

## ORIGINAL ARTICLE

# Multiplex real-time RT-PCR assay for transfusion transmitted viruses in sero-negative allogeneic blood donors: an experience from Southern Pakistan

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### Abstract

**Background:** Blood transfusion safety commences with healthy donor recruitment. The threat of transfusion transmitted infections is greatly minimized by serological tools but not entirely eliminated. Recently, nucleic-acid testing for blood donor screening has virtually eliminated this jeopardy. **Methods:** This prospective study was conducted from February 2015 to February 2016. Samples from seronegative donors were run on multiplex assay (Cobas, S-201 system platform, Roche) in a batch of six [MP-NAT]. In case of reactive pool, tests were run on every individual sample [ID-NAT]. **Results:** Of 16957 donors, 16836 (99.2%) were replacement donors and the remaining 121 (0.7%) were voluntary donors, with a mean age of  $29.09 \pm 7.04$  years. After serologic screening of all 16957 donors, 955 (5.6%) were found to be reactive; 291(1.71%) were reactive for hepatitis-B surface antigen, 361 (2.12%) for antibody to hepatitis C virus (anti-HCV), 14 (0.08%) for antibody to human immunodeficiency virus, 287 (1.69%) for syphilis and 2 (0.01%) for malaria. 14 (0.08%) NAT reactive donors were identified after testing the 16002 seronegative donors, with an overall NAT yield of one reactivity out of 1143 blood donations; 10 donors for HBV-DNA (HBV NAT yield-1:1600) and remaining 4 for HCV-RNA (HCV-NAT yield-1:4000). None were HIV positive. **Conclusion:** NAT has improved the safety attributes in blood products. Although the positivity rate for NAT testing is low but in view of the high prevalence of transfusion transmitted infections in our country, we recommend the parallel use of both serology and NAT screening of all donated blood.

**Keywords:** blood donors, sero-negative, NAT, Pakistan

## INTRODUCTION

Blood safety is a challenging task for a population of over 198 million in the World's sixth most populous country, with limited resources and a high prevalence of hepatitis B (HBV) and hepatitis C (HCV) infections. In Pakistan, the sero-prevalence of HCV, HBV and HIV in blood donors is estimated at 2.6, 1.9 and 0.11% respectively, compared to 0.3, 0.07 and 0.0097% in USA blood donors respectively.<sup>1-3</sup> Healthcare services are not ample and overwhelmed with financial and management constraints. Presently, the blood transfusion services in Pakistan are fragmented and not synchronized, with insufficient regulatory monitoring.<sup>4</sup> Our blood banks are still reliant on family donors and in most centers, testing for transfusion transmitted

infections (TTI) is not quality effective, equipment are neither calibrated nor maintained and results validation is not ensured.

Despite the use of newest generation serological testing of high sensitivities, a considerable residual risk of TTI remains there. Although these tests have shortened the window period, they are still not able to detect a newly infected blood donor.<sup>3</sup> Taking into consideration the high prevalence of TTI in blood donors, there is a need for additional safety measures to minimize the risk of transmission of these infections. This led to the commencement of Nucleic Acid Amplification Testing (NAT) in late 1990s for screening of all sero-negative blood donors. NAT implementation has offered significant advantages for detection of TTI

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during the window period, thus reducing the risk of transmission.<sup>5</sup> NAT donors screening is not obligatory in Pakistan and currently only few blood transfusion centers have voluntarily commenced NAT to further improve on patient safety. There are scanty national studies reported indicating the NAT yields of TTI in healthy blood donors.<sup>6,7</sup> Here we report the preliminary results over 1 year of NAT testing for TTI in allogeneic healthy blood donors in southern Pakistan. The aim of our study was to determine the rate of NAT yield for TTI infections, its spectrum and test specificity.

## MATERIALS AND METHODS

### *Setting*

This prospective cross-sectional descriptive study, extending from February 2015 to February 2016 was conducted at the Liaquat National Hospital & Medical College, Pakistan, which is an over 700-bed tertiary care teaching institute. The hospital caters to a population of about 4 million people. The blood bank comprises of experienced faculty, well-trained technicians and state of the art equipment. The blood bank collects about 17,000 blood units annually. Whole blood units are fractionated to components for 100% of units collected.

### *Study subjects*

Donor's demographical data comprising name, age, gender, contact number and type of donor (replacement or voluntary) were entered into a structured standard questionnaire. All donors were elected according to pre-established defined inclusion criteria consisting of age ( $\geq 18$  years), body weight ( $\geq 50$  kg), hemoglobin level ( $\geq 12.5$  g/dl), hematocrit level (PCV)  $\geq 38\%$ , pulse rate (50–100 beats/min) and with normal blood pressure. Donors were interrogated about medical and prior donation history. Donors with high risk behaviour, male homosexual, extra-marital sexual contact, history of jaundice in the past 1 year, drug abusers, tattooing, recent blood transfusion or recent surgery were deferred from donation. Written consent was obtained from all qualifying donors.

### *Serological testing*

Venous blood samples were collected in 6-mL clot activator tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) from all donors. Tubes were centrifuged for 10 minutes at 8000 rpm. All samples were tested for anti-HIV-1/2 (Combo kit), hepatitis C virus antibody (anti-

HCV), hepatitis B surface antigen (HbsAg) and antibodies to treponema pallidum (TP) by Chemiluminescent Immunoassay (CIA) method on Automated analyzer, Architect i2000 SR (Abbott Diagnostic, USA). Samples were run in batches, with positive and negative controls run simultaneously to validate the results. MP-ICT was used to determine the presence of malaria in all donors.

### *NAT testing protocol*

NAT testing was performed in collaboration with the Department of Molecular Pathology. Exclusively seronegative blood donor samples were subjected to screening for viral genome by NAT by multiplex polymerase chain reaction (PCR) on a 6 unit minipool (MP). This pool size was selected based on previous studies reported from Pakistan and from the studies conducted in highly endemic regions for viral infections.<sup>6-8</sup>

4 ml venous blood samples were collected in Ampulab EDTA tubes which were coated with K3-EDTA (Soyagreen Tec vacutainer tubes). Samples were spun on Thermo Heraeus Mega fuge 8 centrifuge for 15 minutes at 4000 rpm. Automated specimen pooling and all 4 cobas Taq Screen MPX controls pipetting was accomplished by using Hamilton MICROLAB STAR IVD pipettor. NAT screening was performed on a fully automated Roche cobas s 201 system using a multiplex polymerase chain reaction kit, (cobas Taq Screen MPX test, version 2.0, Roche Molecular Systems, Branchburg, NJ).

Viral RNA/DNA isolation and amplification from pooled samples and all four controls were carried out using Roche cobas® Taq Screen MPX Test, v2.0 kit and fully automated Ampliprep module. Roche cobas® Taq Screen MPX, v2.0 was used for the direct detection of Human Immunodeficiency Virus Type 1 (HIV-1) Group M RNA, HIV-1 Group O RNA, Human Immunodeficiency Virus Type 2 (HIV-2) RNA, Hepatitis C Virus (HCV) RNA and Hepatitis B Virus (HBV) DNA in plasma. The assay incorporates an internal control for monitoring test performance in each individual test.

### *Individual-NAT*

In case of a reactive pool results, resolution of the pool was done by individual NAT (ID-NAT) format to identify the reactive donors on the same cobas s 201 system and also identify the individual virus in test samples. All the reactive donors were called, informed of their test results and prohibited from future blood donations and

were advised to visit a gastroenterologist for further management.

#### *Ethical approval*

This study was approved by the Institutional Research Ethical Committee, LNH (0337-2015 LNH-ERC) on 19 January 2015.

#### *Statistical analysis*

Data collected was recorded on Microsoft spreadsheet and later statistical analyses were carried out using IBM statistics SPSS version 22. Results were reported as the mean ( $\pm$  SD) for quantitative variables. Frequency and percentages were calculated for qualitative variables including gender, type of donor and positivity rate.

## RESULTS

#### *Demographic profile*

A total of 18448 blood donors reported to our blood bank for donation, of which 1491 (8.08%) were deferred as per standard criteria. The remaining 16957 blood donors were enrolled over a period of one year. Of these, 16836 (99.2%) were replacement donors and the remaining 121 (0.7%) were voluntary donors. Almost all donors [ $n = 16883$  (99.5%)] were male and only 74 (0.4%) were female. The study population had a mean age of  $29.09 \pm 7.04$  (18-65) years with a median age of 28 years.

#### *Sero-prevalence of TTI*

After serologic screening, 955 (5.6%) were found to be reactive; 291 (1.71%) were reactive for hepatitis B surface antigen, 361 (2.12%) for antibody to hepatitis C virus (anti-HCV), 14 (0.08%) for antibody to human immunodeficiency virus, 287 (1.69%) for syphilis and 2 (0.01%) for malaria.

#### *NAT testing*

All 16002 sero-negative donors were tested. 2767 test pools were analyzed, which yielded 21 reactive minipool (MP). On pool resolution, 7 MPs were NAT nonreactive on the individual donor samples. These pools were labeled as false positive. The overall false positivity rate of NAT for the total (16002) donations tested was 0.04% with a specificity of 99.95%.

14 (0.08%) NAT reactive donors were confirmed after testing 16002 seronegative donors, with an overall NAT yield of one out of 1143 blood donations; 10 donors for HBV-DNA (HBV NAT yield, 1:1600) and remaining 4 were

HCV RNA positive (HCV NAT yield, 1:4000) as shown in Table 1. None were positive for HIV.

## DISCUSSION

Our study showed that virtually all donors were males (99.5%) with a high sero-prevalence of 5.6% for transfusion transmitted infections. The reported positivity rate of TTIs and the male predominance are consistent with previously published studies from the country.<sup>9,10</sup> With regards sero-prevalence, the rate was highest for hepatitis C (2.1%) followed by hepatitis B (1.7%) and syphilis (1.6%) respectively in our series. One recent meta-analysis on Pakistani blood donors also revealed similar serological results which showed a higher prevalence of 2.8% for HCV versus 2.3% for HBsAg in the donor population.<sup>10</sup> The plausible explanation for a lower prevalence of HBsAg in donors may partly be the result of hepatitis B immunization, which became applicable since 2002, as a part of an expanded program of immunization in Pakistan and partly due to increased awareness among general public and health care workers about disease transmission. Distinctly the prevalence of HIV infection (0.08%) was least encountered amongst the various infectious pathogens. A relatively high prevalence of HIV (0.11%) was reported in a recent local study observing donor serology over a decade.<sup>2</sup> Recently, Pakistan has been ranked as a "concentrated epidemic" for HIV in high risk groups as per UNAIDS.<sup>11</sup> Relatively unsafe sexual practices, decline in the use of protective measures, lack of awareness and unscreened infectious transmission may be the causative factors in progressive disease spread.

The crucial role of NAT to ascertain safe blood supply is evident from the results of various studies.<sup>12</sup> NAT accomplishment in our institution led to prohibition of 14 sero-negative donations (10 HBV and 4 HCV positive) during the 1 year duration, consequently increasing the safety index in blood recipients. In practical terms, considering that a single donation is used for 3 components that may be given to 3 recipients, for each unsafe donation prevented, 42 blood recipients were saved. This value, when translated into donations per million, translates to 2624.6 reactive units prohibited from being transfused.

The NAT yield (1 in 1143) in our study is intermediate compared with previous published studies from southern (1 in 845 donation) and northern (1 in 2016 donations) Pakistan.<sup>6,7</sup> Our results showed that NAT yield was highest for

**TABLE 1: Parameters of reactive donors on NAT testing**

S.#	Donor ID	Age	Gender	NAT results			Serology testing		
				HBV	HCV	HIV	HBsAg	Anti-HCV	Anti-HIV
1	4829	28	M	Reactive	Non-reactive	Non-reactive	0.23	0.45	0.07
2	4961	35	M	Reactive	Non-reactive	Non-reactive	0.23	0.38	0.06
3	6351	45	M	Non-reactive	Reactive	Non-reactive	0.15	0.25	0.04
4	10779	20	M	Reactive	Non-reactive	Non-reactive	0.18	0.1	0.16
5	11586	24	M	Reactive	Non-reactive	Non-reactive	0.22	0.08	0.39
6	12194	24	M	Non-reactive	Reactive	Non-reactive	0.17	0.14	0.07
7	14066	34	M	Reactive	Non-reactive	Non-reactive	0.16	0.07	0.08
8	14803	23	M	Reactive	Non-reactive	Non-reactive	0.18	0.04	0.13
9	16749	27	M	Reactive	Non-reactive	Non-reactive	0.2	0.06	0.18
10	16759	32	M	Non-reactive	Reactive	Non-reactive	0.21	0.05	0.17
11	16814	39	M	Reactive	Non-reactive	Non-reactive	0.19	0.06	0.07
12	2779	23	M	Reactive	Non-reactive	Non-reactive	0.24	0.07	0.23
13	3848	40	M	Reactive	Non-reactive	Non-reactive	0.2	0.09	0.12
14	3949	26	M	Non-reactive	Reactive	Non-reactive	0.16	0.08	0.07

HBV followed by HCV genome in Pakistan. One multicenter study reported from neighbouring India has also shown similar findings.<sup>13</sup> However, a Chinese group reported a relatively higher yield for HBV, 1 out of 1056 donors (169 of total 178,447 donations), which is a reflection of a high prevalence of hepatitis B in their general population.<sup>14</sup> Correspondingly, a high yielder for NAT have also been regularly reported from other Asian countries.<sup>8,15</sup>

Similarly, regional reports were available from neighboring countries, India and Turkey. Kosan *et al*, from Turkey, reported 13 cases of 17,792 donations (NAT yield 0.06%), of which 11 were HBV and 2 were HCV reactive.<sup>16</sup> Another

multicenter study from India also reported a NAT yield of 0.06% with 6 HBV cases and 1 each for HCV and HIV reactivity.<sup>13</sup> Our HBV yield (1 in 1600) is more or less similar than that reported in a study from China that found NAT yield of 1 in 1104 donations.<sup>17</sup> A comparison with regional studies is shown in Table 2.

In contrast, in the developed countries, NAT yields for HBV and HCV are reasonably low owing to low prevalence of TTI in their population. We detected the NAT yield of 1 in 1600 for HBV, and 1 in 4000 for HCV which is much higher than that reported from developed countries such as the USA (1/1.6 million units for HCV; 1/260,000 for HBV), Italy (1.8 per

**TABLE 2: Comparison of NAT yield with regional studies**

Country	Author	Year	Tested units	Unit positive	NAT yield (%)	Positivity rate
India	Makroo <i>et al</i> <sup>13</sup>	2008	12,015	8	0.06	1 in 1,528
Turkey	Kosan <i>et al</i> <sup>16</sup>	2010	17,792	13	0.06	1 in 1,384
India	Jain <i>et al</i> <sup>27</sup>	2012	23,779	8	0.03	1 in 2,972
Pakistan	Moiz <i>et al</i> <sup>7</sup>	2014	41,304	49	0.12	1 in 845
China	Dong <i>et al</i> <sup>14</sup>	2014	178,447	169	0.09	1 in 1,056
Pakistan	Niazi <i>et al</i> <sup>6</sup>	2015	54,438	27	0.04	1 in 2,016
Pakistan	Present study	2016	16,002	14	0.08	1 in 1,143

million for HIV: 1/17,301 HBV: 1:400,000 HCV), Germany (1/10.8 million for HCV; 1/360,000 for HBV), Scotland (1/1.9 million HCV:1 in 0.77 million for HIV), Canada (1/13 million for HCV) and Japan (1/51,988 for HBV; 1/348,091 for HCV).<sup>18-23</sup> The decisive reason for this variance seems to be the high prevalence of HCV and HBV and lesser number of voluntary donations in the developing nations. These findings illuminate that NAT screening is more pertinent rather than mandatory in the developing countries owing to the higher prevalence of TTIs.

In view of high HCV sero-prevalence in donors, we anticipated higher numbers of HCV genome than the HBV to be identified by NAT. In contrary, we detect 10 HBV positive donors compared to only 4 HCV reactive donors. Our results are in agreement with other Asian studies which shown a higher HBV reactive donors in different countries.<sup>8,13,15</sup> A previous report by Naizi *et al* also disclosed that 23 HBV and 4 HCV NAT reactive donations were detected in Northern Pakistan.<sup>6</sup> On the other hand, a further regional report postulated, higher yield for HCV (n = 27) cases as compared with HBV (n = 7) cases, the study being conducted in Southern Pakistan.<sup>7</sup> The plausible explanation for higher HBV yield are that DNA viruses are more stable than RNA viruses. DNA structure is more stable than RNA, because RNA forms secondary structures. Also RNA is a single stranded molecule, which is susceptible to hydrolysis. However, these differences in findings need further studies for validation and needs also to look for further explanations.

We did not come across any HIV NAT reactive donation among the sero-negative donors during this period. This is credited to a low prevalence of HIV infection in blood donors in Pakistan.<sup>2,24</sup>

The Food and Drug Administration (FDA) has recommended follow-up testing of all NAT reactive donors and it has been performed in some of the previously conducted studies.<sup>25,26</sup> We did not have a follow-up testing of reactive donors. Lack of awareness about TTI, donor ignorance regarding the screening results and predominance of replacement donors who usually come from far away are major hurdles. Though we have recalled them, and advised them to revisit, only two donors responded and follow-up testing showed similar findings.

Our study had certain limitations: small sample size, fewer voluntary donation, very small numbers of female donors and lack of follow-up data. We also did not perform the viral load in reactive cases to establish the disease burden. Despite the limitations, this is the preliminary report stating NAT yield for TTI in Pakistani blood donors. The study can serve as a baseline for designing future prospective studies in the country. The authors dread that NAT yield identified at our blood bank may be lower and underestimated than the actual existing burden in the country. This is in view of the fact that we have stringent donor selection assessment and very vigilant donor screening tools which usually eliminate high risk donors. In view of the high prevalence of TTI in Pakistan, the implementation of NAT is highly recommended at national level in other blood banks across the country. Furthermore, there should be a nationwide structured functional coordinated blood screening program to integrate and ensure maximum blood recipients safety.

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