

Reassessment of Initial Grey Zone Samples in Enzyme-Linked Immunosorbant Assay by Repeat Test for Blood Donor Screening Of HIV, HBV And HCV at a Teaching Hospital, in the North Eastern India

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Abstract: Enzyme-linked immunosorbant assay (ELISA) is a commonly used serological assay for screening of donated blood units for HIV, HBV and HCV. The decision point for the release of donated blood that is in the grey-zone of ELISA cut-off is a matter of serious concern because the status of true negativity is uncertain. Inclusion of methodologies to enhance the test sensitivity is particularly important for screening purpose. Test specimens with absorbance values within 10% below the ELISA cut-off (grey-zone) were retested in duplicate in the next ELISA run. If both duplicate retest sample absorbance values were less than the cut-off, the sample was considered non-reactive and sample showing one or both absorbance value(s) equal to or greater than the cut-off was considered reactive. Samples showing one or both absorbance value(s) again as grey zone were marked as indeterminate and blood units were discarded, but the donors were documented as non-reactive. The study was designed for three and half years between January 2016 and June 2019. All blood donors were screened for HIV, HBV and HCV by ELISA. A total of 97 (17 for HIV, 32 for HBV and 48 for HCV) blood donor were found to be in the grey-zone out of 23,628 donors sample tested. Results of the grey-zone samples on repeat test in duplicate, 20 (20.62%) samples (1 for HIV, 8 for HBV and 11 for HCV) were found to be reactive; 21(21.65%) samples (5 for HIV, 6 for HBV and 10 for HCV) were indeterminate and remaining 56 (57.73%) samples (11 for HIV, 18 for HBV and 27 for HCV) were non-reactive. A total of 41 potentially infectious blood donors were identified and discarded all the units derived from them. The reassessment of grey zone samples for blood donor screening of is a reasonable and economic testing algorithm.

Keywords: Absorbance value, ELISA cut-off, Grey-zone.

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I. Introduction

The total number of people living with HIV (PLHIV) in India is estimated at 21.40 lakhs (15.90 lakh-28.39 lakh) in 2017 with an adult prevalence rate of 0.22%.[1]Viral hepatitis is also increasingly being recognized as a public health problem in India. Hepatitis B surface antigen (HBsAg) positivity in the general population of India ranges from 1.1% to 12.2%, with an average prevalence of 3-4% and Anti-Hepatitis C virus (HCV) antibody prevalence in the general population is estimated to be between 0.09-15%.[2] The risk of transmission of these infectious agents through blood transfusion is a major concern in blood transfusion services.

It is mandatory to test all donated blood units for HIV I/II antibody, hepatitis B surface antigen and antibody to Hepatitis C by ELISA/Rapid test, VDRL/RPR for syphilis and screening for malarial parasite as per regulations of blood banking in India by Drugs & Cosmetic Act,1945 and Rules1947 . Any technology with similar or higher sensitivity may be used additionally to improve blood safety.[3] World Health Organisation (WHO) and National AIDS Control Organisation (NACO) formulated Standard Operating Procedures (SOPs) for Transfusion Transmissible Infections (TTI) screening of blood donors. NACO recommends the use of ELISA kits with a sensitivity of ≥ 99.5 percent and the specificity of ≥ 98 percent and rapid kits with a sensitivity of ≥ 99.5 percent and the specificity of ≥ 98 percent.[3]

The enzyme-linked immunosorbant assay (ELISA) is a commonly used serological screening assay for blood donors and this is specially cost effective in the blood centers where more sensitive supplementary assay such as NAT has not been adopted universally. So, methodologies that aim in enhancing the sensitivity of

current ELISA technology used for blood donor screening is important. The decision point for the release of donated blood units that are in the grey-zone of ELISA cut-off is a matter of serious concern to the blood banks and there is a need to reassess those units by repeat testing. Literature regarding detection of grey zone and its application in blood donor screening is scarce. The findings on a few studies of “gray-zone” application for improving test sensitivity are encouraging. The inclusion of grey-zone samples for a second test in duplicate as a testing algorithm can be a reasonable and economically feasible strategy to solve partly the problems of uncertainty.[4]

II. Material and Method

This prospective study was conducted during the period from January 2016 to June 2019 in the Blood bank, of a tertiary care teaching Hospital, in the north eastern part of India.

A total of 23,628 blood units were collected and screened during the study period. Five milliliters of whole blood samples were collected from each blood donor in a pilot tube taken at the time of blood collection and serum was separated by centrifugation. The separated sera were used for TTI screening on the same day or were kept frozen in the deep freezer for testing on a later date.

TTI screening of blood donors were done as per departmental SOPs that was prepared in compliance with the documents formulated by NACO and WHO.

Serological assays for blood donor screening for HIV, HBV and HCV were done by semi-automated ELISA instruments using ELISA washer (WELLWASH, Thermo SCIENTIFIC) and ELISA reader (MULTISKAN FC, Thermo SCIENTIFIC). Samples were analyzed for antibodies to HIV-1,2 and HIV p24 antigen (Microlisa HIV Ag & Ab, J. Mitra & Co. Pvt. Ltd. New Delhi, India and Erba Lisa HIV Gen4, TRANSASIA BIO-MEDICALS LTD. Daman, India), HBsAg (HEPALISA, J. Mitra & Co. Pvt. Ltd. New Delhi, India and Erba Lisa SEN HBsAg, TRANSASIA BIO-MEDICALS LTD. Daman, India), antibodies to HCV (HCV Microlisa, J. Mitra & Co. Pvt. Ltd. New Delhi, India and Erba Lisa HCV Gen3 (v2), TRANSASIA BIO-MEDICALS LTD. Daman, India), by ELISA as per manufacturer instructions, a rapid immunochromatographic test for detection of malarial parasite (SD Q Malaria p.f/Pan Ag Test, SD Biosensor Healthcare Pvt.Ltd. Haryana, India and Dx Malaria Pv/Pf, DiaSys Diagnostic India Private Limited, Maharashtra, India) and RPR (Rapid Plasma Reagin) card test for syphilis testing (CARBOGEN, Coral Clinical System, Uttarkhand, India).

The validity of the test was checked as per kit manufacturer’s instructions. Quality control of each run of ELISA testing was done by preparing Levy-Jennings chart using in-house borderline positive controls that ran simultaneously with each run. To ensure efficient and accurate working of the ELISA washer and reader at all times annual maintenance contract (AMC) and calibration at regular intervals were done. The facility also participated in various external quality programs to ensure quality of laboratory practices including proficiency testing for HIV from State reference Laboratory under NACO, and External Quality Assurance Scheme (EQAS) samples from CMC Vellore.

The samples were subjected to one time ELISA for screening purposes of HIV, HBV and HCV. All the samples with absorbance value greater than or equal to the cut-off value were considered reactive and blood units were discarded and donors were notified as per SOP of the blood bank. Test specimens with absorbance value within 10% below the cut-off were assigned as Grey zone (suspect for the presence of antibodies and/or antigens) and were retested in duplicate for their respective viral marker using the same or different manufacturer’s ELISA kits on the next run. If both duplicate retest sample absorbance value less than the cut-off value, the sample was considered as non-reactive. If one or both of the duplicate retest sample absorbance value(s) were equal or greater than the cut-off, the specimen was considered reactive. On repeat testing, the grey zone sample showing one or both absorbance value(s) again as grey zone was marked as indeterminate and the blood unit was discarded, but the donor was documented as non-reactive.

III. Results

During the study period (January 2016 to June 2019) a total of 23,628 donor blood samples were tested as screening test for TTI. The number of donors that were initially reactive for antibodies to HIV-1,2 and HIV p24 antigen, HBsAg and antibodies to HCV were 51(0.216%), 172(0.728%) and 221(0.935%) respectively. A total of 97 (17 for HIV, 32 for HBV and 48 for HCV) blood donor samples were found to be in the grey-zone. Results of the grey-zone samples on repeat test in duplicate, 20 (20.62%) samples (1 for HIV, 8 for HBV and 11 for HCV) were found to be reactive; 21(21.65%) samples (5 for HIV, 6 for HBV and 10 for HCV) were indeterminate and remaining 56 (57.73%) samples (11 for HIV, 18 for HBV and 27 for HCV) were non-reactive. The inclusion of grey zone repeat testing algorithm in TTI screening by ELISA in the present study increased TTI discard rate from 1.88% to 2.05% (Table 2).

Table1.TTI sero-reactivity and grey-zone on initial ELISA runs of blood donor samples (n=23,628)

Test	Number of initially sero-reactive samples(%)	Number of initially grey-zone samples (%)
HIV	51(0.22%)	17 (0.07%)
HBV	172(0.73%)	32 (0.14%)
HCV	221(0.94%)	48 (0.20%)
Total	444(1.88%)	97(0.41%)

Table2.Potential risk of TTI in 23,628 blood donors on reassessment by repeat test

Test	Number of initially sero-reactive donors (%)	Number of initially grey-zone donor's samples	Results of initially grey-zone samples on repeat test			Total number donors with potential risk of TTI (%)
			Reactive	Indeterminate	Non-reactive	
HIV	51(0.22%)	17	1	5	11	57(0.24%)
HBV	172(0.73%)	32	8	6	18	186(0.79%)
HCV	221(0.94%)	48	11	10	27	242(1.02%)
Total	444(1.88%)	97	20	21	56	485(2.05%)

IV. Discussion

There are constraints and challenges on selection of assays for TTI screening of blood donors in developing countries. A number of factors need to be considered in selecting the most appropriate assay such as the screening need and the resources available, including finances, manpower and their expertise, equipment procurement and maintenance, and reagents. [5] Several methods have been devised to improve the sensitivity of \currently use ELISA technology, such as the inclusion of borderline reactive control samples to minimize batch to batch, as well as day to day variation in testing. Another method to enhance the sensitivity of ELISA as screening assay is an estimation of sample lying in a grey zone and its repeat testing. [4]

The residual risk of disease transmission varies according to the incidence of infection in the donor population and the nature of donor screening process in place.[6]There are several other factors such as virus variant, presence of immunologically silent carrier, laboratory errors, window period, as well as limitations in screening test methodology. However, the primary cause of residual transmissions is thought to be donations from individuals in the window period of early infection. The development of highly sensitive and specific test like Nucleic acid amplification test (NAT) can significantly reduce the window period of infection. However, in blood centers with small volume of daily donations coupled with limited resources, the implementation of NAT for mandatory screening of blood donors may not be always feasible considering the minimal incremental gain on blood safety at a considerable cost. In India, serology is still mandatory as per regulations. NAT is used in some centers in the country as an additional layer of safety over and above the serological assays.

In the present study, out of a total of 23,628 blood donors, 444 (1.88%) were initially sero-reactive (Table 1). The sero-prevalence of HIV, HBV, and HCV were 0.22%, 0.73% and 0.94% respectively and this finding is in agreement with other sero-prevalence studies carried out in various parts of India. Prevalence of TTI in India is 1.8-4%, 0.4-1.09%, 0.2-1% and 0.05-0.9% for HBV, HCV, HIV, and syphilis, respectively. [9, 11]

Our study found a total of 97 (0.41%) samples in grey zone for all the three viral markers as compared as compared to 0.04-0.56% in other studies. [10, 12] It was also found that the number of grey-zone for HCV (48) was higher compared to that of HIV (17) and HBV (32) probably due to higher prevalence of HCV and its long window period of infection.

We detected 20 (20.62%) of 97 grey zone samples as repeat reactive, for either of the viral markers another 21(21.65%) were indeterminate. The repeat reactivity in other relevant studies range from 4.6-75%. The 41 potentially infectious donations (20 repeat reactive and 21 indeterminate) that were initially non reactive were effectively discarded after repeat testing of initial grey zone samples. Thus, we discarded a total of 82 units i.e. 13 units whole blood, 28 units PRBC, 28 units of Fresh frozen plasma and 28 units of platelet concentrate that were derived from the 41 potentially infectious donations. The reassessment of grey zone samples by repeat testing in our current study resulted higher blood discard rate which could have been reduced by the use of more sensitive and specific confirmatory test like NAT. However, the higher discard rate due to possible false positivity by the use of grey zone samples of ELISA cut off at the cost improving sensitivity of the assay was felt justifiable considering the impact it would have in reducing the mortality and morbidity of patients due to TTI in an already resource burden nation where universal NAT based assay for blood donor screening being far from reality.

Two limitations of this study were the inability of performing the confirmatory assays and there was no follow up to look for sero-conversion after the maximum window period(s) of infection(s) for the donors whose samples were within grey zone of ELISA cut off. There is no recommended national guideline for notification

and follow up of blood donors whose results are in grey zone of ELISA cut off and it may create even more challenging situations, as the person may either be false positive or require retesting to look for sero-conversion, hence requiring a greater degree of counseling.

National adult (15-49 years) HIV prevalence in India is estimated at 0.22% (0.16% - 0.30%) in 2017.[1] In the state where current study was carried out the adult HIV prevalence (1.43%) is one of the highest in the country, So there are likely a significant number of window period donations that can be identified by NAT. NAT can reduce the average window periods of HBV, HCV, HIV infection and effectively reduce the missed cases caused by virus variants, immune silence and occult infection.[7] In comparative analysis of combined detection of NAT and ELISA in India on a total of 389367 units tested leads to 286 NAT yield. The average NAT yield combining all three viruses is 1:1361 in sero-negative donors.[8] In another study in India, there was high proportion of ELISA positive/NAT negative for HIV (50%) and HBV (15%).[9] The possible causes were false-positive result of ELISA due to nonspecific reactivity and false-negative of NAT. When the virus is suppressed in the human body or during antiviral therapy the load of viral nucleic acid is lower than the detection limit, but ELISA detection can still detect the presence of antigen and /or antibodies.[8,9,10] No test is 100% sensitive and considering the paramount importance of high sensitivity assays for blood donor screening, implementing combined use of ELISA and NAT for blood donor screening can be a “gold standard” for determining the presence and absence of infection.

V. Conclusion

The detection of true positive or true negative individuals is always difficult when individual absorbance values are close to the cut-off values in ELISA. In the resource limited blood centers, assays that are sensitive and are easy to use together with low cost of the equipment, kit and consumables can be a valid alternative to NAT-based test. The use of more sensitive IV generation ELISA kit within a quality environment and employing methodologies such as reassessment of initial grey zone samples of ELISA cut-off by repeat test on the next run to enhance the sensitivity of current ELISA will be of importance for ensuring blood safety.

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