Can We Improve the Management of Blood Donors With Nonspecific Reactivity in Viral Screening and Confirmatory Assays?

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Donors with nonspecific reactivity in viral screening or confirmatory assays are problematic for blood services because of donor management issues and product loss. Considerable experience has now accumulated in the use of screening and confirmatory assays; therefore, it is timely to examine the ways in which donors with nonspecific reactivity are managed. In this review, we summarize the causes and characteristics of nonspecific reactivity in blood donors and approaches for reducing the number of nonspecific reactive results and we offer some suggestions for improving the management of these donors.

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Just 2 decades ago, hepatitis B surface antigen (HBsAg) was the sole viral marker available for blood donor screening. By the early 1990s, viral screening of blood donors in developed countries had expanded to include anti-human immunodeficiency virus 1 and 2 (anti-HIV-1 and 2, respectively), anti–hepatitis C virus (anti-HCV), and, in a number of countries, anti–human T-lymphotropic virus (anti–HTLV-1 and 2, respectively). More recently, some countries have also implemented nucleic acid testing (NAT) for HIV-1, HCV, and, in a few cases, HBV. Contemporary immunoassays (IAs) for serological screening have excellent performance characteristics, both in terms of sensitivity and specificity. However, 100% specificity remains elusive and arguably will not be achieved with current technologies. Therefore, IAs, when used to screen low-risk populations such as voluntary blood donors, have a low positive predictive value because most reactive results are due to nonspecific reactivity. Management of donors with nonspecific reactivity poses a number of challenges for blood services. In many countries, donations that test repeatedly reactive on a screening IA must be discarded because of regulatory requirements, even if subsequent confirmatory testing reveals the result to be due to nonspecific reactivity. In addition, donors whose results are consistently nonspecifically reactive require counseling, possible deferral from donating, and in some cases, additional testing and medical evaluation.

The purpose of this review is to examine the issue of nonspecific reactivity in viral screening and confirmatory assays in voluntary blood donors and to discuss possible strategies for improving donor management while minimizing product loss.

Terminology

An IA is a qualitative test where capture molecules (antigen or antibody) are immobilized onto a solid phase to which analyte in the test sample (corresponding antibody or antigen) will bind if present. A detection system indicates the presence of captured analyte. IAs are the method of choice for serological screening of donors because they are relatively economical, can be adapted for high-throughput testing, and, as indicated, have excellent sensitivity and specificity. Sensitivity is a measure of the assay’s ability to give a reactive result for an infected donor and is expressed as 100% minus the percentage of infected donors who test negative. In contrast, specificity is a measure of the assay’s ability to give negative results for uninfected donors. The specificity of an assay is expressed as 100% minus the percentage of unconfirmed repeatedly reactive results. Manufacturers of contemporary screening IAs generally aim for a specificity of 99.9% or higher.

Donor samples are screened in singlicate by IA and, if reactive, retested in duplicate on the same assay. Up to half of these initially reactive results are not reproduced upon retesting and the sample is considered negative. However, samples that remain reactive upon retesting are referred to as repeatedly reactive and subject to confirmatory testing to
clarify the status of the donor. Repeatedly reactive samples that are negative by confirmatory testing are usually referred to by terms such as biologic false-reactive (BFR) or false-positive. These samples are regarded as negative for the marker in question and the reactive result is considered to be due to “nonspecific” or “false” reactivity. In this review, we will refer to samples reactive by IA but negative by confirmatory testing as BFR.

Confirmatory testing of samples repeatedly reactive on IAs used for viral antibody screening usually involves further testing by immunoblot, sometimes in conjunction with an additional IA. Most laboratories use commercially available immunoblots that incorporate nitrocellulose strips onto which various viral proteins separated into distinct bands have been transferred. Diluted test sample is incubated with a strip so that viral antibodies, if present in the test sample, will bind to the appropriate viral protein. The bound antibodies can then be detected using a system similar to an enzyme immunoassay (EIA). The appearance of bands on the nitrocellulose strip allows identification of specific antibodies. Immunoblots require interpretative criteria to define the band combinations that represent a confirmed positive result. Band patterns that do not meet the confirmed positive criteria, referred to as indeterminate, create problems for donor management. Like IAs, immunoblot assays do not have 100% specificity and can therefore show nonspecific indeterminate reactivity. For example, it has been shown that even some low-risk donors who tested negative on IA, and are therefore presumably uninfected, can give indeterminate results on immunoblot.

POSSIBLE CAUSES OF BFR RESULTS

The primary cause of BFR results appears to be nonspecific antibody reactivity, either against viral or contaminating nonviral epitopes. This is indicated by the frequent association of BFR results with increased antibody production experienced during an immune response, including the following:

- vaccinations such as influenza or rabies
- recent acute infections or allergies
- immunologically related agents
- transplantation antigens and autoantibodies

The involvement of antibodies not specific for the viral antigens used in screening IAs is further suggested by the association of biologic false reactivity with IgM and IgG antibody production, heterophile antibodies, and natural nonspecific polyreactive antibodies with low affinity.

A unique situation can occur with HBsAg screening because of the very sensitive IAs now available for screening. Donors recently vaccinated for HBsAg can give reactive results because of the presence of HBsAg epitopes in the vaccination preparation. To avoid this phenomenon, it is recommended that donors wait up to 7 days after vaccination before donating.

CHARACTERISTICS OF BFR RESULTS

Assay Related

A sample with a BFR result on an IA will usually give a negative result when retested on an alternate assay because there is often (but not always) minimal overlap between the BFR populations of different IAs. Therefore, BFR results are often assay specific.

Changes in BFR Rate With Assay Master Lots

The BFR rate of an IA can vary significantly between different assay master lots. For example, Sharma et al found that the repeat reactive rate for their anti-HIV EIA varied from 0.028% to 0.111% between different master lots, but the prevalence of anti–HIV-confirmed positive donations did not show any significant change.

Variation in BFR Rate due to Assay Modifications

BFR rates can also vary with changes in assay configuration. For example, in our blood service, we observed an increase in the BFR rate of the PRISM HIV assay, from 0.069% to 0.084%, when the assay was reconfigured to incorporate HIV O epitopes. Further analysis of these donors indicated that the modified assay detected the same BFR population as the previous version, and an additional BFR group as well, presumably as a result of the new epitopes incorporated in the assay.

Transient BFR Results

In keeping with presumed etiology, biologic false reactivity is sometimes only transient, with donors testing negative on IA after several months. In a longitudinal study of donors with BFR results,
we found that of those who gave an index BFR result for anti-HIV, anti-HCV, or anti-HTLV, about 34%, 23%, and 28%, respectively, gave negative results at subsequent donations. The mean time for donors to become negative on IA after a BFR result was 7.1 months (range 2.5-35 months). However, this figure is almost certainly an overestimate because of the long interdonation periods of a number of donors.

IA Sample Signal Strength/Assay Cutoff Ratios

IA sample signal strength/assay cutoff ratios (S/CO) can be indicative of whether the final result will be BFR or confirmed positive after confirmatory testing. HCV screening provides a good example of this because of the relatively large number of confirmed HCV-positive donors. In a study of Australian donors, the mean S/CO for the anti-HCV BFR group was 1.524, compared with 4.546 for the anti-HCV-positive/RNA-negative and 6.467 for the HCV RNA-positive groups. In another study of donors in our blood service, we found that most BFR results showed IA S/CO less than 3.000 whereas most confirmed positive donors showed values higher than 3.000. For anti-HIV, 95.4% of BFR results showed S/CO less than 3.000 compared with 0% for confirmed positive donors; for anti-HCV, the figures were 82.6% vs 0%, and for anti-HTLV, they were 96.2% vs 13.0%.

Our study also found that for donors who had given negative results before becoming BFR, the IA S/CO of their previous negative donations was relatively high (>0.400) for those who became anti-HIV BFR (64.6% of donations), anti-HCV BFR (74.8% of donations), and anti-HTLV BFR (74.8% of donations). The appropriate negative control groups (who had never given a BFR result) showed S/CO higher than 0.400 in only 0.4%, 2.3%, and 18.3% of donations for anti-HIV, anti-HCV, and anti-HTLV, respectively. Similar results were also obtained for BFR donors who subsequently became negative, the majority of whom continued to give IA S/CO higher than 0.400. This observation may explain in part the variation in repeat reactor rates sometimes seen between master lots of the same assay. Interlot variations may result in a small upward shift in S/CO distribution, in turn, causing donors with previously high negative S/CO to give BFR results.

Donor Demographics

Ownby et al found that BFR and indeterminate results in donors are associated with certain demographic groups including women, nonwhites, younger age groups, and first-time donors. The authors suggest that age-, sex-, or race-related proteins may cross-react with assay antigens and that elucidation of the cross-reacting antigenic determinants and their removal from assays may improve specificity.

NONSPECIFIC REACTIVITY IN IMMUNOBLOT ASSAYS

Anti-HIV–Indeterminate Results

It has now been well established that the majority of donors with indeterminate anti-HIV Western blot results are not truly infected with HIV. Most do not have detectable HIV DNA or clinical symptoms of HIV infection and have not been associated with HIV transmission. Follow-up studies have demonstrated that anti–HIV-indeterminate donors rarely progress to a positive pattern and therefore do not represent seroconverters. Indeterminate results are either not associated with risk factors or show similar patterns of risk factors as serologically negative controls. When Western blot–indeterminate samples are tested by alternate confirmatory assays, they either remain indeterminate or are resolved as negative rather than giving confirmed positive results. The most common cause of indeterminate results on anti-HIV Western blot assays in voluntary blood donors is nonspecific reactivity to the gag proteins, particularly p24, but also p55 and p17.

Anti–HTLV-Indeterminate Results

In voluntary blood donors reactive by anti-HTLV IA, between 50% and 75% give indeterminate results when tested by anti-HTLV Western blot. Anti–HTLV-indeterminate results in blood donor populations show many of the same characteristics as anti–HIV-indeterminate results, indicating that most are due to nonspecific reactivity. Most anti–HTLV-1–indeterminate donors are negative for DNA, not confirmed by an immunofluorescence assay, and can be resolved as negative by an alternate confirmatory assay. Indeterminate donors rarely show evidence of seroconversion upon follow-up, even in endemic areas. Like anti–HIV-indeterminate results, the
most common cause of anti–HTLV-indeterminate results in blood donors is nonspecific reactivity to one or more of the \textit{gag} proteins.\textsuperscript{34}

\textbf{Anti-HCV–Indeterminate Results}

Unlike HIV and HTLV, between 15\% and 45\% of HCV-infected individuals (depending on the population studied) apparently clear the infection, with plasma RNA becoming undetectable and anti-HCV remaining as a marker of past infection. For anti-HCV confirmatory testing, one of the most widely used immunoblots is the Chiron third-generation recombinant immunoblot assay (RIBA-3). A positive result on RIBA-3 is defined as reactivity to at least 2 virus-specific proteins, whereas reactivity to only 1 virus-specific protein is defined as indeterminate. Because most donors with anti–HCV-indeterminate results do not have detectable circulating RNA, they could, therefore, represent either past cleared infection or nonspecific reactivity.

There is no definitive method of determining the significance of anti–HCV-indeterminate/RNA-negative results in voluntary blood donors. A number of approaches have been reported, including analysis of risk factors, alanine aminotransferase levels, and IA S/CO, as well as the use of synthetic peptides and/or anti-E2 assays.\textsuperscript{40-42} We have attempted to clarify the significance of RIBA-3–indeterminate/RNA-negative results in our donor population based on an analysis of IA S/CO, RIBA-3 band strength and type, risk factors, and previous donation history.\textsuperscript{26} We concluded that RIBA-3–indeterminate/RNA-negative donors with relatively high IA S/CO (\(>2.000\)), RIBA-3 band intensities of 2+ or higher for either c22p (core) or c33c (NS3), no history of previous negative or BFR donations, and an identifiable risk factor most likely represent true anti-HCV rather than nonspecific reactivity. Although not definitive, these guidelines provide a basis for assessing the likelihood as to whether an anti–HCV-indeterminate result represents true anti-HCV and may assist with donor counseling.

\textbf{MANAGEMENT OF DONORS WITH NONSPECIFIC REACTIVITY}

As a result of regulatory requirements in most jurisdictions, donations that are repeatedly reactive on a screening IA must be discarded, regardless of whether the final donor status is BFR, indeterminate, or confirmed positive. It is unacceptable to allow donors with persistent BFR results to continue donating indefinitely while their donations are being discarded, and it is a waste of resources to collect donations that cannot be used. The challenge for the blood services is to determine when to inform these donors of their results and how this can be done while minimizing donor anxiety. It can be difficult to explain to donors that although their results do not indicate infection, their blood is not suitable for transfusion. Additionally, if such results were misinterpreted by donors as “positive,” there could be medicolegal implications in areas such as health or life insurance.

Development of strategies for managing BFR donors is in part dependent upon whether BFR results are transitory or persist for an extended period. We recently performed an analysis, covering a period of 3.5 years, of donors from our blood service who gave BFR results on the Abbott PRISM ChLIA (HBsAg, anti–HIV-1 and 2, anti-HCV, or anti–HTLV-1 and 2).\textsuperscript{26} Of donors who gave an index BFR result on the anti–HIV-1 and 2 assay, some 66\% continued to give BFR results at subsequent donations, whereas 77.5\% and 71.6\% did so for anti-HCV and anti–HTLV-1 and 2, respectively. For those donors who gave a second BFR result for anti–HIV-1 and 2, anti-HCV, or anti–HTLV-1 and 2, 80.6\%, 84.6\%, and 74.5\%, respectively, continued to give BFR results at subsequent donations. In contrast, for the HBsAg assay, about 86\% of donors with an index BFR result gave negative results at subsequent donations. Based on these results, we concluded that the most effective way to manage donors with BFR results was, upon an index BFR result, not to inform the donors but allow them to continue donating. Most donors with HBsAg BFR results will give IA-negative results at subsequent donations, and a substantial minority (22.6\%-34.0\%) of those with anti–HIV, anti-HCV, or anti–HTLV BFR results will give subsequent IA-negative results. However, after a second BFR result, donors should be informed, counseled, and advised not to donate because they are unlikely to give subsequent negative results. They should be informed that their reactive results are related to the testing system and do not indicate infection with the virus in question. They can also be advised that they may be eligible to return to donate should there be a change of screening IA in the future. In blood
services where NAT screening for HIV-1 and HCV has been implemented, the remote possibility that a BFR result could represent an early seroconversion can be almost completely eliminated.

It is not certain whether these findings can be extended to other screening technologies such as EIA and/or other donor populations. Therefore, each blood service should perform an analysis of their own data before implementing a strategy for managing BFR donors. It would also be advisable to perform a review when there is a change of screening assay. There has been at least 1 report that up to 88% of BFR donors may become IA negative if retested after a period of several years. However, the study involved a relatively small number of donors and it was not stated whether there had been a change in screening IA during the period before retesting the BFR donors.

The use of an alternate screening IA as a strategy for managing donors who give persistent BFR results has been reported. According to this strategy, if a donor gives 2 BFR results at least 6 months apart, upon giving a third BFR result, the donor is tested by an alternate IA and if negative, product can be released without restriction. At subsequent donations, the donor is screened only on the alternate IA. This approach minimizes donor and product loss due to BFR results. However, for this strategy to be effective, it is important to ensure that the alternate IA is of a similar sensitivity as the primary IA and the 2 assays have significantly different BFR populations. However, a potential difficulty with this strategy is that screening laboratories usually screen large numbers of donations using a primary IA for each marker on a common platform. To maintain an alternate assay, which may also require an alternate testing platform, for a relatively small number of donors may not be cost-effective.

**MANAGEMENT OF DONOR REENTRY**

Donors who have given BFR results on previous generations of screening IAs are often negative when retested on contemporary versions. For example, many donors who gave BFR results on the first-generation anti-HCV EIAs (which incorporated a single antigen from the NS4 region of the viral genome) were negative on the second- and third-generation multiple-antigen assays. Similarly, donors who have given anti-HIV or anti-HTLV BFR results on viral lysate–based IAs may test negative on contemporary assays using recombinant proteins or synthetic peptides. Donors who have given BFR results on earlier versions of screening IAs provide a potential pool of donors suitable for reentry.

The effectiveness of a reentry program is dependent upon the previously and currently used assays having significantly different BFR populations. Ideally, archive samples from BFR donors should be tested on the current assay before initiating a donor recall. If the archive sample is negative, it is highly probable that the donor will test negative if recalled. If however, the archive sample is reactive, there is a high probability that the donor would remain BFR at recall.

A similar approach can also be used for donors with nonspecific indeterminate immunoblot results. Donors with indeterminate results on earlier versions of these assays often give negative results when retested on the current version because of improved specificity. For example, Busch et al found that use of a third-generation anti-HCV EIA can resolve the status of a number of donors who have previously given second-generation anti-HCV EIA-reactive/RIBA-2–indeterminate results. Of 33 anti-HCV EIA-2–reactive/RIBA-2–indeterminate donors, 18 (55%) were negative when retested on a third-generation EIA and showed no signs of HCV infection on supplemental testing (RIBA-3 or NAT).

One approach to reducing the number of indeterminate results is to reduce the number of samples tested by immunoblot. This can be achieved by use of a sequential immunoassay (SI) strategy, whereby donors who are reactive on a primary screening IA are further tested by a secondary IA, and only if reactive on both assays are they tested by immunoblot (Fig. 1). Donors who are reactive on the primary IA but negative on the secondary IA are assessed as BFR and no further testing is performed. We have effectively applied this strategy to all 3 of our viral antibody IAs (anti–HIV-1 and 2, anti-HCV, and anti–HTLV-1 and 2). Comparing data from a period before the implementation of the secondary IA (June 7, 1999, to February 5, 2000) to the period immediately after the implementation of the secondary IA (June 7, 2000, to February 9, 2001), we found that the SI strategy reduced the number of immunoblots from 242 to 54 for anti–HIV-1 and 2, from 851 to 187 for anti-HCV, and from 206 to 18 for anti–HTLV-1 and 2. The use
of SIs has been increasingly used in blood service and reference laboratories for anti-HIV,49,50 anti-HCV,51 and anti–HTLV-1 and 2.52,53

The use of an SI strategy without incorporating an immunoblot has been proposed for anti-HCV testing, whereby samples reactive on both IAs would be classified as “confirmed” positive for anti-HCV.54,55 However, we would caution against such a strategy, because reactivity on 2 IAs can represent nonspecific reactivity, with such samples showing negative or indeterminate results on immunoblot.2 An analysis of Australian donors with reactivity on 2 IAs and indeterminate RIBA-3 results has indicated that a number most likely represent nonspecific reactivity.26 The use of an SI strategy without incorporating an immunoblot would result in a number of donors with nonspecific reactivity being assessed as confirmed positive. Such an outcome would subject the donor to unnecessary stress and could even have legal implications.

The efficacy of an SI strategy requires minimal overlap between the BFR populations of the 2 IAs. Because the BFR overlap between IAs can vary significantly, assays to be used in combination should be selected carefully to ensure minimal overlap. As a guide, assay combinations should be selected on the basis of differences in assay format, capture antigen preparations, and detection systems. However, it is also important, before full-scale implementation of an SI strategy, to perform an evaluation of candidate IAs.

CONCLUSIONS

It is now well established that BFR results in voluntary blood donors represent nonspecific reactivity. Screening IAs have improved significantly since first-generation anti-HIV IAs were developed and implemented for donor screening. In addition, NAT screening for HIV-1 and HCV has been introduced in many countries, virtually eliminating the possibility that a BFR result could be due to seroconversion. With these improvements, along with a better understanding of the performance of screening and confirmatory assays,
it is timely to review the management of donors with nonspecific test results. BFR donors can be reassured that they are not infected and could be eligible for future reentry. However, indeterminate immunoblot results are somewhat more problematic and the use of an SI strategy will not entirely eliminate this problem. For anti–HIV- and anti–HTLV-indeterminate donors, retesting after 3 to 6 months without evidence of seroconversion (including a negative NAT result) strongly suggests that the indeterminate result is due to nonspecific reactivity. Therefore, these donors can also be reassured and considered for future reentry. Anti–HCV–indeterminate results remain difficult to interpret because there is no definitive method for determining whether they represent nonspecific reactivity or true anti–HCV. Therefore, at present, there is little choice but to defer anti–HCV-indeterminate donors. Improving the specificity of anti–HCV immunoblot assays and developing guidelines for interpreting indeterminate results should be a priority.

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