NAT for HBV and anti-HBc testing increase blood safety

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BACKGROUND: Routine HBV PCR screening of blood donations to our institutes was introduced in January 1997 to complete the NAT screening program for transfusion-relevant viruses. Testing was successively extended to customer transfusion services with a total of 1,300,000 samples tested per year.

STUDY DESIGN AND METHODS: Minipools of 96 blood donation samples were formed by automatic pipettors. HBsAg-reactive samples were included. HBV particles were enriched from the minipools by centrifugation. Conventional and in-house TaqMan PCRs were successively applied for HBV amplification. Sensitivity reached 1000 genome equivalents per mL for each individual donation. Confirmatory single-sample and single-sample enrichment PCRs were established with sensitivities of 300 and 5 to 10 genome equivalents per mL, respectively.

RESULTS: After screening of 3.6 million donor samples, 6 HBV PCR-positive, HBsAg-negative donations were identified. Two samples were from infected donors who had not seroconverted and four were from chronic anti-HBc-positive low-level HBV carriers. Retesting by single-sample PCR of 432 samples confirmed positive for HBsAg identified 37 donations that were negative in minipool PCR. Donor-directed look-back procedures indicated that no infected donor who had not yet seroconverted was missed by minipool PCR. However, recipient-directed look-back procedures revealed two anti-HBc-positive recipients of HBsAg-negative minipool PCR-negative, anti-HBc-positive and single-sample PCR-positive blood components. After testing randomly selected 729 HBsAg-negative minipool PCR-negative, anti-HBc-positive donors by single-sample enrichment PCR, 7 were identified with ≤10 HBV particles per mL of donor plasma.

CONCLUSION: Minipool PCR testing after virus enrichment was sensitive enough to identify HBsAg-negative donors who had seroconverted and HBsAg-negative, anti-HBc-positive chronic HBV carriers. HBV NAT in conjunction with anti-HBc screening would reduce the residual risk of transfusion-transmitted HBV infection.

The most transfusion-relevant viruses that are currently tested for are HCV, HIV-1/2, and HBV. For HBV, blood safety relies on sensitive HBsAg testing. HBV infections from transfusions became very rare after the introduction of the HBsAg test in the early 1970s. Recent estimates based on prevalence and incidence rates among American multiple-time blood donors revealed a residual risk ranging between 1 in 30,000 and 1 in 147,000.1 Similar estimates were published for German multiple-time donors based on nationwide surveys.3

Routine NAT for HBV suitable for labile blood components was introduced at our blood transfusion service in January 1997.3 HBV by NAT has not yet been imposed in Europe and the US although a considerable rate of HBV DNA-positive, HBsAg-negative individuals has been described.4-22 It was shown that plasma from those individuals transmitted HBV to recipients and to experimentally infected chimpanzees.4,7,11,15,17,18,22 The majority of these HBV transmissions could be attributed to chronic low-level HBV carriers. Although anti-HBc testing could eliminate these carriers from the donor population, it was introduced only in some countries (e.g., in the US15,23) and not in Germany. However, anti-HBc testing would not detect HBsAg-negative donors in the preseroconversion window phase. Due to the slow ramp-up in viral load and the short diagnostic window phase, NAT was expected to require a very high sensitivity to identify those donors.24 Therefore, it was questioned whether minipool

ABBREVIATION: anti-HBs = antibody to HBsAg; geq = genome equivalents.

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HBV NAT would add any benefit on top of sensitive HBsAg screening.25

We now report on the sensitivity of minipool PCR and the number of HBsAg-negative infected donors who had not seroconverted and of anti-HBc-positive chronic low-level HBV carriers after screening of 3.6 million donations to routine blood bank settings in central Europe.

**MATERIALS AND METHODS**

**Minipool PCR**

Pooling of 96 samples was carried out as described previously.3 HBsAg-reactive samples were included in the pools. To reduce the risk of HBV-contaminated pools, first-time donor samples were pooled separately. Pools were centrifuged at 48,000 \( \times g \) for 1 hour to quantitatively force viruses into the pellet. Virtually virus-free supernatants were decanted and the pellets enriched for the viruses were subjected to nucleic acid extraction by use of a viral RNA extraction kit (Qiagen, Hilden, Germany). Nucleic acid was eluted from the Qiagen columns in a final volume of 75 \( \mu \)L of water. One aliquot of 20 \( \mu \)L was subjected to amplification in HBV PCR. The final volume of plasma introduced into the PCR was 26.7 \( \mu \)L.

For HBV amplification, we established a nonnested in-house PCR test in conjunction with agarose gel electrophoresis of amplicons until April 1998.3 Thereafter, a real time PCR test (TaqMan, Applied Biosystems, Foster City, CA) was introduced and performed on a sequence detection system (ABI Prism 7700, Applied Biosystems, Weiterstadt, Germany) in plate-read detection mode.26 Primers and probes were as described.27 The detection limit at 95-percent probability was 1000 genome equivalents (geq) per mL for each individual donation.3,27 Validity and sensitivity of each individual PCR reaction were controlled by coamplified competitive HBV amplicon DNA with a modified probe-binding site inserted into a plasmid vector. This internal control sequence was coextracted and coamplified with the viral sequences at a concentration allowing for monitoring of the sensitivity of each individual HBV PCR at the detection limit.

**Single-sample PCR and single-sample enrichment PCR**

For single-sample PCR testing of HBV, we developed a more sensitive extraction method that utilized proteinase K digestion before standard extraction by a viral RNA extraction kit (Qiagen). Briefly, 100 \( \mu \)L of plasma was incubated with 100 \( \mu \)L lysis buffer (100 mM Tris, pH 7.0; 75 mM EDTA, pH 8.0; 10 mM NaCl; 1% SDS) and 20 mg per mL proteinase K for 10 minutes at 56°C. Lysates were processed according to the standard Qiagen protocol. The DNA was eluted in a volume of 30 \( \mu \)L water of which 20 \( \mu \)L was introduced into the TaqMan PCR. The 95-percent detection probability reached 300 geq per mL donor plasma.

To increase sensitivity, we performed enrichment of viruses from 9.6 mL of single-donor plasma by centrifugation applying 48,000 \( \times g \) for 1 hour before extraction (single-sample enrichment PCR). Nucleic acid extraction and PCR were performed as described for single-sample PCR.

**Serologic testing**

An antibody assay was used for HBsAg screening (Prism, Abbott Laboratories, Delkenheim, Germany). A second antibody assay (Axsym, Abbott) was used for repeat testing of reactive samples in an alternative test environment and also for screening of small sample numbers. To confirm the screening results, one of the following assays had to be positive: the hepatitis HBe-antigen, anti-HBc, or neutralization assay (all Abbott). ALT testing was performed according to the German guidelines on two chemical analyzers (Bayer Opera, Bayer AG, Wuppertal, Germany; and Epos Eppendorf, Hamburg, Germany). All data based on serologic testing were generated at the Hesse and Bavarian Red Cross Transfusion Services.

**Quantitative PCR**

HBV load in plasma samples was quantified in triplicate by single-sample TaqMan PCR according to the instructions outlined in the user manual for the ABI Prism 7700. Calibration curve was generated by use of the HBV Europe 2 standard preparation in 1 in 10 dilution steps. The lower limit of detection for quantitating HBV DNA was 300 geq per mL of each individual donor plasma.27

**Sequencing and phylogenetic analysis**

HBV surface gene-, core gene-, and \( x \) gene-specific primers (nucleotide positions according to Galibert et al.28 254-273 and 412-433; 2268-2286 and 2421-2438; 1426-1449 and 1652-1675, respectively) were used for sequencing plus and minus strands, respectively. Amplification products were directly sequenced by use of the cycle sequencing kit from Applied Biosystems. Sequence was determined on a sequencer (ABI Prism 310, Applied Biosystems).

Phylogenetic analysis was performed by use of a computer program (Treecon for Windows, Vs.1.3b).29 The matrix was calculated by use of Kimura correction,30 whereas the tree was estimated by the neighbor-joining method.31 Bootstrap analysis resampling was utilized as a pseudoempirical test on the reliability of the tree topology.32

**Look-back procedures**

Look-back procedures were performed according to the German guidelines. Briefly, donor-directed look-back procedures were initiated by donations that were repeat reactive in screening assays and positive by confirmatory
assays. A search was started in our database to retrieve the corresponding donors and their previous donations. Archived samples from the last negative donation and from donations within 6 months before the last negative donation were thawed and repeatedly tested by antibody screening and confirmatory assays as well as by single-sample PCR. Recipients were traced and tested for the presence of viral markers.

Recipient-directed look-back procedures were started when we received the notification of a suspected virus transmission to a recipient of our blood components. Donors were traced and those who tested positive either in a following donation or in a freshly drawn blood sample initiated a donor-directed look-back procedure as indicated.

RESULTS

Performance of HBV PCR
The Red Cross Blood Transfusion Service Hesse, Frankfurt, Germany, started NAT of all donations to the state Hesse in January 1997. Subsequently, testing was extended to customer transfusion services. The Bavarian Red Cross Blood Transfusion Service commenced testing of donations from their own institutions and from customer transfusion services in January 1999 at the institution in Wiesentheid, Germany. The applied methods are identical at both test sites. A total of 3.6 million donations were tested until September 2000. The rate of false-positive pools was significantly reduced from 1.4 to 0.1 percent after the introduction of the TaqMan PCR technique and changing to positive-displacement pipets rather than pipets with filter tips.

Yield of HBV minipool PCR in HBsAg confirmed-positive samples
We found 195 of 292 (66.8%) and 103 of 140 (73.6%) HBsAg confirmed-positive samples that were positive in minipool PCR at Frankfurt and Wiesentheid, respectively. Confirmatory testing by single-sample PCR of HBsAg confirmed-positive samples that were negative in minipool PCR testing started in July 1998 in Frankfurt and in January 1999 in Wiesentheid and resulted in 37 minipool PCR-negative, single-sample PCR-positive samples (Frankfurt, 20; Wiesentheid, 17). Taking minipool PCR and single-sample PCR results together, a total of 298 (69.0%) of 432 HbsAg confirmed-positive samples were NAT positive, for both institutes.

Yield of HBV minipool PCR in HBsAg-negative samples
We identified 6 HBV PCR-positive, HBsAg-negative donations after screening of 3.6 million. This results in 1 infectious donation in 600,000 for central Europe (Germany, Austria, Luxembourg) that would have been missed without PCR testing. Separating the frequency by state and country, we found one PCR-positive donation among 390,000 Hesse, 1 in 820,000 German (including Hesse), and 1 in 153,000 Austrian donors.

All donors who were positive only in HBV PCR had a normal ALT below 22 U per L. Two donors were in the preseroconversion window phase and four were chronic anti-HBc-positive, anti-HBs-negative, low-level HBV carriers. For one HBV carrier, we could show by follow-up and retrospectively by testing of archived samples that viremia was transient and lasted at least for 6 months with declining plasma virus concentrations (Table 1). Recipients of previous donations that were negative in minipool PCR were HBV PCR negative.

One infected donor who had not seroconverted revealed a short transient viremia constantly below the detection limit of the Prism and AxSYM HBsAg tests (Fig. 1A,B). Development of antibody to HBsAg (anti-HBs) and anti-HBc (seroconversion) occurred 3 and 6 months after the initial positive PCR result, respectively (Fig. 1C). The donor became infected by sexual intercourse with his fiancée, a chronically infected Thai woman with high-titer viremia. His platelets were released without the HBV PCR result due to a shortage of platelets and a coincidental heavy contamination of the laboratory with HBV amplons that delayed test results. Packed RBCs and quaran-tine plasma were to be retained and discarded. The recipient of the platelets became transiently positive for HBsAg and was HBV PCR positive and seroconverted without clinical symptoms. Sequencing and phylogenetic analysis were performed on the virus isolates from the recipient, the donor, and the donor’s fiancée. All three isolates revealed 100-percent sequence identity in the surface, core, and X-genes, thus proving transmission (data not shown).

Sensitivity of HBV minipool PCR
To check whether the sensitivity of the HBV minipool PCR is sufficiently high to detect all those infectious window-phase donations that would be missed by the present HBsAg screening assays, we performed look-back procedures for all donors to the Frankfurt institute who seroconverted since the introduction of HBV minipool PCR testing in January 1997. A total of 15 donor-directed look-back procedures were initiated and 16 archived samples from preseroconversion donations could be retrieved. They were all negative in single-sample PCR upon retesting. Thirteen samples could be tested again for anti-HBc and 8 were positive. Blood components of the 8 anti-HBc-positive donations were transfused to 10 recipients. One recipient was anti-HBc-negative, 1 was anti-HBc-positive before transfusion, and 8 have died due to their underlying disease.

Since the introduction of NAT in 1997, we retrieved
and tested by PCR and serology samples from 276 donors involved in 27 recipient-directed look-backs initiated by suspected HBV transmission. Thirteen donors were anti-HBc-positive. Only 5 of them donated blood after the introduction of PCR testing. Archived samples were available from 11 donors only. They were all anti-HBc-positive. Only 1 sample, dating back to 1996, was positive in HBV single-sample PCR. The HBV sequence was identical to the HBV sequence of the recipient of the corresponding component, indicating transmission. For the blood components negative in single-sample PCR on archived samples, transmission of HBV to the recipients could not be established.

We tested the single-sample PCR-positive donor sample by minipool PCR after spiking a negative minipool. Minipool PCR was positive, indicating that the contaminated donation would have been identified if PCR had been in place in 1996. The donor donated blood six times after the introduction of PCR testing. Donor-directed look-back procedures were initiated and all six archived samples that were negative in minipool PCR and HBsAg testing were anti-HBc-positive. In single-sample PCR testing, four were negative and two were intermittently positive and negative as could be expected for samples with a virus concentration close to the detection limit. The recipients of the corresponding blood components were anti-HBc-positive, HBsAg-negative, and single-sample PCR negative. Since sequencing could not be performed, transmission could not be confirmed.

After having identified 4 HBsAg-negative, anti-HBc-positive chronic low-level HBV carriers by minipool PCR screening and 2 by single-sample PCR retesting of archived samples during look-back procedures, we were interested in whether we could find additional HBsAg-negative, low-level HBV carriers among those with anti-HBc-positive samples by use of a more sensitive PCR. Seven of 729 randomly selected anti-HBc-positive donations were identified that were exclusively positive in the highly sensitive single-sample enrichment PCR with an input of 9.6 mL donor plasma (Table 2). Based on the fact that our PCR detects an input of 30 geq (0.1 mL plasma at a concentration of 300 geq/mL), the calculated virus particle concentration was below 10 geq per mL of donor plasma (30 geq sedimented by centrifugation from 9.6 mL donor plasma).

### DISCUSSION

HBV PCR was started simultaneously with HCV and HIV-1 PCR in 1997. The yield of minipool PCR screening of 3.6 million blood donations from central Europe (Germany, Austria, Luxembourg) for HBV was below the theoretical estimates\(^1,2\) and only two of the six samples that gave a positive HBV PCR result were from donors who had not seroconverted (infectious window-phase donations). One donor showed a very low transient viremia not sufficient to become positive in HBsAg testing. Seroconversion (development of anti-HBc and anti-HBs) was delayed by several months. Usually, antibodies to the core antigen appear earlier than antibodies against HBsAg.\(^3,3\) The results in this case did not follow that pattern, and it was the first where the sequence of a sexual transmission from a chronic female carrier to a male and from him via transfusion to a platelet recipient could be confirmed by follow-up and sequencing of the respective HBV isolates. It shows that 300 geq of HBV per mL plasma was sufficient to transmit HBV infection and that donors may be infectious without ever developing symptoms or detectable antigenemia. It is conceivable that HBV spreads silently in this way into a significant proportion of anti-HBc-positive blood donors who cannot remember any risk behavior or clinical symptoms. They can only be identified retrospectively by anti-HBc testing.

The identification by minipool PCR testing of four HBV PCR-positive, HBsAg-negative donations that were anti-HBc-positive argues in favor of the introduction of anti-HBc and/or NAT screening, neither of which are mandatory in Germany. Moreover, in an HBsAg-negative, anti-HBc-positive donor we showed that viremia may transiently emerge and last for several months at a level below the detection limit of minipool PCR and even single-sample PCR. This phenomenon was reported previously.\(^9,14,15,19,33\) Only single-sample enrichment PCR utilizing high sample volumes enabled the detection of this low-level viremic donation 41 days after the single-sample PCR became negative. Even routine screening by single-sample PCR would not be sensitive enough to identify those low-level carriers. The necessity of anti-HBc testing is further supported by our finding that 7 of

### TABLE 1. Follow-up testing of an anti-HBc-positive chronic HBV carrier with low-level viremia*

<table>
<thead>
<tr>
<th>Days after donation</th>
<th>Minipool PCR</th>
<th>Single-sample PCR (0.1 mL)</th>
<th>Single-sample enrichment PCR (9.6 mL)</th>
<th>ALT HBsAg U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Donation +</td>
<td>NT</td>
<td>NT</td>
<td>– 15</td>
</tr>
<tr>
<td></td>
<td>FFP +</td>
<td>NT</td>
<td>NT</td>
<td>– 14</td>
</tr>
<tr>
<td>13</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>– 13</td>
</tr>
<tr>
<td>20</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>– 16</td>
</tr>
<tr>
<td>34</td>
<td>–</td>
<td>+</td>
<td>NT</td>
<td>– 16</td>
</tr>
<tr>
<td>48</td>
<td>–</td>
<td>+</td>
<td>NT</td>
<td>– 15</td>
</tr>
<tr>
<td>62</td>
<td>–</td>
<td>+</td>
<td>NT</td>
<td>– 12</td>
</tr>
<tr>
<td>90</td>
<td>–</td>
<td>–</td>
<td>NT</td>
<td>– 15</td>
</tr>
<tr>
<td>131</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>– NT</td>
</tr>
</tbody>
</table>

* Archived samples of previous donations were negative in single-sample PCR.
† Results were negative, negative, positive in three successive extractions and amplifications.
729 HBsAg-negative, anti-HBc-positive donations were found to be positive only by highly sensitive single-sample enrichment PCR. The fact that 6 were positive for anti-HBs is in accordance with previous findings utilizing sensitive PCR methods.5-7,10,12-14,20 However, 3 of them had an antibody titer higher than 100 IU/mL which is thought to be immunologically protective and to inhibit transmission.34 Whether this would hold true in these cases is questionable and can only be demonstrated by look-back procedures as discussed below or by chimpanzee experiments.

The primacy of single-sample PCR over minipool PCR with respect to sensitivity was demonstrated by the 37 HBsAg-positive donations that were missed by the minipool PCR. The main reason for the increased sensitivity of single-sample PCR is the additional proteinase K digestion of the plasma sample before the nucleic acid extraction which is advantageous for the liberation of HBV DNA. However, retesting of archived samples showed that no single-sample PCR-positive donation had passed minipool PCR testing. This does not necessarily indicate sufficient sensitivity for all preseroconversion donations.

In a recipient-directed look-back dating back to 1996 before the introduction of minipool PCR screening we found transmission of HBV by a donation that retrospectively tested positive in minipool PCR. However, the donor continued to donate after the introduction of minipool PCR testing and two of six archived specimens were found that were intermittently positive and negative by single-sample PCR upon retesting. Although the recipients were anti-HBc positive, transmission could not be confirmed by sequencing because they were HBV PCR-negative. The recipients of the single-sample PCR-negative donations, however, were anti-HBc-negative.

Our data indicate that minipool PCR reduces the residual risk of HBV transmission by infected donors who have not seroconverted as well as by chronically infected low-level carriers. Although we could show that HBV minipool PCR reliably identified infected donors who have not seroconverted, there is evidence that a significant proportion of chronic low-level carriers were
missed. Moreover, our data show that the frequency of HBV infected donors who have not serconverted is low among central European blood donors compared to the frequency of chronic, anti-HBc-positive, low-level carriers and that anti-HBc screening would significantly reduce the residual risk. Minipool PCR and even single-sample PCR would not be sensitive enough to provide a risk-reduction factor with respect to chronic low-level carriers similar to that of anti-HBc screening. However, HBV transmission by preseroconversion donations can only be reduced by NAT. Therefore, screening by anti-HBc and a sensitive NAT procedure (detection limit below 300 geq/mL donor plasma [Fig. 1A]) would provide the highest safety level achievable at present. The remaining benefit of ALT and HBsAg testing must then be reconsidered.

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REFERENCES

### TABLE 2. Seven low-level HBV carriers identified by single-sample enrichment PCR*

<table>
<thead>
<tr>
<th>Donor</th>
<th>Minipool PCR (0.1 mL)</th>
<th>Single-sample enrichment PCR (9.6 mL)†</th>
<th>HBsAg‡ (sample/cutoff value)</th>
<th>Anti-HBc§ (sample/cutoff value)</th>
<th>Anti-HBc// (sample/cutoff value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>+++—</td>
<td>0.17</td>
<td>0.29</td>
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</tr>
<tr>
<td>2</td>
<td>–</td>
<td>+—</td>
<td>0.25</td>
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<tr>
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<td>0.08</td>
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</tr>
<tr>
<td>4</td>
<td>–</td>
<td>+</td>
<td>0.13</td>
<td>0.15</td>
<td>&gt;1000.0</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>++</td>
<td>0.17</td>
<td>0.17</td>
<td>23.0</td>
</tr>
<tr>
<td>6</td>
<td>–</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>++</td>
<td>0.19</td>
<td>0.05</td>
<td>—</td>
</tr>
</tbody>
</table>

* Tested donations: 729 (446 were HBsAg-negative, minipool PCR-negative, anti-HBc-positive and anti-HBs-positive; 283 were HBsAg-negative, minipool PCR-negative, anti-HBc-positive and anti-HBs-positive).
† Each symbol (+ or −) indicates an independent enrichment, extraction, and amplification process.
‡ Positive result is a sample/cutoff value ≥ 1.0.
§ Positive result is a sample/cutoff value ≤ 1.0.
// Positive result is a sample/cutoff value ≥ 10 mIU/mL.


