# Comparison of two algorithms to confirm and discriminate samples initially reactive for nucleic acid amplification tests

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**Abstract:**

**BACKGROUND:** Blood centers in India have published individual donor nucleic acid testing (ID-NAT) data based on an algorithm (Algorithm A) where serologically negative, NAT reactive sample was subsequently tested with discriminatory NAT (d-NAT), and on the basis of d-NAT, initial reactive samples were classified as "NAT yield" or inconclusive. We followed Algorithm B based on replicate testing and Ultrio Plus assay and compared the results with Algorithm A with Ultrio assay.

**MATERIALS AND METHODS:** Results of ID-NAT using two algorithms were analyzed.

**RESULTS:** A total of 88,583 (31,844 with Algorithm A and 56,739 with Algorithm B) samples were tested. Among serology nonreactive donations, NAT inconclusive results came down from 95.2% in Algorithm A to 73.1% in Algorithm B ($P = 0.0001$). Discriminated yield (DY) rate went up from 4.7% in Algorithm A to 21.9% in Algorithm B ($P = 0.001$).

**CONCLUSION:** The study data suggest that replicate testing strategy and Ultrio Plus reduce the number of "inconclusive results" seen with earlier commonly used algorithm. We recommend a replicate testing strategy in ID-NAT testing since it will increase the DY and will eliminate the unnecessary discriminatory tests.

**Keywords:**

Algorithm, nucleic acid test, replicate testing

## Introduction

Nucleic acid testing (NAT) is a highly sensitive technique for viral nucleic acids based on the amplification of targeted regions of viral ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). This has been added as an additional layer of safety to routine serological screening methods (Enzyme immunoassay, Chemiluminescence immunoassay, Microparticle enzyme immunoassay), narrowing the window period of HIV, hepatitis B virus (HBV), and hepatitis C virus (HCV) infections. Benefits of NAT have been demonstrated through

the detection of units known as NAT yields (serology nonreactive but NAT reactive). In India, many blood centers have started using NAT for screening of blood donors to provide safer blood to their patients and published their experience of NAT yield[1-8] [Table 1]. These results are based on the "Algorithm A" [Figure 1] where, if the individual donor sample was reactive on a multiplex NAT (ID-NAT) after negative serological screening test, the donation was subsequently tested with discriminatory NAT (d-NAT) [Table 2]. If the d-NAT was positive, it was considered as d-NAT yield (DY). If the d-NAT was negative, the initial test was considered

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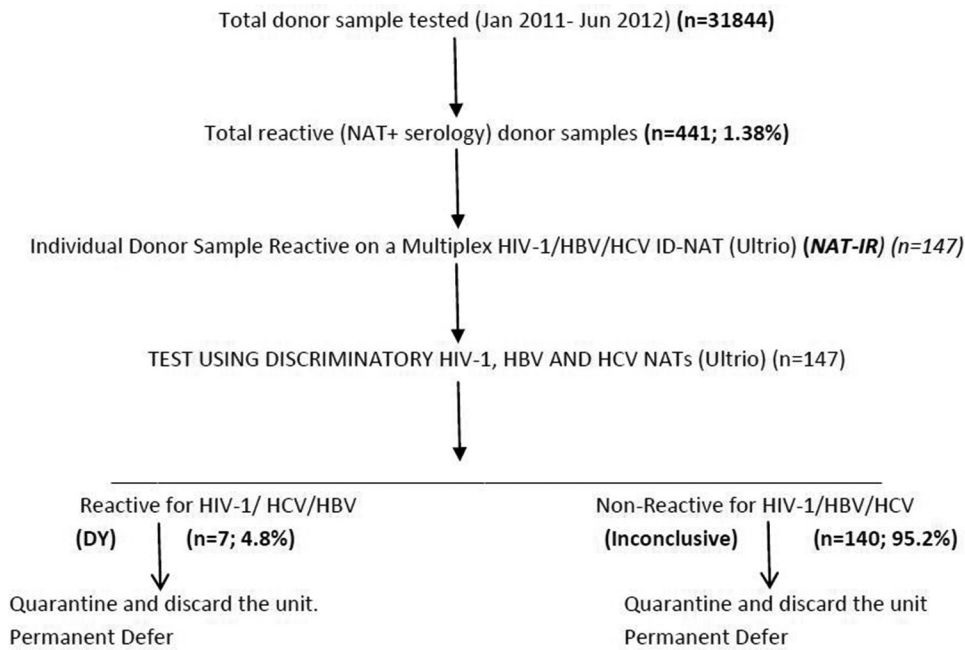
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**Figure 1:** Results of Algorithm A. NAT-IR: Donations showing reactivity in initial multiplex assay. (Nonreactive in serological assays; anti-HIV, anti-HCV, and HBsAg). DY: NAT-IR samples showing reactivity in discriminatory assays. Inconclusive: NAT-IR samples not showing reactivity in discriminatory assays. NAT-IR: Nucleic acid testing initial reactive, DY: Discriminated yield, HCV: Hepatitis C virus, HBsAg: Hepatitis B surface antigen

**Table 1: Studies published on nucleic acid testing yields from India**

| Year of publication | Authors | Samples studied | NAT yields (HBV + HCV + HIV) | Total NAT yields | Yield rate (total NAT yields/samples studied) |
|---|---|---|---|---|---|
| 2008 | Makroo *et al*.[1] | 12,224 | 6+1+1 | 8 | 1:1528 |
| 2009 | Menon[2] | 24,530 | 9+0+0 | 9 | 1:2726 |
| 2012 | Chatterjee *et al*.[3] | 18,354 | 4+3+0 | 7 | 1:2622 |
| 2013 | Agarwal *et al*.[4] | 73,898 | 73+37+1+10* | 121 | 1:610 |
| 2014 | Marwaha *et al*.[5] | 51,593 | 33+13+1+1* | 48 | 1:1066 |
| 2014 | Doda *et al*.[6] | 28,134 | 25 | 25 | 1:1125 |
| 2015 | Chigurupati and Murthy[7] | 8000 | 4 | 4 | 1:2000 |
| 2015 | Makroo *et al*.[8] | 10,302 | 15 | 15 | 1:687 |

*HBV and HCV co-infection. HBV = Hepatitis B virus, HCV = Hepatitis C virus, NAT = Nucleic acid testing

**Table 2: Algorithm A (testing, product disposition, and donor management for an individual donor sample that is reactive on a multiplex nucleic acid testing after negative serological screening tests)**

| If | Then | After that if | Then |
|---|---|---|---|
| Individual donor sample reactive on Multiplex HIV1/HBV/HCV ID-NAT | Test the sample for discriminatory HIV1/HBV/HCV | Reactive for HIV1/HBV/HCV (DY) | Quarantine and discard the unit, defer |
| | | Nonreactive for HIV1/HBV/HCV (Inconclusive) | Quarantine and discard the unit, defer |

NAT-IR = Donations showing reactivity in initial multiplex assay (nonreactive in serological assays; anti-HIV, anti-HCV, and HBsAg), DY = NAT-IR samples showing reactivity in discriminatory assays, Inconclusive = NAT-IR samples not showing reactivity in discriminatory assays. DY = Discriminated yield, NAT = Nucleic acid testing, NAT-IR = NAT initial reactive, HBsAg = Hepatitis B surface antigen, HCV = Hepatitis C virus, ID-NAT = Individual donor nucleic acid testing

"inconclusive." When these samples were re-tested with supplementary other molecular tests such as polymerase chain reaction, they would sometimes test reactive or nonreactive. Similar inconclusive results were also observed in the published reports using single replicate discriminatory testing.[9] To resolve this confusion, a meeting of Indian NAT users with the manufacturers was held and a consensus evolved to change the test algorithm to "Algorithm B" where the initial sample that would test reactive would be submitted to retest in three replicates [Table 3].[10] The Algorithm B was based on a previous publication in another country.[9]

We introduced Algorithm B (replicate testing) with Ultrio Plus assay in July 2012. We analyzed the effect of this change and compared it with the earlier algorithm (Algorithm A) with Ultrio assay in blood donors with an aim to assess the concordance between serology and NAT test results and NAT yield rates.

**Table 3: Algorithm B (testing, product disposition, and donor management for an individual donor sample that is reactive on a multiplex nucleic acid testing after negative serological screening tests)**

| If | Then | After that if | Then | After that if | Then |
|---|---|---|---|---|---|
| Individual donor sample reactive on multiplex HIV1/HBV/ HCV ID-NAT (NAT-IR) | Repeat multiplex HIV-1/HBV/ HCV ID-NAT in triplicate from primary sample tube | If all NR | (NRR) | No further testing | Quarantine and discard the unit, defer |
| | | Even if one is reactive (RR) | Repeat multiplex HIV-1/HBV/ HCV ID-NAT in triplicate from plasma bag + test sample for discriminatory HIV-1, HBV, and HCV NAT in triplicate | Reactive for HIV1/HBV/ HCV (DY) | Quarantine and discard the unit, defer and notify |
| | | | | Nonreactive for HIV1/ HBV/HCV (NDY) | Quarantine and discard the unit, defer and notify |

NAT-IR = Donations showing reactivity in initial multiplex assay (nonreactive in serological assays, anti-HIV, anti-HCV, and HBsAg), NRR = Subset of NAT-IR showing nonreactivity in replicate testing (multiplex tests run in triplicate from tube), RR = Subset of NAT-IR showing reactivity in replicate testing (multiplex tests run in triplicate from tube and/or bag) but may or may not be reactive in triplicate discriminatory assays, DY = Subset of RR showing reactivity in triplicate discriminatory assays, NDY = Subset of RR showing nonreactivity in triplicate discriminatory assays, NAT = Nucleic acid testing, NAT-IR = NAT initial reactive, NRR = Nonrepeatable reactive, RR = Repeatable reactive, DY = Discriminated yield, NDY = Nondiscriminated yield, ID-NAT = Individual donor nucleic acid testing, HBsAg = Hepatitis B surface antigen, HCV = Hepatitis C virus

## Materials and Methods

### Setting
This retrospective analysis was done in the department of transfusion medicine in a large tertiary care hospital in National Capital Region - India, from January 2011 to August 2014. Ethics committee approval was not required as per our institutional policy for retrospective analysis and when no personal identifiers of participants were revealed.

### Serological testing
All blood donor samples were tested for HIV (anti-HIV1/2), hepatitis B surface antigen (HBsAg), and hepatitis C (anti-HCV) by enhanced chemiluminescence method on Vitros EciQ (Ortho Clinical Diagnostics, Johnson and Johnson, USA) using donor's serum sample.

### Individual donor nucleic acid testing
Simultaneously, dedicated EDTA blood sample of the donor was subjected to ID-NAT for HIV 1, HBV, and HCV. ID-NAT test was performed using the eSAS system Procleix Ultrio/Ultrio Plus Assay (Novartis diagnostics, CA, US).

Algorithm A: [Table 2] with Ultrio (April 2011 - June 2012)

Algorithm B: [Table 3] with Ultrio Plus (July 2012 - August 2014)

Both algorithms have used multiplex assays for detecting the presence of any or all of HIV, HCV, and HBV infections in individual donation testing utilizing

transcription-mediated amplification of target viral RNA or DNA. All tests were undertaken in accordance with the manufacturers' instructions.

### Definitions in Algorithm A
*Nucleic acid testing-initial reactive*
Donations showing reactivity in initial multiplex assay (nonreactive in serological assays; anti-HIV, anti-HCV, and HBsAg).

*Discriminated yield*
Nucleic acid testing-initial reactive (NAT-IR) samples showing reactivity in discriminatory assays.

*Inconclusive*
NAT-IR samples not showing reactivity in discriminatory assays.

### Definitions in Algorithm B
*Nucleic acid testing-initial reactive*
Donations showing reactivity in initial multiplex assay (nonreactive in serological assays; anti-HIV, anti-HCV, and HBsAg).

*Nonrepeatable reactive*
Subset of NAT-IR showing nonreactivity in replicate testing (multiplex tests run in triplicate from tube). Nonrepeatable reactive is considered equivalent to inconclusive of Algorithm A.

*Repeatable reactive*
Subset of NAT-IR showing reactivity in replicate testing (multiplex tests run in triplicate from tube and/

or bag) but may or may not be reactive in triplicate discriminatory assays.

### Discriminated yield
Subset of repeatable reactive (RR) showing reactivity in triplicate discriminatory assays.

### Nondiscriminated yield
Subset of RR showing nonreactivity in triplicate discriminatory assays.

### Data analysis
Results of routine nucleic acid testing using the Algorithm A from January 2011 to June 2012 and the Algorithm B from July 2012 to August 2014 were analyzed. Percentage of concordant positives (serology and NAT reactive), concordant negatives (serology and NAT nonreactive), sero-yield (serology reactive and NAT nonreactive), and DY (serology nonreactive and NAT reactive) was compared between the two algorithms.

### Statistical analysis
Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA). "Z-test" was used for comparing signal-to-cutoff ratios and Chi-square test was used for comparison of proportions. $P < 0.05$ was considered statistically significant.

### Results
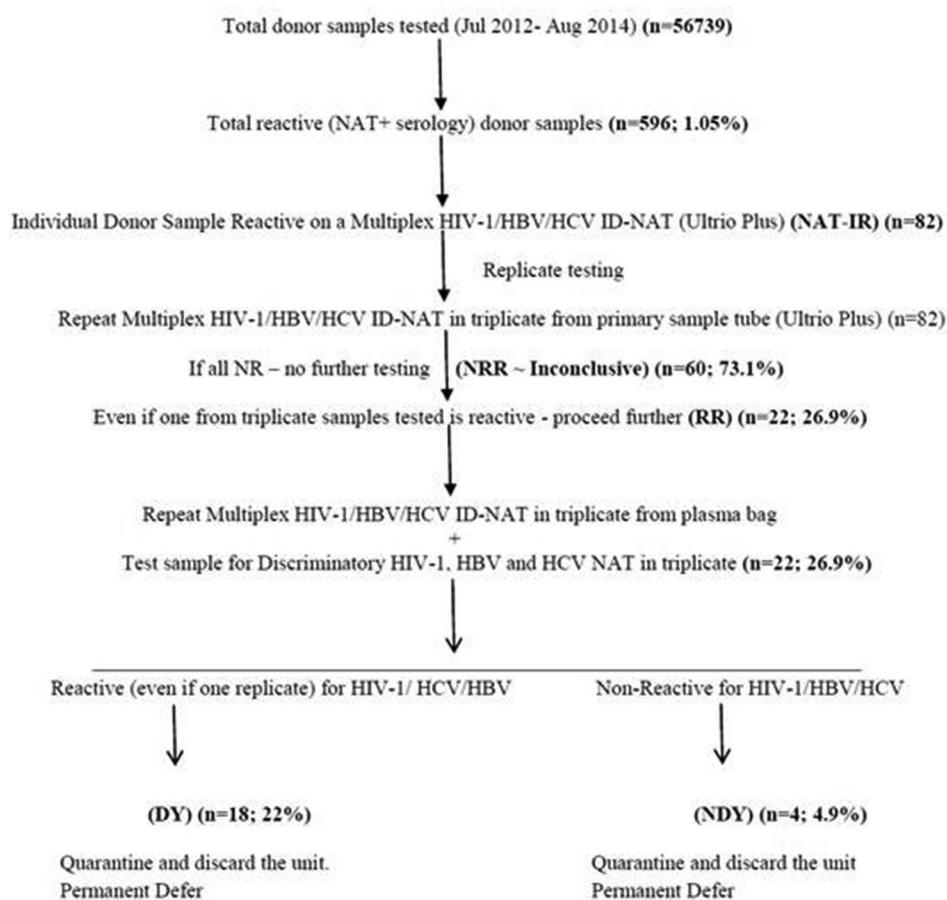
### Participant demographics
During the period of observation (January 2011-August 2014), a total of 88,583 blood donors donated blood. Mean age of the donors was 32.1 years; majority of donors were male replacement donors [Table 4].

### Prevalence of transfusion-transmitted infections
A total of 88,583 blood donor samples were tested during the study period by Enhanced Chemiluminescence Assay and NAT assay simultaneously. About 1037 donor samples (441 [1.38%] with Algorithm A and 596 [1.05%] with Algorithm B) were reactive during the study period in one or both the assays [Table 5]. About 698 donor samples were concordant serology and NAT reactive while 339 samples were discordant reactive. About 252/441 (57%) and 446/596 (74%) donor samples were concordant between Algorithm A and B, respectively, and 42/441 and 68/596 were discordant, respectively, with significant *P* value ($P = 0.0001$; two-proportion *Z*-test), which shows there is a significant increase in concordant rates while a significant decrease in discordant (NAT-IR) rates with Algorithm B as compared to Algorithm A. On further analysis, of concordant test results, the pattern was HBV > HCV > HIV, while in discordant test, the result pattern was HCV > HBV > HIV [Table 5].

### Nucleic acid testing-initial reactive
Totally 229 (147 + 82) NAT-IR donor samples were analyzed using Algorithms A and B. NAT inconclusive rate of 95.2% and 73.1% with Algorithms A and B, respectively, was statistically significant ($P = 0.0001$). DY rate was 4.8% and 22% in Algorithms A and B, respectively, which was statistically significant ($P = 0.001$; two-proportion Z-test). Four donor samples were nondiscriminated though initial NAT reactive in Algorithm B yielding the nondiscriminated yield (NDY) rate of 4.9% [Figures 1 and 2].

### Table 4: Demographics of donors

|  | Algorithm A (%) | Algorithm B (%) | Total |
|---|---|---|---|
| Age (mean) | 31.8 | 32.3 | 32.1 |
| Gender (*n*=88,583) |  |  |  |
| Male | 29,614 (93) | 53,522 (94.1) | 83,136 |
| Female | 2230 (7) | 3217 (5.9) | 5447 |
| Type of donors (*n*=88,583) |  |  |  |
| Voluntary | 2866 (9) | 4540 (8) | 7406 |
| Replacement | 28,978 (91) | 52,199 (92) | 81,177 |

Stats for statistically insignificant

### Table 5: Total samples tested, total reactive samples, and concordance of nondiscriminated yield and serology with Algorithms A and B

|  | Total | Algorithm A (April 2011 to June 2012) | Algorithm B (July 2012 to August 2014) | *P* |
|---|---|---|---|---|
| Total samples tested | 88,583 | 31,844 | 56,739 | NA |
| Total reactive (serology + NAT) (%) | 1037 (1.17) | 441 (1.38) | 596 (1.05) | 0.876 |
| Concordant |  |  |  |  |
| Serology and NAT reactive | 698 (67.3) | 252 (57.2) | 446 (74.8) | 0.0001 |
| HBV + HCV + HIV | 504+150+44 | 182+54+16 | 380+76+10 |  |
| Discordant |  |  |  |  |
| Serology reactive and NAT nonreactive; sero-yield (%) | 110 (10.6) | 42 (9.5) | 68 (11.4) | 0.0001 |
| HBV + HCV + HIV | 23+70+17 | 9+27+6 | 15+43+10 |  |
| Serology nonreactive and NAT reactive; NAT-IR (%) | 229 (22.1) | 147 (33.3) | 82 (13.8) | 0.0001 |

HBV = Hepatitis B virus, HCV = Hepatitis C virus, NAT = Nucleic acid testing, NAT-IR = NAT initial reactive, NA = Not available

**Figure 2:** Results of Algorithm B. NAT-IR: Donations showing reactivity in initial multiplex assay. (Nonreactive in serological assays; anti-HIV, anti-HCV, and HBsAg). NRR: Subset of NAT-IR showing nonreactivity in replicate testing (multiplex tests run in triplicate from tube). NRR is equivalent to inconclusive of Algorithm A. RR: Subset of NAT-IR showing reactivity in replicate testing (multiplex tests run in triplicate from tube and/or bag) but may or may not be reactive in triplicate discriminatory assays. DY: Subset of RR showing reactivity in triplicate discriminatory assays. NDY: Subset of RR showing nonreactivity in triplicate discriminatory assays. NRR: Nonrepeatable reactive, RR: Repeatable reactive, DY: Discriminated yield, NDY: Nondiscriminated yield, HCV: Hepatitis C virus, HBsAg: Hepatitis B surface antigen

## Discriminated yield and nondiscriminated yield

In the analysis of 22 (18 DY + 4 NDY) donations by Algorithm B, 10 donor samples were reactive in all the test runs (triplicate from sample tube, triplicate from bag sample, and triplicate in discriminatory run) while the other 8 donor samples show variable reactivity as shown in Table 6. Out of the ten donor samples, eight were discriminated as HBV reactive while two were HCV reactive. The other eight samples with variable reactivity were also discriminated as HBV reactive. Four donor samples were reactive in initial repeat triplicate test runs while nonreactive in bag sample and discriminatory test runs NDY [Table 6].

## Discussion

Since January 2011, all blood donations at our center have been screened for HBV DNA, HIV-1 RNA, and HCV RNA by ID-NAT format using the Procleix Ultrio and Ultrio Plus systems in conjunction with Vitros EciQ serology screening for anti-HIV, anti-HCV, and HBsAg.

Donors were tested using two different algorithms and assays of ID-NAT within the specified period. Overall, NAT reactivity of donors was 1.04% (not shown in table) which was similar to 1.09, 1.02, and 1.49, respectively, in other published Indian reports.[1,3,4]

## Algorithm A versus B

Two hundred and twenty-nine NAT-IR donor samples were analyzed using respective algorithms yielding statistically significant ($P$ = 0.0001) difference in the NAT inconclusive rate of 95.2% and 73.1% with Algorithms A and B, respectively. It is possibly a combined effect of increased sensitivity of Ultrio Plus and new algorithm (Algorithm B) which has led to a significant decrease in inconclusive rates. Changing to new algorithm (Algorithm B) increases the specificity of the Ultrio Plus assay because replicate testing decreases the random effect. This was also observed by Marwaha *et al*.[5] and in the second part of the study done by Grabarczyk *et al*. in Poland.[9] Significant increase in the DY rate (21.9%) in Algorithm B was also observed in our

**Table 6: Replicate testing of 22 (18 discriminated yield and 4 nondiscriminated yield) samples of Algorithm B**

| Sample tube | Bag sample | Discriminatory | Final interpretation |
|---|---|---|---|
| 3/3 | 3/3 | 3/3 | HBV |
| 3/3 | 3/3 | 3/3 | HBV |
| 3/3 | 3/3 | 3/3 | HBV |
| 3/3 | 3/3 | 3/3 | HBV |
| 3/3 | 3/3 | 3/3 | HBV |
| 3/3 | 3/3 | 3/3 | HBV |
| 3/3 | 3/3 | 3/3 | HBV |
| 3/3 | 3/3 | 3/3 | HBV |
| 1/3 | 1/3 | 1/3 | HBV |
| 1/3 | 0/3 | 1/3 | HBV |
| 1/3 | 0/3 | 1/3 | HBV |
| 1/3 | 1/3 | 1/3 | HBV |
| 2/3 | 2/3 | 2/3 | HBV |
| 2/3 | 3/3 | 2/3 | HBV |
| 2/3 | 3/3 | 3/3 | HBV |
| 3/3 | 3/3 | 1/3 | HBV |
| 3/3 | 3/3 | 3/3 | HCV |
| 3/3 | 3/3 | 3/3 | HCV |
| 1/3 | 0/3 | 0/3 | NDY |
| 3/3 | 2/3 | 0/3 | NDY |
| 1/3 | 1/3 | 0/3 | NDY |
| 1/3 | 0/3 | 0/3 | NDY |

HBV = Hepatitis B virus, HCV = Hepatitis C virus, NDY = Nondiscriminated yield

study which was due to the new algorithm (Algorithm B), as this algorithm is based on replicate testing which increases the probability of detection of DY samples. Even if one of the replicate tests would test reactive, it would be counted as yield, and at the same time, if none of the replicates are reactive, it would not be subjected for discriminatory assay. While in Algorithm A, where the sample was subjected to discriminatory testing without any replicate testing, and if this would not discriminate, it would be considered inconclusive. In other words, if it was possible to submit inconclusive samples of Algorithm A period to replicate testing, few of these could have resulted in DY. Out of 18 DY samples in Algorithm B, two samples which finally discriminated for HCV were reactive in all six replicates (triplicate from sample tube and triplicate from bag sample) discriminated in all the three discriminatory replicates. In another 16 which were discriminated as HBV, 8 donor samples were reactive in all six replicates (triplicate from sample tube and triplicate from bag sample) and discriminated in all the three discriminatory replicates while the other 8 samples exhibited variable reactivity ranging from one to six in six replicates. None of these 8 donor samples which were discriminated shows uniform reactivity in all the three discriminatory replicates. Variable results in tube and bag sample replicates also reflected similar

variability in discriminatory assays. This variable pattern was seen only with HBV not with HCV. This could be because of low levels of HBV DNA close to the assay's limit of detection. This is in concert with the findings of Charlewood and Flanagan.[11] Many countries have implemented different testing and blood release algorithms in the blood donor screening with a wide variation on whether nonrepeat reactive donations are transfused or discarded and their future eligibility for blood donations. In our study, all nonrepeat reactive donations were discarded and donors were not eligible for future donations.

### Concordance of nucleic acid testing and serology with Algorithms A and B

In our study, concordant rates were significantly higher while there was a significant decrease in discordant rates between serology and NAT-IR with Algorithm B as compared to Algorithm A. This difference was mainly observed in the detection of HBV which may be because of improved sensitivity of Ultrio Plus assay for HBV.[11] This increased sensitivity of Ultrio Plus is due to addition of the Target Enhancer Reagent which gives an alkaline shock to the virus particles during the target capture step in the NAT assay. This improved sensitivity was not seen for HCV and HIV.[11] This improved effect of Ultrio Plus was best observed in the work done by Grabarczyk *et al*. where Ultrio Plus assay was 3.3 (2.4–4.7) times more sensitive than Ultrio in all HBV genotype standard dilution panels that were analyzed.[9] This was also reiterated by Enjalbert *et al*.[10] whose study data confirmed higher sensitivity of Ultrio Plus over Ultrio assay.

### Limitations

This study has few limitations; first, it is difficult to discern whether the differences observed were only because of the algorithm change *per se* or a combination of change in algorithm and Ultrio Plus. Second, Algorithms A and B comprised two different donor populations. Third, no alternative NAT or follow-up donor testing was used for inconclusive samples. Inconclusive results could be because of false-positive NAT-IR, occult hepatitis B infection, or window period donation, as reported by Charlewood and Flanagan,[12] Kiely *et al*.,[13] and Allain and Candotti[14]

### Conclusion

Our data suggest that replicate testing algorithm possibly reduces the number of "inconclusive results" seen with earlier commonly used algorithm. We suggest a replicate testing strategy in ID-NAT testing since it will increase the DY and will eliminate the unnecessary discriminatory tests. Studies analyzing the same sample by both algorithms are needed to reconfirm these initial findings.

## Conflicts of interest

There are no conflicts of interest.

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