

True positivity of anti-Hepatitis C Virus Enzyme-linked immunosorbent assay reactive blood donors: A prospective study done in western India

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Abstract:

Background: A significant number of safe donations are removed from the blood supply, because of the reactive anti-HCV screening test results. This study aimed to assess if the HCV (Hepatitis C Virus) seropositive donors were confirmed positive or not. **Materials and Methods:** More than 68,000 blood donors' samples were routinely screened and 140 samples were found to be anti-HCV ELISA reactive. These 140 samples were tested by NAT. The NAT negative samples were tested by RIBA. Analysis of samples reactive in single ELISA kit vs. two ELISA kits was done. **Results:** Out of 140 anti-HCV ELISA reactive samples, a total of 16 (11.43%) were positive by NAT. The results of 124 RIBA showed 6 (4.84%) positive, 92 (74.19%) negative, and 26 (20.97%) indeterminate results. None of the sample which was reactive in only single ELISA kit was positive by NAT or RIBA. **Conclusion:** Only a minority of blood donors with repeatedly reactive anti-HCV screening test is positive by confirmatory testing, but all these blood units are discarded as per existing legal provisions in India. Efforts should be made to retain these donors and also donor units.

Key words:

Anti-Hepatitis C Virus Enzyme-linked immunosorbent assay, nucleic acid amplification test, recombinant immuno blot assay

Introduction

The quality and safety of blood transfusion therapy is of continuing concern all over the globe, particularly in developing countries where 80% of world population lives.^[1] In India, all blood banks test blood units for Hepatitis C Virus (HCV) by ELISA (enzyme-linked immunosorbent assay) as per mandatory law. Increase in test sensitivity of transfusion transmitted infections (TTI) is highly desirable for ensuring recipient safety. However, increased sensitivity of testing kits has often been associated with decreased specificity and consequently an increased rate of false-positive test results.^[2] Biological false-reactive results in blood donors are problematic due to both component loss and donor management issues. There is a need for a more standardized approach to the screening of blood donors with the aim of minimizing the number of false-reactive screening test results.^[2] This study was conducted to assess if the blood donors who were repeatedly reactive on enzyme immunoassay (EIA) were actually carrying HCV infection or not by testing with NAT and or RIBA method. The study also correlated the results of samples reactive for anti-HCV by single ELISA kit only vs. those by two ELISA kits with Nucleic Acid Amplification Test (NAT) and or Recombinant Immuno Blot Assay (RIBA) results.

Materials and Methods

This prospective study was conducted primarily at Prathama Blood centre, a Regional Blood Transfusion Center (RBTC) in Ahmedabad in Western India. Blood samples were also collected from two other associated blood centers at Ahmedabad. A total of 68,951 blood units were screened by anti-HCV ELISA routinely at the three blood centers, over a period of 15 months. Two ELISA kits were primarily used, i.e., kit A (Murex 4 anti-HCV) and kit B (Heptest HCV Ultra). A total of 350 (0.51%) samples were initially reactive in single kit (i.e., either by kit A or kit B). When tested in duplicate in the same kit, only 203 (0.29%) samples were reactive. Of these, only 62 (0.09%) samples were reactive by second ELISA kit. For this study, a total of 140 samples (which met the inclusion criteria) were included. These were 87 (62.14%) samples which were reactive in single ELISA kit only and 53 (37.86%) samples which were reactive by both the ELISA kits.

When a donor sample was tested reactive by ELISA, plasma from anticoagulant Ethylene Diamine Tetra Acetic Acid (EDTA) tube of that sample was separated and stored at - 30°C deep freezer in duplicate vials for further testing by NAT. NAT-negative samples were further tested by RIBA.

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Statistical Analysis

While evaluating the data whether it was significant or not, the statistical tests applied were the “Chi square” and the “standard error of proportion (SEP)”. If the “P” value was equal to or less than 0.05 ($P < 0.05$), then it was assumed that the comparison was significant.

Results

Out of 68,951 blood donor samples, 140 samples were tested by NAT. A total of 16 (11.43%) were positive by NAT and 124 (88.57%) were negative. The 124 NAT negative samples were tested by RIBA. The RIBA results showed 6 (4.84%) positive, 92 (74.19%) negative, and 26 (20.97%) indeterminate results. So, out of 140 samples, HCV confirmed positive samples by NAT or RIBA was 22 (15.71%).

Out of 140 samples, 87 (62.14%) samples were reactive by only one (single) ELISA kit and 53 (37.86%) samples were reactive by both the ELISA kits. None of the samples reactive in single ELISA kit (87) was positive by NAT or RIBA. Of the 53 samples reactive in the two ELISA kits, 16 (30.19%) were NAT positive. In the 37 NAT negative samples, RIBA was positive in 6 (16.22%) samples, negative in 14 (37.84%) samples, and indeterminate in 17 (45.85%) samples [Table 1 and Figure 1].

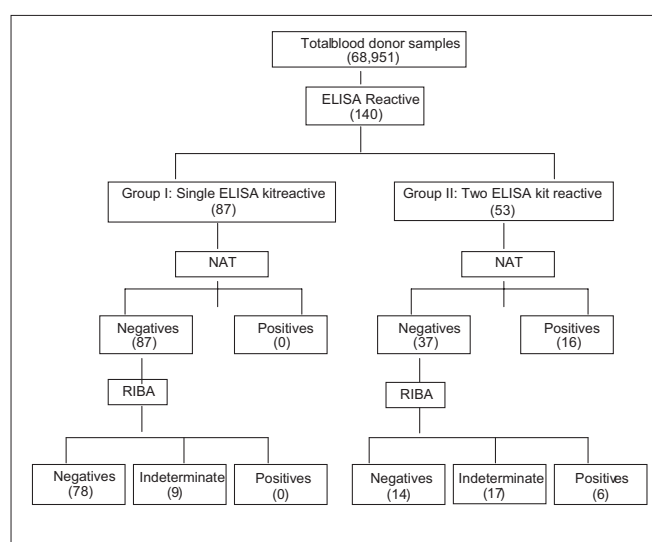


Figure 1: Flow diagram of testing done by enzyme-linked immunosorbent assay, nucleic acid amplification test and recombinant immuno blot assay in the study group donor samples

Table 1: Nucleic acid amplification test or recombinant immuno blot assay results of all (single and two kits) enzyme-linked immunosorbent assay reactive samples

ELISA reactive with single or two kits	No. of ELISA reactive samples	NAT negative (%)	NAT positive (%)	RIBA negative (%)	RIBA ID ^s	RIBA positive (%)	NAT or RIBA positive (%)
Group I Single kit	87 (62.14)	87	00	78	09	00	00
Group II Two kits	53 (37.86)	37	16	14	17	06	22
Total	140	124 (88.57)	16 (11.43)	92 (74.19)*	26 (20.97)*	06 (4.84)*	22 (15.71)

^sID: Indeterminate, *Percentage calculated out of 124, N.B. - Out of 140 ELISA reactive samples, 16 (11.43%) were NAT positive. Out of 124 NAT negative samples, 6 (4.84%) samples were positive by RIBA

Discussion

This study was conducted with the aim to study the number of samples which were reactive by anti-HCV ELISA and were negative by NAT and or RIBA. The study was planned to evaluate whether the samples reactive for anti-HCV in single ELISA kits or those reactive in two ELISA kits had a better correlation with NAT and or RIBA results.

Correlation with nucleic acid amplification test/recombinant immuno blot assay results

In the present study, 16 (11.34%) of the 140 anti-HCV ELISA reactive samples were positive by NAT. Similar findings were found in a study in South Africa in which 13.6% (37 out of 275) ELISA reactive samples were positive by NAT.^[3] However, in a study by Kim *et al.*, 202 (57.1%) out of 354 subjects were positive for HCV by Reverse Transcribed Polymerase Chain Reaction (RT-PCR).^[4] The reason may be that the study population had high prevalence of liver diseases and subjects older than 60 years were also included. In another study by Ren *et al.*, 53 samples (34%) out of 156 ELISA reactive donor samples were positive by NAT.^[5] It was observed that the number of samples positive by NAT were less in the present study as compared to the other studies. One of the reasons may be that in the present study, majority of the donors were voluntary donors and about 35% were repeat donors.

If the NAT result is negative in persons with a positive ELISA screening test results, the anti-HCV antibody or infectious status cannot be determined. In these persons, additional testing with RIBA is necessary to verify the anti-HCV result^[6] In the present study, RIBA was done on all the 124 NAT negative anti-HCV ELISA reactive samples. RIBA results showed 6 (4.84%) positive, 92 (74.19%) negative, and 26 (20.97%) indeterminate results. Similar results were reported by Piro *et al.* in which RIBA was positive in 28 (10.3%), negative in 178 (65.7%), and indeterminate in 65 (24%) of the 271 anti-HCV ELISA reactive samples.^[7] In contrast, in a study by Kleinman *et al.* of 47,041 ELISA repeat reactive donations, 49.3% were RIBA positive, 33.5% were RIBA negative, and 17.1% were RIBA indeterminate. Out of the total RIBA positive, 79.2% were NAT positive.^[8] The reason for finding less number of RIBA positive samples in the present study may be that RIBA was done on NAT negative samples only and not on all the ELISA reactive samples, as done in the other study.

One of the important findings in the present study was that out of 124 NAT negative samples, 6 samples were positive by RIBA. Dow *et al.* have shown that when 177 RIBA positive samples were tested by NAT, 54 (30.51%) were RNA negative.^[9] The reason may be that the circulating HCV RNA titre may vary considerably. While a single qualitative assay for HCV RNA confirms active viral

replication, a single negative test does not exclude viremia and may reflect only a viral load below the detection limit of the assay.^[10] So the US, CDC has mentioned that the significance of a single negative HCV RNA result is unknown and the need for further medical evaluation is determined by verifying anti-HCV status.^[6]

In the present study, the positive result by either NAT or RIBA was interpreted as confirmed HCV infection. A total of 22 samples (15.71%) out of 140 anti-HCV ELISA reactive samples were confirmed positive by NAT or RIBA. The above observations imply that the number of false-positive results of anti-HCV ELISA positive may vary depending on the ELISA kit used and also on the type of study population and the HCV prevalence in that area. Numerous studies have shown that donors who have repeat reactive test results by screening assays, but are negative or indeterminate upon further testing are usually not infected with the viral agent under question. However, donations obtained from these donors are discarded and the donor are deferred as per legal requirements from donating blood. Large numbers of precious blood units are discarded due to false-positive results in screening test. False-positive screening test results have also been reported to cause psychological distress and confusion because the clinical significance of this results is some time not known.^[2]

Single enzyme-linked immunosorbent assay kit vs. two enzyme-linked immunosorbent assay kits

Adverse test results can be minimized in at least two ways: by the selection of highly specific primary screening immunoassays to minimize the number of false-positive results and by the use of confirmatory testing strategies to minimize nonspecific indeterminate results. An alternative approach to clarifying anti-HCV results, referred to as the sequential immunoassays strategy, is to test samples that are repeat reactive on one immunoassay by alternative screening immunoassay and subject only those samples reactive on both assays to further confirmatory testing.^[11]

Samples reactive in single enzyme-linked immunosorbent assay kits

In this study, it was noted that from a total of 140 anti-HCV ELISA reactive samples, 87 were reactive in single ELISA kit (i.e., reactive by one kit and negative by the other kit) and none of these was positive by NAT or RIBA. This is similar to the pattern in the study in China, in which 156 samples were tested by seven ELISA kits and those with discrepant results in the different ELISA kits were negative by both NAT and RIBA.^[5] Kita *et al.* have also shown in a study done in Japan that in specimens that were positive by only a single screening reagent, the RIBA III did not test positive, suggesting that the incidence of false positives may be high. The author has suggested that each anti-HCV antibody screening reagent in use has unique features, and it is suggested that caution be used when diagnosing HCV infection on the basis of the results of a single screening test.^[12]

Samples reactive by two enzyme-linked immunosorbent assay kits

However, among the samples reactive by both the ELISA kits, the percentage of samples positive by NAT or RIBA in the present study was less as compared to the study by Ren *et al.* (2005). In the present study, it was observed that of the 53 samples reactive in both the kits, 16 (30.19%) were positive by NAT and 6 (16.22%) were NAT negative but RIBA positive. Of this group, 53 (63.1%) were positive by NAT and 23 of NAT negative samples were RIBA positive. The percentage (53.8%) of samples positive by NAT and

RIBA is more in the study by Ren *et al.*^[5] than in the present study. One reason may be that the study group samples in the study by the same author were reactive in seven ELISA kits and in the present study by two ELISA kits. The probability of the samples reactive by seven ELISA kits to be positive was likely to be higher than the samples reactive in the two kits. In a study done in Hungary, by Hejjas *et al.*^[13] in blood donors, 11 samples out of 32 (34.37%), which were concordantly reactive in five ELISA kits, were positive for HCV RNA by PCR. These results were similar to the present study (30.19% positive by NAT).

Comparison of results of samples reactive in single enzyme-linked immunosorbent assay kits vs. those reactive in two enzyme-linked immunosorbent assay kits

From the above studies, it can be concluded that the samples which were reactive by single ELISA kit only have a high probability of being negative by NAT or RIBA. Sample found to be repeatedly reactive by two different ELISA tests for anti-HCV had a high probability of being positive by NAT or RIBA. The reason may be that two assays applied sequentially can increase the positive predictive value of the process because samples reactive on both assays have a higher probability of representing true reaction rather than those reacting only in one. Even WHO Guidelines (2002) mentioned that if confirmatory testing is not available, use an alternate assay, which is as sensitive as the primary assay, for use in confirming the status of the samples that are found to be repeatedly reactive by the primary assay.^[11,14]

Anti-hepatitis C virus reactive but nucleic acid amplification test and recombinant immuno blot assay negative blood donors may be considered for re-entry in the donor pool

Blood donors who are reactive by anti-HCV ELISA but negative by NAT and RIBA need not be permanently deferred. They may be considered for re-entry in the donor pool later. Readmission of these donors can make a considerable contribution as it allows regular valuable motivated donors, whose blood has been shown to be safe to continue their support. It was also observed in a look back study by Vrieling *et al.* that none of the recipient's of blood products from previous donations of anti-HCV ELISA positive, cDNA – PCR negative, and RIBA 2 indeterminate or negative donors were HCV infected. Such donors were not infected and the author had suggested that these donors could re-enter the donor pool, provided that future donations were anti-HCV ELISA negative.^[15] In another study by Moore *et al.*, those donors whose samples were reactive both in the routine screening test and in the alternate assay were not withdrawn permanently from donation, but removed from the donor panel for an arbitrary period of 3 years.^[16]

Conclusion

In conclusion, this study demonstrated that:

1. Donor samples reactive for anti-HCV in single ELISA kit should be tested by a second ELISA kit. The second assay must be of equivalent sensitivity to the first assay. Samples reactive in one ELISA kit, but not reactive in the other ELISA kit, may be considered to be false positive. These units may not be discarded though this needs regulatory approval.
2. Donors who are reactive in two ELISA kits should be tested by NAT and or RIBA. The donors reactive in two ELISA kits and also positive by NAT or RIBA should be permanently

deferred. These donors should be informed about the results and permanently deferred from donating in the future. These donors should be advised to consult clinician for future medical advice.

- The donors reactive in two ELISA kits but negative by NAT and RIBA should not be permanently deferred from donating blood. They should be followed in the future and if they are nonreactive by at least the same two kits on two occasions (at interval of 6 months), they may be included in the donor pool.

False-reactive screening test results are of great concern because they often lead to deferral of donors and may cause psychological adverse effects in donors. Only a minority of donors with repeatedly reactive screening tests is positive by confirmatory testing, but all blood units obtained from donors with reactive screening test results are discarded as per existing legal provisions. So efforts should be made to retain these donors and also donor units.

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