Nucleic acid testing (NAT) in high prevalence–low resource settings

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A B S T R A C T

Blood screening by NAT for major transfusion transmitted viral infections (TTIs) was originally intended to complement serology for detection of infected donations. Reports from developed countries showed limited marginal value to NAT blood screening in improving blood safety. Reports on NAT results from Europe indicated yield of 1:0.6 million donations for HBV, ≤1:M for HCV and HIV-1-related to low prevalence of TTI. In contrast, prevalence of TTI in resource-limited countries is almost always high. As a result, more incident cases can be expected among first-time blood donors. Most reports of NAT blood donation screening in these countries showed NAT confirmed yield as high as 1/2800 for HBV and 1/3100 blood donations for HCV as reported from Thailand and Egypt, respectively. The issues for low resource countries are mostly the high cost of NAT but also the requirements of staff qualification, adequate facilities, reagent procurement and maintenance of delicate equipment. Alternatives to commercial NAT are the use of combos antigen-antibody for HIV and HCV, anti-HBc for HBV and in-house NAT. Most of these alternatives have been reported but very few comparisons are available. Once yield data is available, models for estimation of feasibility and cost-effectiveness are proposed to help decision-making.

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1. Introduction

NAT blood screening for key TTIs was originally intended to complement serological screening for detection of donations infectious for those viruses. The main advantage of NAT screening is interdiction of new incident cases during the window period infections. An additional benefit, which was identified as a result of accumulated experience of the use of the NAT was the identification of occult hepatitis B carrier status, which can potentially be infectious. Most populations in resource-limited regions suffer from high prevalence rate of these TTIs, and are expected to have more frequent incident cases, as well as more occult carriers. Consequently NAT screening of TTIs in these populations would be expected to identify more yield cases as compared to the developed world and thus to be more cost-effective.

The introduction of NAT should be considered only when an efficient and effective program of serological testing is in place [1] and there is clear evidence for additional benefits of NAT. Although NAT reduces the window period of infection, in many cases the incremental gain is minimal as the actual reduction in the window period may be very small and the number of donors in the window period at the time of donation is generally very low. Only in countries with a high prevalence and incidence of infection are likely to yield a significant number of window period donations. Thus although the risk of transfusing a blood unit collected during the window period may be decreased using NAT, the actual benefit in most populations should be determined and balanced against the complexity and high cost of setting-up, performing and sustaining NAT [2–4].

The implementation of NAT blood screening at the national or blood establishment level requires an appropriate infrastructure, defined test algorithm, existing quality assurance and quality control, confirmatory assays for reactive results, management procedures for donors with confirmed positive test results. In addition, an evaluation of cost-effectiveness should be performed before considering such a screening program. Several developing countries have recently implemented NAT screening and have published their experience and presented their conclusions on the value of this strategy according to locally or nationally defined criteria. This review summarizes these experiences and their conclusions.

1.1. Blood screening program for TTIs

A screening program may be developed to implement the national policy on blood screening to identify and prevent the
release of any donations reactive for specific TTIs using the most reliable and cost-effective approach [5]. As a general philosophy, it is understood that for any country, particularly those with scarce resources, the most important priorities should be considered. First is the adequacy of the blood supply for patient requirements. There is no definitive number establishing the extent of such need but in developing countries where most blood demand is for emergency indications of massive blood loss or acute anemia, a supply ranging between 10 and 20 units/1000 inhabitants is generally cited. Second, this blood should be as safe as possible within the limits of affordability. Depending on resources, blood testing might be only for anti-HIV, or anti-HIV and HBsAg or for all three main markers (HIV, HBV, anti-HCV). The use of combo antibody/antigen to improve HIV or HCV safety depending on local epidemiology can be considered at relatively modest increase in costs over antibody testing alone. Envisaging the introduction of NAT requires a number of preliminary steps described below. These include: (1) defining local or regional epidemiology; (2) performance of pilot studies to generate data as well as to establish operational and financial parameters; and (3) determination of strategy taking into consideration not only technical and operational issues but most importantly cost-effectiveness. These preliminary steps usually imply the existence of a national blood policy that should address national requirements for the screening of whole blood and apheresis donations prior to their release for clinical or manufacturing use. Such a policy should define mandatory screening for specific infections markers and screening for other TTIs, based on national epidemiological data on blood-borne pathogens. It should also outline the measures taken to ensure that all screening is performed in the context of effective, quality-managed blood transfusion services and require the most efficient use of available resources. The need for, and the role of, confirmatory testing should also be clearly defined.

2. National blood collection and screening strategy

Blood safety depends on donor selection and donor/blood testing. In areas of high prevalence of viral infection, donor-dependent blood safety may rely more upon assuring repeat donation than on the classical distinction between volunteer and replacement donors. Laboratory screening of blood donations is the second step that determines whether or not a donor or a donation are non-reactive for specific markers of infection and blood is therefore safe to release for clinical or manufacturing use. In most cases, laboratory screening is performed after the blood donor selection and the donation process. The expectation is that the majority of the donations collected would be from healthy, uninfected, individuals and would be non-reactive. However, some donations will be repeat reactive and procedures must be in place to distinguish between true and false reactivity by the process of confirmation.

Consideration should also be given to the management of donors who are deferred after their blood is reactive in screening tests and deferred and then of those confirmed infected. Reliable confirmation of screening reactivity is essential, helping both in the management of donors and in the understanding of the epidemiological profile of blood-borne infections in the donor population. In addition to the screening strategy, the blood screening program should define the confirmation algorithm for repeat reactive test results. Each country must decide on the TTIs to be tested as part of the blood screening program and select a screening strategy appropriate to its specific situation. This will be influenced by the incidence and prevalence of infection, the costs of testing, the capacity and infrastructure of the blood transfusion service and the available resources.

3. Collecting epidemiological evidence

Epidemiological evidence should be collected on a relatively large, representative, cross-section of the donor population including first-time volunteer and replacement donors, as well as repeat donors. In many areas, only screening data is available but this may not provide accurate information as only confirmed positive results are informative. For antibody or antigen assays, reactivity with two separate assays is the minimum approach for confirmation, particularly where prevalence is high. When combo assays are used, the presence of antigen only (separate antibody assay negative) indicates window period infections, which can be used to determine the incidence of infections. The next step would be to collect NAT data, within or prior to the pilot stage to determine the potential added efficacy of the method. NAT data collected with a Triplex system provides data on HIV, HCV and HBV in a single study. For HBV safety, identifying window period infection is important but it is also important to detect HBV DNA in HBsAg negative, anti-HBc positive donations. The alternative strategy for additional HBV safety is screening for anti-HBc. This approach may lead to discarding large proportions of donations unless anti-HBc positive samples are also tested for anti-HBs and only donations without anti-HBs or with anti-HBs titer <100 IU/L are discarded. In such circumstances, the cost of discarding collected units and of recruiting new donors to compensate for such loss of supply as well as the percentage of repeat donors have to be taken into consideration in the costing of the strategy [6–8]. For instance, a unit of blood may cost $20 in Sub-Saharan Africa when collected mostly from replacement donors, $50 in India or in African centralized volunteer only systems, making these estimations widely different. Preliminary calculations indicate that at a $20 blood cost, NAT becomes cost-effective above 30% anti-HBc positive units but at 15% discard rate when blood cost reaches $50 or above (van Hulst, personal communication).

4. Pilot phase

The pilot phase is critical as it provides not only the additional epidemiologic data needed but also an evaluation of the technology(ies) available, exposure of staff to equipment and methods, assessment of algorithms for screening and confirmation, and defines the changes in facilities and operations created by the new method. In some cases such as in Thailand, Hong Kong or Taiwan, both commercial triplex assays were successively or concomitantly evaluated [9–11]. In these three countries, centralized high throughput systems were already in place and some funding was available for the evaluation. As HBV was the only high prevalence infection, screening in pools of six to eight or in individual unit samples revealed differences in the frequency of yield cases both window period and occult HBV infection (OBI) as well as in the frequency of HBsAg confirmed positive cases negative for HBV DNA. The data were then presented to the authorities for decision-making in which both testing efficacy and cost of screening were taken into consideration. In other evaluations, only one assay was assessed, either commercial or in-house [12–15]. In situations where resources and expertise are limited, it may be appropriate to utilize evaluation data from external sources to assess potential assays and systems. In all cases, however, it is essential that an effective process is defined and put in place to ensure that new assays and systems are considered and possibly introduced only following extensive investigation, evaluation and validation.
5. Laboratory quality systems

Effective quality systems are essential for the overall effectiveness of the blood screening program and to prevent the transmission of infection through transfusion. Quality systems should not be limited to laboratories only but should encompass all BTS activities to ensure that all donations are screened correctly and handled appropriately before and after laboratory testing. For instance, in blood services where the number of blood samples collected is modest, screening is performed at several day intervals during which part of the blood stock is quarantined. The disturbances of such quarantine might create for storage of blood and to the blood availability must be carefully evaluated. Meeting defined quality standards will ensure the safety and clinical efficacy of blood and blood products as well as protecting the health and safety of all staff that come into contact with them.

6. Human resources

A sufficient number of qualified, trained and competent staff should be available to perform the laboratory activities associated with blood screening, including the implementation of quality systems. Such training prior to the pilot phase of the evaluation will be provided either on-site supervised by commercial company staff or, probably more effectively, through in-depth training in an external laboratory for people already knowledgeable in molecular biology would be very beneficial as it would provide the blood center with new skills that may be applied to a range of targets. During the weeks required for such training, not only the screening method(s) but also methods for confirmation and in depth understanding of the methodologies involved is needed as most of the time, staff will be without external support when problems occur. Continued support by electronic communication for data interpretation or technical issues by the specialists at the training site are often psychologically and technically very helpful. Blood transfusion services would benefit from working with national health and education authorities to ensure that education and training institutions provide suitable opportunities for staff qualification and training. In-service training programs established and reviewed at appropriate intervals and measures adopted to retain experienced staff will help to ensure that laboratories function effectively.

7. Financial resources

Experience has demonstrated that in terms of blood safety, either economic or political criteria are usually critical in the decision-making process. It is therefore essential that detailed and evidence-based information be given to the decision-making bodies. Recently, several articles have been published examining cost-effectiveness issues related to HIV and HBV blood safety since they are the most critical in most developing countries. The case of Egypt is unique since HCV is the most frequent infection in blood donors. The Working Party on Infectious Diseases of the International Society of Blood Transfusion has developed a program which uses the input of basic operational and epidemiologic information to determine the cost-efficiency of a given intervention. Preliminary results obtained with this program indicated that HIV NAT in Ghana where the prevalence is around 4% in the general population and 2% in blood donors was not cost efficient [16]. In contrast, it demonstrated that an in-house Triplex NAT in pools of 10 samples following pre-donation screening for anti-HIV, anti-HCV and HBsAg performed with rapid tests was more cost efficient than microritet plate EIAs (Van Hulst, 2009, submitted). One of the main uncertainties in the assessment of cost-efficiency is the infectivity of HBV outside of the window period. The implementation of new systems for screening is best undertaken in a stepwise fashion with appropriate data collected, cost-effectiveness calculated and resources allocated for establishing long-term operations and quality systems [16].

8. Procurement and supply of test kits and reagents

Once the decision is made to implement a strategy including NAT, continuity in the supply test kits, reagents and consumables required for quality testing depends on reliable procurement and supply systems is needed. Interruptions in the supply of test kits and reagents may result in the temporary inability of screening facilities to screen for TTIs thus resulting in issuance of unscreened blood for transfusion. Similarly, maintenance of the delicate equipment required to perform the assays needs to be assured. Formal procurement processes, where appropriate using existing national procurement systems are recommended to ensure the sustainability of the screening program. A reliable procurement and supply system not only benefits the BTS, but also ensures that each supplier is fully aware of the test kits and reagents required, the usage rates and the quantities needed. Transportation and storage of reagents (some of them need to be kept frozen), particularly during airport transit in tropical conditions can be a challenging problem. The manufacturer and the BTS should ensure that reliable cold chain systems are in place to assure compliance at all times [17,18].

9. Testing approaches

9.1. Commercial and in-house NAT blood screening assays

Since the late 1990s several European blood centers implemented NAT blood screening. These centers used either in-house or commercial assays. The technologies used were either direct and reverse transcription-mediated amplification by polymerase chain reaction (PCR) or transcription-mediated amplification of particular genome sequences. In-house assays must be carefully validated, should include positive, negative, and internal controls and should always be monitored by the use of external quality assurance panels [19]. In contrast to the situation in developed countries, in many developing countries, no specific requirements are in place to make commercial assays mandatory. In-house assays can be more easily implemented at a fraction of the cost offered by western commercial companies. However, as mentioned above, assay quality and quality assurance rules must be equally applied all assays whether commercial or in-house.

9.2. NAT blood screening experience in resource-limited countries

Several resource-limited countries have implemented NAT blood screening either on a national or center level. For example, the Thai National Blood Center collected 486,676 seronegative blood donations in 2007 tested by NAT using the Novartis TIGRIS/Procleix Ulitro test and the Roche Cobas s 201/Cobas TaqScreen multiplex (MPX) test (Table 1). The NAT yield rate for human immunodeficiency virus Type 1 (HIV-1), hepatitis C virus (HCV), and hepatitis B virus (HBV) was 1:97,000, 1:490,000, and 1:2800, respectively. A majority of occult HBV infections, detected by both tests, were identified. HIV-1 and HCV window period cases were detected with both tests [9]. In Taiwan (which is not a developing country but a country with high HBV infection rate) among 10,727 seronegative donations, 12 HBV NAT yield cases (0.11%) and one HCV NAT yield case (0.01%) were detected. Follow-up results for 1–8 months showed that the HCV yield case was a window case and
all HBV NAT yield cases were occult carriers. The authors concluded that Triplex NAT detected occult HBV and reduced the HCV window period. The yield rate, especially occult HBV, was 10- to 100-fold higher than that in developed, HBV non-endemic, countries. Therefore, NAT implementation for routine donor screening in a more cost-effective manner should contribute to safer blood transfusion in Taiwan [10].

In South Africa, NAT and serology yield rates in first-time, lapsed, and repeat donors were analyzed (Table 1). The HIV, HBV, and HCV ID-NAT window phase yield rates in 732,250 blood donations tested as single samples were 1:45,765, 1:11,810, and 1:732,200, respectively [20]. In Ghana, an in-house Triplex NAT for donations tested as single samples were 1:45,765, 1:11,810, and 1:60 when tested in individual donations. This rate increased to 1:60 when tested in individual donations. Of note, Ghana is one of the countries in the world with the highest rate of HBV seropositivity (80%) and chronic HBV infection (15%). Despite a 4% prevalence of HIV infection in the general population (2% in blood donors), no HIV window period infections were identified.

In Brazil, an “in-house” RT-PCR method was developed that allows the simultaneous detection of the RNA of HCV and an external control. Samples were analyzed in pools of six to 12 donations, each donation included in two pools, one horizontal and one vertical, permitting the immediate identification of a reactive donation, obviating the need for pool resolution. The whole process took 6-8 h and results were issued in parallel with serology. The method was shown to detect all six HCV genotypes with a sensitivity of 500 IU/mL. Until July 2005, 139,678 donations were tested and 315 (0.23%) were found reactive for HCV RNA. Except for five false-positives, all reactive samples were also antibody positive and no WP donations were identified (Table 2). Detection of WP donation in the population studied would probably require testing of a larger number of donations [12]. This method detects all HIV-1 group M subtypes, plus group N and O, with a detection threshold of 500 IU/mL. After validation, the method replaced p24 Ag testing which had been in use for blood donation screening since 1996. From July 2001 to February 2006, 102,469 donations were tested and 41 (0.04%) were found HIV-1 RNA reactive (Table 2). One window period infection was observed giving a yield of 1:102,469 [13].

In Mexico an exploratory comparison of the performance of the standard immunoassay-based screening tests for the HBV and HCV and NAT, in blood donations was conducted [26]. From January 1999 to March 2005, 94,806 blood donors were screened for hepatitis B surface antigen (HBsAg). Molecular screening was performed on 100 consecutive blood donations to detect HBV DNA and HCV RNA by home-made PCR

### Table 2: Pilot screening for single virus by NAT in blood donors from developing countries.

<table>
<thead>
<tr>
<th>Country</th>
<th>Author</th>
<th>Dominant genotypes</th>
<th>Serology</th>
<th>NAT</th>
<th>Mode</th>
<th>N samples</th>
<th>Sero-/ NAT+</th>
<th>Sero-/ DNA+</th>
<th>Sero-/ DNA+</th>
<th>WP infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil</td>
<td>Wendel [12]</td>
<td>D</td>
<td>HCV</td>
<td>In-house</td>
<td>MP6</td>
<td>250</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Brazil</td>
<td>Levi [13]</td>
<td>B</td>
<td>HIV</td>
<td>In-house</td>
<td>MP6</td>
<td>483</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Mexico</td>
<td>Chiquete [20]</td>
<td>D/F</td>
<td>HBV</td>
<td>In-house</td>
<td>ID</td>
<td>100</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mexico</td>
<td>Chiquete [20]</td>
<td>I</td>
<td>HBV</td>
<td>In-house</td>
<td>ID</td>
<td>100</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mexico</td>
<td>Garcia-Montalvo [26]</td>
<td></td>
<td>HBV</td>
<td>In-house</td>
<td>ID</td>
<td>11,240</td>
<td>&gt;13</td>
<td>&lt; 1:865</td>
<td>&lt; 1:865</td>
<td>1</td>
</tr>
<tr>
<td>Lebanon</td>
<td>El-Zaatari [27]</td>
<td>D</td>
<td>HBV</td>
<td>In-house</td>
<td>ID</td>
<td>5111</td>
<td>11</td>
<td>1:501</td>
<td>1:501</td>
<td>0</td>
</tr>
<tr>
<td>Lebanon</td>
<td>Ramia [28]</td>
<td>D</td>
<td>HBV</td>
<td>In-house</td>
<td>ID</td>
<td>2505</td>
<td>7</td>
<td>1:358</td>
<td>1:358</td>
<td>0</td>
</tr>
<tr>
<td>Mongolia</td>
<td>Tatsaralt-Od [29]</td>
<td>D</td>
<td>HBV</td>
<td>In-house</td>
<td>ID</td>
<td>403</td>
<td>5</td>
<td>1:81</td>
<td>1:81</td>
<td>0</td>
</tr>
<tr>
<td>China</td>
<td>Ren [30]</td>
<td>B/C</td>
<td>HBV</td>
<td>S201</td>
<td>MP6</td>
<td>14,305</td>
<td>10</td>
<td>1:1430</td>
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<tr>
<td>Malaysia</td>
<td>Lam [31]</td>
<td>B/C</td>
<td>HBV</td>
<td>Ultrio</td>
<td>ID</td>
<td>5354</td>
<td>12</td>
<td>1:3616</td>
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</tr>
<tr>
<td>India</td>
<td>Makroo [7]</td>
<td>D</td>
<td>HBV</td>
<td>Ultrio</td>
<td>ID</td>
<td>12,224</td>
<td>6</td>
<td>1:2037</td>
<td>1:2037</td>
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<tr>
<td>Pakistan</td>
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<td>1/3</td>
<td>HCV</td>
<td>Ultrio</td>
<td>ID</td>
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<td>2</td>
<td>1:6112</td>
<td>1:6112</td>
<td>1</td>
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<tr>
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<td>Behzad-Beihabani [6]</td>
<td>D</td>
<td>HBV</td>
<td>In-house</td>
<td>ID</td>
<td>24,674</td>
<td>&gt;40</td>
<td>&lt; 1:617</td>
<td>&lt; 1:617</td>
<td>1</td>
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<tr>
<td>Kuwait</td>
<td>Al Radwan [9]</td>
<td>D</td>
<td>HBV</td>
<td>In-house</td>
<td>ID</td>
<td>121,384</td>
<td>5</td>
<td>1:24,275</td>
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<tr>
<td>Pakistan</td>
<td>Bharti [31]</td>
<td>D</td>
<td>HBV</td>
<td>In-house</td>
<td>ID</td>
<td>966</td>
<td>5</td>
<td>1:193</td>
<td>1:193</td>
<td>0</td>
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<tr>
<td>Mozambique</td>
<td>Cunha [32]</td>
<td>A1</td>
<td>HBV</td>
<td>In-house</td>
<td>ID</td>
<td>1577</td>
<td>23</td>
<td>1:69</td>
<td>1:69</td>
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techniques without sample pooling. In the 75-month period of serologic screening, HBsAg was detected in 219 donors (0.23%; 95% CI, 0.20–0.26%) and anti-HCV antibodies in 922 (0.97%; 95% CI, 0.90–1.03%). The annual trend for HBsAg prevalence had a significantly decreasing pattern over the years, whereas that for anti-HCV did not. In the molecular screening of seronegative samples, HBV DNA was detected in one donor (1%) and HCV RNA in another (1%). Conclusion was that NAT can detect cases of HBV and HCV infections that standard immunoassay techniques cannot, even in a highly selected population such as blood donors. Large-scale studies are warranted for NAT to be considered as a method for routine screening of HBV and HCV in Mexican blood banks [21].

As shown in Table 2, a relatively large number of preliminary studies of NAT applied to blood donors in developing and high prevalence countries have been published. Most studies targeted HBV because of its high prevalence in many areas. The frequency of HBV DNA in HBsAg seronegative blood donations was as high as 1:69 in Mozambique. These numbers however do not distinguish between different donor populations, particularly between repeat and first-time donors. Only three studies addressed HIV or HCV NAT testing and yield was generally low.

While most reports on NAT blood screening in resource-limited countries have focused on the NAT yield in serology negative blood donors, there were no studies comparing the possible three scenarios of NAT only yield, NAT/serology concordant yields and serology only yield in a cohort of blood donors. A recent study conducted in Shabwari Blood Transfusion Center, Cairo, Egypt compared the NAT (Procleix Ultrio ID_NAT on Tigris, Chiron, Novartis) and serology (CLIA on Architect, Abbott) yields by screening 15,655 first-time blood donors for both. While HIV yield was identical by both NAT and serology, there were differences in the 23 NAT-negative carriers was 3.4 cps/mL (<1–230) and 22 ng/mL (0.3–6253), respectively. Sequencing indicated all samples to be wild type genotype D. The sensitivity of Q-PCR, ULTRIO Plus and Taqscreen was similar. The estimated risk of HBV transmission by ULTRIO Plus screened RBCs was reduced to 0–13% in 17/23 donations with viral load <25 cps/mL [22].

In the same population of first-time donors 701 (4.5%) were reactive to either HBV antibody or RNA. HBV Ab and ID-NAT reactivity of 444 samples (63.3%) were concordant, five samples (0.7%) were only ID-NAT reactive and probably WP infections and 252 (35.9%) were anti-HCV CLIA reactive, but ID-NAT non-reactive of whom 130 (18.5%) had anti-HCV S/CO > 2.0. For red cell transfusions of first-time Egyptian donors the residual HCV transmission risk was estimated to be 1:3100 or 1:89,800 if anti-HCV CLIA or ID-NAT alone was used, respectively. The residual risk after combined anti-HCV screening and ID-NAT was estimated at 1:150,000 [23].

10. Discussion and conclusions

Reports from developed countries have shown the limited value of NAT blood screening in improving blood safety. The Scottish BTS reported a NAT yield rate for HIV and HCV of 1 per 1.9 and 0.77 million donations [18]. Reports on NAT yield of screening 3.6 million blood donations from continental Europe for HBV, HCV and HIV-1 were 1 per 0.6 million donations for HBV, HCV and 1 per 1.9 million for HIV-1 [24,25]. This is primarily due to the low prevalence of HIV-1, HBV and HCV in these countries. In contrast to this, the prevalence of these viral infections in resource-limited countries is generally high. In these circumstances, more incident cases can be expected among first-time blood donors. Most of the reports of NAT screening in these countries showed NAT yield cases as high as 1:60 blood donation [15] for HBV and 1:3100 blood donations for HCV [23]. Also of importance in the consideration of NAT blood screening in resource-limited areas is the importance of assessing infectivity for HCV and HBV [22,23]. While these reports and their projections suggest to support recommending the use of the NAT blood screening in resource-limited countries, there are many critical elements to carefully evaluate before any recommendation can be made. The level of development of the blood services in these countries and their ability to adapt this complicated and expensive technology as well as its integration in the general blood service is very challenging. Most importantly, cost/effectiveness and affordability of implementation are paramount in making such decision.

References


