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Multicenter evaluation of individual donor nucleic acid testing (NAT) for simultaneous detection of human immunodeficiency virus -1 & hepatitis B & C viruses in Indian blood donors

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***Background & objectives*: India has a high prevalence of HIV-1, hapatitis C and B virus (HCV and HBV) in the blood donors but has yet to implement nucleic acid testing (NAT) in blood screening. We undertook a multicentre evaluation of blood donor testing by NAT for simultaneous detection of HIV-1, HBV and HCV in a single tube and also to determine the feasibility of NAT implementation in India's low volume setting.**

***Methods*: A total of 12,224 unlinked samples along with their serological results were obtained from representative eight blood banks in India and were individually manually tested by the Procleix® Ultrio® Assay (Chiron Corp. Emeryville, CA) for simultaneous detection of HIV-1, HCV, and HBV.**

***Results*: Of the 12,224 samples tested, 209 (1.71%) were seroreactive. One hundred thirty three samples (1.09%) were reactive by Ultrio assay, 84 samples were seroreactive but NAT non reactive. There were eight NAT yield cases: 1 HIV, 1 HIV-HCV co-infection, and 6 HBV.**

***Interpretation & conclusions*: Our observed NAT yield for all three viruses was 1 in 1528 (0.065%). We estimate NAT could interdict 3272 infectious donations a year among our approximate 5 million annual donations**.

Key words Blood screening - hepatitis B and C viruses - human immunodeficiency virus 1 - India - nucleic acid testing (NAT)

Human immunodeficiency virus 1 (HIV-1), hepatitis C virus (HCV), and hepatitis B virus (HBV) are easily transmitted through infected blood, thus considerable, successful effort has been devoted in recent years to interdict their transfusion transmission[1]. Currently the seroprevalence of anti-HIV-1, anti-HCV,

and HBsAg in Indian blood donors is 0.5, 0.4, and 1.4 per cent, respectively[2] compared to 0.0097, 0.3, and 0.07 per cent in the US blood donors respectively[3]. In our country, blood collection, testing and distribution are carried out by 2212 licensed blood banks[4] that are operated both privately and by the government and testing for potential transfusion transmitted infections is unsatisfactory and poorly regulated in most blood banks, regardless of their type and location[5]. Different serological tests are used for blood screening. Antibody to hepatitis B core antigen (anti-HBc) is not used as a screening tool, as given the relatively high HBV endemicity rate, it would probably be of limited value. Even after implementing the more sensitive, newest generation of serological tests, a considerable residual risk of transfusion transmission of these viruses remains. Although the more sensitive serological tests have shortened the pre-seroconversion window period, they still are not able to identify a number of newly infected donors[6]. These undetected window period infections are responsible for most of the transfusion transmission of these viruses[3,7]. Other reasons that the serological tests may fail to identify some infected donors include occult infection, viral variants, non-seroconverting or delayed seroconverting carriers, procedural testing errors and false negatives[8-11]. Nucleic acid testing (NAT) is currently used in conjunction with serological tests in the four continents, North America, Europe, Australia and Asia[12]. Although NAT screening cannot completely eliminate the risk of transfusion transmitted infection, it has reduced the risk for HIV-1 and HCV where it has been implemented[1,13]. Japan was the first country to implement routine HBV NAT in addition to HCV and HIV-1 NAT screening and observed a significant reduction in transfusion transmission of this virus as well[14]. South Africa and in a number of EU countries also employ the NAT[15-18]. As a screening tool, individual donor NAT detects infection before serological tests - 10-16 days earlier for HIV-1, 49 - 65 days for HCV, and 25-36 days for HBV[6,17]. In addition, NAT is also useful for determining the incidence of active infection by these viruses in blood donor populations. This knowledge is critical to the successful planning of measures to increase blood safety[6]. As a first step in improving the safety of blood supply, we need to determine the current prevalence of active infection by these viruses in our blood donors. It is also necessary to determine if NAT was feasible in a developing country with a fragmented blood banking system characterized by low donor volumes in each centre. In this study, we tested blood donations from different types of blood collection agencies from geographically diverse regions of India, using the Procleix® Ultrio® Assay (Chiron Corporation, Emeryville, CA. USA) a multiplex NAT, which allows the simultaneous detection of HIV-1, HCV, and HBV in a single tube.

Material & Methods

Study design: Between June 2004 and January 2005 a total of 12,794 samples were obtained from eight blood centres in seven major Indian cities. Of these four centers were from the northern part of the country, two were from western region, and one each was from the eastern and southern part of the country. Also four types of blood centres, stand alone, hospital based, government and non government operated were represented. Only collection agencies in cities with a population of over 1 million were included. The following centres participated in the study: Indraprastha Apollo Hospital Blood Bank (New Delhi), Rotary Blood Bank (New Delhi), Rotary TTK Blood Bank (Bangalore), Prathama Blood Centre (Ahmedabad), Life Care Blood Bank (Kolkata), Post Graduate Institute of Medical Education & Research (PGIMER, Chandigarh), Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS, Lucknow) and Bombay Hospital Blood Bank (Mumbai).

Fifteen centres were approached to take part in this study, but only eight participated.

Donor samples studied: From June 2004 to January 2005 a total of 12,794 samples were collected from consecutive donors in 5 ml Vacutainer Plasma Preparation Tubes (PPT tubes - Becton Dickenson) after normal donor selection screening at the different blood banks. All donors were included, but some samples were excluded on the basis of volume or integrity. Of the 12,794 samples collected, 12,224 were included in the study. Samples were excluded for the following reasons: inadequate sample volume (n=350), no barcode or mislabeled (n=176), indeterminate results (n=22), haemolysis (n=22). All donors gave their informed consent and the study was approved by the Ethics Committee of the Indraprastha Apollo Hospital and by other participants where required. Additional information obtained along with the donations included: demographic data, including donor's age, sex, marital status, and donor status, first time or repeat, and volunteer or replacement (Table I).

Sample handling: Samples were centrifuged within two hours of collection. After obtaining relevant donor

Table I. Demographic description of donors (%)

Sex	Marital status (n= 12,224)		Replacement donors (n= 8, 999)		Volunteer donors (n=3,225)	
	Married (n=7501)	Unmarried (n=4723)	1st time (n=4463)	Repeat (n=4536)	1st time (n=1686)	Repeat (n=1539)
Male (n=11,280)	92	93	93	94	88	92
Female (n=944)	8	7	7	6	12	8

Reported percentages are column percentages

Table II. Serological kits used by different blood banks

Serological kits used	Country of origin	Screening
Micro Elisa J.Mithra & Co.	India	HIV Ab
Abbott Axsym	Germany	HIV 1/2
Ortho HIV ½ Ab Capture Elisa	USA	HIV1/HIV2 Ab
Bio-Rad GENSCREEN plus HIV Ag-Ab	France	HIV Ag-Ab
BioMerieux Vironostika v. 3.0	The Netherland	HIV Ag/Ab
Micro Elisa J.Mithra & Co	India	HCV Ab
Abbott Axsym Anti HCV Version 3.0	Germany	HCV Ab
Abbott Murex anti HCV version 4.0	South Africa	IgG Ab to HCV
Ortho HCV version 3.0	USA	HCV Ab
LG HCD 3.0	Korea	HCV Ab
Abbott AXSYM	Germany	HBs Ag
Ortho Vitros ECI	USA	HBs Ag
Micro Elisa J.Mithra & Co.	India	HBs Ag
Murex HBsAg	South Africa	HBs Ag
Bioelisa v. 3.0	Spain	Hbs Ag
Hepalisa v. 3.0	India	HBs Ag
BioRad MONOLISA Ag HBs Plus	France	HBs Ag
BioMerieux Hepanostika HBsAg Uniform II	The Netherland	HBs Ag
Ortho HBs Ag Test system 3.0	USA	HBs Ag
Abbott Auszyme monoclonal qualitative v. 3.0	Germany	HBs Ag

demographic information and serological results, samples were transported to the Department of Transfusion Medicine, Indraprastha Apollo Hospital, New Delhi, at a temperature of 2-8 ^{0}C and refrigerated immediately on arrival. Samples that were not to be tested within 24 h, were stored at -20 to -25 ^{0}C.

Serological screening: Routine serological screening for HIV antibody, HCV antibody; and hepatitis B surface antigen (HBsAg) was conducted at each blood bank using different ELISA kits (Table II). Ranges of sensitivity for various tests were as follows: HIV antibody (88-99.8%), HIV Ag/Ab (100%), HCV antibody (81-99.9%), and HBs Ag (93.4-100%). For the most part, serological results were sent separately to the Department of Transfusion Medicine at the Apollo Hospital so as to not prejudice the NAT testing. Blood banks followed their regular protocol for release of blood products.

Nucleic acid testing: NAT was conducted at the Department of Transfusion Medicine, Indraprastha Apollo Hospital, New Delhi, using the Procleix® Ultrio® Assay (Chiron Corporation, Emeryville, CA) according to manufacturer's instructions. All samples were tested individually. The Procleix® Ultrio® Assay is a multiplex test which provides simultaneous detection of HIV-1 RNA, HCV RNA and HBV DNA in human plasma using transcription mediated amplification technology (TMA).

The TMA assay involves three main steps utilizing three proprietary technologies: *(i)* target capture based sample preparation, *(ii)* transcription-mediated amplification, and *(iii)* hybridization protection assay, all performed in a single tube. All three assays incorporate an internal control to validate each reaction[19,20]. During sample preparation, viral RNA and DNA are isolated from plasma specimens via the use

of target capture reagent. Target capture reagent contains an internal control RNA, capture oligonucleotides complementary to specific sequences of HIV-1, HCV RNAs, HBV DNA and super paramagnetic particles. Target capture reagent causes lysis of the viral particles and inactivates nucleases, thus enhancing viral RNA stability[19]. Oligonucleotides (capture oligonucleotides) that are homologous to highly conserved regions of HIV-1, HCV, and HBV are hybridized to the HIV-1 or HCV RNA or HBV DNA target, if present, in the test specimen. The hybridized target is then captured onto magnetic microparticles that are separated from plasma in a magnetic field. Wash steps are utilized to remove extraneous plasma components from the reaction tube. Magnetic separation and wash steps are performed with a Target Capture System. Target amplification occurs via TMA. The GenProbe TMA technology exponentially amplifies the capture viral nucleic acids. The Procleix® Ultrio® Assay utilizes the TMA method to amplify regions of HIV-1 RNA, HCV RNA, and HBV DNA.

Detection is achieved by hybridisation protection assay (HPA)[19] using single-stranded nucleic acid probes with chemiluminescent labels that are complementary to the amplicon. The labeled nucleic acid probes hybridize specifically to the amplicon. The selection reagent differentiates between hybridized and unhybridized probes by inactivating the label on unhybridized probes. During the detection step, the chemiluminescent signal produced by the hybridized probe is measured in a luminometer and is reported as Relative Light Units (RLU). Internal control is added to each test specimen, external control, or assay calibrator tube via the working target capture reagent that contains the internal control. Internal control specific amplicon is detected using a probe with rapid emission of light (termed flasher signal). Amplicon specific to HIV-1/HCV/HBV is detected using probes with relatively slower kinetics of light emission (termed glower signal). The Dual Kinetic Assay (DKA) is a method used to differentiate between the signals from flasher and glower labels. When used for the simultaneous detection of HIV-1, HCV, and HBV, the Procleix® Ultrio® Assay differentiates between internal control and combined HIV-1/HCV/HBV signals but does not discriminate between individual HIV-1, HCV, and HBV signals.

Samples found reactive in the Ultrio test were later retested for HIV-1, HCV and HBV using discriminatory assays. The Procleix® HIV-1, HCV, and HBV discriminatory assays utilize the same steps as the Procleix Ultrio Assay (target capture, TMA, HPA and DKA) with one difference: HIV-1 specific, HCV specific, or HBV specific probe reagents are used in place of the Procleix Ultrio Assay probe reagent. As an initially reactive sample is only confirmed after discriminatory testing, the discriminatory test acts as a confirmatory test. The process of testing initially reactive samples twice reduces the chances of errors or false positive results. Samples initially reactive by the Ultrio assay, but non-reactive by discriminatory testing, were re-tested using the multiplexed Ultrio protocol. If the samples were non-reactive in the repeat Ultrio test, they were treated as unconfirmed reactive. However, if they were repeat reactive on re-test, they were re-tested using the discriminatory assays. If found reactive by the discriminatory assays, they were classified reactive, but if found non-reactive, they were considered non-discriminating reactive.

The sensitivity of Procleix® Ultrio® Assay has been described in Table III.

Table III. Analytical sensitivity of Procleix® Ultrio® Assay*

Panel tested	Assay	Detection probabilities	
		50% (95% fiducial limits)	95% (95% fiducial limits)
HIV-1 B copies/ml	Procleix® Ultrio® Assay	13.9 (12.0-15.9)	37.7 (33.6-43.0)
HIV-1 B copies/ml	dHIV-1	12.9 (11.2-14.6)	35.4 (33.8-36.9)
HIV-1 WHO (97/656) IU/ml	dHIV-1	7.5 (6.4-8.7)	18.1 (16.1-20.8)
HCV WHO (96/790) IU/ml	Procleix® Ultrio® Assay	1.3 (1.0-1.5)	3.7 (3.3-4.2)
HCV WHO (96/790) IU/ml	Dhcv	1.0 (0.9-1.2)	2.4 (2.1-2.7)
HBV WHO (97/746) IU/ml	Procleix® Ultrio® Assay	3.3 (3.0-3.8)	8.0 (7.1-9.3)
HBV WHO (97/746) IU/ml	dHBV	3.0 (2.7-3.4)	6.8 (6.0-7.7)

* These numbers are National Institute for Biological Standards and Control (NIBSC) code for HIV, HBV and HCV NAT International Standard

Results

All 12,224 study samples were serologically screened at the blood centres where these were collected. Of these, there were 209 (1.71%) seroreactive samples, including 137 (1.12%) HBsAg positive samples, 40 (0.33%) anti-HCV reactive samples and 32 (0.26%) anti-HIV reactive samples.

Of the 12,224 study samples, 133 (1.09%) were reactive by the Ultrio Assay. Among these, 106 were reactive for HBV DNA (0.87%), 15 for HCV RNA (0.13%) and 13 for HIV-1 RNA (0.10%). One sample was reactive for both HIV-1 and HCV. There were 84 (0.69%) samples which were seroreactive but NAT non reactive: 36 (0.29%) HBsAg positive, 27 (0.22%) anti-HCV reactive, and 21 (0.17%) anti-HIV reactive. Some of these were weakly serologically reactive but could not be confirmed whether they were false reactive or not. Inadequate sample volume precluded repeat serological testing. For HCV and HBV, it was possible that the sample was taken at the time of resolving infection with waning HCV antibody or HBsAg and resolved or very low level viraemia. A total of 125 (1.02%) samples were reactive by both serology and NAT: 101 HBV (0.83%), 13 HCV (0.11%) and 11 HIV (0.09%) (Table IV).

There were 27 samples which were Ultrio reactive but discriminatory negative. Of these, only five samples had sufficient volumes to retest on Ultrio and all these were negative on repeat testing. These five samples represented 0.04 per cent of the all 12,224 tests performed. It is possible that this percentage of Ultrio reactive/discriminatory non-reactive results could be as high as 0.22 per cent (27 of 12,224) if the remaining 22 samples could have been repeat tested and were all negative on retest.

A summary of the demographic data from the reactive donors is given in Tables V and VI. There were almost 12 times the number of male donors then the female donors and the percentage of reactive male donors (1.85 %) was almost twice that of female donors (0.85%). Among the 12,224 donors, only 3225 (26.5%) were voluntary donors. The reactive rate among voluntary donors was 1.46 per cent compared to 1.9 per cent in replacement donors. First time voluntary donors had a 1.66 per cent reactive rate compared to a 1.23 per cent reactive rate observed in repeat voluntary donors.

Yield: The combined yield (seronegative /NAT reactive) for HIV-1, HCV and HBV was 8 (0.065%), and included 6 HBV, 1 HCV and 2 HIV-1 NAT-only positive samples,

Table IV. Reactive donors by serology and/or NAT

% of 12224	HIV-1	HCV	HBV	Combined total %
% total seroreactive	0.26 (n=32)	0.33 (n=40)	1.12 (n=137)*	1.71 (n=209)
% seroreactive/NAT non-reactive	0.17 (n=21)	0.22 (n=27)	0.29 (n=36)	0.69 (n=84)
% total NAT reactive and seroreactive	0.09 (n=11)	0.11 (n=13)	0.83 (n=101)	1.02 (n=125)
% total NAT reactive	0.11 (n=13)	0.12 (n=15)*	0.87 (n=106)	1.09 (n=133)
% NAT reactive/seronon-reactive (NAT yield)	0.016 (n=2)	0.008 (n=1)**	0.05 (n=6)	0.065 (n=8)

* One HCV reactive sample was negative for anti HCV but positive for HBsAg. This sample was not a NAT yield
**One sample was reactive to both HCV and HIV-1 and also counted in HIV-1 tally

Table V. Percentage distribution of reactive donors by gender and marital status

	Gender		Marital status	
Per cent* reactivity by virus	Male (n=209)	Female (n=8)	Married (n=152)	Unmarried (n=65)
HIV seroreactive and/or NAT reactive	15 (n=32)	25 (n=2)	14 (n=22)	18 (n=12)
HIV NAT yield	1 (n=2)	0 (n=0)	1.3 (n=2)	0 (n=0)
HCV seroreactive and/or NAT reactive	20 (n=41)*	0 (n=0)	16 (n=25)	25 (n=16)
HCV NAT yield	0.5 (n=1)	0 (n=0)	0.65 (n=1)	0 (n=0)
HBV seroreactive and/or NAT reactive	65 (n=137)	75 (n=6)	69 (n=106)	57 (n=37)
HBV NAT yield	3 (n=6)	0 (n=0)	2.61 (n=4)	3 (n=2)
% of all donors in that category	1.85 (209/11,280)	0.85 (8/944)	2.03 (152/7501)	1.38 (65/4723)

Rounded to nearest whole number - percentages are column percentages.
*Sample reactive for both HCV and HIV-1 and also included in HIV-1 tally

Table VI. Donor type profiles of reactive donors

	Replacement donors (n=170)		Voluntary donors (n=47)	
% reactivity by virus	First time (n=85)	Repeat (n=85)	First time (n=28)	Repeat (n=19)
HIV serology and/or NAT+	19 (n=16)	8 (n=7)	32 (n=9)	11 (n=2)
HIV NAT yield	1.2 (n=1)	1.2 (n=1)	0 (n=0)	0 (n=0)
HCV serology and/or NAT+	15 (n=13)	21 (n=18)	18 (n=5)	26 (n=5)
HCV NAT yield	0 (n=0)	1.2 (n=1)*	0 (n=0)	0 (n=0)
HBV serology and/or NAT+	66 (n=56)	71 (n=61)	50 (n=14)	63 (n=12)
HBV NAT yield	4.7 (n=4)	1.2 (n=1)	3.6 (n=1)	0 (n=0)
% of all donors in that category	1.9 (85/4463)	1.9 (85/4536)	1.66 (28/1686)	1.23 (19/1539)

*Sample reactive for both HIV-1 and HCV and also included in HIV-1 tally
Values are given as percentage.

Table VII. Yield by collection centre type

Type of blood bank	Total no. of samples contributed	% yield for all 3 viruses	
Stand alone/hospital based:			
Stand alone (n=4)	2724	0.037	(n=1)
Hospital based (n=4)	9500	0.074	(n=7)
Government/non government:			
Government (n=2)	1692	0.059	(n=1)
Non government (n=6)	10532	0.066	(n=7)

where the one HCV positive sample was co-infected with HIV and counted in the HIV-1 tally. The individual NAT yields for the three viruses were as follows: HIV (0.016%), HCV/HIV co-infected (0.016%) and HBV (0.049%). The yields observed in different types of collection centers are shown in Table VII.

Discussion

Blood safety is a challenge in India because of the high prevalence of HIV, HCV, and HBV, the relatively low percentage of volunteer donors[2] and the lack of standardization of screening procedures among the multitude of blood collection centres[5]. Of the 12,224 samples tested from 8 centres, there were 8 NAT yields (1 in 1528) (0.065%): 1 in 12,224 (0.008%) for both HIV-1 mono-infection and HCV-HIV-1 co-infection and 1 in 2037 (0.049%) for HBV. Among the 12,224 donor samples tested, 209 (1.71%) were screen reactive by either serology and/or NAT. Similar studies in other countries have also demonstrated high yields[14-21-22]. The potential for NAT yield in India is staggering when compared to other countries that have already implemented the technology. Data from our study suggested that the NAT yield for all three viruses in India could be 29 times higher than that observed in Japan, and even higher for HIV-1 alone. Our observed HIV-1 yield was over 515 times that observed in the US and Canada, 89 times that observed in Italy. Our HCV yield was 21.5 times that observed in the US and Canada, 26.5 times that of Italy and 125.6 times that of France[14,21,22]. The higher observed yield in India is not surprising given the prevalence of these viruses in the population; 5.7 million[23] with HIV, 12 million with HCV[24], and 40 million with HBV which represents 10 per cent of the world's HBV infected population[25]. In addition, India has a high percentage of replacement blood donors who are associated with higher infection rates compared to volunteer donors[26]. Many countries, such as Japan and the US, have mostly all volunteer donors[1,14].

In most developed countries, the majority of blood donors are repeat voluntary donors, while in India volunteers only constitute 50 per cent of all blood donors[2]. In our study, we had only 26.4 per cent voluntary donors, the remainder being replacement donors. Voluntary donors appear to have a lower prevalence of viral infection than do replacement donors[26]. Another interesting finding of our study was that the percentage NAT yield observed in hospital-based blood banks (0.074%) was approximately twice that observed in stand alone blood banks (0.037%). This could be done to the differences in collection practices. Most hospital-based blood banks collect largely from replacement donors, whereas most stand alone banks collect the majority of their blood through voluntary blood donation camps.

We were also able to demonstrate the feasibility of NAT implementation in a relatively small volume setting with a semi automated system. One well trained operator could test 182 samples individually in 6-7 h for all 3 viral markers and a few thousand tests annually.

Our study showed that blood donors with a low viral load can sometimes go unrecognized by the discriminatory assays. The discrepancies, between

multiplex and discriminatory assays observed in the present study, should be attributed rather to the low-viraemia content of the sample tested than to false positive results or to decreased sensitivity of the discriminatory assays. The most likely explanation of discrepant results is stochastic sampling variation in low viral load samples[27]. Regardless the outcome of the discriminatory probe assay or multiplex repeat assays, it has been recommend to discard all initial NAT reactive donations in order to avoid infusion of a very low-level viraemic unit that was originally detected as reactive by the primary screening assay but missed in the repeat assays[28].

The Ultrio Assay could improve the screening of the approximately 5 million units of blood collected annually in India. Based on the results of this study, Ultrio screening would be predicted annually to interdict 3,272 infectious donations (5 million/ 12,224 = 409 x 8 (study yield) (95% CI 1400-6450) including 818 HIV infected, 409 HCV, and 2454 HBV infected donations. These results suggest India stands far more to gain from implementation of NAT screening. Since many of our modern blood banks process multiple components including packed red cells, fresh frozen plasma, and platelets, a single infected unit may transmit the infection to 2 or 3 recipients, further increasing the number of potential transfusion transmitted infections even after serological screening, if NAT is not used. Although cost-effectiveness in our country still needs to be evaluated, it is usually high when the yield is great. In addition, India still has some blood banks that perform HIV antigen tests, usually p24 antigen, in addition to routine serological screening. As NAT is associated with a shorter window period than HIV antigen tests, it appears that implementation of routine NAT blood screening would allow elimination of HIV antigen tests[29].

In conclusion, our findings showed that NAT allowed rapid detection of three prevalent viruses that cause serious infections and its semi-automated platform allowed individual testing in our low volume setting. This tool could provide the next large step in improving the safety of blood supply in our conutry. In addition to interdicting infected donations, NAT can also help provide valuable epidemiological data regarding the incidence and prevalence of these serious viral infections.

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