Original Article

Seroprevalence of human immunodeficiency virus in north Indian blood donors using third and fourth generation Enzyme linked immunosorbent assay

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Abstract:

Background: The percentage of HIV cases attributable to blood transfusion has decreased significantly in the last decade. The newer 4th generation Enzyme linked immunosorbent assay (ELISA) has been shown to have increased sensitivity compared to 3rd generation ELISA. **Objectives:** To estimate the seroprevalence of HIV among blood donors using 4th generation ELISA assay and to compare it with the 3rd generation ELISA. **Materials and Methods:** This prospective study involved 10,200 blood donors- 6,800 were voluntary donors (3400-students and 3400-non students) and 3400 were replacement donors. All blood units were tested with 3rd as well as 4th generation ELISA. All samples found reactive or in grey zone with either 3rd or 4th generation ELISA were retested by Western blot (WB). **Results:** The seroprevalence of HIV was estimated to be 1.37/1000 donations (0.14%) with 3rd generation ELISA compared to 3.62/1000 donations (0.36%) with 4th generation ELISA ($p>0.05$). The seroprevalence of HIV among voluntary donors was estimated to be 1.32/1000 donations (0.13%) with 3rd generation ELISA and 3.67/1000 donations (0.36%) with 4th generation ELISA. The prevalence of HIV among replacement donors was 1.47/1000 donations (0.15%) with 3rd generation ELISA and 3.52/1000 donations (0.35%) with 4th generation ELISA. **Conclusion:** 4th generation HIV ELISA detects a higher number of seroreactive donors compared to 3rd generation ELISA. However, larger studies are required with confirmatory tests for both 3rd and 4th generation ELISA for making any policy changes.

Key words: Enzyme linked immunosorbent assay, Human immunodeficiency virus, window period, transfusion associated HIV/AIDS, blood donors

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Introduction

Transfusion associated HIV/AIDS is defined as "AIDS" occurring in a person who has received transfusion after 1977, but has no other risk factors for HIV infection.[1] Transmission of HIV through blood and blood products can be reduced to a great extent by efficient and reliable screening of the blood to be transfused. An ideal screening test should be highly sensitive, easy to perform, not require sophisticated instruments, cost-effective and able to distinguish between HIV-1 and HIV-2 infections. Due to the currently prevalent stringent screening practices, the percentage of HIV cases attributable to blood transfusion has decreased considerably from 8% in mid-nineties to 1% in 2009.[2] However, there is still a need for improved screening and diagnostic methods for HIV so as to further reduce the window period transmission.

The 4th generation ELISA assays simultaneously detect antibodies against HIV-1 and 2 and the presence of p24 antigen and thus shorten the window period to about 14 days, as compared to about 22 days with 3rd generation Enzyme linked immunosorbent assay (ELISA) assay.[3] This study was undertaken to estimate the seroprevalence of HIV among blood donors at a tertiary care institute in India using a 4th generation ELISA (antigen + antibody) assay and to compare it with the 3rd generation ELISA (antibody) assay, presently in use. Such data is required to be generated in the country so as to review screening strategies for HIV in transfusion services, as these services are under regulatory control and hence subject to operational uniformity.

Materials and Methods

The Department of Transfusion Medicine, PGIMER Chandigarh collects approximately 50,000 units of blood annually. Of these, approximately 42,000 are voluntary donors and 8,000 are replacement donors. Of all these donations, 10,200 blood donors were included in this study. Taking incidence of HIV seroprevalence among blood donors as 0.3% from previous study[4] and using EPIINFOVERSION 6 software, we calculated the sample size to be 10,200 at more than 80% power and 95% confidence limits. The donors were divided into two groups - voluntary donors (6,800) and replacement donors (3,400). The voluntary donors were further divided into 2 subgroups – student and non-student donors (of

3,400 donors each). No additional sampling was done apart from that which was routinely collected in pilot tubes at the end of phlebotomy for pre transfusion testing. The test was performed on serum. The serum was separated from the clot as soon as possible to avoid any hemolysis. Specimens with observable particulate matter were centrifuged prior to testing as suspended fibrin particles or aggregates may yield false reactive results. The samples were frozen at - 20°C till the time of testing.

Methodology

As per Drugs and Cosmetics Act (3rd amendment 2001),[5] Govt. of India, all blood units were tested for HIV antibodies using 3rd generation ELISA (Microlisa – HIV microwell ELISA kits manufactured by J. Mitra and Co. Pvt. Ltd.). In addition 10200 donor units were screened with 4th generation HIV Ag-Ab ELISA (Eliscan HIV advance 4th generation ELISA kits manufactured by RFCL). Manufacturer's instructions were strictly followed while performing each assay.

Calculation and interpretation of the results

The presence or absence of detectable HIV antigen or antibodies to HIV-1 and/or HIV-2 was determined by comparing the absorbance measured for each sample to the calculated cut-off value. Samples with absorbance values less than the cut-off value were considered to be ELISA non-reactive. Sample with absorbance values equal to greater than the cut-off value were initially considered to be ELISA reactive. Sample with absorbance values within 10% of cut off value were considered in grey zone. Samples initially found in grey zone were retested using the same ELISA kit and if again found in grey zone were termed as possibly reactive and included in analysis.

All samples found reactive or possibly reactive with either 3rd or 4th generation ELISA were further tested by Western Blot (WB) (kits manufactured by J. Mitra and Co. Pvt. Ltd.) since it is considered confirmatory for 3rd generation ELISA. The results of Western blot were interpreted as reactive, non-reactive or indeterminate [Table 1]. All the details regarding demographic profile of the donors (age, sex, number of donations), whether voluntary or replacement donors and the results of HIV seroreactivity with 3rd or 4th generation ELISA was recorded. HIV seroprevalence among blood donors was estimated by both the 3rd and 4th generation ELISA as percentages with confidence limits of 95%. Performance of 4th generation ELISA was compared against 3rd generation ELISA using chi square test.

Table 1: Calculation and interpretation of the results with Western Blot

Interpretation	Pattern
Reactive	
HIV-1 positive	2 ENV ± 1 GAG/ 1 POL
HIV-1 reactive with HIV-2 indicated	2ENV ± 1 GAG/ 1 POL + HIV-2 band
HIV-1 non-reactive with HIV-2 indicated	Only control band + HIV-2 band
Indeterminate	
Viral specific bands present but pattern does not meet the criteria of positive	1 ENV ± 1 GAG ± 1 POL 1 GAG ± 1 POL Only GAG Only POL
Indeterminate with HIV-2 indicated	Viral specific bands present but pattern does not meet the criteria of reactive HIV-2 band
Non-reactive	Only control band or control band with p51/55 band
Invalid	No control band

Results

Of the 10,200 samples tested, 14 were found to be seroreactive for HIV using 3rd generation ELISA and result of 4 samples were in grey zone. On repeat testing, these 4 samples were negative, thus giving a prevalence of 14/10200 i.e; 1.37 per 1000 donations (0.14%) with 3rd generation ELISA, or the yield of 3rd generation ELISA can be estimated to be 1.37 per 1000 donations (0.14%). The prevalence of HIV among student subgroup was 0.29/ 1000 donations (1/3400 donations or 0.03%) and among non-student subgroup, it was 2.35/ 1000 donations (8/3400 donations or 0.23%). Combining the results of the subgroups showed the seroprevalence of HIV among voluntary donors to be 1.32/ 1000 donations (9/6800 donations or 0.13%). The prevalence of HIV among replacement donors was 1.47/ 1000 donations (5/3400 donations or 0.15%).There was no statistically significant difference in HIV seroprevalence between replacement donors and student donors (P=0.1), replacement and non-student donors (0.4), and replacement and voluntary donors (0.85). HIV seroprevalence among first time donors and repeat donors was estimated to be 1.61 per 1000 donations (6/3710 donations) and 1.23 per 1000 donations (8/6490 donations) respectively (P = 0.32). Of the 10,200 samples, 30 were found to be seroreactive for HIV using 4th generation ELISA and result of 7 samples were in grey zone. On repeat testing, these samples were again seen in grey zone (possibly reactive), thus giving a prevalence of 37/10200 i.e.; 3.62 per 1000 donations (0.36%) or yield of 3.62 per 1000 donations (0.36%). Although 4th generation ELISA could detect significantly higher number of seroreactive samples (37 vs 14 per 10200 donations; P = 0.002), yet the difference in seroprevalence expressed per 1000 donations was not statistically significant (1.37/1000 Vs 3.62/1000 donations; P = 0.53). The prevalence of HIV among student subgroup was 1.76/ 1000 donations (6/3400 donations or 0.17%) and among non-student subgroup, it was 5.55/ 1000 donations (19/3400 donations or 0.55%). Combined seroprevalence of HIV among voluntary donors was found to be 3.6/ 1000 donations (25/6800 donations or 0.36%) and among replacement donors, it was 3.5/ 1000 donations (12/3400 donations or 0.35%). Similar to the results with 3rd generation ELISA, there was no statistically significant difference in HIV seroprevalence between replacement donors and student donors (P = 0.23), replacement and non-student donors (0.27), and replacement and voluntary donors (1.0) with 4th generation ELISA. HIV seroprevalence among first time donors and repeat donors was estimated to be 3.60 per 1000 donations (10/3710 donations) and 4.16 per 1000 donations (27/6490 donations) respectively (P = 0.24). The difference in the seroprevalence of HIV among blood donors in various groups and subgroups using 3rd and 4th generation ELISA was not found to be significant (Student donors 0.29/1000 Vs 1.76/1000; P = 0.76, Non student donors 2.35/1000 Vs 5.55/1000; P = 0.41), Voluntary donors 1.32/1000 Vs 3.6/1000; P=0.52, Replacement donors 1.47/1000 Vs 3.5/1000; P=0.6). Table 2. shows the number of donor samples reactive for HIV in individual groups and subgroups using both 3rd and 4th generation ELISA.

Comparing 3rd generation ELISA with WB, it was seen that of the 14 samples found reactive with 3rd generation ELISA, 11 were confirmed to be reactive and 3 were non-reactive with Western blot. A similar comparison between 4th generation ELISA and WB observed that of the 37 samples found reactive or possibly

Table 2: HIV seroreactive samples in various groups

Sample source	3rd generation Reactive	4th generation Reactive	Grey Zone (4th generation ELISA)	WB Reactive	WB Indeterminate
Student (n = 3400)	1	5	1	2	0
Non-student (n = 3400)	8	17	2	11	2
Replacement donors, (n = 3400)	5	8	4	4	4
Total samples (n = 10,200)	14	30	7	17	6

Table 3: Comparison of ELISA and Western blot results

Reactive ELISA Results	Western Blot Results		
	Reactive	Non-reactive	Indeterminate
3rd generation ELISA, n = 14	11	3	0
4th generation ELISA only, n = 30	15	10	5
Grey zone (with 4th gen ELISA), n = 7	2	4	1

reactive with 4th generation ELISA, 17 were Western blot reactive, 14 were non-reactive and 6 were indeterminate. Table 3 shows the comparison of ELISA reactive samples using 3rd generation ELISA and 4th generation ELISA with WB.

Additional yield with 4th generation HIV ELISA

Of the 26 samples which were tested non-reactive with 3rd generation ELISA, 19 were tested reactive and 7 samples were found to be possibly reactive with 4th generation ELISA.

Discussion

Transfusion is a very efficient method of transmitting the HIV virus. Estimates indicate that up to 95% of persons receiving HIV seroreactive blood become infected.[6] Recent findings demonstrate that in primary HIV infection, random blips of low level viremia occur which can last up to 25 days with HIV concentration in plasma between 1 and 10 copies/ml.[3] Plasma donations during this stage can be infectious though HIV transmission through sexual contact is relatively improbable, since the threshold for heterosexual transmission of HIV is 1500 copies/ml. It is thus important for us to reduce the transfusion transmitted HIV to minimum possible limits and to stop further addition to already growing population of PLHA (people living with HIV/AIDS). The most critical component of blood safety is the screening of blood for infectious markers. Testing blood donors for HIV was introduced in 1985[7] and has been mandated for blood screening ever since. HIV kits have undergone a considerable range of performance improvements over this time with the aim of shortening the window period between infection and the detection of HIV and of ensuring that the various HIV subtypes are detected. In the late 1990s, fourth generation or combined antigen/antibody ELISA assays were introduced, which incorporate in a single assay the advantages of sensitive anti-HIV detection as well as p24 antigen detection.[8] The p24 antigen is detectable in blood several days before anti-HIV appears. This window period can be shortened to about 2 weeks using p24 antigen assays.

In the present study, HIV seroprevalence was estimated to be 1.37 per 1000 donations using 3rd generation ELISA. Alvarez *et al.*, estimated the HIV incidence rate to be 3.23 per 100,000 donor-years in Spain using 3rd generation kits.[9] The HIV incidence among blood donors in the above study (as in most studies from developed world) is much lower compared to our study probably because of better donor education and increased donor awareness. Table 4. shows the results of various studies from India estimating HIV seroprevalence.[10-17] In a previous study from our institute, Sharma *et al.,* screened 2, 35,461 donors between 1996 and 2002 using third generation ELISA. The prevalence of HIV ranged from 0.16% in 1996 to 0.3% in 2002. In our study, HIV seroprevalence was 0.14%, hence lower than the previous study from our institute. More awareness and better screening of donors may account for this trend.

In our study, although seroreactivity was less in voluntary as compared to replacement donors, the results were not statistically significant. Similarly student voluntary donors had less seroreactivity as compared to non-student voluntary donors. Our results are consistent with the study by Sharma *et al.*, where among voluntary donors, student donors had a lower seroreactivity rate (0.07%) as compared to non-student donors (0.14%).

Using the 4th generation ELISA, HIV seroprevalence was estimated to be 3.62 per 1000 donations. Sudha, *et al.,* evaluated the TRI-DOT Rapid HIV test for the early detection of human immunodeficiency virus (HIV) infection in comparison with a 4th generation ELISA (Vironostika HIV Uniform II) in 23609 samples between January 2003 and April 2004.[17] In the case of discordance, sera were retested by Western Blot, and qualitative RT-PCR. The overall prevalence of HIV-1 and HIV-2 in the sera studied was 4.7% and 0.2%, respectively, and 19 (1.7%) of the 1150 HIV-reactive patients were infected with both HIV-1 and HIV-2. The seroprevalence is high as compared to our study i.e. 0.36% (overall prevalence) and 0.058% (HIV-2) because the author's study is from a region of high HIV prevalence (prevalence of HIV in Andhra Pradesh is 0.9% vs 0.33% in Chandigarh).[2] In our study, of the 11 samples which were ELISA reactive and WB reactive using 3rd generation ELISA, all were found to be reactive with 4th generation ELISA. It is estimated that use of combined antigen-antibody assay would give a yield of 0.58 window period units per 1000 donations additional to those which are tested reactive by the current 3rd generation assays. Of the 37 samples reactive or possibly reactive with fourth generation ELISA in the present study, 17 were also reactive with WB. However, true yield cannot be estimated without NAT or repeat fourth generation ELISA testing. It may be mentioned here that studies from China and Spain have shown conclusively that introduction of 4th generation ELISA significantly reduces the residual risk per unit transfused.[9,18]

Currently Nucleic acid amplification testing (NAT) is considered to be the gold standard for detecting the HIV infected persons during the pre-seroconversion period as it decreases the window period to around 5 days with ID (individual donation) NAT and 9 days with MP (mini-pool) NAT.[19] In our study, NAT was not done to confirm the results of 4th generation ELISA due to financial

Table 4: Studies on HIV seroprevalence in Indian blood donors

Author	Year	Donors (n)	Seropositivity	Test used	Province
Makroo, *et al.*[10]	1989–93	566 928	3·0/1000	ELISA	Delhi
Nanu, *et al.*[11]	1989–96	132 093	5·5/1000	ELISA	Delhi
Sharma, *et al.*[4]	1996-2002	235 461	3.0/1000	ELISA	Chandigarh
Kapoor, *et al.*[12]	1989–97	333 054	0·72/1000	ELISA and WB	Delhi
Khurana, *et al.*[13]	1988–97	79 553	0·8/1000	ELISA and WB	Punjab
Bhushan, *et al.*[14]	1988–93	14 084	1·9/1000	ELISA and WB	Tamilnadu
Choudhury, *et al.*[15]	1993–98	65 288	0·2/1000	ELISA and WB	UP
Thakral, *et al*[16]	2003-05	39 764	1.6/1000	ELISA and WB	Chandigarh
Sudha, *et al.*[17]	2003-2004	23 609	47/1000 HIV1 2/1000 HIV2	ELISA and RTPCR	Hyderabad

constraints. There are few studies in literature that have directly compared the performance of NAT assay and fourth generation HIV ELISA assay. In a retrospective study on first time blood donors by Barreto *et al.*, from Brazil from 1995-2001,[20] it was estimated that addition of p24 antigen, minipool NAT, and individual donation NAT assays would detect 3.9, 8.3 and 10.8 window period units per 10,00,000 first-time donations, respectively. In contrast, Nantachit *et al.*, did not find any additional yield for HIV 1 with NAT assay compared to 4th generation ELISA.[21] In a meta-analysis by Kucirka *et al.*, estimating the risk of window period infection in high risk donors among deceased transplant donors,the risk was 0.086 per 10000 donations when ELISA was used, and 0.035 when NAT was used which is low but not insignificant.[22] However, the limitations regarding the universal use of NAT in a developing country like ours include the higher cost, availability and time required to run the test.

Limitations of our study include relatively small sample size. Secondly NAT was not applied to confirm the results of 4th generation ELISA. Another limitation of the 4th generation ELISA is the relatively high false reactive rate. This may be of special concern in low HIV seroprevalence regions and paradoxically may have a negative impact on the blood donation services.[23] Jarvis *et al.*, recommended HIV 4th generation assays as an alternative to NAT in low prevalence countries as the latter is costly and only reduces but does not completely eliminate the risk of TTHIV infection.[24] In contrast, during a 9 month survey in France covering areas with high HIV seroprevalence, it was observed that 17 patients who were negative for both 3rd generation ELISA and Western blot tested reactive with 4th generation ELISA.[25]

To conclude, this is the first study from India comparing the HIV seroprevalence among blood donors with 3rd and 4th generation ELISA on the same donor population. Results of our study show better performance of 4th generation ELISA compared to 3rd generation ELISA in terms of HIV seroreactivity. Although both 4th generation ELISA and NAT are suitable for testing sizeable number of samples, can be easily adapted to automated platforms and have high stability, the former offers an advantage over NAT in that it is relatively less expensive and simple to perform.

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