Transfusion-transmitted HBV infection in an endemic area: the necessity of more sensitive screening for HBV carriers

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BACKGROUND: By NAT, HBV DNA is occasionally detectable in blood donors with past HBV infection but negative for HbsAg. Whether or not these donors can cause transfusion-transmitted HBV infections is uncertain.

STUDY DESIGN AND METHODS: To determine whether or not donors with past HBV infection but negative for HbsAg can cause HBV transfusion-transmitted infections, recipients followed for blood transfusion in a university medical center in Taiwan were studied. HBV DNA and serologic markers were tested in donors and recipients.

RESULTS: Of 1038 enrolled recipients, 910 completed the 6-month post-transfusion follow-up visit. Of these, only 39 patients (4.3%) tested negative on the pretransfusion sample for HBsAg, anti-HBs, anti-HBc, and HBV DNA by PCR. These 39 HBV-naive recipients had been transfused with blood from 147 donations for which stored samples were available for HBV DNA testing by PCR; 11 of these HbsAg-negative samples tested positive for HBV DNA and anti-HBc. Two of the 11 patients who received the HBV-DNA-positive donations (18%) became positive for HBV DNA, and one seroconverted to anti-HBc and finally to anti-HBs, with a mild transient elevation of serum ALT activities. Based on the one confirmed case of HBV transmission, a projection was made that approximately 200 post-transfusion HBV infections could occur in one million units of transfused blood in Taiwan.

CONCLUSIONS: In HBV-endemic areas like Taiwan, where blood donors are screened for HbsAg only, the risk of transfusion-transmitted HBV appears to be substantial. Implementation of NAT for blood screening in these settings warrants consideration.

ABBREVIATIONS: anti-HBs = antibody to HbsAg; TTV = TT virus.

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nates blood-transmitted HBV, with the rare exception of pre-HBsAg window-period donations. Such a practice is feasible in developed countries in which the HBV infection rate is low (<3%). It also eliminates most of the blood-transmitted HBV. In contrast, in developing countries that are endemic for HBV, approximately 90 percent of adults have either past or ongoing HBV infections, and such a criteria would disqualify most of the volunteer blood donors. Therefore, in Taiwan, blood donors are screened only for evidence of ongoing infections by HBsAg or serum ALT levels but not for past infections (by anti-HBc). This protocol has been carried out for more than 20 years in Taiwan and has significantly reduced the risk of blood transfusion-transmitted HBV. However, the advent of new viral detection technology, especially NAT, led to the conclusion that approximately 10 percent of people with past HBV infection actually harbor viral DNA in their blood or blood cells. Even in people positive for antibody to HbsAg (anti-HBs) and anti-HBc, conventional criteria for a full recovery from past HBV infections, 5 to 15 percent of patients are reportedly positive for HBV DNA by NAT in endemic areas, though at a very low titer. However, the incidence of HBV-DNA positivity might be lower in blood donors in nonendemic areas.

These observations call for re-evaluating the current protocol for screening blood donors in Taiwan. It is imperative to know how many blood donors, qualified by the current HBV screening protocol, are still positive for HBV DNA. More importantly, what are the consequences of transfusion of those HBV-DNA-positive blood units into recipients? This is a very unique setting for understanding the infectivity of the transfusion of blood containing low-titer HBV DNA. This information will not only provide much-needed data to be used in deciding whether current practices are in need of revision, but will also be helpful in determining whether new tests, such as NAT, are required in the future.

**MATERIALS AND METHODS**

**Blood recipients**

From June 1987, we conducted a prospective study of post-transfusion hepatitis in the National Taiwan University Hospital. Results for hepatitis virus infection, HTLV-I, and HGV have been reported in detail previously. In brief, patients who received blood transfusion and met the following criteria were recruited: normal liver function tests before transfusion; no transfusions in the past year; no previous history of liver diseases; and no history of alcoholism, drug addiction, or exposure to hepatotoxic drugs. After transfusion, the recipients were followed up every 2 to 4 weeks for 6 months. Patients with elevated ALT during the 6 months were followed every month for 1 year and then every 3 months for as long as possible. They were diagnosed with post-transfusion hepatitis if ALT levels exceeded 2.5 times the upper limit of normal between 1 and 26 weeks after transfusion, at least twice at an interval of 1 or more weeks apart; and if other possible causes of the elevated ALT (such as shock, hemochromatosis, autoimmune hepatitis, etc.) were excluded.

**Donors**

The blood or blood components used were donated by volunteers who were negative for HBsAg tested by ELISA (Evernew Biotech, Hsin-Chu, Taiwan), anti-HIV, and who had serum ALT activities less than 45 IU per L (normal, <31 IU/L). Anti-HCV testing with a second-generation assay was added to the screening list in July 1992. Plasma samples of donors were also collected from transfused FFP or whole blood before transfusion where as possible. The plasma samples were aliquoted and kept at –80°C until testing.

**HBV-DNA PCR**

Given the high background infection rate of HBV in Taiwan, we used serologic markers to identify recipients without a previous HBV infection, and we further confirmed this by HBV-DNA PCR. HBV-DNA nested PCR procedures were performed as previously described. Viral DNA was purified from 100 μL of plasma samples. Briefly, each serum sample was incubated at 55°C for 2 hours in 25-mM sodium acetate (pH 6.5), 2.5-mM EDTA, 0.5-percent SDS, and 2.5 mg per mL proteinase K. After phenol and chloroform extraction, the viral DNA was precipitated with ethanol and the pellet was redissolved in water. The PCR was carried out in a 50-μL mixture containing 10-mM tris-HCl (pH 8.3), 50-mM KCl, 1.5-mM MgCl2, 0.01-percent gelatin, 2.5 units recombinant Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), 200 μM of each dNTP, 0.6 μM of each outer primer, and 5 μL of the DNA mixture (DNA from 20 μL of plasma). A fast temperature cycling was performed as described previously to improve sensitivity. Briefly, 40 cycles of 96°C for 30 seconds, 56°C for 15 seconds, and 74°C for 30 seconds were done, and the temperature was maintained at 74°C for 10 minutes at the end of the last cycle. One microliter of the mixture after this first PCR was transferred to the Round 2 reaction mixture containing 0.6 μM of each inner primer and the same buffer as in the Round 1 PCR. The temperature cycling for the Round 2 PCR was the same as for the Round 1 PCR. Samples from the same patient were tested in aliquots of the same reagents and under the same reaction conditions. Cloned HBV DNA was used as a positive control, and serum samples from healthy persons and reagents without DNA were used as negative controls in PCR. We followed the suggestions of Kwok et al. to avoid possible contamination. In addition, reagents were stored in small aliquots. All the pipet
DNA sequencing

DNA sequences were determined in PCR products from the seroconverted recipients and their donors. For each sample, two independent PCR products were sequenced. For sequencing reactions, 40 μL of the specific biotinylated amplification product (amplified by primer pairs from both regions ORF1) was used with streptavidin to generate a single-stranded template for sequencing (Dynabeads M280 streptavidin, Dynal AS, Oslo, Norway). The single-stranded, biotinylated PCR products were directly sequenced using a cycle-sequencing protocol and reagents supplied with a cycle-sequencing kit (Taq Dye Terminator Cycle Sequencing Kit, ABI, Foster City, CA). The thermal cycling conditions for TT virus (TTV) sequences were 35 cycles of 94°C for 50 seconds, 60°C for 50 seconds, and 72°C for 50 seconds, after the initial denaturation step of 94°C for 5 minutes. After PCR, the reaction mixtures were extracted with phenol and chloroform (4:1) twice and precipitated with 95-percent ethanol and 3-form (4:1) twice and precipitated with 95-percent ethanol and 3-form (4:1). After centrifugation for 25 minutes, each pellet was dried in a vacuum (Speed-Vac, Savant, New York, NY), and stored at -20°C until electrophoresis. Dried pellets were resuspended in 4 mL of loading buffer (5:1 [vol/vol] deionized formamide/50 mM EDTA pH 8.0) and were heated at 95°C for 3 minutes before loading on a 6-percent polyacrylamide gel containing 7 M urea. PAGE was performed on an automated sequencer (ABI 373).

RESULTS

Recipient enrollment and follow-up

From June 1987 through June 1994, a total of 1038 blood samples were obtained during each visit and kept frozen at -80°C until tested. Additional samples were obtained in some patients when they were seen for follow-up for their underlying diseases. The samples were carefully handled and aliquoted to avoid contamination and degradation of viral nucleic acid. As of June 1994, a total of 1038 patients were enrolled. Of these, 910 recipients completed the 6-month follow-up, and 128 patients did not. Among the 910 recipients, 665 received transfusion when anti-HCV was not screened in blood donors, and 245 patients were transfused after the anti-HCV screening that was initiated on July 1, 1992. Most of the blood recipients were patients receiving cardiac surgery, and the 910 recipients had a mean donor number of 18.6 ± 14.6 (range, 1-97). The mean age of the 910 recipients was 48.7 years.

Identification of naive recipients

To find the acute HBV infections, we first needed to identify recipients with no evidence of prior HBV infection. Such a population constitutes only a very small proportion of our blood donors because of hyperendemicity of HBV in Taiwan. Epidemiologic studies have found this proportion to be less than 10 percent for those living in Taiwan, up to the average age of the recipients in our study. To identify these recipients, we measured HBsAg, anti-HBs, and anti-HBc, and tested HBV DNA by PCR on the blood samples taken before transfusion of 910 recipients. Only 39 recipients were negative for the three HBV seromarkers and HBV DNA. We therefore considered the 39 recipients to be truly naïve to HBV infection.

Detection of HBV DNA in the plasma of donors

The sensitivity of PCR was approximately 100 to 1000 copies and approximately 1 to 10 copies of cloned DNA in first and nested PCR, respectively. The 50-percent and 95-percent detection limits were 50 copies per mL and 500 copies per mL, respectively. The PCR results were the same for both primer pairs (Fig. 1). All blood donors were identified for these 39 recipients. We recovered 147 plasma samples for 21 of the 39 recipients. Ten of the other 14 recipients received platelets or RBCs. Six whole-blood donors to the remaining four recipients were missed during collection. We repeated HBsAg testing in

![Image](image-url)

**Fig. 1.** PAGE of nested PCR products from two positive samples and a negative sample. Sample 1, Lanes 1, 4; Sample 2, Lanes 2, 5; Sample 3, Lanes 3, 6. M = marker. Lanes 1-3, PCR using primers in core region; Lanes 4-6, PCR using primers in surface region. Arrow = 179 bp. Black line = 381 bp.
the 147 samples with another ELISA (Abbott Laboratories, Chicago, IL). They remained all negative. HBV PCR was done to detect any HBV DNA in these HBs-Ag-negative, ALT-normal plasma samples. Of the 147 plasma samples, 11 were shown to contain residual HBV DNA (approx., 7%). HBV DNA was subjected to semiquantification by 10-fold serial dilution of DNA samples. Titers were defined by nested PCR at positive maximum dilutions. They were tested for anti-HBc and anti-HBs (Abbott Laboratories). The results are shown in Table 1. Our data showed that a proportion of HbsAg-negative, healthy blood donors indeed retained HBV DNA, as previously noted.

Follow-up of naive recipients transfused with HBV-DNA-containing blood

Among the 21 patients with a traced donor history, 11 received transfusion from blood positive for HBV DNA by PCR. The serial blood samples of each recipient were tested for HBsAg, anti-HBc, anti-HBs, and also ALT. In addition, PCR for HBV DNA, HCV RNA, HGV RNA, and TTV DNA was done. Only two recipients were found to be positive for HBV DNA after transfusion. One appeared to be an abortive infection or HBV-DNA carry-over, in which HBV DNA disappeared later and without seroconversion to anti-HB (Fig. 2). The other patient followed a course of mild and transient acute HBV. With a slightly elevated ALT level, the HBV DNA persisted for 2 months and then disappeared. Finally, the patient seroconverted to anti-HBc and anti-HBs (Fig. 3). Interestingly, none of the remaining nine recipients who had received HBV-DNA-containing blood became positive for serum HBV DNA or other HBV markers after transfusion. The HBV either failed to replicate or was immediately cleared by the host after transfusion. Nevertheless, one patient did present with a de novo HGV infection and three patients presented with TTV infections without any evidence of hepatitis.

Comparison between HBV-DNA sequences of a post-transfusion HBV patient and the implicated donor

To establish the direct transmission of HBV from the implicated donor to the recipient, we did DNA sequencing on HBV fragments amplified from the core region of the seroconverted recipient and his donor (Table 1, Figs. 3 and 4). This fragment is in the nucleocapsid gene of HBV genome. The 160 nucleotide fragments sequenced were all identical. The identical DNA sequences suggest that the two viruses came from a common origin.

<p>| TABLE 1. Clinical data of 11 recipients who received HBV-positive donations |
|-----------------------------|----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/age</th>
<th>Viral genome detected*</th>
<th>Peak ALT†</th>
<th>Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/36</td>
<td>HBV</td>
<td>52</td>
<td>5 x 10⁷</td>
</tr>
<tr>
<td>2</td>
<td>M/56</td>
<td>HBV</td>
<td>21</td>
<td>5 x 10⁷</td>
</tr>
<tr>
<td>3</td>
<td>F/31</td>
<td>NA</td>
<td>27</td>
<td>5 x 10⁷</td>
</tr>
<tr>
<td>4</td>
<td>F/40</td>
<td>TTV</td>
<td>30</td>
<td>5 x 10⁷</td>
</tr>
<tr>
<td>5</td>
<td>M/59</td>
<td>NA</td>
<td>19</td>
<td>5 x 10⁷</td>
</tr>
<tr>
<td>6</td>
<td>M/67</td>
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<td>40</td>
<td>5 x 10⁷</td>
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<td>7</td>
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<td>5 x 10⁷</td>
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<td>8</td>
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</tr>
<tr>
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<tr>
<td>11</td>
<td>F/45</td>
<td>NA</td>
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</table>

* Detection by PCR.
† Upper limit of normal 31 IU/L.
DISCUSSION

In this large series of prospectively followed recipients, we evaluated the risk of transfusion-transmitted HBV infection from units of blood that tested negative for HBsAg and had normal ALT levels. The results provide important information concerning transfusion safety. Initially, we found that 7 percent of these blood samples contained HBV DNA as detected by the sensitive PCR assay. This finding was consistent with previous reports from Taiwan and Japan, as well as a US study of Sardinian donors. Therefore, in people negative for HBsAg but positive for seromarkers indicating past HBV infection, the HBV-DNA-positive rate is still relatively high. The HBV-DNA prevalence rate in our blood donors is high (7%), especially in comparison to some low endemic areas where donors positive for anti-HBc are disqualified and rejected. In blood donors from low endemic areas, HBV DNA may only be present in people in the window period after acute infection, and the HBV-DNA-positive rate in blood donors is as low as approximately 3 in 1 million donations. Clearly, the problem of HBV-DNA positivity among our blood donors needs to be further addressed.

The more important question concerns the outcome after transfusion of this HBV-DNA-containing blood into recipients. Of note in our blood donors is that despite HBV-DNA positivity, the viral titer is usually very low. This differs from samples from patients in the window period of acute infection, as these samples carry a very high viral titer. Therefore, the infectivity of blood with low-titer HBV is quite unique and must be investigated. Small amounts of plasma and MNCs from HBV-DNA-positive, HbsAg-negative, and anti-HBs-positive blood do not appear to be infectious in chimpanzees; however, in transfusion settings, the amount is usually much larger. In our series, among 11 naive recipients, there were only two positive for HBV DNA after transfusion. The remaining nine were negative, even immediately after transfusion. It is now well known that the human body can rapidly clear a large number of HBV virions (about 10^{11} in 1 day) through innate immunity. That may help to reduce the infectivity of HBV-DNA-containing blood. Despite this defense mechanism, we still detected an 18-percent chance of transmitting HBV DNA to recipients. Eventually, one person (approx., 9%; this recipient received 250 mL of whole blood from the HBV-DNA-positive donor) indeed developed mild acute HBV infection. The infection in the other recipient with only a very short period of viremia and no seroconversion is difficult to document. We therefore conclude that our HbsAg-negative, HBV-DNA-positive blood donor still carried an approximately 10-percent risk of transmitting HBV to susceptible recipients.

Furthermore, based upon these numbers, we could evaluate the magnitude of this problem by estimating transfusion-transmitted HBV infections taking place in Taiwan's blood recipients. Calculated from our results, there could be 70,000 HBV-DNA-positive donations per million. This number is quite alarming, but not well noticed. Because the majority of our recipients have past HBV infections, about 80 percent of our recipients harbor anti-HBs and the other 15 percent carry HbsAg. Transfusion of HBV-DNA-positive units into recipients with anti-HBs will perhaps not cause infections unless a large number of viruses are transferred. Transmission of HBV into HbsAg carriers may also go unnoted or may be assumed to be reactivation of the pre-existing HBV infection. Nevertheless, whether this reinfection aggravates the HBV disease remains unknown.

**Fig. 3.** Clinical course of the patient who seroconverted to HBV viral markers.

**Fig. 4.** DNA sequences of the donor and the patient who seroconverted to HBV viral markers, from nucleotide 2240-2396 of the HBV genome. D = donor; R = recipient.
The most significant finding of our study is the confirmed transmission of HBV to a naive recipient transfused with an HBV-DNA-positive, HBsAg-negative unit. Based on the estimated number of HBV-naive recipients in Taiwan and the HBV-DNA positivity and transmission rates by HBsAg-negative units documented in this study, we estimate that 200 to 300 donations per million units may be infectious for HBV in endemic regions. The number is indeed high in comparison with the projected yield of HBV infections by NAT in the US and in Germany, at levels of 6 to 7 per 1 million units and 3 per 1 million units respectively. However, the actual incidence may vary due to limited case numbers in our prospective study. Nevertheless, the risk of post-transfusion HBV infection can be documented.

Finally, from our results, it is evident that HBV NAT appears to be the best option to prevent de novo transfusion-transmitted HBV infection in Taiwan. After exclusion of all HBV-DNA-positive donors, we can better ensure the safety of blood transfusion. The reasoning is quite obvious in considering the experience in the US, Germany, and Japan using NAT to reduce transfusion-transmitted viral infections. In these countries, the implementation of NAT for HCV, HIV, and HBV eliminated transfusion-transmitted HBV infection in Taiwan. After exclusion of HBV-DNA-positive donors, we can better ensure the safety of blood transfusion.

In comparison, if HBV NAT is implemented in Taiwan, the predicted yield is 20-fold higher than these rates, and, thus, is worthy of advocating in our country. Of particular note is that by doing this, we can also eliminate as much as possible occult HBV transmission into anti-HBs-positive recipients or to HBsAg carriers. Therefore, unless the sensitivity of the assay of HBsAg can be greatly enhanced, screening of HBV DNA should be considered in hyperendemic areas of HBV infection like Taiwan.

REFERENCES

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