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State of the art

Occult hepatitis B infection and transfusion-transmission risk

Hépatite B occulte et risques transfusionnels

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Abstract

Advances in serology and viral nucleic acid testing (NAT) over the last decades significantly reduced the risk of transfusion-transmitted hepatitis B virus (HBV). The combination of HBsAg testing and NAT efficiently prevents the majority of HBV transmission. However, a specific residual risk remains associated with extremely low viral DNA levels in blood donors with occult HBV infection (OBI) that are intermittently or not detectable even by highly sensitive individual donation (ID) NAT. Studies have reported HBV transfusion-transmission with blood components from donors with OBI that contained low amount of viruses (< 200 virions). HBV transfusion-transmission seems to depend on a combination of several factors including the volume of plasma associated with the infected blood components transfused, the anti-HBV immune status of both recipient and donor, and possibly the viral fitness of the infecting HBV strain. Models based on clinical and experimental evidences estimate a residual transmission risk of 3–14% associated with OBI donations testing HBsAg and ID-NAT non-reactive. Anti-HBc testing has the potential to improve further blood safety but it may also compromise blood availability in settings with medium/high HBV prevalence. Pathogen reduction procedures might be considered.

Keywords: Hepatitis B virus; Blood safety; Transfusion-transmission; Occult HBV infection; Nucleic acid testing

Résumé

L'amélioration des performances des tests sérologiques et le développement des techniques de détection du génome viral ont réduit significativement le risque de transmission du virus de l'hépatite B (VHB) par transfusion. L'association du dépistage de l'AgHBs et du génome viral s'avère efficace pour prévenir la grande majorité des infections à VHB. Cependant, un risque résiduel transfusionnel lié aux donneurs porteurs d'une infection B occulte et présentant des niveaux extrêmement faibles d'ADN viral indétectables ou détectés de façon intermittente par les tests DGV unitaires les plus sensibles persiste, particulièrement dans les pays ne dépistant pas les anti-HBc. Des cas de transmission par des produits sanguins provenant de tels sujets et contenant de faibles quantités de virus (< 200 virions) ont été rapportés. La transmission de l'infection B semble dépendre de plusieurs facteurs non-exclusifs tels que le volume de plasma associé au produit sanguin infecté transfusé, le statut immunitaire anti-HBV du receveur et/ou du donneur, et éventuellement les propriétés réplicatives de la souche virale infectante. Des modèles développés sur la base de données cliniques et expérimentales estiment un risque de transmission virale lié aux dons infectés par le VHB et échappant au dépistage de l'AgHBs et de l'ADN viral entre 3 % et 14 %. Le dépistage des anticorps anti-HBc en combinaison avec le DGV pourrait renforcer la sécurité transfusionnelle mais en compromettant la disponibilité des produits sanguins dans des zones de moyenne et forte endémie pour le VHB. Le recours à des méthodes de réduction des agents pathogènes dans les dons peut être envisagé.

Mots clés : Virus de l'hépatite B ; Sécurité transfusionnelle ; Transmission par transfusion ; Infection B occulte ; Détection du génome viral

1. Introduction

Despite the availability of a vaccine and the continuous development of antiviral treatments, persistent hepatitis B virus

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(HBV) infection remains a global major public health issue. It is estimated that more than two billion people worldwide have been in contact with the virus at some time in their lives and 257 million of them remain chronically infected [1]. HBV chronic infection is usually defined by detectable levels of HBV surface antigen (HBsAg) in the blood. The development of molecular diagnostic assays uncovered another form of active persistent/chronic HBsAg-negative HBV carriage identified as occult HBV infection (OBI) [2].

HBV transmission involves direct exposure to infected blood or organic fluids containing infected blood. Before 1970, it is estimated that HBV transfusion-transmission occurred in ~6% of multi-transfused patients. Over the last four decades, blood safety has been continuously improved by the constant development of sensitive and specific serologic assays to detect the HBV surface antigen (HBsAg) and anti-HBc antibodies against the HBV Core antigen (anti-HBc) in blood donations. Global implementation of nucleic acid testing (NAT) for HBV DNA in 2004–2008 significantly reduced further the residual risk of HBV transfusion-transmission by reducing the diagnostic pre-seroconversion window period and by detecting occult HBV infection/carriage [3]. Yet, HBV transmission remains the most frequent transfusion-transmitted viral infection. The residual risk of HBV transfusion-transmission appears to be mainly related to blood donations negative for HBsAg but containing extremely low levels of viral DNA potentially infectious that may escape detection by the currently most sensitive NAT assays.

2. Reducing the risk of HBV transfusion-transmission

2.1. Blood donor selection

Recruitment of voluntary non-remunerated donors and risk behavior-based selection of donors prove essential for blood safety. These processes reduce the risk for transfusion-transmissible infections (TTIs) by temporarily or indefinitely deferring high-risk and therefore potentially infectious candidate donors. The pre-donation evaluation usually consists of a self-administrated written questionnaire about a range of risk exposures for TTIs and a confidential interview with a medical counselor. Effectiveness is shown by the significant lower prevalence of TTIs observed among eligible donors compared to the general population, but it also strongly depends on both donor education and accurate and truthful risk behavior disclosure. However, post-donation interviews revealed that 22% to 28% of infected donors subsequently admitted risk exposures that would have result in permanent deferral [4,5]. Reasons behind donor questionnaire noncompliance can be complex varying from deliberate (e.g., test seeking, social discomfort, disagreement with deferral criteria, misunderstanding of the pre-donation screening purpose since donations are tested further) to genuine (e.g., misinterpretation of questions, failure of recall, erroneous no-risk belief associated with temporally remote exposure). A recent study reported a 10% rate of non-compliance in HBV-infected blood donors from the Netherlands that was mainly associated with male-to-male sex risk factor, whereas the main infective risk exposure identified in the whole donor population with confirmed HBV infection was to originate or to be related to a HBV endemic country [4,5].

2.2. HBsAg testing

HBsAg testing remains the first-line of HBV screening in blood donors. Commercial enzyme immunoassays (EIAs), including enzyme linked immunosorbent assays (ELISAs), and chemiluminescent immunoassays/chemiluminescent enzyme immunoassays (CLIAs/CLEIAs) are the most commonly used assays for blood screening. These assays are currently implemented on (semi)-automated testing platforms combining high throughput and high analytical sensitivity ranging between 5 and 50 mIU/mL. HBV genotypes and mutations associated with structural changes and/or with reduced synthesis or secretion of HBsAg may negatively affect the analytical and clinical performance of HBsAg detection [6]. The formation of circulating immune complexes between HBsAg and hepatitis B surface antibodies (anti-HBs) may also cause detection failure when they are not or poorly displaced by the HBsAg capture antibodies used in the manufactured assays.

The high cost and considerable equipment requirements of these commercial HBsAg assays may limit their use in resource-limited settings. Over the last decade, several reliable and sensitive rapid diagnostic tests (RDTs) have been successfully developed that do not require laboratory infrastructure, are easy to perform with minimal training, and provide conclusive results within a few minutes [7].

2.3. Anti-HBc testing

Antibodies to the HBV core protein (anti-HBc) are associated with HBV natural infection and remain detectable throughout the entire course of chronic infection at least in immunocompetent individuals. They also persist for life after recovery even when anti-HBs become undetectable and can be the only serological marker, associated or not with low levels of viral DNA, in rare long-standing chronic infection. However, anti-HBc assays show mediocre specificity despite continuous improvements and the lack of proper confirmatory tests necessitates retesting reactive samples with an alternative assay to discriminate true from false-positive samples [8]. Despite these limitations, anti-HBc screening has been implemented in HBV low-endemic countries where donor definitive deferral was considered sustainable in terms of donation wastage, but it adds to the already considerable list of donor exclusion. Introducing anti-HBs testing in anti-HBc only positive donations has been considered to mitigate donor lost in countries that have a slightly elevated HBV prevalence like Japan where donations with high anti-HBs titers ($\geq$ 200 IU/L) presumably non-infectious are considered acceptable for plasma fractionation, and donations with low anti-HBc and anti-HBs titers are rejected [9]. Nevertheless, anti-HBc testing cannot be implemented in high-endemic countries without compromising blood availability (e.g. Sub-Saharan Africa, Southeast Asia).

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2.4. HBV nucleic acid testing

HBV nucleic acid testing (NAT) of blood donations has been successfully implemented in most countries of Europe, East Asia, North America, Australia and South Africa as part of multiplex assays for the simultaneous detection of human immunodeficiency virus (HIV) and hepatitis C virus (HCV) RNAs [3]. Commercial NAT assays are based on PCR or TMA and show specificity >99% and 95% detection limits ranging between 4 and 12 IU/mL [10]. Two strategies are commonly used worldwide: testing in minipool of 6 donor plasmas (MP-NAT) or in individual donor (ID-NAT). The implementation of NAT uncovered two distinct types of HBsAg-negative/HBV DNA positive donors. First, donors in the pre-seroconversion "window period" (WP) defined as the time between infection and detection of viral antigen or antibody markers, early window period with the most sensitive serological assays. The high sensititvity of ID-NAT allows to significantly reduce this WP to 13–15 days that includes the so-called eclipse phase that can last for ten days during which HBV DNA is usually undetectable even with the most sensitive NAT assays [11]. Second, donors with persistent low-level chronic infection so-called occult HBV infection (OBI) [12]. The frequency of detected WP and OBI cases is approximately 28% and 72%, respectively [10]. NAT can also detect immunovariant viruses escaping serological screening [13,14].

Performance of HBV DNA detection depends not only on the intrinsic sensitivity of the NAT assays, but also on the sample volume tested as well as the dilution factor introduced when plasma pooling is used [15,16]. Three separate virus-specific so-called discriminatory assays are usually used to identify the origin of a multiplex NAT reactivity. However, discriminatory assays based on the same technology and reagents as the screening assay do fully qualify for confirmation. This procedure also introduces the problem of samples reactive with the initial screening assay but non-reactive with the discriminatory assay. Non-reproducible reactivity (NRR), defined as multiplex reactive but discriminatory assay non-reactive and repeat multiplex non-reactive, has been extensively reported [17]. Confirmation of HBV-NAT true reactivity with both MP- and ID-NAT can prove particularly challenging for OBI donors with very low HBV DNA levels that may be only intermittently detectable over time even by ID-NAT. Therefore, alternative non-exclusive investigational NAT approaches should be considered: multiple repeat testing, improved assay sensitivity by increasing the nucleic acid extraction volume, confirmation by alternative assays (real-time PCR, nested PCR) targeting different regions of the viral genome, and ultimately viral DNA sequencing [18].

Confirmation of inconclusive NAT by complementary serological testing of index and follow-up donations might not be sufficient due to anti-HBc background prevalence especially in high-endemic areas and to discrepancies between serological and molecular markers (Fig. 1). The frequency and nature of these discrepancies is difficult to evaluate as they largely depend on the sensitivity of the assays used and to some extend on HBV genotypes. Recently, data from a large-scale multi-regional study using a comparable HBV screening algorithm showed that 2% and 4% of OBI donors presented no detectable serological markers or an anti-HBs only profile, respectively (Fig. 1). In addition, 6% (range: 3–10%) of HBV-infected donors were HBsAg reactive and HBV DNA ID-NAT non-reactive but anti-HBc reactive when tested.

2.5. Alternatives to HBV blood screening

Pathogen inactivation or reduction methods and large-scale HBV vaccination have the potential to reduce TTI. Different pathogen inactivation systems are currently applied to plasma and platelet concentrates with an estimated relatively modest inactivation yield of two to four log [19,20]. Further developments are still needed for red cell components. Over the past two decades, systematic vaccination at birth or early childhood in several countries with moderate or high HBV endemicity resulted in a decrease of HBV incidence. However, vaccination may also favor the development of escape mutants and neutralizing anti-HBs antibodies level decrease over time in vaccinated people who may become susceptible to HBV infection, especially to infection with genotypes different from the one used in the vaccine (HBV genotype A2) [21].

3. Occult HBV infection in blood donors

OBI is defined by the presence of low levels of HBV DNA in the liver (with detectable or undetectable HBV DNA in the serum) of individuals testing HBsAg-negative by currently available assays, with or without detectable anti-HBc and/or anti-HBs antibodies, outside the pre-seroconversion window period [2]. Donors with OBI tend to be ≥ 45 years old, except in sub-Saharan Africa, suggesting a late stage of chronic HBV carriage. OBI is a worldwide-disseminated entity irrespective of HBV genotypes although its prevalence varies between different geographic areas and populations according to the corresponding general HBV epidemiology. Regional OBI detection rates vary between 1:3400 and 1:59,000 in first time donors and between 1:6000 and 1:64,500 in repeat donors [10].

OBI donors show no evidence of liver damage but OBI has been associated with higher risk of chronic infection progression and hepatocellular carcinoma (HCC) when other causes of liver damage co-exist (e.g. HIV or HCV co-infection) [22]. In addition, mutations in the *HBV X* gene associated with HCC in patients have been described in the genome of some OBI strains [23]. Long-term follow-up studies are still needed to establish the risk of clinical progression in blood donors associated with the OBI status.

Sustained viral replication with very low levels of HBV DNA in blood characterizes OBI. The viral load median level in OBIs varies generally between 10 and 50 IU/mL [2,12,18]. Different mechanisms have been proposed to explain these persistent low levels of viraemia observed in OBI including (1) interference with other viruses (i.e. HIV, HCV), (2) epigenetic changes (i.e. methylation of cccDNA), (3) genomic integration resulting in disruption of the HBV genome, (4) virus-specific mutations impairing replicative competence of HBV, and (5) incomplete immunological control of HBV infection in associa-

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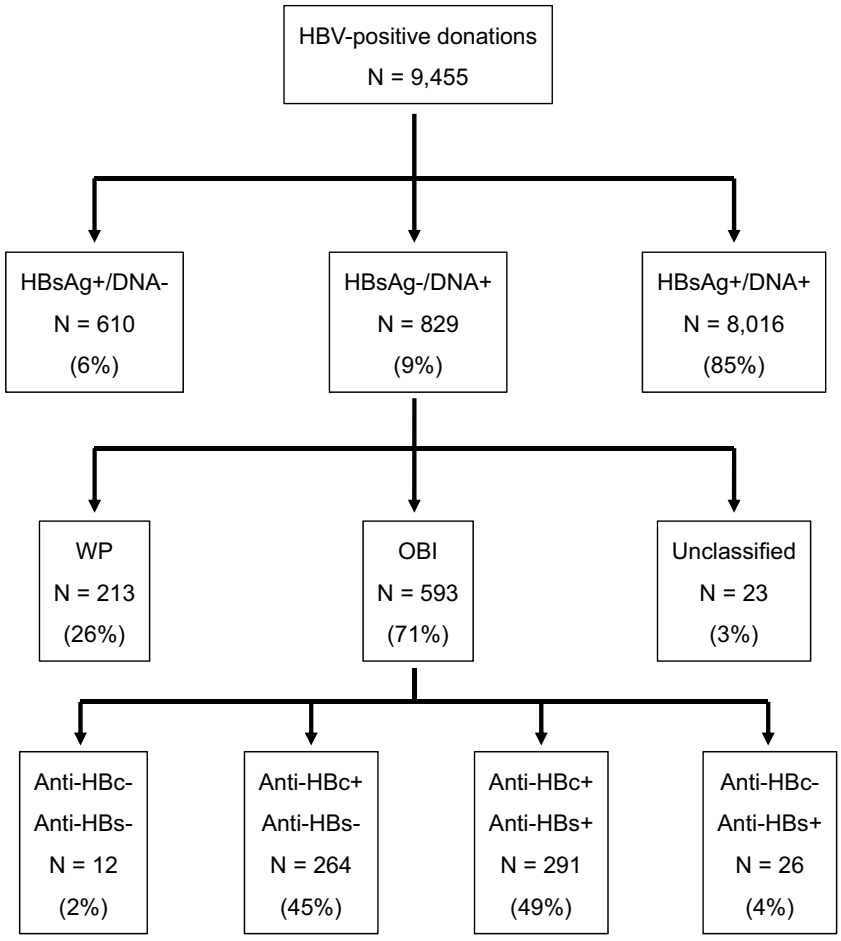

Fig. 1. HBV markers distribution in infected blood donors. HBsAg, anti-HBc antibodies, anti-HBs antibodies, and HBV DNA were investigated in confirmed HBV-infected donors from South Africa ($n = 3416$), the Mediterranean region ($n = 1608$), Central and Northern Europe ($n = 503$), South East Asia ($n = 3754$), and Oceania ($n = 174$). Initial HBV screening was performed by using ID-NAT with the procleix Ultrio assay on the Tigris platform (Grifols Diagnostic Solutions Inc. and Hologic) and HBsAg screening with the Abbott PRISM or ARCHITECT chemiluminescent assays. Data derived from Lelie et al., 2017 [11].

tion with adaptation of the virus to this immunological pressure by selection of escape mutants [12,13]. The latter mechanism is supported by the presence of detectable anti-HBs in 49% of OBI donors, and by the high genetic variability observed within both cellular and humoral epitopes located in the HBV surface proteins of OBI viral strains compared to strains from patients with overt HBV infection [24]. In addition, mutations outside the major epitopes may also contribute to OBI by affecting HBV infectivity, cell tropism, virion morphogenesis, and HBsAg excretion [25]. The implication of the host immune system in the genesis of OBI is indirectly supported further by numerous reports of HBV reactivation in OBI patients undergoing severe immunosuppression [26]. However, the characterization of a large number of OBI strains with little or no variation in their surface protein epitopes suggests that immune pressure may not be the only major factor involved in OBI genesis. Genetic analyses identified multiple mutations affecting viral structural (Core, Pol, S) and non-structural (X) genes, various critical cis-regulatory elements (i.e. promoters, ε and ϕ elements), and/or critical RNA secondary structures within the HBV pre-genomic RNA and mRNAs that might negatively affect viral replication and/or gene expression [23,24,27,28]. However, the majority of studies investigated the genetic variability of OBI strains that provided only indirect evidences while functional analyses especially those based on full-length HBV genome remain rare.

In France, since the implementation of a systematic HBV-NAT in 2010 to the end of 2015, 0.24% of HBsAg-negative/anti-HBc positive donations were found HBV DNA positive. From 2005 to 2015, 72% (43/60) of HBV-NAT yield cases were OBI. When tested, 14 out of 32 (42%) of these OBIs had low levels of anti-HBs (11–227 IU/L). HBV DNA was quantified in 33 OBI donations: 22 (67%) had viral load < 6 IU/mL (limit of quantification), 7 (21%) had 8 to 1074 IU/mL, and no viral DNA was detected in 4 (12%) (data from EFS, CTSA, SPF and INTS).

4. Transfusion-transmission of OBI

While HBV transmission from HBsAg-negative donations is supported by clinical observations for decades, it remains a not frequently reported event. Reasons are that HBV transmission can only be assessed by look-back or trace-back investigations. The former consists in systematic identification and testing of recipients transfused with donations preceding the index donation identifying the donor as OBI and the latter is trig-

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gered by a clinical infection in a transfused patient for whom a transfusion cause is investigated. Trace-back investigations rely on clinical evidence and proper diagnostic of HBV infection. However, primary HBV infection is asymptomatic in 65–80% of patients with an incubation period that can extend over six months in recipients with an impaired immune system or with active/passive neutralizing antibodies. Look-back studies are often limited because a lack of donor archive samples and the difficulty in tracing recipients since a ~50% mortality rate within 6–12 months post-transfusion from underlying conditions has been reported [29]. A major limitation remains the lack of pre-transfusion HBV test results in most recipients to exclude previous HBV exposure, particularly in areas of medium/high HBV prevalence where community acquired, nosocomial, or previous recovered infection should be considered [30,31]. HBV transfusion-transmission can be definitively confirmed only by sequence homology (> 99%) of viral strains infecting the donor and the recipient. Such molecular confirmation may be difficult to obtain due to the undetectable or intermittently detectable HBV DNA levels in OBI donors and/or the absence of detectable viral DNA in the recipients because of viral clearance by the time investigations are launched. Nevertheless, HBV transfusion-transmissions associated with blood products from OBI donors were fully confirmed or deemed highly probable by indirect serological evidences in several studies [29,32,33].

Higher transmission rates were consistently reported with fresh-frozen plasmas (FFP) and platelet concentrates (PC) suspended in 200 mL of plasma than with red blood cells (RBC) preparations containing approximately 20–50 mL of plasma [32]. This association between infectivity and the volume of plasma transfused suggests that viral load, and more importantly the amount of infectious virions transfused, are critical factors in transmission. This is further supported by the observation that transmission by blood products collected from donors in the WP is over than 10-fold higher than transmission by products from OBI donors [29]. The extremely low viral load observed in most of OBI carriers may explain the apparent discrepancy between relatively high OBI prevalence and few transmission cases documented even before NAT implementation. However, transmission has been reported associated with viral DNA levels < 10 IU/mL or unconfirmed ID-NAT [33]. The HBV infectious dose by transfusion is still largely unknown. Look-back studies estimated the minimum 50% infectious dose by transfusion to be somewhere between 20–200 IU (100–1000 virions) in the absence of anti-HBs antibodies [32,34].

Nevertheless, there is no clear relationship between infectivity and viral load in blood since similar viral loads were observed in transmitting and non-transmitting OBI donors and blood components from the same OBI donor were infectious in some recipients but not in others [29]. This suggests that other factors may be involved in HBV transmission. A body of evidence suggests that HBV transmission rate is significantly reduced when neutralizing anti-HBs antibodies are concomitantly present in either donors or the recipients. In some countries, donors testing anti-HBc reactive with anti-HBs > 100 IU/L are considered eligible. However, the protective level of anti-HBs is still a matter of debate. A study reported HBV transfusion-transmission related to OBI donors with high anti-HBs titers whereas no evidence of transmission with components containing < 100 IU/L anti-HBs was observed in another one [32]. Few cases of HBV transmission in the presence of anti-HBs (< 50 IU/L) were also described suggesting that in anti-HBs-positive OBI donors the fluctuating viral load may even temporarily overcome a low level of anti-HBs [29]. The presence of anti-HBs in ~50% of OBI donors may appear reassuring but it is counterbalance by the concomitant presence of immune escape HBV variants potentially infectious.

Lack of infectivity may be also related to possible altered viral fitness due to the accumulation of mutations across the whole genome of most OBI strains. Mutations in regulatory elements and/or structural genes may affect the replication properties of the virus. The particularly high mutation rates persistently reported within the pre-S1 and S hepatocyte ligand domains of OBI strains may also interfere with virus binding to the target cells [2,23,24,27]. However, OBI strains showed no evidence of altered replication properties in newly infected individuals when transmitted either by transfusion or naturally [12,22]. Another possibility might be the presence of non-infectious defective viruses generated by alternative splicing of the HBV pre-genomic RNA [27]. The frequency of spliced genome in OBI donors is unknown. Nevertheless, viral particles containing a full-length and apparently functional genome were identified in more than 50% of OBI donors [18]. So far, a number of studies have examined the genetic variability in OBI strains but functional analysis are still needed.

The immune status of the recipient may be also involved in the risk of acquiring HBV infection. Naturally immunocompromised elderly and patients receiving intensive immunosuppressive treatments or undergoing solid organ/haematopoietic stem cell transplantation may be susceptible to infection with lower infectious dose even in the presence of anti-HBs. Approximately 50% of transfused recipients in Western Europe present some degree of immunodeficiency.

5. Residual transmission risk of OBI

Several studies have reported transmission rates for blood products from OBI donors that can vary significantly between 1.2% and 85% for FFP, 2.2% and 24% for RBC, and 1.2% and 51% for PC (Table 1). Differences in screening methodology, HBV epidemiology, and intrinsic limitations of each study may account for these discrepancies. The transmission rate may be underestimated in studies that investigated a limited number of cases and confirmed HBV transfusion-transmission by sequence analysis with a donor/recipient sequence homology > 99% [31,34–36]. Similarly, a lower transmission rate might be expected when candidate donors are tested for anti-HBc and only donors showing low anti-HBc titres or high anti-HBc titers with high anti-HBs titers are eligible [35]. Inversely, transmission rate might be overestimated when considering possible or probable HBV transmission based on indirect serological evidence in the absence of genetic analysis and pre-transfusion data. To overcome this limitation, some studies analyzed the prevalence of markers for past and current HBV infection in recipients and

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Table 1
HBV transmission rates from OBI donations.

Studies	Blood components	Nb transmission cases/Nb transfused components	Transmission rate[a]
Wang et al., 2002	NA	2/11	18.2%
Satake et al., 2007	FFP	1/15	6.7%
	RBC	0/17	
	PC	0/1	
	FFP + RBC + PC	1/33	3%
Yuen et al., 2011	FFP	0/8	
	RBC	1/21	
	PC	0/16	
	FFP + RBC + PC	1/45	2.2%
Allain et al., 2013	FFP	16/19	84%
	RBC	23/74	31%
	PC	6/12	50%
	FFP + RBC + PC	45/105	43%
Seed et al., 2015	FFP	7/55	13%
	RBC	11/163	6.7%
	PC	2/36	5.6%
	FFP + RBC + PC	21/254	8.3%
Lieshout-Krikke et al., 2016	FFP	1/1	100%
	RBC	2/56	3.6%
	PC	1/23	4.3%
	FFP + RBC + PC	4/80	5%

[a] Based on confirmed transmission by donor/recipient sequence homology > 99% (Wang et al., Satake et al., Yuen et al. and Lieshout-Krikke et al.) and on indirect evidence and adjusted rate according to the background anti-HBc prevalence in the general population (Allain et al. and Seed et al.).

adjusted this prevalence to account for the background anti-HBc prevalence in the general population [32,33].

Following the implementation of HBV-NAT, the overall residual risk of HBV transmission by transfusion was estimated to 1.2–17.4 per million donations according to HBV prevalence. However, few reports indicated that OBI donors with HBV DNA levels undetected by ID-NAT might transmit HBV in the absence of anti-HBc testing [33,37,38]. A mathematical model was recently developed to estimate the HBV residual transmission risk specifically associated with OBI [20]. This model is based on the probability distribution of the viral load in randomly selected OBI donors, the probability that a given viral load remains undetected by NAT, and the probability that this viral load causes infection in the recipient. The model estimates that 3.3% of OBI donations that are not detected by ID-NAT (95% LOD: 3 IU/mL) may cause infection by RBC containing 20 mL of plasma. The estimated risk increases up to 14% for 200-mL FFP transfusion. Another model based on look-back data provided similar 2–3% residual estimates of OBI transmission with blood products screened by ID-NAT (95% LOD: 3–12 IU/mL) [33]. These risk estimates may vary according to the donation-testing algorithm used, the sensitivity of NAT, and the epidemiological context.

6. Conclusion

The risk of HBV transfusion-transmission has been steadily reduced over the last decades by improving donor selection and both molecular and serological HBV screening assays. The association of HBsAg and ID-NAT testing intercepts the majority of HBV-infected donations. However, a limited risk of HBV transmission persists in relation with blood components from OBI donors with extremely low viral load undetectable by ID-NAT. More studies are needed to evaluate properly this residual risk. Anti-HBc testing and/or pathogen reduction procedures might be considered to improve further blood safety. In countries where anti-HBc testing is implemented like France, OBI appears to be no threat to blood safety. However, understanding the molecular mechanisms associated with OBI and their putative implication in the clinical evolution of the infection, including the risk of HBV reactivation, may help donor counseling.

Disclosure of interest

The authors declare that they have no competing interest.

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