Original Article

Impact of grey zone sample testing by enzyme-linked immunosorbent assay in enhancing blood safety: Experience at a tertiary care hospital in North India

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Abstract:

Background: Enzyme-linked immunosorbent assay (ELISA) used for screening blood donors for transfusion transmitted infections (TTIs) can sometimes fail to detect blood donors who are recently infected or possessing the low strength of pathogen. Estimation of a grey zone in ELISA testing and repeat testing of grey zone samples can further help in reducing the risks of TTI in countries where nucleic acid amplification testing for TTIs is not feasible. **Materials and Methods:** Grey zone samples with optical density (OD) lying between cut-off OD and 10% below the cut-off OD (cut-off OD × 0.9) were identified during routine ELISA testing. On performing repeat ELISA testing on grey zone samples in duplicate, the samples showing both OD value below grey zone were marked nonreactive, and samples showing one or both OD value in the grey zone were marked indeterminate. The samples on repeat testing showing one or both OD above cut-off value were marked positive. **Results:** About 119 samples (77 for hepatitis B virus [HBV], 23 for human immunodeficiency virus [HIV], and 19 for hepatitis C virus [HCV]) were found to be in grey zone. On repeat testing of these samples in duplicate, 70 (58.8%) samples (45 for HBV, 12 for HIV, and 13 for HCV) were found to be reactive. Six (5%) samples (four for HBV, one for HIV, and one for HCV) were found to be indeterminate. **Conclusion:** Seventy donors initially screened negative, were found out to be potentially infectious on repeat grey zone testing. Thus, estimation of grey zone samples with repeat testing can further enhance the safety of blood transfusion.

Key words:

Enzyme-linked immunosorbent assay, grey zone, transfusion transmitted infection

Access this article online

Website: www.ajts.org

DOI: 10.4103/0973-6247.164272

Quick Response Code:

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Introduction

Every year millions of lives are saved through blood transfusions. In spite of advanced screening test technologies, recipients still have an increased risk of becoming infected with human immunodeficiency virus (HIV) and other transfusion transmitted infections (TTIs) such as hepatitis B virus (HBV) and hepatitis C virus (HCV).[1] The situation becomes graver in developing countries, as improvised testing technologies for screening of TTIs leads to increased cost of blood components for the patients. Hence, transmission of HIV and other viral infections continues to be a serious threat to safe blood transfusion in these areas. The fact that these developing countries account for more than 90% of all new HIV cases worldwide makes the task of screening blood donors more challenging.[2]

The risk of TTIs is estimated to be 1 in 6,77,000 units for HIV, 1 in 1,03,000 for HCV, and 1 in 63,000 for HBV.[3] Blood transfusion is an effective mode of transmission of TTIs as it allows entry of large quantity of infective virions into the recipients. In multiply transfused hemophiliac patients, the prevalence of HCV was found to be as high as 23.9%.[4] In India, mandatory screening for HCV was implemented quiet late in 2002.[5]

Several tools have been implemented for preventing TTIs ranging from donor selection to donor testing. Screening of blood donors for infectious markers is done by immunoassay in form of antigen/antibody detection methods such as latex agglutination,

Cite this article as: Solanki A, Singh A, Chaudhary R. Impact of grey zone sample testing by enzyme-linked immunosorbent assay in enhancing blood safety: Experience at a tertiary care hospital in North India. Asian J Transfus Sci 2016;10:71-4.

column chromatography, and enzyme-linked immunosorbent assay (ELISA), or by genetic tests like nucleic acid amplification technology (NAT) assay. Drug and Cosmetic Act in India has made testing of HIV, HBV, HCV, syphilis, and malaria mandatory on all blood donations done in blood banks.[6] In India, screening of all blood donors is done complying with the strategy I as laid down by World Health Organization (WHO). Strategy I of WHO mentions subjecting all blood donors sample to one time ELISA for screening purposes and marking samples with OD above or equal to cut-off OD as reactive or positive and samples below cut-off OD as nonreactive or negative.[7] Literature regarding detection of grey zone sample and its application in TTI screening procedures in blood bank set up is scarce. Therefore, it becomes very prudent to assess the utility of grey zone calculation and its role in improvising the current screening methodologies. We present here our experience of testing grey zone samples and its role in enhancing the sensitivity of current ELISA technology used for blood donor screening at our set up.

Materials and Methods

This prospective study was performed on blood donors coming for blood donation in the department of Transfusion Medicine and was conducted for a period of 1-year. Donors passing the proper donor selection criteria as laid down by drug and cosmetic act were included in the study after obtaining their informed consent.[6] The study was approved by ethical committee of the institution. Immunoassay in the form of ELISA was done for screening all blood samples by an automatic instrument (Davinci, Biomerieux) strictly following the manufacturer's guideline. HIV screening was performed by fourth generation ELISA kits (Vironostika, HIV Ag/Ab, Biomerieux, Netherland) for HBV (Hepanostika, HBsAg ultra, Biomerieux, Netherland) and HCV (Monalisa Anti-HCV PLUS version 2, Biorad, France) third generation ELISA kits were used. Validation of each test was performed according to manufacturer's instruction. Quality control of ELISA testing was done by preparing Levy-Jennings chart by simultaneously running the in-house borderline positive controls for 30 consecutive runs as mentioned in National Aids Control Organization guidelines.[7] All the samples with optical density (OD) more than the cut-off were considered reactive and blood units were discarded and donors were notified as per departmental standard operating procedure. Grey zone was calculated as 10% below the cut-off OD. All the samples with OD between cut-off value and 0.9 × cut-off value were marked as grey zone sample and were quarantined. All the grey zone samples were retested in duplicate for their respective viral marker using the same ELISA kits the next day. On repeat testing, the grey zone samples showing both OD values below 0.9 × cut-off value were marked as nonreactive and the blood units were included in the inventory. If on repeat testing the grey zone sample showed one or both OD value above the cut-off value it was marked as reactive and blood units were discarded and donor notified. The grey zone sample showing one or both OD value again as grey zone on repeat testing was marked as indeterminate and blood unit was discarded, but the donor was documented as nonreactive and notified for repeat testing after 6 months. The algorithm used for processing the grey zone sample is illustrated in Figure 1.

Results

Of 21,252 healthy donors screened for mandatory infectious markers, HIV positivity was found in 82 (0.38%) donors with HBV and HCV in 382 (1.79%) and 108 (0.5%) donors, respectively. Cumulative overall positivity for all infectious markers was found to be 2.67%. Excluding all reactive samples, about 119 (0.56%) more samples were found to lie in a grey zone with 77 belonging to HBV, 23 belonging to HIV, and 19 belonging to HCV. On repeat testing of these grey zone samples, 70 were found to be reactive with 45 for HBV, 12 for HIV, and 13 for HCV. Six grey zone samples showed indeterminate results on repeat testing with four for HBV, one for HIV, and one for HCV [Table 1]. The inclusion of criteria of grey zone calculation in routine ELISA screening test at our center increased the positivity of infectious markers from 2.67% to 3% [Table 2].

Discussion

HBV, HCV, and HIV are the most important agents responsible for TTIs and thus their testing on blood donors is mandatory worldwide due to potential serious clinical complications associated with these agents.[8,9] With advances in screening techniques in the form of NAT, the risk of TTI's has decreased considerably.[10] Still TTI's remains a threat to blood safety due to several factors such as genetic variations of infectious agents, presence of immunologically silent carriage, laboratory errors, and variations in the window period of the infectious agent, as well as limitations in screening testing methodology.[8] In developing countries where NAT test is not routinely practiced for screening due to non-affordability, immunological assays like ELISA serves as a main screening tool in blood bank setup. Several methods have been devised to improve the sensitivity of ELISA such as the inclusion of borderline reactive control samples in each run to minimize batch to batch, as well as day to day variation in testing. These borderline reactive samples are also able to detect minor variation in the assay procedure.[7] Another method to enhance the sensitivity of ELISA as screening assay is an estimation of the sample lying in a grey zone and its repeat testing. It has been very well-illustrated by Pereira *et al*.[11] that ELISA-based screening test for TTI in blood banks does involve a certain amount of uncertainty especially around the cut-off zone used for calculating the reactive samples. Hence,

Table 1: TTD marker seroreactivity in grey zone samples (*n* = 119)

TTD marker	Grey zone sample	Repeat testing of grey zone samples		
		Repeat nonreactive	Repeat seroreactive	Repeat grey zone (indeterminate)
HBV	77	28	45	4
HIV	23	10	12	1
HCV	19	5	13	1
Total	119	43	70	6

HBV: Hepatitis B virus; HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; TTD: Transfusion transmitted diseases

Table 2: Total yield of seroreactivity on grey zone sample assessment

TTD marker	First time seroreactive of 21,252 donors (%)	Repeat reactive of 119 grey zone donors (%)	Total yield on grey zone testing of 21,252 donors (%)
HBV	382 (1.79)	45 (37.8)	427 (2)
HIV	82 (0.38)	12 (10.1)	94 (0.44)
HCV	108 (0.5)	13 (10.9)	121 (0.56)
Total	572 (2.67)	70 (58.8)	642 (3)

HBV: Hepatitis B virus; HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; TTD: Transfusion transmitted diseases

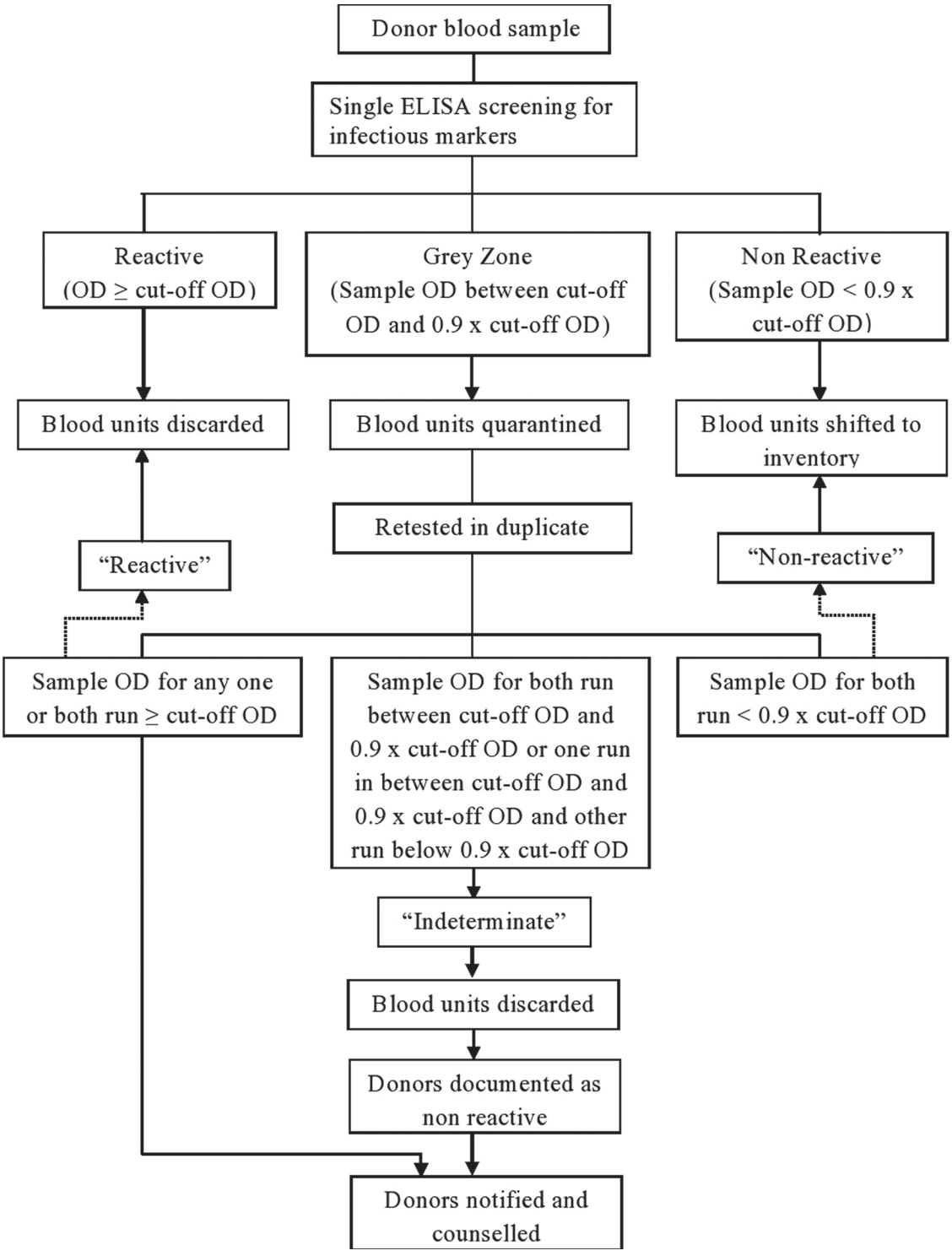

Figure 1: Algorithm for evaluating grey zone samples

they have emphasized on the measurement of this uncertainty around the cut-off zone in the form of grey zone sample testing. Presently, there are no such existing guidelines for grey zone sample testing in any regulatory authority in India and most of the blood bank in India follow the strategy I of one time ELISA testing as screening procedure as per WHO guidelines.[7] Grey zone sample testing might not have gained much relevance due to the issues of false positivity on repeat testing wherein a study conducted in Turkey have reported 70% false positivity on testing grey zone samples.[12] It has also been estimated that on applying the confirmatory test to grey zone samples resulted up to only 2% of true positivity.[13] We found a total of 119 (0.56%) samples in grey zone area for all three viral markers as compared to 0.14-0.29% found by other authors.[13,14] We observed a greater percentage of

grey zone samples than that observed elsewhere of 0.35% where samples lying in extended grey zone (30% below the cut-off OD value) were also included,[15] this may be due the differences in the type of ELISA kits used for performing the test. We detected 58.8% of 119 grey zone samples showing repeat reactivity, for either of the viral markers, on repeat testing as that of 4.6-75% in relevant studies.[13-15] One of shortcoming of our study is the inability of performing the confirmatory assay. Hence, we are not able to comment on the overall effectiveness of repeat grey zone sample testing in improving the transfusion safety. However, repeat reactivity in grey zone sample testing is an alarming indication for mandatory implementation of more sensitive testing technologies like NAT in developing countries. Since at our center we separate all blood units into at least three components, we effectively discarded 210 infectious components considering 70 grey zone samples showing positive infectious status on repeat testing. On the inclusion of grey zone sample testing, we observed the prevalence of HBV, HIV, and HCV to be 2%, 0.44%, and 0.56%, respectively. Recently Acar *et al*. have also reported similar findings of 1.76%, 0.17%, and 0.50% for HBV, HIV, and HCV in Turkey.[13]

Although the risk of TTIs today is lower than ever, but to achieve an era of zero risk transfusion still remains a challenge as the supply of safe blood remains subjected to contamination known and yet to be identified pathogens. Continuous improvement in the form of diligent donor screening and implementation of sensitive screening assay and effective pathogen inactivation procedures can ensure the elimination or at least reduction of the risk of acquiring TTIs.[9]

Implementation of cost-effective measures to improve the sensitivity of screening assays can be practiced especially in areas where TTI are highly prevalent. Higher discard rate of reactive blood units and minor increase in cost due to new testing methodologies can be justified by the impact it would have in reducing the mortality and morbidity of patients due to TTIs in an already resource burden nation. NAT technology for TTI screening being far from reality in developing nations, hence several alternative methods, in the form of assessment of grey zone samples to improve the sensitivity of the current screening procedure holds special importance.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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