In a recent paper published in the respected gastroenterological journal *Gut*, Candotti et al. report the transmission of hepatitis B virus (HBV) to nine recipients of blood products which were tested for HBV by the highest current diagnostical standards (1). How could that happen?

Candotti et al. identified three multiple donors who had occult HBV infection (OBI) with particularly low and variably detectable numbers of HBV particles in their blood. Locarnini and Raimondo re-iterate in their comment to that paper (2) the generally accepted definition of OBI (3): “OBI is defined as the presence of the virus in the liver, with detectable or undetectable HBV DNA in the serum of individuals testing hepatitis B surface antigen (HBsAg) undetectable in blood, using the most sensitive commercial assays.” This somewhat complicated definition reflects the complications in reliably detecting OBI. The extreme variability of manifestations in HBV-infected individuals prevents the generation of universally valid definitions and dependable algorithms for identification of all forms of HBV infection. But a consensus has evolved on the definition of at least five major forms of HBV infections (4,5). Here, we discuss the potential consequences for the safety of blood donations in view of the difficulties to detect all forms of active HBV infections including OBI and the so-called early window phase.

**Chronic low symptomatic HBV infections**

HBV is able to establish a chronic, so-called high replicative, low inflammatory state (previously called “immunotolerant”) during which infected liver cells produce huge amounts of HBV antigens and virus particles but remain largely functional without being attacked by the immune system. Seemingly healthy individuals with persistently high HBV replication and low inflammatory activity can be easily recognized by current HBsAg assays (4,5).

**Early phase HBV infections**

However, not all HBV-infected individuals have very high concentrations of HBsAg. Donors in the early phase of the infection (the so-called window phase) have still low viremia and a not-yet activated immune response to HBV. It is obvious that in this phase the most sensitive assays for HBsAg are required, and nevertheless a recently infected donor may be missed. Here, we will briefly discuss the strengths and weaknesses of current HBsAg tests and the efforts to standardize HBsAg assays for objective external quality control.

**Current status of HBsAg screening**

Since 25 years, WHO provides International Standards (IS) for HBsAg with defined content of HBsAg which allowed the quantitative assay of HBsAg in International Units (IU). Since HBsAg may be derived from various HBV genotypes A-I, the WHO genotype panel was created in collaboration with the Paul-Ehrlich-Institute, consisting of 15 native plasma-derived HBsAg samples with eleven different (sub)genotypes: A1, A2, B1, B2, C2, D1, D2, D3, E, F2 and...
The panel was distributed to nine national reference laboratories and six laboratories of leading test producers for determination of the detection limits of 19 widely used HBsAg test kits. The 2nd WHO IS for HBsAg was provided as internal reference. Six of the 19 licensed HBsAg test kits had a limit of detection around 20 [18–22] milli-International Units (mIU) per mL but most assays were less sensitive; the least sensitive detected only 105 mIU/mL (6). One point is: the 2nd IS for HBsAg consists of heat-denatured HBsAg, the so-called “Dutch” HBV vaccine (7). Unfortunately, one of the 18 assays was optimized for exactly that HBsAg and therefore recognized non-heated native HBsAg at 108 vs. 30 mIU/mL much less sensitive (5). Another point is that the 2nd IS contains only HBsAg from HBV genotype A2 (7). This genotype is predominant in USA and Northern Europe, but 99% of all HBV-infected individuals worldwide have other HBV genotypes. The international trial did not identify significant deviations of the mean detection limits around 20 mIU/mL with various (sub)genotypes, but two assays had detection limits for subgenotype F2 and H >100 mIU/mL (6). Recently, a plasma-derived HBV vaccine made from at least two donors in Vietnam was used as the stock solution for the 3rd WHO standard. It was not as strongly heated as the 2nd IS and has genotype B typical for the highly endemic region Southeast Asia (8). In another WHO international trial, it yielded results well comparable to the 1st and 2nd IS, but certain assays reacted slightly different with the partially denatured HBsAg present in the three IS preparations provided by WHO when compared to native HBsAg of the same genotype in plasma (9). In conclusion, the HBsAg assays have reached a very good level but there is potential for improvement: Recently, a so-called “ultra-sensitive” prototype version of Abbott’s Architect HBsAg assay with a detection limit of 5.2 mIU/mL was described (10). Although the improvement by the factor 4 does not really deserve the attribute “ultra-sensitive”, the new assay reduces the early window phase as shown for 27 seroconversion panels. The new assay detected 191 of 364 early phase sera vs. 144–160 of the 364 detected by the other Abbott assays samples and shortened the HBsAg negative window phase for one seroconversion case from 94 to 51 days. A much greater improvement would, however, be the introduction of a sensitive nucleic acid amplification technique (NAT) as exemplified by the result that NAT with a detection limit of 34 copies/mL or 10 IU/mL HBV DNA detected all samples of the one mentioned seroconversion case from day 0–51 and, furthermore detected 246 of the 364 samples of all seroconversion series.

Patients in the late acute or chronic phase

These patients are in the transition from the high replicative, low inflammatory phase to the immune clearance phase with decreasing viremia and HBs-antigenemia. Immune clearance is connected with inflammation and consecutive fibrosis. Initially most patients with chronic hepatitis B are still HBeAg positive. Since HBeAg is non-essential, patients eliminate the HBeAg-expressing HBV wildtype, but HBeAg negative variants are selected which are often faster replicating. Absence of HBeAg indicates the breakdown of the “immune tolerance” to HBV and an enhanced immune clearance combined with intensified pathogenicity. Clinical observations suggest that levels of viremia usually decrease faster than the levels of HBs-antigenemia (11). Thus, HBsAg assays are sufficiently sensitive enough for detection of virtually all donors in these phases whereas HBV DNA screening would not identify many additional HBV carriers if any.

Inactive HBsAg carriers

Finally, many patients reach the inactive carrier state which is characterized by low or undetectable HBV numbers <10⁴ per mL while HBsAg is usually readily detectable (4). It appears that immune clearance often targets hepatocytes expressing all HBV antigens while cells expressing only HBsAg due to integrated HBV DNA fragments encoding HBsAg are saved (11). Such cells cannot generate infectious HBV but they are indicators for the few remaining hepatocytes which still carry viable HBV genomes. Furthermore, HBsAg in plasma indicates insufficient amounts of protective or neutralizing anti-HBs antibodies. Escape mutants with altered HBsAg epitopes may have been selected during the immune clearance phase. Some assays cope well with such mutants, e.g., the above mentioned but others show seriously reduced sensitivity for these mutants (10).

Occult HBV infection (OBI)

Most if not all HBV infections virtually never become completely cured in the sense that all viable HBV genomes got eliminated. A surrogate for complete cure is functional cure by which an effective immune control suppresses generation and release of infectious HBV particles and subviral HBsAg particles below the level of detectability. By definition, OBI is characterized by absence of detectable HBsAg (4,5). However, during persistent OBI, HBV particles

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often are still expressed to the point that recipients of blood products may be infected (1,12). According to current knowledge HBV requires an envelope consisting of HBsAg and preS1 domains to be infectious (13). Thus, the question arises where is the HBsAg? In some cases, mutations of the S gene encoding HBsAg may cause non-reactivity in the serological assays but as pointed out above, most of the improved HBsAg assays cope well with these mutants. True OBI is characterized by low viremia <200 IU/mL (or ca. <1,000 HBV genome equivalents, ge/mL) and low HBsAg (3). Masking by anti-HBs is a plausible explanation, even if anti-HBs is not detectable. In a longitudinal analysis of the HBV quasi-species present in an anti-HBs negative OBI positive donor, the S gene was highly variable in the HBs-antigenic loop with 10–14% altered amino acids suggesting presence of unrecognized selection-relevant anti-HBs. Five donations from that donor within 10 months contained 9–59 ge HBV DNA/mL. All eleven recipients of 200 mL fresh frozen plasma from that donor were found to be anti-HBC positive by look back suggesting persistent infectivity of the plasma while only 5 of the 12 recipients of red cell concentrates (containing ca. 20 mL plasma) from that donor were anti-HBC positive ([14] and M. Saniewski, PhD thesis). In a study from Europe reporting about this and 23 further OBI donors, 11 infected recipients with the same HBV strains as in the corresponding donor were identified, plus 34 probably/possibly infected recipients who were anti-HBC positive. Using this data, the equivalent of the HBV particle number per donation to the 50% infectious dose was calculated to be 1,049 ge or “copies”/donation (95% CI: 117–3,441) (12). This estimate is compatible with the observation that the transmission rate with 200 mL plasma was 85% and “only” 24% with red cell concentrates and it translates to a virus concentration of 5 ge/mL plasma (95% CI: 0.5–18 ge/mL) (12). The large CI interval was due to the small number of cases and to the high variability of the viremia during the course of OBI. The great majority of the 45 certainly or potentially infected recipients were not notably sick. But 4 recipients in whom the same strain as the donor was identified developed acute liver failure. Surprisingly, no typical acute hepatitis B or transient HBs-antigenemia was observed. This is consistent with the observation that OBI develops mostly on the basis of HBeAg-negative persistent HBV infection. HBeAg-negative strains are eliminated more rapidly, but if not, they replicate faster and induce severe immune pathogenesis up to acute liver failure, as has been observed in many HBV transmissions due to unhygienic blood sampling [e.g., (15)].

In view of the potentially fatal consequences many countries introduced measures to screen blood donors for OBI including testing for anti-HBc and/or NAT for HBV DNA. Given the rather high virus dose observed to be necessary for transmission in most studies, detection limits of NAT were considered sufficient at 10 or 3.4 IU/mL HBV DNA (1) even when testing minipools of samples (16).

**Gaps left by current NAT testing**

Transmission of HBV by NAT negative donations was repeatedly reported as summarized by Candotti and Laperche (17). Infection experiments with chimpanzees or humanized mice suggest that one single HBV particle from early phase sera may induce a full HBV infection whereas particles from later still HBsAg positive phases are usually less infectious [summarized in (18)]. The recent paper from Candotti et al. (1) provides deeper insight because it shows that in certain OBI cases HBV particles may be as infectious as particles from the early phase. Furthermore, the study illuminates the difficulties to follow-up and identify infected recipients unless the follow-up of recipients and look-back of donors with suspected donors is consequently pursued (2). This observation takes the minimum requirements for HBV screening procedures to a new dimension if optimum safety at all economic cost is pursued: NAT screening should be applied to all donations at a limit of 95% detection of 0.8 ge/mL (or 0.15 IU/mL) which would require testing of several mL of single donations and virtually exclude minipool testing.

**Neglected potential of HBsAg screening**

The potential of HBsAg is probably not sufficiently exploited. In many resource-limited countries, very insensitive HBsAg tests are used with detection limits >1,000 mIU/mL (19). The use of a truly ultra-sensitive HBsAg test could identify samples with down to 0.5 mIU/mL (or 0.5 pg/mL) HBsAg and was reported to be as sensitive as HBV DNA in patients with reactivated HBV infection (20). Unfortunately, this test is not (yet?) validated in blood donors. As long as 3.4 or 10 IU/mL HBV DNA is considered a satisfactory sensitivity for blood donor screening, an ultra-sensitive HBsAg screening which detects also the major escape mutants could possibly replace NAT for HBV. During most phases of HBV infection HBsAg is produced in large excess over complete HBV particles. During the characterization of the WHO genotype panel for HBsAg (6) a ratio of virion-
bound HBsAg to HBsAg present in subviral particles of 1:2,700±1,300 was determined for all analyzed genotypes (not valid for G) in HBeAg positive samples from asymptomatic carriers. At a detection limit of 0.5 pg/mL HBsAg, such samples would contain ca. 0.0002 pg/mL as virion-bound surface antigen. Assuming the number of HBsAg subunits in the HBV envelope at 240 (based on the known symmetry of the capsid) with 25000 Dalton mol. weight each, the entire MW of the envelope would be 6,000,000 Dalton and after division by Avogadro’s number (6×10^{23}) the weight of HBsAg protein in one HBV particle would be 10^{-17} g or 0.00001 pg. Thus, an HBsAg test with a detection limit of 0.5 mIU/mL could detect in first approximation 20 virus particles/mL if accompanied by the typical excess of subviral HBsAg particles. This corresponds, according to the calibration from Heermann et al. (21) to 4 IU HBV DNA/mL.

Role of HBV vaccination

Locarnini and Raimondo (2) express in their commentary the expectation that universal hepatitis B immunization will more often protect recipients against HBV in blood donations. Even passively administered anti-HBs by concomitant donations from immunized donors protects against donations from OBI donors (1,12). The downside of vaccination is that it does not completely protect and that vaccinated donors with low (<100 mIU/mL) or absent anti-HBs may develop after exposure an acute quasi-occult HBV infection which can only be detected by NAT. This incomplete protection occurs rarely but in increased frequency when the infecting HBV strain has a genotype different from the vaccine strain (16,22). This side effect should definitely not deter blood donation services to accept vaccinated donors with low anti-HBs. A better consequence would be that responsible institutions like pharmaceutical industry and WHO would consider the possible improvements of current HBV vaccines like use of the regional predominating HBV genotypes or preS1 containing vaccines (23). Unnoticed OBI of vaccinated individuals detectable by anti-HBc occurs very often in highly endemic countries. In the worst case only highly sensitive NAT can detect a recent occult HBV infection in anti-HBs positive vaccinated individuals [briefly reviewed in (24)].

Acknowledgements

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

References

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