ISSN: 2581 – 3633
PubMed - National Library of Medicine - ID: 101745081

International Journal of Medical Science and Advanced Clinical Research (IJMACR)

Available Online at:www.ijmacr.com

Volume – 6, Issue – 2, March - 2023, Page No. : 709 - 714

Reassessment of initial grey zone samples in enzyme – linked immunosorbent assay (ELISA) by repeat test for blood donor screening of HIV, HBV AND HCV.

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How to citation this article: Dr. Sanehaanjum Shaikh, Dr. Farzana Kothari, "Reassessment of initial grey zone samples in enzyme – linked immunosorbent assay (ELISA) by repeat test for blood donor screening of HIV, HBV AND HCV", IJMACR- March - 2023, Volume – 6, Issue - 2, P. No. 709 – 714.

Type of Publication: Original Research Article

Conflicts of Interest: Nil

Abstract

Purpose: TTI in blood Centre involve a certain amount of uncertainty especially around the cut-off zone used for calculating the reactive samples. There is limited literature on grey-zone available in the country & its repeat testing. To ensure safety of blood transfusion in resource limited countries.

Method: A Cross-sectional study conducted over a period from January 2020 to August 2021 in the Department of Immuno hematology and Blood Transfusion. ELISA is done by semi-automatic technique at our center. Grey Zone samples are sample OD between cut-off OD and 0.9 * Cut off OD. The units were quarantined & samples retested in duplicate. Results were Reactive, On reactive or grey- zone accordingly decision was taken.

Result: During the study period from January 2020 to August 2021 over 16,972 donors were tested for HIV-I/ II (anti-HIV-I/ II), hepatitis B (HBsAg), and hepatitis C (anti-HCV). Out of 16,972 donor samples, 74 samples were in grey zone by ELISA: 7 (9.5 %) of HIV, 57 (77.02%) for HBV and 10 (13. 51%) of HCV were in grey zone. On repeat testing, in HBV out of 57, 2 were reactive, 2 were in grey zone & 53 non-reactive. In HIV 7 were non-reactive. In HCV out of 10, 1 was reactive, 2 were in grey zone and 7 non-reactive.

Conclusion: 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.

Keywords: Grey-zone, TTI, ELISA

Introduction

Safe transfusion of blood and blood components saves millions of lives, but unsafe transfusion practices put millions of people at risk of TTIs. Blood is one of the major sources of transmission of infectious diseases, viz. HIV, HBV, HCV, syphilis, and many other infections in India. The enzyme–linked immuno sorbent 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 supple mentary assay such as NAT has not been adopted universally. 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.

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.[1] Literature regarding detection of grey zone and its application in blood donor screening is scarce. The findings on a few studies of "grey-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.

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.

Material and Method

This is a Cross-sectional study conducted over a period from January 2020 to August 2021 in the Department of Immunohematology and Blood Transfusion, SSG Hospital and Medical College, Vadodara, Gujarat. Study was approved by the respective Institutional Ethical Committee. Inclusion Criteria was to imclude Sample whose absorbance value lies between cut off and 10% below cut off during ELISA testing i.e Grey Zone samples.

Exclusion Criteria was Sample whose absorbance value is more than cut off considered Reactive while whose absorbance value lies below cut off considered as non-Reactive. These samples will be excluded from study.

ELISA is done by semi-automatic technique at our center. Third-generation assays using an antigen–anti body–antigen sandwich technique, have led to a big improvement in Sensitivity and Specificity. Fourth-generation Elisa combines detection of antibodies with detection of antigens. Enzyme-linked Immuno sorbent Assay (ELISA) is an enzymatic immuno - assay technique of the "sandwich" type for the detection of HBsAg, HCV, and HIV in human serum or plasma. The test uses mono clonal antibodies selected for their ability to bind themselves to the antigen of HBsAg, HCV, and HIV in now recognized by the World Health Organization (WHO) and the most part of variant HBV strains.[4] ELISA uses combination of the specificity of antibodies or antigens with the sensitivity of enzyme assays to detect HBsAg, antibodies to HCV and HIV 1 and 2 in human serum. These antigen antibody reactions occur in a coated micro well with a positive and negative control being run with each batch of tests following the principle of 'Sandwich ELISA'. ELISA was validated by the acceptance criteria laid down by the manufacturers.

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. 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

Grey zone samples whose OD lies between 0.9* Cut off OD and cut off OD were retested. The units were quarantined & samples retested in duplicate. Results were Reactive, Non-reactive or grey- zone accordingly decision was taken.

Results

The present study attempted to analyze number of grey zone samples and retesting them by Enzyme Linked Immunosorbent Assay. During the study period from January 2020 to August 2021 over 16,972 donors were tested for HIV-I/ II (anti-HIV-I/II), hepatitis B (HBsAg), and hepatitis C (anti-HCV). The hospital's blood center uses semi-automated Enzyme linked immunosorbent assay (ELISA) for detection of HBsAg, anti-HCV and anti-HIV antibodies.

All blood donors were examined for blood pressure, pulse; hemoglobin concentration and other general health indicators. Apparently, healthy persons of ages 18 to 65 years with body weight above 45 kg would qualify for donations.

A 16, 972 total blood donors who satisfied the qualifying criteria for the donation were enrolled in this study.

During the study of 16,972 donor samples,74 samples were in grey zone by ELISA. During the study period, 7 (9.5 %) of HIV, 57 (77.02%) for HBV and 10 (13.51%) of HCV were in grey zone.

Table 1: Repeat testing of grey zone TTI samples.

TTI marker	Grey zone	Repeat reactive	Repeat Non-reactive	Repeat Grey zone
HBV	57 (77.02%)	02 (3.50%)	53 (92.98%)	02 (3.50%)
HCV	10 (13.51%)	01 (10%)	07 (70%)	02 (20%)
HIV	07 (9.5%)	-	07	-
TOTAL	74	03	67	04

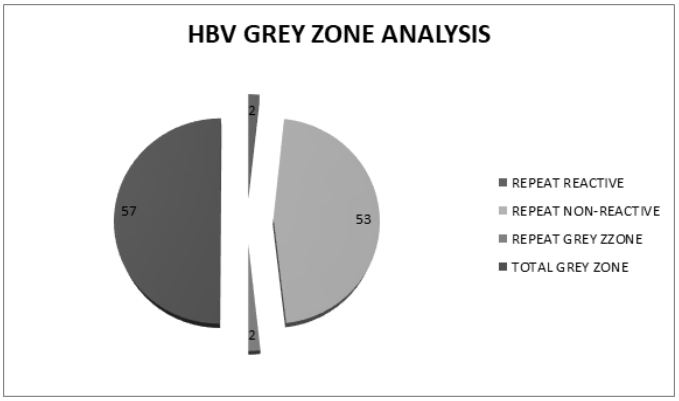
TTI= transfusion transmissible infections; HBV= hepatitis B virus; HCV=hepatitis c virus; HIV= human immuno-deficiency virus.

Table 2: Sero reactivity after grey zone sample assessment.

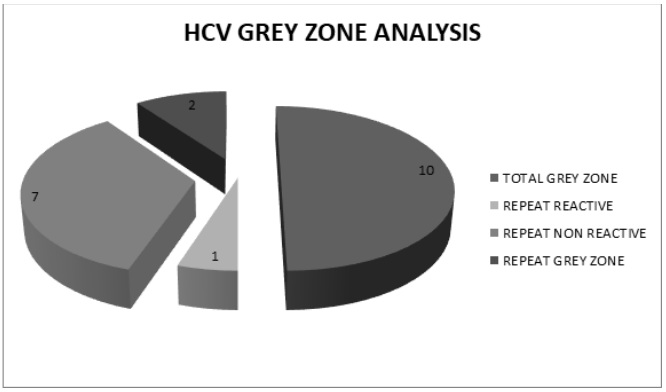
TTI marker	First time Seroreactivity of 16,972 donors (%)	Repeat reactive of 74 grey zone donors (%)	Total yield on grey zone testing of 16,972 donors (%)
HBV	76 (62.80%)	02 (66.6%)	78 (62.90%)
HCV	25 (20.66%)	01 (33.3%)	26 (21.48%)
HIV	20 (16.52%)	-	20 (16.52%)
TOTAL	121	03	124

TTI= transfusion transmissible infections; HBV= hepatitis b virus; HCV=hepatitis c virus; HIV= human immuno-deficiency virus.

Graph 1:



Graph 2:



Discussion

With advances in screening techniques in the form of NAT, the risk of TTI's has decreased consider ably. [3] Still TTI's remains a threat to blood safety due to several factors such as genetic variations of infectious agents, presence of immuno logically silent carriage, laboratory errors, and variations in the window period of the infectious agent, as well as limitations in screening testing metho dology.

In developing countries where NAT test is not routinely practiced for screening due to non-afford ability, immuno logical assays like ELISA serves as a main screening tool in blood bank setup. [10]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. [10]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. 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, they have emphasized on the measurement of this uncertainty around the cut-off zone in the form of grey zone sample testing.[9]

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 NACO guidelines. [10] 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 It has also been estimated that on applying the confirmatory test to grey zone samples resulted up to only 2% of true positivity. [10] Repeat reactivity in grey zone sample testing is an alarming indication for mandatory implementation of more sensitive testing technologies like NAT in developing countries. Recently Acar et al. have also reported similar findings of 1.76%, 0.17%, and 0.50% for HBV, HIV, and HCV in Turkey.

Implementation of cost-effective measures to improve the sensitivity of screening assays can be practiced especially in areas where TTI are highly prevalent. [14] 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. [10] 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. [14] An unsafe blood transfusion is very costly from both human and economic points of view. Morbidity and mortality resulting from the transfusion of infected blood have far-reaching consequences, not only for the recipients themselves, but also for their families, their communities and the wider society. [10]

Serological and Molecular assays are predominant and reliable methods for HBV detection. CLIA is more sensitive than ELISA. Rapid tests are also dependable and useful for screening purpose, especially in resource poor settings.[14] Quantitation is important for monitoring. Real time PCR, b DNA assays are principal methods used for this purpose. Automated systems are more sensitive when compared to in house assays.

In the present study, out of a total of 16,972 blood donors, 121(0.71%) were initially sero-reactive (Table 1). The sero-prevalence of HIV, HBV, and HCV were 0.11%, 0.44% and 0.14% 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. Our study found a total of 74 (0.43%) 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 HBV (57) was higher compared to that of HIV (7) and HCV (10) probably due to higher prevalence of HBV. We detected 3 (4.05%) of 74 grey zone samples as repeat reactive, for either of the viral markers another 4 (5.40%) were indeterminate. The repeat reactivity in other relevant studies range from 4.6-75%. The 9 potentially infectious donations (3 repeat reactive and 4 indeterminate) that were initially non reactive were effectively discarded after repeat testing of initial grey zone samples.

Thus, we discarded a total of 20 units i.e. 7 units PRBC, 7 units of Fresh frozen plasma and 6 units of platelet concentrate that were derived from the 7 potentially infectious donations.

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 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.

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