatocellular carcinoma (HCC) in OBI.[16] Integration of HBV DNA into chromosomal DNA and subsequent rearrangement of genes may lead to a loss of HBsAg gene expression and reduced virion production. Processing defects in core antigen synthesis may block virus assembly and release from the cell, and lead to the accumulation of HBV DNA replicate intermediates in the liver.[17] Their adventitious integration may increase the risk of HCC development.

Other factors may hamper the expression of HBsAg. Mutations introduced during the error-prone reverse transcription of a pregenomic RNA (pgRNA) intermediate in HBV replication increase the likelihood of modulating HBsAg's expression, secretion, and synthesis.[18] Mutations in antigenic determinants in the surface gene may result in an escape of detection of HBsAg by currently available assays. The most common mutants reside in the exposed major hydrophilic region on the S protein, where the 'a' determinant is found.[19] Mutations in the 'a' determinant alter the disulphide bonds in cysteine residues, preventing immune recognition of HBsAg by specific anti-surface antibodies (Antibodies against the hepatitis B virus surface antigen (anti-HBs))[19] and resulting in the escape of detection by assays using monoclonal tracers.[20] Another issue is the failure to detect immune-escape mutations of HBsAg in individuals with HBV reactivation in the presence of anti-HBs from past HBV exposure[21] or vaccination.[22] The overlapping polymerase (P) and surface (S) open reading frames also enable drug-selected mutations in the reverse transcriptase/polymerase to hinder HBsAg detectability.[23] These challenges necessitate highly sensitive assays utilising anti-HBs probes targeting multiple epitopes of HBsAg to detect these rarer S variants consistently. Assays that use multivalent polyclonal tracer antibodies that bind a range of mutated HBsAg's have been developed,[24] but the sensitivities of these assays remain variable.[20]

Although OBI is defined as HBV DNA positivity in the absence of detectable HBsAg, failure to detect HBsAg because of escape

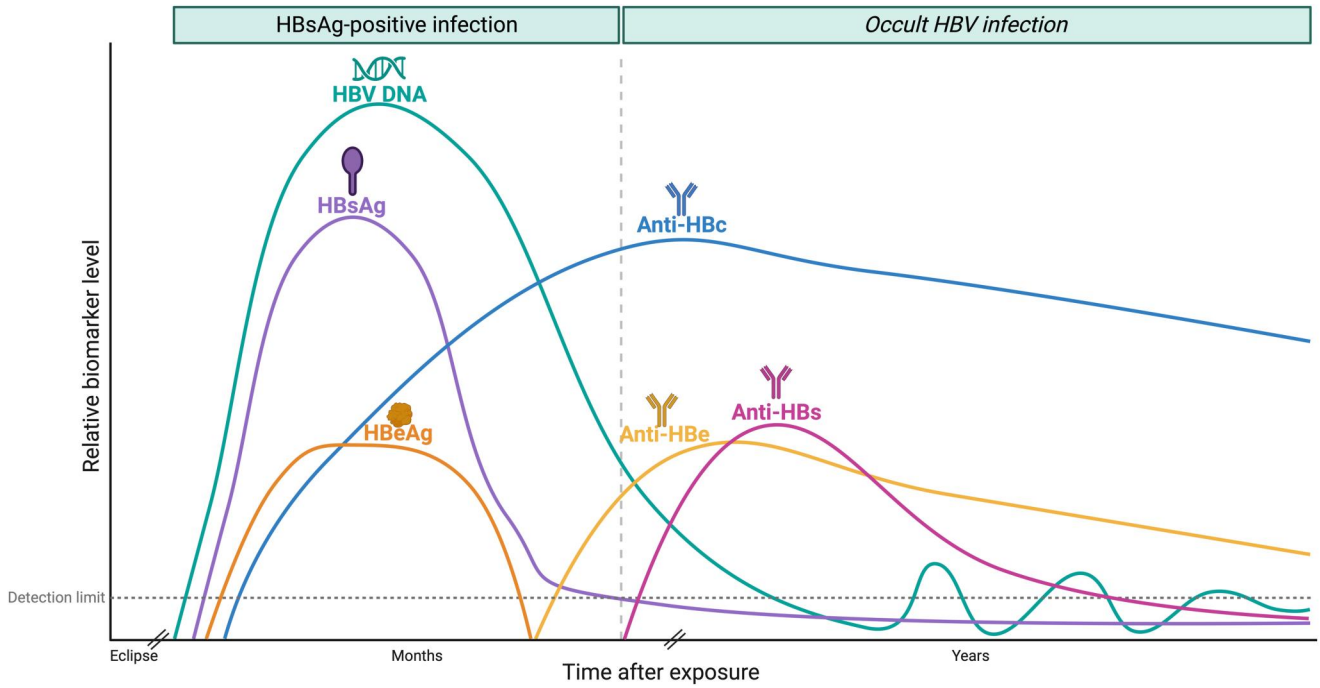

FIGURE 1 Schematic depiction of the trends in the relative levels of the virological blood biomarkers in the natural history of occult hepatitis B virus (HBV) infection. HBV DNA is the hallmark of HBV infection, indicating levels of replication. Hepatitis B surface antigen (HBsAg) constitutes the outer envelope component of HBV, where positivity of at least 6 months indicates chronicity. HBsAg is the first serological marker to appear, where seroconversion to HBV anti-surface antibodies (anti-HBs) occurs into occult HBV infection. Hepatitis B e antigen (HBeAg) is associated with viral replication. Antibodies against the core antigen (anti-HBc) are detectable throughout the natural history of infection, constituting the most sensitive marker of HBV exposure. Antibodies against the e antigen (anti-HBe) may be detectable independent of HBsAg or anti-HBs, but not without anti-HBc. *Up to 20% of Occult hepatitis B virus infection (OBI) donors may have an absence of all HBV serological markers.

mutations represents a very different infection outcome from typical OBI where HBsAg non-detection is the result of suppressed expression of antigenically normal HBsAg.[25] HBsAg in the former may be present in high titres comparable to those HBsAg-detectable infections and potentially with higher HBV DNA viral loads,[3] conferring high transmissibility by transfusion.

Non-detection of HBsAg may also result from intracellular retention of viral proteins, leading to low-level or absent extracellular HBsAg in the bloodstream. Mechanistically, rare mutations in the small S envelope protein were found to prevent HBsAg secretion, resulting in accumulation in the ER-Golgi apparatus.[26,27] Deletions in the S-promoter region also reduced transcript levels of middle and small S proteins, where subsequent overexpression of large S protein led to intracellular retention of non-secretable HBsAg.[28]

Finally, excess anti-HBs antibodies in the presence of anti-HBs/HBsAg immune complexes may mask the detection of HBsAg[29] since HBsAg assays typically can only bind non-immune complexed protein. Immune complexed HBsAg has been detected in patients with HCC previously diagnosed with OBI[30] and in acute and chronic HBV infections,[31] where non-detection was not the result of mutants in major HBsAg epitopes.[32] Indeed, a subset of OBI patients may have detectable HBsAg in assays that could pre-dissociate HBsAg from immune complexes.[33]

The development and wider application of HBsAg assays that are either more sensitive for the target protein, those capable of detecting S gene mutants, and HBsAg dissociation assays will significantly affect OBI diagnosis and differentiation from patients with more active HBV infection. While their use in donor screening may identify more significant numbers of donors with detectable HBsAg, there has been evidence of acute asymptomatic HBV infections in blood donors who never develop detectable HBsAg despite persistently low HBV DNA levels and anti-HBc seroconversion.[34] Thus, not all OBI may follow the natural history of HBV infection with HBsAg seroconversion, and nucleic acid test (NAT) screening would be required to detect these donors and prevent HBV TTI.

1.1.2 | Hepatitis B virus DNA

In line with the recent consensus definition of OBI,[3] the optimal diagnostic test for HBV DNA is the analysis of DNA extracts from liver tissues using highly sensitive techniques.[25] HBV DNA is only intermittently detectable in the blood,[6,35,36] usually with concentrations of less than 200 IU/mL.[6,37,38] However, liver biopsy is rarely available and unfeasible for most individuals due to its invasive nature, especially in generally healthy blood donors, and the small biopsy volumes may limit HBV detection due to irregular distribution of HBV DNA in the liver.[39] Serum or plasma analysis is currently the most utilised sample to identify OBI cases.

Occult hepatitis B virus infection diagnoses depend not only on HBsAg but also on HBV DNA assay sensitivities. When mini-pools are used for NAT (MP-NAT) testing of blood donors, those with OBI are often not detected since HBV DNA levels are usually lower than the pooled limit of detection (LOD) of current assays.[40] Using a prospective repository of donor-recipient pairs in Italy, 50% of DNA-positive samples detected by individual donation nucleic acid test (ID-NAT; LOD 3.7 IU/mL) were not identified by 6-MP-NAT (LOD 22.2 IU/mL).[38] As a result, two cases of HBV transmission were confirmed by donor-recipient sequence identity. One recipient received a red blood cell (RBC) unit from an OBI donor with a viral load <12 IU/mL and developed acute liver failure 22 weeks post-transfusion. The second recipient was on long-term immunosuppressive treatment and experienced HBV reactivation with acute life-limiting liver failure.[38] There may have been more confirmed transmissions than found in this study due to the lack of samples from several donor-recipient pairs for ID-NAT testing. This study emphasises the need for increased ID-NAT sensitivity compared to MP-NAT to detect OBI in donors, to allow sequence analysis of donor-recipient viruses and to prevent HBV transmission to recipients.

However, many OBI donations remain undetected, even with ID-NAT.[40] HBV transmission was confirmed from a donor with undetectable DNA (ID-NAT LOD: 8 IU/mL).[41] With a 95% LOD of 3.4 IU/mL, mathematical modelling suggested that 3.3% and 14% of undetectable OBI donations may result in transmission with 20 and 200 mL of plasma, respectively.[42] This modelling was corroborated by a recent study of 24 anti-HBs negative recipients of components from three HBsAg-negative and DNA-negative at screening (LOD 3.4 IU/mL) repeat donors in Slovenia that, after centrifugation with increased volumes of plasma, managed to confirm nine recipients newly HBV-infected with >99% donor-recipient sequence homology.[6] 37.5% of susceptible recipients being transmitted with HBV from OBI donors highlights an urgent need to develop more sensitive HBV DNA PCRs to exclude infectious donations with low viral loads. This study revised the estimated minimal infectious dose to 3 IU of HBV DNA in 20 mL of plasma, requiring an estimated LOD of 0.15 IU/mL[6]. Such sensitive detection is challenging, considering current NAT assays have 95% LODs ranging from 4 to 12 IU/mL.[43] We recently developed an ultrasensitive HBV DNA Polymerase chain reaction (PCR) system with a 50% LOD of 0.12 IU/mL, where assay sensitivity was improved by increasing sample representation and extraction volume to 5 mL of plasma.[44] Indeed, testing DNA extracts from at least 1 mL of sample was also previously recommended.[3] Increasing the sample volume in automated testing in diagnostic services would significantly improve the sensitivity of HBV DNA detection, enabling further DNA-positive OBI donors to be identified. Other methods to improve DNA testing include confirmation by alternative PCR assays targeting different HBV genome regions and viral DNA sequencing to confirm true NAT reactivity.[45] Multiple repeat extraction and testing may also increase the likelihood of detecting a low number of HBV template sequences under the assumptions of the Poisson distribution.[46]

As supported by findings of absent HBV DNA in archived samples of infectious OBI donors by transfusion,[6] OBI donors are often characterised by intermittent viraemia, where periodic testing of HBV DNA is necessitated.[36,47] Such testing may be impractical, especially in many LMICs where HBV is highly endemic, and the cost of performing ID-NAT is already economically prohibitive. When ultrasensitive HBV DNA testing is not feasible in low-endemic countries, anti-HBc is currently advocated as a surrogate marker to identify potentially infectious donations with fluctuating viraemia and improve blood safety,[25] since almost all OBI donors are anti-HBc positive.[37]

1.1.3 | Antibodies against the hepatitis B virus core antigen

Anti-HBc antibodies usually persist during HBV infection and remain detectable after recovery.[48] Anti-HBc can be the only detectable HBV serological marker in OBI blood donors.[49,50] What proportion of healthy individuals, such as blood donors who test anti-HBc positive but NAT-negative in blood, harbour replication-competent cccDNA in the liver is currently unknown. Similarly, it is not well-understood what factors trigger their replication beyond immunosuppression.[51] Without liver biopsies to ascertain cccDNA presence, anti-HBc screening may be necessary to exclude potentially infectious donations.

We recently showed that a higher anti-HBc titre was predictive of DNA presence in the plasma of anti-HBc positive donors,[44] corroborating other findings that higher anti-HBc levels were independently associated with detecting cccDNA.[52] Increased anti-HBc levels suggest the production of more HBV core antigens and may correlate with increased production of infectious virions, translating to higher TTI risk. Anti-HBc quantification would be a cheap and convenient method to identify OBI donors, complementing NAT testing. However, one study has shown that low anti-HBc titre donors could also carry HBV DNA and recommended the rejection of all anti-HBc reactive donors,[53] suggesting that anti-HBc titre may not be as discriminating a biomarker as previously thought.

Whilst anti-HBc assays are more economical and practically more straightforward to implement than ID-NAT testing, the high false-positive rate of current assays results in the unsustainable deferral of otherwise healthy donors,[54] particularly donors with rarer blood types.[55] Anti-HBc testing becomes a particular issue in HBV-endemic countries where the prevalence of anti-HBc can approach 50% and only a tiny proportion of anti-HBc positive donations have detectable HBV DNA.[54] Some blood establishments re-test reactive samples with an alternative assay to discriminate between true and false positives. However, many of these assays are still not fully discriminatory; hence, developing a highly specific confirmatory test would help reduce this diagnostic uncertainty.

Anti-HBc screening does not identify all OBI donations; extreme NAT sensitivity is also required to eliminate DNA-containing donations.[46] For example, the absence of anti-HBc does not rule out seronegative OBI. Estimated to comprise between 1% and 20% of all OBI individuals,[3] this status may result from the progressive disappearance of antibodies following the resolution of acute HBV

infection or from a lack of circulating antibodies from the beginning of HBV infection, as shown in the woodchuck *hepadnavirus* model.[56] However, the prevalence of seronegative OBI in many countries is unknown, and its presence is debatable, requiring future work to study the clinical significance of seronegative OBI, its risk for TTI, and the utilisation of non-serological markers to detect these cases.

1.1.4 | Antibodies against the hepatitis B virus surface antigen

Accumulating evidence suggests that the infectivity of blood transfusions from OBI donors is significantly reduced and potentially neutralised when anti-HBs antibodies are present compared to donors with isolated anti-HBc status.[6,8,53,57–60] Indeed, studies found HBV DNA detection highest in anti-HBc positive and anti-HBs negative blood donors,[37,44] where anti-HBs in the bloodstream may neutralise infectious HBV particles.[54] TTI studies corroborate this, where a European study found all recipients of anti-HBs-negative fresh frozen plasma (FFP) were infected, whilst none of the three FFP recipients of anti-HBs positive FFP had markers of infection.[8] Similarly, no transmission was reported in 22 recipients of anti-HBs positive blood components, whereas 10 of 37 recipients of anti-HBs negative components were HBV infected.[53] A follow-up study of a donor with intermittently low DNA levels <10 IU/mL and anti-HBs >2000 IU/L found no posttransfusion HBV from RBC or FFP components over 7 years.[61] In chimaeric mice, transfusion of anti-HBs negative donor blood confirmed HBV transmission with detectable cccDNA and blood HBV DNA.[62] Conversely, the other transfused anti-HBs positive blood (34 IU/L) did not transmit HBV.[62] Therefore, identifying anti-HBs negative but anti-HBc positive donors is crucial in preventing TTIs from donors with OBI. These transfusions could lead to acute fulminant HBV with fatal outcomes in immunosuppressed recipients and recipients with sepsis, as shown by recipient-induced investigations.[59]

However, anti-HBc screening does not identify isolated anti-HBs OBI, hypothesised to occur from long-term persistence of OBI from vertical transmission, vaccine breakthrough infection[63] that may have extremely low viral loads below NAT LODs.[64] Previous studies had shown 5% of anti-HBc negative but vaccinated donors to have detectable HBV DNA,[65] whilst long-term loss of anti-HBc may occur before anti-HBs loss after acute HBV infection with up to 11% of OBI donors were found to be DNA-positive.[43,66] There has been a recent report of HBV TTI from an isolated weakly anti-HBs positive donor (10.7–95.8 IU/L) with transient detectable levels of HBV DNA (LOD: 3.1 IU/mL) to a recipient not immunocompromised that was HBV-negative pre-transfusion but developed typical HBV infection after transfusion with confirmed sequence identity.[64] Although the vaccination status of the donor was unknown, this novel case highlights that potentially infectious isolated anti-HBs OBI donors may be missed by screening strategies currently utilised by blood establishments. Alternative biomarkers and screening strategies are needed to identify these cases.

Although anti-HBs presence may significantly reduce the TTI likelihood,[8] the protective level of anti-HBs is not well-defined, and anti-HBs presence does not invariably preclude infectivity. Neutralising anti-HBs prevents the intracellular spread of HBV but does not block the replication of the intracellular virus.[67] Indeed, several cases of transmission with anti-HBs positive donations (<50 IU/L) have been described. Recipient-triggered lookback in Australia found a pretransfusion serology-negative recipient who developed acute HBV infection post-transfusion, identifying one donor who was anti-HBc positive and NAT-negative (LOD 10.4 IU/mL) with an anti-HBs titre of 36 IU/L.[68] Although sequence homology could not be assessed to confirm TTI, this case highlights that low anti-HBs levels may not prevent transmission and that NAT assays must be more sensitive to detect the low viral loads characterising OBI.

The infectivity of anti-HBs containing blood components for immunodeficient recipients has yet to be systematically investigated; caution should be taken when these components are transfused to immunodeficient/unimmunised recipients,[69] especially since transfusion of around 50% of blood components in Western Europe is to patients with some degree of immunodeficiency.[70] However, one study found that an anti-HBs positive (12 IU/L) OBI donor infected two immunocompetent recipients with 180 IU/mL of HBV DNA,[71] suggesting higher viral loads in OBI may overcome the relatively weak neutralising capability of low anti-HBs levels and transmit HBV, even in the case of immunocompetent recipients. Thus, current evidence shows that low anti-HBs levels may not be protective from infectivity when HBV DNA is present. Donors with anti-HBs >100 IU/L, incorporating a substantial safety margin,[68] could be considered eligible for donation. However, the latter group may still harbour risks of infectivity, and it is often difficult to ascertain whether the detected anti-HBs response is due to natural HBV infection or HBV vaccination. It may be that a combination of low anti-HBs levels and high anti-HBc titres would identify donors at higher risk of active infection, an approach shown to be effective for predicting high risks of HBV reactivation in patients with lymphoma and resolved HBV infection receiving rituximab-containing chemotherapy.[72] However, our recent findings suggested that his approach had relatively low sensitivity and specificity for predicting detectable HBV DNA.[44]

1.1.5 | Limitations of studies

There is much variability in donor demographics across existing studies, sensitivities of assays used, and whether blood samples were collected longitudinally or cross-sectionally. Therefore, it is difficult to compare studies investigating OBI transmission. The current review is further limited in part by the lack of literature examining the transmissibility of HBV infection from donors with OBI. Various factors contribute to this, mostly from underreporting HBV transmission to recipients. Identifying post-transfusion HBV infection primarily relies on the clinical presentation of acute infection and subsequent detection of HBV markers. It is known that >90% of acute HBV infections in adults are asymptomatic and resolve

spontaneously, whilst only 5%–10% develop into chronic HBV infection. Transfusion-transmitted infections may not be recognised if investigations do not occur within the acute infection period or if the sensitivity of assays used is insufficient. Only four of nine infected recipients of OBI-containing donations in a study were symptomatic,[6] suggesting many transmissions may not be identified and/or reported due to asymptomatic subclinical infection and lack of apparent hepatitis in recipients.[59] The only evidence of potential TTI may be an anti-HBs spike that demonstrates likely HBV exposure,[41] reinforcing the need for clinicians to have a heightened awareness of the likelihood of resolved acute infections in recipients with HBsAg seroconversion at the time of investigation.

The challenges of lookback exercises further limit current studies. Since OBI donors may be intermittently infectious, donor lookback investigations are warranted. The development of chronic HBV infection in an exposed recipient may result in severe consequences for them and their close contacts if the infection remains undiagnosed. Recipient lookback is also essential to ascertain whether recipients were already HBV-infected pre-transfusion; however, it can be challenging to differentiate new infection and HBV reactivation in immunocompromised recipients.[73] The lack of recipient pretransfusion samples renders this challenging,[59] combined with difficulty and reluctance to trace recipients.[66] Lookback is often conducted years after transfusion, where approximately a 50% mortality rate at 6 months post-transfusion decreases the number of informative cases.[74] When lookback is performed, viral clearance at the time of lookback restricts definitive confirmation of TTI by demonstrating donor-recipient sequence homology, which is lacking in the current evidence on OBI transmissibility. Limited volumes of donor archive samples and undetectable or intermittently detectable HBV DNA also limit this virological characterisation to confirm OBI transmission.[66]

Taken together, the challenges and needs identified for current blood biomarkers of transfusion-transmitted OBI highlight the potential value of considering alternative biomarkers to predict potentially infectious OBI blood donors and reduce TTIs without compromising the sustainability of the blood supply (Table 1). These alternative biomarkers should have high sensitivity and specificity and be accessible by all blood establishments.[75]

1.2 | Novel occult hepatitis B virus infection biomarkers

1.2.1 | Hepatitis B virus core-related antigen

Hepatitis B virus core-related antigen (HBcrAg) is a composite of HBeAg, HBcAg, and a truncated HBcAg coded by the pre-core/core ORF[76] (Figure 2). HBcrAg was suggested as a potential intrahepatic cccDNA transcriptional activity marker for distinguishing phases of HBsAg-positive infection.[77] Indeed, HBcrAg detection in 65% of patients 10 years after HBsAg seroconversion[78] suggests low levels of HBV protein expression in HBsAg-negative patients. However, a recent study that longitudinally sampled chronic hepatitis C co-infected patients after treatment cessation did not detect HBcrAg despite HBV DNA detectability.[79] Further evidence suggested HBcrAg as less sensitive than anti-HBc quantification for predicting cccDNA presence.[52] These findings may be confounded by assay sensitivity, where the recent development of ultrasensitive HBcrAg has shown promising results for the early detection of HBV reactivation.[80] However, the HBcrAg assay currently has limited availability in research or clinical practice, and its use in risk-stratifying OBI donors has yet to be studied. Additionally, since HBcrAg consists of multiple antigens, developing a core-specific biomarker would eliminate confounding factors such as pre-core and basal core promoter mutants that affect HBcrAg detection.[81]

1.2.2 | Pregenomic RNA

PgRNA is exclusively transcribed from cccDNA[82] (Figure 2) and may indirectly measure cccDNA activity. The pgRNA intermediate functions as the mRNA for HBcAg and polymerase and is also reverse-transcribed into relaxed circular DNA for secretion from cells or for replenishment of intranuclear cccDNA.[83] Previous evidence has shown delayed decreases in pgRNA compared to HBV DNA levels following antiviral cessation,[84] where both HBcrAg and HBV RNA levels, but not HBV DNA, were shown to predict HBV reactivation.[85] These preliminary findings suggest that pgRNA levels may be a useful surrogate biomarker to reflect cccDNA transcriptional activity and possible infectivity compared to HBV DNA. Detecting pgRNA in blood via RNA PCR assays is yet to be explored in OBI. Investigations into combined pgRNA and HBcrAg predictive value for intrahepatic activity need to be undertaken to assess these biomarkers' suitability to identify potential TTI. However, failure to detect HBV RNA by currently available assays in patients with low HBV DNA levels following treatment[85] suggests that improved sensitivities are required to detect HBV RNA in OBI donors with often very low viral loads. Using primers that target the 5′ end of pgRNA in PCR assays is recommended to increase detection sensitivity and avoid RNA derived from integrations.[86,87]

1.2.3 | MicroRNA

MiRNAs may regulate gene expression in the translational process (Figure 2) or through the degradation of HBV transcripts via imperfect base pairing.[88] Previous evidence shows that four miRNAs (Lethal-7c, miR-23b, miR-122, and miR-150) are differentially expressed in OBI compared to healthy controls.[89] MiR-122, a liver-specific miRNA, downregulates HBV gene expression and, subsequently, HBsAg and HBeAg expression,[90] suggesting potential value in predicting the activity of OBI. However, with a small sample size of 11 OBI sera from a specific region and age group, further studies are necessitated to provide data on whether miRNAs could be a novel biomarker for OBI detection, where additional miRNAs such as miR-125a-5p may reduce HBsAg expression and secretion.[91]

TABLE 1 Summary of the challenges and needs of blood biomarkers of transfusion-transmitted occult hepatitis B virus infection (OBI) currently utilised in diagnostic practice.

Biomarker	Challenges in OBI detection	Future needs
HBsAg	Extremely low levels of HBsAg expression resulting in undetectability	More sensitive assays with a limit of detection ≤0.5 mIU/mL to be validated in OBI donors and adopted by blood establishments
	Rare mutations in the surface gene result in the escape of detection by currently available assays	Widely available assays using multivalent polyclonal anti-HBs probes to identify donors with HBsAg mutants
	Anti-HBs/HBsAg immune complexes mask the detection of HBsAg	Investigations into the potential use of HBsAg dissociation assays in diagnostic practice
	Some donors may never develop detectable HBsAg	Screening for other biomarkers such as HBV DNA and anti-HBc
HBV DNA	Viral loads below the detection limit of MP-NAT and ID-NAT	ID-NAT testing with commercialised ultrasensitive assays
	Economically prohibitive, especially in low-and-middle-income countries	Development of point-of-care assays or novel biomarkers that are accessible for blood establishments globally
	Intermittent detection in the blood	Use of surrogate biomarkers such as anti-HBc to exclude potentially infectious donations
	Not an accurate reflection of cccDNA activity	Development of novel biomarkers that more accurately reflect intrahepatic activity
Anti-HBc	Low specificity of currently available assays results in the deferral of large numbers of donors	Re-testing reactive samples with alternative assays, development of highly specific assay, use of anti-HBc quantification
	Anti-HBc may not detect all OBI donors and may not be practical in HBV high-endemic countries	Ultrasensitive HBV DNA testing or the use of alternative biomarkers
Anti-HBs	Protective titre is not well-defined	Further investigations into the virological and immunological responses in OBI donors with differing anti-HBs levels
	Anti-HBs titre has suboptimal sensitivity and specificity to risk-stratify potentially infectious donations	Development of more sensitive biomarkers to identify potentially infectious donations

Abbreviations: anti-HBc, antibodies against core antigen; anti-HBs, antibodies against surface antigen; cccDNA, covalently closed circular DNA; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; ID-NAT, individual donation nucleic acid testing; IU/mL, international units per millilitre; MP-NAT, mini-pool nucleic acid testing.

1.2.4 | T-cell reactivity

Host immune responses are critical in controlling HBV replication but remain poorly understood in OBI.[3] With the maintenance of cccDNA infecting hepatocytes in OBI[4] and reactivation of typical HBsAg-positive infection under host immunosuppression,[92] it is plausible that the host immune system strongly suppresses HBV replication (Figure 2), leading to low viral loads and lack of detectable HBsAg.[93,94] Limited evidence suggests that OBI individuals have potent, HBV-specific T-cell responses, where type 1 T helper cell responses are quantitatively stronger than in inactive HBsAg-positive infection and are similar or higher against HBV antigens than in resolved HBV infection.[95] T-cell responses may also help distinguish seropositive and seronegative OBI, where anti-HBc positive OBI had T-cell responses comparable to resolved HBV infection, whilst seronegative OBI had minimal HBV-specific T-cell expansion, suggesting a lack of protective memory.[96] Simple cytokine stimulation and IFN-release assays have been utilised for SARS-CoV-2[97] and tuberculosis[98] detection, respectively. These assays could be adapted to detect HBsAg-specific cell-mediated responses that diminish HBsAg levels,[99] where HBV-specific T-cell responses could predict periodic reactivation of OBI comparable to that characterised in other DNA viruses[100] and thus minimise the risk of TTI whilst ensuring the sustainability of the blood supply.

1.2.5 | Human leucocyte antigen

Human leucocyte antigen (HLA) molecules are important in epitope presentation to T-cells (Figure 2), promoting T-cell allorecognition and peptide binding, stimulating immune clearance of HBV infection.[101] A Chinese study found that the frequencies of specific HLA phenotypes associated with infection were higher in OBI than in healthy controls, whilst other phenotypes related to DNA clearance were lower.[102] Over-representation of these HLA alleles may confer enhanced T-cell responses against HBV peptides in OBI compared to HBsAg-positive infection, comparable to that recently shown in asymptomatic SARS-CoV-2 infection.[103] This over-representation may also occur in certain ethnic minorities with differing HLA frequencies,[104] but validation of this in OBI donors is necessitated. Further, homozygous compared to heterozygous HLA alleles may hamper antigen presentation[102] and impair HBV immune clearance in OBI. Specific HLA-DP single nucleotide polymorphisms were also associated with OBI.[60] If shown to be sensitive to OBI, identifying

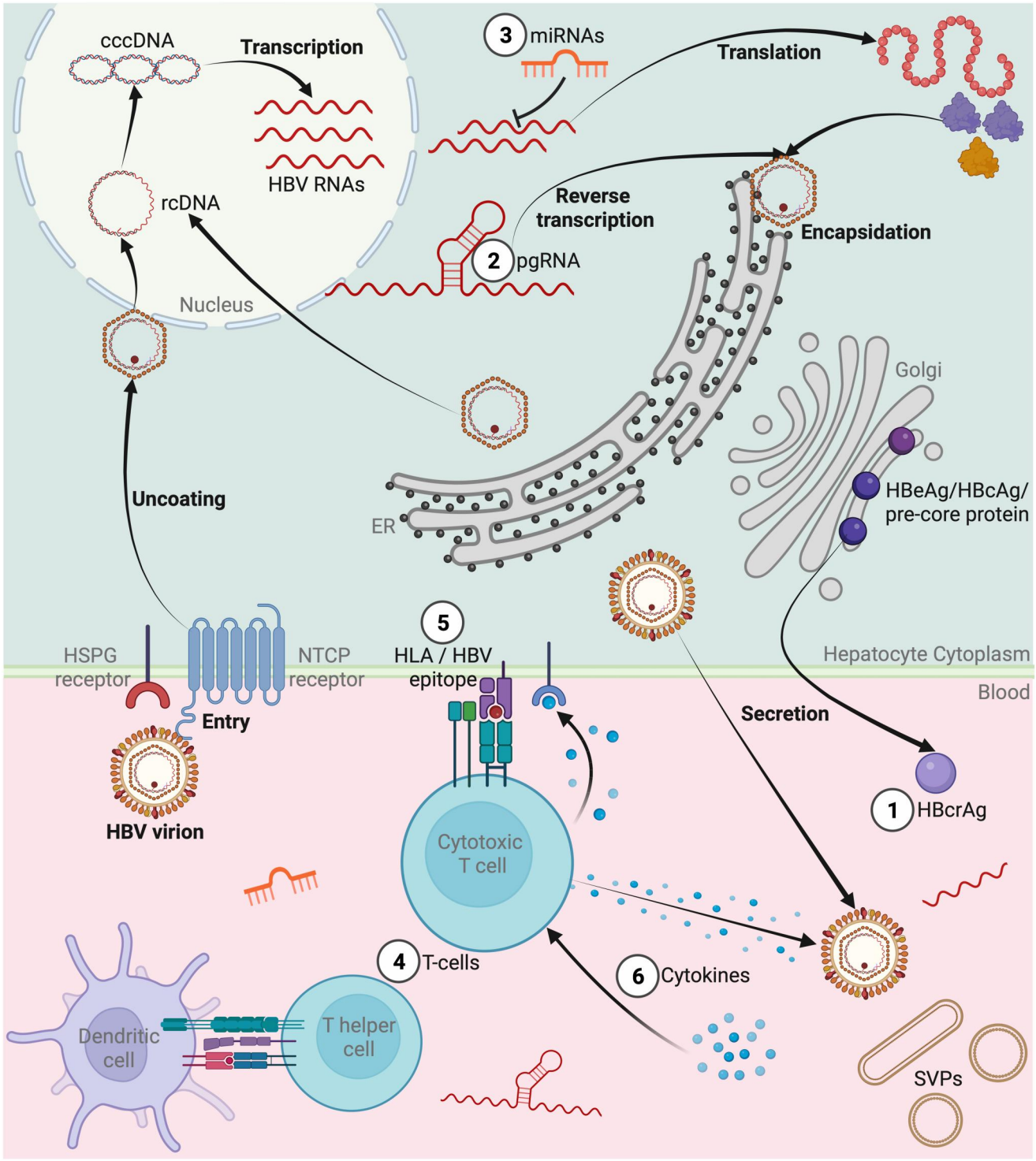

FIGURE 2 The life cycle of hepatitis B virus (HBV) and immunological interactions in occult HBV infection, showing the various novel markers hypothesised in this review: (1) HBcrAg, HBV core-related antigen; (2) pre-genomic RNA (pgRNA), pregenomic RNA; (3) miRNAs, microRNAs; (4) T-cells; and (5) HLA, human leucocyte antigen. cccDNA, covalently closed circular DNA; ER, endoplasmic reticulum; HBeAg, HBV e antigen; HSPG, heparan sulphate proteoglycan; IFN-γ, interferon-gamma; NTCP, sodium taurocholate co-transporting peptide; rcDNA, relaxed circular DNA; SVPs, sub-viral particles.

specific host gene variants and HLA alleles could be a valuable screening method to risk-stratify donors with enhanced immune responses against HBV replication who are at increased risk of reactivating HBV periodically.

1.2.6 | Cytokines and chemokines

Signalling pathways of innate immunity may be induced to varying extents in different stages of HBV infection[105] (Figure 2). Interleukin

(IL)-10 is the major cytokine in limiting cell-mediated immune responses against pathogens,[106] whilst IL-17 upregulates anti-apoptotic molecules in hepatocytes.[107] A study showed that IL-10 and IL-17 levels were higher in OBI than in resolved HBV infection, suggesting more suppressed immune responses and enhanced survival of virus-infected hepatocytes than in resolved infection.[108] Polymorphisms within these cytokines were suggested to hamper their expression and weaken host immune clearance of HBV infection in OBI.[109] Immune checkpoint molecules may also be somewhat affected in OBI, where the upregulation of programed cell death protein 1, the most highly expressed inhibitory receptor on HBV-specific T-cells,[110] resulted in T-cell dysfunction in HBsAg-positive infection.[111] Investigations into the cytokines and chemokines that may regulate periodic HBV reactivation and mild liver inflammation would provide novel perspectives on identifying donors with ongoing immunological activity in the liver, where utilisation of a multiplexed assay for screening blood donors would be able to measure hundreds of such biomarkers with small volumes of blood.[112]

2 | CONCLUSION

Although the risk of TTI from HBV-containing blood components has been reduced over the last decades through donor selection and screening assays, residual risk remains. Anti-HBc screening results in the deferral of valuable donors and may not detect all OBI donors, whilst ultrasensitive HBV NAT is technologically demanding and expensive. Investigations into novel biomarkers, such as further virological and emerging immunological markers, are necessitated to predict the presence of OBI with ongoing intrahepatic activity and increased probabilities of reactivating HBV periodically. Combining novel and current blood biomarkers to improve sensitivity and specificity for identifying OBI could help reduce donor deferral via current screening algorithms and improve blood safety in blood establishments globally.

AUTHOR CONTRIBUTIONS

All authors conceptualised the manuscript. MXF performed the narrative review and draughted the manuscript. HH, PS, and MA finalised the manuscript and provided supervision. All authors approved the final version of the manuscript submitted.

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CONFLICT OF INTEREST STATEMENT

No conflicts of interest declared.

DATA AVAILABILITY STATEMENT

No original data was used in this manuscript.

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