Comparative evaluation of a triplex nucleic acid test for detection of HBV DNA, HCV RNA, and HIV-1 RNA, with the Procleix Tigris System

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\textbf{A B S T R A C T}

Nucleic acid testing (NAT) is valuable for screening blood donors for occult hepatitis B virus (HBV) infection and infection during the window period in countries where HBV is endemic and/or non-endemic. An "in-house" NAT (Triplex NAT) was developed for screening for HBV DNA, hepatitis C virus (HCV) RNA, and the human immunodeficiency virus type 1 (HIV-1) RNA. Using the Triplex NAT, a head-to-head comparative clinical evaluation was carried out against the most common commercial NAT used for blood screening in China: the Procleix Tigris System. A total of 33,025 specimens which were negative for Hepatitis B surface antigen, HCV antibody and HIV-1 antibody/antigen from potential blood donors were tested for HBV DNA, HCV RNA, and HIV-1 RNA by both the in-house Triplex assay and the commercially available Procleix Tigris System. Eleven specimens were detected as HBV positive by both NATs. Twelve specimens were detected as HBV positive by the Procleix Ulitrio assay and the discriminatory assays, and not the Triplex. Twenty-eight specimens were detected as HBV positive by the Triplex and not the Procleix Ulitrio. This study, combined with other data obtained in China, suggest that at least 50% HBV surface antigen negative but DNA-positive blood donations would be undetected using the current commercial NATs because of their insufficient sensitivity and/or Mini-Pool formatting strategies.

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1. Introduction

The prevalence of occult HBV infection and infection during the window period in blood donations has become the primary reason for transfusion-transmitted HBV infections in many Asian countries where nucleic acid test is not implemented (Allain, 2004; Li et al., 2008). While anti-HBc screening can eliminate the residual risk of occult HBV transmission by transfusion in low endemic areas, it would be impractical in most parts of the world where the prevalence of anti-HBc is greater than 10%, as too many otherwise healthy donors will be ineligible to donate blood (Allain, 2004; Liu et al., 2006a). The risk of transfusion-transmitted HBV infection in Asian countries is significantly higher than in non-endemic countries in Europe and North America. A study in Taiwan indicates that the incident of post-transfusion acute HBV infection is 0.9% (100 per million units of donated blood), which is 7 to 40-times higher than in non-endemic countries (Liu et al., 2006a,b). These endemic areas require better sensitivity of HBV screening to minimize the risk of post-transfusion HBV infection. In addition, antigen and/or antibody assays of HBV, HCV, and HIV-1 are not sensitive enough to detect viremia in the window period.

In recent years, NATs for HBV, HIV, and HCV have been implemented gradually within the blood centers located in more urban areas of China. Two commercial automated NAT screening systems are used widely in most blood centers across China: the Procleix Ulitrio Assay, using the Procleix Tigris automated instrument (Novartis), and the TaqScreen MPX assay using the Cobs s 201 instrument (Roche). Both assays are designed to detect HCV, HIV and HBV in a multiplex assay. Analytical sensitivity, clinical and operational performances have been demonstrated by several studies (Jarvis et al., 2008; Liu et al., 2006a; McCormick et al., 2006; Ohhashi et al., 2010; Schmidt et al., 2010).

Comparisons of the two NAT systems indicate that the Tigris system has superior sensitivity in detecting HCV RNA and HIV RNA than does the Cobs s 201 system. And the difference in analytical and clinical sensitivity was not significant for HBV testing, despite the 1:6 dilution factor in s 201 (Assal et al., 2009a,b; Margaritis et al., 2007).

The clinical performances of the individual donor NAT have been reported in a few studies within China, revealing relatively high

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; LOD, limit of detection; NAT, nucleic acid test.

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rates of HBV DNA positive samples among HBsAg-negative samples. Specifically, HBsAg-negative blood donors tested positive for HBV-NAT at a rate of 0.12% by the Procleix Ultra. Rates vary when using the TaqScreen MPX assay, depending on whether the samples are pooled (0.055% by mini-pool NAT) or not (0.195% by individual donor NAT) (Gong et al., 2010; Song et al., 2010).

The aim of this study was to conduct a head-to-head comparative clinical evaluation of the in-house Triplex NAT against the Procleix Tigris System using the same samples. The intention of this study is to focus on HBV DNA, but the HIV-1 RNA and HCV RNA analyses were included as well.

2. Materials and methods

2.1. In-house Triplex real-time PCR NAT system

2.1.1. Overview of in-house NAT

The in-house Triplex NAT system utilizes the Chemagic Star platform which combines Chemagen's NA extraction system with a Hamilton liquid handling system for fully automated sample pooling of blood donations and automated NA extractions. The in-house NAT assay has been named “Triplex”, to indicate that this real time PCR test is capable of simultaneous detection of HBV DNA, HCV RNA, and HIV-1 RNA in human plasma.

The primers and probes designed to detect HBV, HCV, HIV-1 are specific for the HBV S gene, HCV 5’ UTR and HIV-1 gag gene, respectively. Modified Primers (TriLink, San Diego, CA) containing thermolabile phosphotriester modification groups were used in the Triplex assay to improve the sensitivity and specificity of PCR amplification (Lebedev et al., 2008). In this way, HBV primers and HCV/HIV-1 forward primers were blocked until higher temperatures of PCR were reached. Unmodified primers for reverse transcription of HCV/HIV-1 RNA and TaqMan probes for HBV, HCV, HIV-1 and IC detection were purchased form Takara (Dalian, China). Sequences and modifications of primers and probes are listed in Table 1. The PCR mixture with a final volume of 50 μl contains 1 × RT-PCR Buffer, 1.25 mM dNTPs each (Toyobo, Japan), 6 mM MgCl₂, 200–400 nM of each primer, 200 nM of each probe, 5U Hot-Switch Taq polymerase (Takara), 5U AMV reverse transcriptase (Promega), and 30 μl aliquots of the extracted eluates. Amplification and detection was performed using 7500 Sequence Detection System (Applied Biosystems), using the following thermal cycling conditions: 1 cycle at 50 °C for 20 min and 95 °C for 10 min; 5 cycles at 95 °C for 60 s, 55 °C for 20 s, and 20 s at 72 °C; 45 cycles at 95 °C for 10 s and 60 °C for 45 s.

An artificial IC sequence bearing the same sequences of HIV-1 group M primers was inserted into an expression plasmid with Escherichia coli phage MS2 packaging sequences for manufacturing Armored RNA IC reagent. IC was diluted to the appropriate concentration in SM buffer and injected into each sample for monitoring the entire detection process including steps of NA purification and amplification. Results with failed IC detection are regarded as invalid and associated samples are retested in the next batch.

One test batch could be either 24 individual donor samples (or positive/negative controls) or 24 mini-pooled samples, where 1 pool consists of 8 donor samples. The final volume of pooled or individual samples is 1.2 mL. The viral type (HBV, HCV or HIV) of a positive sample is directly determined by using TaqMan probes with distinct fluorescent dyes. ICs are also determined by using labeled probes with distinct fluorescent dyes. When a pool is identified as positive, all eight related individual donations are individually re-tested.

Repeatedly reactive samples in mini-pool and individual donor NAT are quantified further by in-house single real-time PCR tests for HBV, HCV or HIV-1, which were calibrated using the WHO international standards mentioned below.

2.1.2. Analytical sensitivity

The analytical sensitivity was determined as 95% and 50% limit of detection (LOD) using serial dilutions of the following reference standards: WHO 2nd HIV-1 RNA International Standard (NIBSC code: 97/650), WHO 3rd Hepatitis C Virus (HCV) RNA International Standard (NIBSC code: 06/100), and 2nd WHO International Standard for Hepatitis B Virus DNA (NIBSC code: 97/750). Each viral standard was diluted with normal (virus-negative) human plasma to give the following concentrations:

- HIV-1 group M: 6, 20, 40, 50, 60, 200 IU/mL;
- HCV: 10, 20, 30, 40, 50, 100 IU/mL;
- HBV: 0.5, 1.3, 3.3, 4.0, 5.0, 12.0 IU/mL.

Each dilution series was tested using three different lots of the Triplex NAT assay for a total of 48 replicates per concentration (16 replicates per lot). Probit analysis (SPSS, vision 19; IBM) was used to determine the 95% LOD and the two-sided fiducial confidence intervals from the combined data of all replicates tested for each virus.

2.1.3. Genotype inclusivity

The ability of the Triplex NAT to detect variable genotypes was evaluated using three genotype panels: NIBSC HIV-1 Genotype panel (08/358); NIBSC HCV Genotype panel (08/264); 1st WHO International Reference Panel for Hepatitis B Virus Genotypes (PEI code 5086/08).

The HIV panel consists of 11 different HIV-1 genotypes diluted in human plasma (A, B, C, D, AE, F, G, AG-GH, group N and group O), and a negative control. Since the concentration of the panel is not given, each specimen was diluted five-fold and tested by the Triplex NAT.
The HCV panel consists of six vials representing each of the major genotypes (1a, 2b, 3a, 4a, 5 and 6a) at an assigned concentration of 500–1500 IU/mL. Each member was diluted 10-fold with normal (virus-negative) plasma and tested by the Triplex NAT.

The HBV panel consists of the following genotypes: three of A, three of B, three of C, three of D, one of E, one of F, and one G. All were diluted to 10 IU/mL with normal (virus-negative) plasma and tested by the Triplex NAT.

### 2.1.4. Clinical specificity

The clinical specificity of the Triplex NAT was evaluated by testing 33,112 blood donations in 4139 pools of 8. When a mini-pool was reactive initially, then all eight samples were re-tested by the Triplex NAT individually. If all the samples were negative upon re-testing, the mini-pool result was called a false positive.

### 2.2. Procleix Tigris System (Novartis)

The Procleix Tigris system (Tigris) is a fully automated system for nucleic acid amplification and detection based on transcription-mediated amplification (TMA) technology. The Procleix Ultrio assay is used to make a preliminary detection of whether a sample is positive for any of the transfusion transmitted viruses. If a sample is deemed positive, a second step requires three discriminatory probes assays to assign viral type.

As a routine NAT process at the Shenzhen blood center, all plasma specimens are parallel tested by the Procleix Ultrio and Enzyme Linked Immunosorbent Assays (ELISAs). If a sample is positive by the Procleix Ultrio assay and negative by the ELISAs, it is characterized further using Procleix discriminatory probe assays for HBV, HCV, and HIV-1 identification. Samples with positive results on either discriminatory assay are classified as discriminatory test reactive. Samples with negative results on all three discriminatory assays are classified as discriminatory test nonreactive.

### 2.3. Comparative clinical study

The clinical performance of the Triplex NAT system was evaluated at the Shenzhen Blood Center. Plasma specimens were collected between July and December 2011. Each serology test (ELISA) was performed in parallel using two different commercial kits: HBsAg test (InTec, Xiamen, China; DiaSorinS.p.A.); Anti-HCV test (Livzon, Zhuhai, China; Ortho-Clinical Diagnostics); HIV Ab/Ag test (Wantai, Beijing, China; Bio-Rad). If the two results for each type of test were discrepant, another assay would be used to confirm the results.

Overall, 33,112 blood donation samples were tested individually by the Procleix Ultrio and in pools of 8 by the Triplex NAT. If a sample tested positive by the Procleix Ultrio, it was re-tested individually by the Triplex NAT. Of these donation samples, a portion of HBsAg-positive samples were also screened by the Triplex NAT in pools since HBsAg-positive samples often had undetectable viral loads. Most of them were nonreactive by the Procleix Ultrio. All the HCV/HBV-1 seropositive samples and the rest HBsAg-positive samples, which were also reactive by the Procleix Ultrio, were not screened by the Triplex mini-pool NAT.

### 3. Results

#### 3.1. Analytical sensitivity

Results of the Probit analysis to determine the 95% LOD and 50% LOD of the Triplex NAT system for individual viruses are shown in Table 2.

### Table 2

95% LOD and 50% LOD of the Triplex NAT for target viruses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>International standard (NBSC code)</th>
<th>Units</th>
<th>Average 95% LOD (95% CI)</th>
<th>Average 50% LOD (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1</td>
<td>WHO 2nd (97/650)</td>
<td>IU/ml</td>
<td>53.6 (41.9–75.8)</td>
<td>11.8 (9.1–14.5)</td>
</tr>
<tr>
<td>HCV</td>
<td>WHO 3rd (06/100)</td>
<td>IU/ml</td>
<td>37.9 (31.4–50.6)</td>
<td>12.2 (9.7–14.4)</td>
</tr>
<tr>
<td>HBV</td>
<td>WHO 2nd (97/750)</td>
<td>IU/ml</td>
<td>3.9 (3.9–6.9)</td>
<td>1.2 (0.95–1.4)</td>
</tr>
</tbody>
</table>

#### 3.2. Genotype inclusivity

All the diluted members in HIV-1, HCV and HBV panel were detected by the Triplex NAT. A summary of the Genotype inclusivity is shown in Table 3.

### Table 3

Genotype inclusivity by the in-house NAT.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Genotype</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1</td>
<td>A, B, C, D, AE, F, G, AG-GH, group N, group O</td>
<td>100–400 IU/mL (estimated)</td>
</tr>
<tr>
<td>HCV</td>
<td>1a, 2b, 3a, 4a, 5, 6a</td>
<td>50–150 IU/mL</td>
</tr>
<tr>
<td>HBV</td>
<td>A, B, C, D, E, F, G</td>
<td>10 IU/mL</td>
</tr>
</tbody>
</table>

#### 3.3. Clinical study

##### 3.3.1. Seronegative population study

Of the total 33,112 samples tested by the two NATs, 33,025 were seronegative. Summary of the results is shown in Fig. 1. Twenty-three repeat reactive samples were detected as HBV DNA positive by the Procleix Ultrio and the discriminatory assays. Fifty initially reactive samples were positive with the Procleix Ultrio assays, but nonreactive in the discriminatory assays. Thirty-nine samples were HBV-DNA positive by the Triplex NAT.

Twelve samples were HBV-DNA positive by the Procleix Ultrio but not detected by the Triplex NAT. Twenty-eight samples were HBV-DNA positive by the Triplex NAT but were not detected by the Procleix Ultrio. Eleven samples were positive by both assays.

All the Tigris react repeat reactive or initially reactive samples detected as negatives by the Triplex mini-pool NAT were retested individually by the Triplex. Of the 12 HBV NAT positives only by the Procleix Ultrio, 8 were positive and 4 remained negative when tested individually by the Triplex NAT. Of the 50 initially reactive samples, 4 were HBV DNA positive in pools by the Triplex NAT, and 12 were detectable only in undiluted.

The viral loads of a total 59 HBV-DNA positive specimens were determined by the in-house quantification assays: 51 (93.2%) had a viral load of below 50 IU/mL, and 4 above 100 IU/mL, and 4 were nonreactive.

One clinical sample was HCV NAT positive and detected by the Triplex NAT with a viral load of 10^4 IU/mL. Undiluted sample could not be restested by the Procleix Ultrio due to insufficient sample volume. However, it was reactive in 1:100 diluted by the following tests of the Procleix Ultrio and discriminatory assay of HCV. The donation was probably made during the donor’s window period, but further investigation was not conducted for donor’s request. In addition, one HIV-1 RNA positive was detected on both NATs with a very high viral load (4.5 × 10^5 IU/mL).
33.025 Seronegative samples

32,972 NAT negatives

53 NAT positives (repeat reactive)

12 Concordant positives

11 HBV positives

1 HIV-1 positives

41 Discordant positives

40 HBV positives

1 HCV positives (Triplex)

12 Tigris positives

28 Triplex Mini-Pool NAT positives

**Fig. 1.** Summary of comparative clinical data from seronegative donations.

### 3.3.2. HBsAg-positive donations

A total 167 HBsAg-positive samples were tested by ELISAs and Procleix Ultro. Eighty-seven of these samples were tested by the Triplex NAT and most (74 of 87) were nonreactive in the Procleix Ultro. Thirteen HBsAg-positive donations were detected to be HBV-DNA positive by both the Procleix Ultro and the Triplex NAT. Nine HBsAg-positive donations were detected as HBV DNA positive by the Triplex NAT (nonreactive in the Procleix Ultro) and were in the mini-pool format. All of those HBV DNA positives had viral loads below 60IU/mL tested by the in-house quantification assays.

### 3.3.3. Clinical specificity

A total of 4139 mini-pools from the Triplex NAT with an IC valid result were chosen for the evaluation of specificity. A total 78 mini-pools were initially reactive and 62 of those were re-tested and associated with a positive individual result. Sixteen (0.39%) initially reactive pools (all were HBV positives) were considered false positive results and the specificity on initially reactive results was 99.61%.

### 4. Discussion

Results of this study reveal the rates of HBV-NAT-positives in HBsAg-negative donations were 0.069% (Procleix Ultro individual NAT) and 0.118% (Triplex mini-pool NAT) and 0.154% (Procleix Ultro individual NAT/Triplex mini-pool NAT) in the HBsAg-negative donations. These results are similar to a Beijing study on the Procleix Tigris system, with a rate of 0.121% (1/826) (Song et al., 2010). It is notable that of the total 51 HBV DNA positives, only 11 (21.56%) were both detected by the two tests, and the viral level of these positives were often below 50IU/mL. As HBV viral load of the chronic donations were close to the NAT cutoff (approximate 15IU/mL for the Procleix Ultro assay (McCormick et al., 2006) and 40IU/mL for the Triplex test), it is well noted that both of the NATs could only exclude a proportion of the HBV-DNA-positives from the HBsAg-negative donations due to stochastic sampling issues.

The influence of pool size on the HBV DNA detection rate was also evaluated in this study and discussed here below. The differences were not significant for the rates of HBV-NAT-positives on the Tigris and the Triplex NAT, despite the dilution of 1:8 in the Triplex NAT. Of the 23 HBV-DNA-positives with the Tigris, 11 (47.8%) were detected by the Triplex mini-pool NAT, nevertheless, 8 (34.7%) were only detected by individual donor NAT upon retest. Furthermore, since the sensitivities of the Procleix Ultro assay and the discriminate probe assay are similar for using the same method and same sample volume, the initially reactive samples, which were initially reactive but not detected by the discriminatory assays, should be either true positives (mainly for HBV DNA), or true false positives. Of the 50 initially reactive, but discriminatory nonreactive samples, 4 (8%) were detected by Triplex mini-pool NAT while 12 (24%) were only detected by Triplex individual donor NAT. Although abundant donations were not tested by the Triplex individual donor NAT, this study suggests Triplex individual donor NAT would significantly increase the rate of HBV-NAT-positives in HBsAg-negative donations. This result corresponds with another Chinese study on the Cobas s 201 system (Roche) which revealed the ratio of HBV-NAT positive rates of individual donor NAT to mini-pool NAT (in pools of 6) to be 3.58:1 (Gong et al., 2010). These data suggested that more than 50% HBsAg-negative but DNA-positive donations were overlooked by currently licensed NATs due to insufficient sensitivity or pooling format for HBV screening.

A study of donor samples with chronic HBV infection indicates a weak correlation between HBsAg and HBV DNA concentrations (Kuhns et al., 2004). In the study, it is notable that numerous HBsAg-positive donor samples were Procleix Ultro assay nonreactive and that most of these samples had extremely low viral loads. Of the 74 NAT-negatives by the Procleix Ultro assay, 9 were reactive in the Triplex mini-pool NAT. Viral loads of these discordant positives
were all below 60 IU/mL by the quantification assay. These results indicated that HBV DNA levels in HBsAg-positive donations could be extremely low or undetectable. Currently, it is inappropriate to discontinue HBsAg screening following implementation of HBV NAT in China.

Since infection may occur even if the viral load in the blood is very low, it is necessary to enhance the sensitivity of the current NATs to minimize the risk of HBV post-transfusion infection in highly endemic Asian countries. To that end, the priority is to enhance the sensitivity of the assay and mutated genotype detection abilities. However, due to the high prevalence of occult HBV infections with extremely low viral loads, it is still a great challenge to exclude blood donations with occult HBV infection by the current NAT systems. Triplex individual donor NAT, with enhanced sensitivity, is needed in highly endemic countries like China, now, and in the foreseeable future.

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Conflict of interest

The authors have no conflict of interest.

Ethical approval

Not required.

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