Original Article

Half a decade of mini-pool nucleic acid testing: Cost-effective way for improving blood safety in India

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Abstract

Background and Objectives: It is well established that Nucleic acid testing (NAT) reduces window phase of transfusion transmissible infections (TTI) and helps improve blood safety. NAT testing can be done individually or in pools. The objectives of this study were to determine the utility, feasibility and cost effectiveness of an in-house minipool-NAT(MP-NAT). **Materials and Methods:** Blood donors were screened by history, tested by ELISA and sero-negative samples were subjected to an in-house NAT by using reverse transcriptase-polymerase chain reaction (RT-PCR). Testing was done in mini-pools of size eight (8). Positive pools were repeated with individual samples. **Results:** During the study period of Oct 2005-Sept 2010 (5 years) all blood donors (n=53729) were screened by ELISA. Of which 469 (0.87%) were positive for HIV-1, HBV or HCV. Sero-negative samples (n=53260) were screened by in-house MP-NAT. HIV-NAT yield was 1/53260 (n=1) and HBV NAT yield (n=2) was 1/26630. **Conclusion:** NAT yield was lower than other India studies possibly due to the lower sero-reactivity amongst our donors. Nevertheless it intercepted 9 lives including the components prepared. The in-house assay met our objective of improving blood safety at nominal cost and showed that it is feasible to set up small molecular biology units in medium-large sized blood banks and deliver blood within 24-48 hours. The utility of NAT (NAT yield) will vary based on the donor population, the type of serological test used, the nature of kit employed and the sensitivity of NAT test used. The limitations of our in-house MP-NAT consisted of stringent sample preparation requirements, with labor and time involved. The benefits of our MP-NAT were that it acted as a second level of check for ELISA tests, was relatively inexpensive compared to ID-NAT and did not need sophisticated equipment.

Key words:

Blood donor testing, blood safety, in house assay, mini-pool nucleic acid testing, real-time-polymerase chain reaction

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Introduction

It has long been known that nucleic acid testing (NAT) reduces the window phase of transfusion transmissible infections (TTIs) and helps improve blood safety.[1] Many transfusion centers around the world have introduced mini-pool (MP) NAT to reduce the risk of Hepatitis B virus (HBV), Hepatitis C virus (HCV), and Human Immunodeficiency virus (HIV) transmission by blood donations made in the infectious window phase.[2]

The introduction of NAT for screening pooled or individual donations has led to improved blood safety. The size of MP NAT is considered critical for identification of HIV-1-infected donors, during the preseroconversion phase of infection. A very small size of the pool helps greater reduction in the serological window phase.[3] However, the feasibility of NAT for a developing country like India or its application to Indian blood transfusion service has been a topic of debate. To the best of our knowledge, this is one of the largest reports of TTI screening by mini-pool NAT in India till date.

The risk of viral infection is lower today than ever before, thanks to improvements in donor screening and testing practices. NAT has lowered this risk even further in few centers where this has been adopted. However, this additional benefit comes at additional cost to the health care system.[4]

Most reports from high prevalence low resource countries showed a yield as high as 1/2800 for HBV and 1/3100 blood donations for HCV with NAT testing.[5]

The limiting factors[6] for implementation of NAT in India relate to infrastructure, staff, and cost. NAT requires three separate rooms for reagent preparation, sample handling, and amplification to prevent cross contamination. Staff working in transfusion service is alien to molecular biology techniques and needs to be trained. High cost of available assays-INR 750-1000(US $ 15-20) compared to INR 150-300 (US $ 3-6) for serology [enzyme-linked immunosorbent assay (ELISA)] is another limiting factor.

The seroprevalence of HIV, HBV, and HCV has shown a wide variation in various parts of the country.[7-10] HCV has a longer window period (80 days) than HBV (56 days) and HIV (16 days). Although HCV has been the primary focus of NAT

testing, subsequently many centers around the world chose to include HIV along with HCV as the two were both RNA viruses and both could be tested together without a major cost increment. However, subsequently HBV NAT has been added to HIV/HCV MP-NAT and the major companies of NAT systems (Roche and Novartis-Chiron/Gen-Probe) have been developing triplex assays including all the three markers. Considering the widespread prevalence of HBV in India,[7] our in-house assay was developed and implemented to include all three markers-HIV-RNA, HCV-RNA, and HBV-DNA for routine blood donor screening.

Our transfusion services began blood donor screening by an in-house MP-NAT in Oct 2005.

The primary objective of our study was to determine utility, feasibility, and cost-effectiveness of MP-NAT for blood donor screening in India.

Materials and Methods

All blood donors both in the blood center and in camps were counseled one to one, using donor questionnaire, and direct questioning, that elicited high risk sexual behavior. All eligible donors who donated blood during the period Oct 2005-Sept 2010 (n = 53729) were tested by serology (ELISA) using Qualisa kits from Tulip Diagnostics. All samples testing negative by ELISA (n = 53260) were tested by using an in-house MP-NAT within 24-48 h of serological testing. MP-NAT developed was a multiplex assay for HIV-1, HBV, and HCV. Thus, all seronegative samples (n = 53260) were subjected to MP-NAT in small pools of eight (8) samples. When there were fewer than 8 samples, testing was still carried out with a reduced pool size to avoid delays in issue of blood. In case of a positive MP, individual samples were retested and all the bags in the pool were quarantined. To ensure that sensitivity is not greatly compromised by pooling and also to avoid delays due to retesting of positive pools, we restricted the donor pool size to eight.

Pooling of samples

Pilot ethylenediaminetetraacetic acid samples were collected at the time of blood donation and stored at ambient temperature. Plasma (500 uL) for pooling was separated within 4 h of blood collection by centrifugation. Pooling of samples was done using separate aerosol tips (certified free from RNase, DNase, and pyrogen) under the laminar hood using aseptic precautions and stored at –20°C.

Viral extraction

Viral RNA/DNA was extracted using QIAGEN extraction kit. (QIAmp MinElute Virus spin Kit cat No.57704). Viral RNA/DNA was extracted from the pooled sample tube using the MinElute Virus Spin procedure comprising of four steps-lyses, bind, wash, and elute. Lysis was accomplished by using heat denaturation combined with enzyme protease in the viral extraction kit.

Reverse transcription

Reverse transcriptase was used to produce a complementary DNA copy (cDNA) of the target RNA viruses-HIV and HCV. Reverse transcriptase was from Qiagen [one-step real-time polymerase chain reaction (PCR) kit Cat Nos 210210, 210212 and 210215].

Amplification

Recombinant Taq DNA Polymerase from Thermo Scientific (Cat no EP0402) was used for amplification along with nucleotide primers from Sigma Life Sciences. Thermal cyclers (Peltier Thermal cycler Model 1196, MJ Research, Biorad) were used for amplification.

Detection

The amplified product was detected using agarose gel electrophoresis and documented using a gel doc (TGM1717, CBS Scientific company).

Results

Nearly 53,729 samples were screened for HIV, HBV, and HCV by ELISA. Of these 469 samples were seroreactive for HIV, HBV, or HCV. Samples that were seronegative by ELISA (n = 53260) were tested by MP-NAT. NAT testing showed three (3) positive pools in the MP NAT; one of which was HIV and the remaining two were HBV. Occasionally seroreactive samples were included in the pool as part of quality control. However, these pools have been excluded from the study. Whenever the MP was positive, the reactive sample was identified by testing individual samples. Due to small size of the pool (n = 8), this was not a problem. No discriminatory assay was needed as the multiplex assay gave three separate bands-one each for HBV, HCV, and HIV. The HIV band corresponds to 129 bp (using Pol primer), HCV to 162 bp (using 5'UTR primer), and HBV band is at 272 bp corresponding to the core region of HBV as shown in Figure 1. Results of blood donor testing by ELISA and MP NAT are shown in Table 1. The NAT yield (Seronegative/NAT reactive) was 3 out of 53,729 donors screened. On the basis of the results, it is evident that MP NAT was able to detect one HIV and two HBV infections that went undetected by ELISA. Thus, 469 (0.87%) samples tested reactive by ELISA and an additional 3 (0.006%) were NAT reactive.

Discussion

Mandatory MP-NAT that has been in use in Germany since 1996 and the assays used have a minimal sensitivity limit of 5000 IU HCV RNA/mL and 10,000 IU HIV-1 RNA/mL, where a pool size of 96 was employed.[11] Subsequently, many countries including Japan and Germany added HBV NAT to HIV/HCV MP-NAT.[12]

This in-house PCR assay we developed included all three markers from day 1 and was validated using samples confirmed positive for HIV-1, HBV, or HCV. To determine sensitivity, log dilutions of samples with known viral loads were run. The sensitivity of the assay was standardized to detect about 100 HIV copies/mL

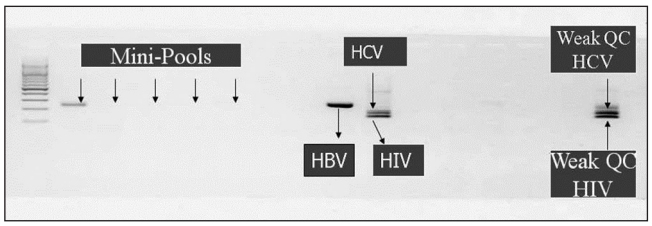

Figure 1: Multiplex minipool nucleic acid testing: Detection by agarose gel electrophoresis

(200 IU/mL), 100 HBV copies/mL (18 IU/mL), and nearly 170 HCV copies/mL (63 IU/mL). Repeatability of results by the same technologist and also a different technologist using a different PCR was ensured by interlab comparisons. Reactive samples were included in the pool for validation of assay as part of quality control. Commercially available panels were not used due to the prohibitive costs. The reagent cost per test (per donor cost) by use of this in-house MP NAT was in the range of INR150-200 (3-4 US$).

Although the MP NAT assay picked up only one HIV infection and two HBV infections out of 53,260 donor samples screened, it intercepted thrice the number of infections in recipients taking into account the components prepared from these units. We were, thus, able to intercept three HIV infections and six HBV infections in patients. Our assay did not pick up any HCV infection possibly because the seroreactivity among our blood donors for HCV was very low (0.09%).

We compared our data with other data available as shown in Table 2. Our study shows a NAT yield of 1:53260 for HIV and 1:26630 for HBV. The lower NAT yield compared to Makroo *et al.*, study and Jain *et al.*, study[10] is probably on account of the lower seroreactivity rate in our study (0.87%) as opposed to 1.71% and 2.62% in Makroo *et al.*, and Jain *et al.*, study , respectively. *P* value comparing our data with Makroo *et al.*, study using chi square with yates correction (chi square 18 with one degree of freedom) was found to be less than 0.0001 and comparison with Jain *et al.*, study gave a *P* value equal to 0.007 (chi square with Yates correction 7 with one degree of freedom) showing that the comparison was statistically significant. Stringent donor screening measures may also have contributed to lower seroreactivity and consequently lower NAT yield in our study.

The utility of NAT (NAT yield) will vary based on the donor population, the type of serological test employed, the nature of the kit employed and also based on the sensitivity of NAT test employed. Patterns of infections among blood donors in our country also vary widely and TTIs continue to be a threat to safe transfusion practices.[8] TTIs is still a major concern to patients, physicians, and policy makers who seek a risk-free blood supply. Results of NAT testing vary significantly based on these factors.

While studies in developing countries have shown high NAT yields,[5,9] as high as 1/2800 for HBV and 1/3100 blood donations for HCV, other studies in developed countries of Central Europe[13] have shown that yield of NAT using sensitive MP-NAT assays is less than expected. After screening 3.6 million donations for HCV and HIV, the NAT yield was found to be 1:600,000 for HCV-RNA and 1 in 1.8 million for HIV-RNA. Another large study in USA[14] screened 66 million donations by NAT over a 10-year period beginning in 1999 and identified additional 32 HIV cases (NAT yield for HIV 1:2 million) and another 244 HCV cases (NAT yield for HCV 1:270,000).

The limitations of our in-house MP-NAT consisted of stringent sample preparation, labor, and time involved in sample processing and testing which was approximately 5-6 h. Training and skill were critical to optimum assay performance. Further, it is felt that employing viral concentration techniques in case of home brews, such as ours, will further enhance the NAT yield and consequently the utility of NAT.

The benefits of our MP-NAT were that it acted as a second level of check for ELISA tests, it was relatively inexpensive compared to ID-NAT, did not need sophisticated equipment, and was as easy to perform as ELISA after appropriate training. We were able to complete MP-NAT within 24-48 h and the additional NAT testing did not significantly upset the logistics of blood supply.

What this study demonstrates unequivocally is the feasibility and cost-effectiveness of MP-NAT as a cost-effective adjunct to ELISA. As against the commercial assays which cost 750-1000 Indian rupees, the cost of in-house assays is about 150-200 Indian rupees (3-4 US$), roughly the same as the cost of testing by ELISA. However, while designing a MP NAT it is important to factor in the sample size, the additional QC samples to be run with every assay, the cost of repeat testing of positive pools and not merely the reagent cost.

It is also evident that the benefits of NAT testing multiply as we split blood into more and more components. Studies have shown that addition of NAT tests to blood donor screening, will reduce residual risk further by up to 50%, depending on the sensitivity of the NAT assay and whether individual or pooled blood donations are screened.[15]

Conclusion

The in-house assay met our objectives of improving blood safety with only a nominal cost increment of INR150-200 (2-3 US $) without the logistic problems like delay in issues due to additional testing as the testing was carried out at the blood center within 24-48 h of blood collection. This study shows that it is feasible to set up a small molecular biology unit for MP NAT testing in most medium/large size blood banks. However, technical improvements and newer developments in immunology have greatly enhanced the reliability

Table 1: Blood donor screening results

Blood donor testing	Sample size	HIV	HBV	HCV	Cumulative TTI
Reactive by serology (ELISA)	53729	56 (0.1%)	366 (0.68%)	47 (0.09%)	469 (0.87%)
Reactive by MP-NAT	53260	*n*=1 (0.002%)	*n*=2 (0.004%	*n*=0	*n*=3 (0.006%)

TTI = Transfusion transmissible infections, MP-NAT = Minipool NAT

Table 2: Comparison of in-house minipool-nucleic acid testing with other nucleic acid testing studies (India)

NAT	Sample Size	Seroreactive	NAT reactive			NAT yield*
			HIV	HBV	HCV	(Total)
Makroo *et al.*, (ID NAT)[9]	12224	1.71%	2	6	1	*n*=8 (1/1528)
Jain *et al.*, (MP-NAT)[10]	23779**	2.62%	0	8	0	*n*=8 (1/2972)
Present study (in-house MP-NAT)	53260	0.87%	1	2	0	*n*=3 (1/17753)

*NAT yield = Reactive by NAT only but negative by serology; **In Jain *et al.*, Study serology sample size was 47558 and NAT was done on half these units (*n* = 23779)

of serological assays and contributed to a reduction in the window period for TTIs (HIV). They cannot be given up. It is, therefore, felt that MP NAT combined with serology may be a cost-effective adjunct in improving blood safety for resource constrained countries like India. Hence, it is felt that MP NAT could possible take us one step closer to blood safety. What we probably need are more of such assays and better viral concentration techniques for improving blood safety.

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