ORIGINAL REPORT

A unique approach to screen for blood donors lacking high-prevalence antigen In^b of the Indian blood group system

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The In^b antigen of the Indian blood group system is a high-prevalence antigen. The presence of alloanti-In^b in a recipient may pose a problem in finding compatible blood for transfusion. The aim of this study was to screen blood donors for In^b and to include individuals found to be In(b–) in our rare donor registry. To save resources, a unique study design was constructed. Blood group O donors were tested for In^b because their red blood cell (RBC) units could serve recipients across all ABO groups. EDTA blood samples were used for serologic and genomic testing. These samples were first tested serologically for In^a, and samples typed as In(a+) were then tested both serologically and molecularly for In^a and In^b to find homozygous *IN*01/01* [i.e., the predicted In(b–) phenotype]. A cost-conservative approach in using recycling of antibody was adopted to economize available resources. Of 6300 donors, 196 donor samples typed as In(a+) and were also found to be In(b+) when tested by serologic and genomic methods. Although none of the donors typed as In(b–), the statistical analysis suggests the expected prevalence for this rare phenotype to be 0.02 percent among the total number of donors tested. In conclusion, this report presents a unique cost-conservative approach using limited reagents to screen a large number of donors for the rare In(b–) phenotype. ***Immunohematology*** **2021;37:126–130. DOI: 10.21307/immunohematology-2021-019.**

Key Words: donor screening, high-prevalence antigen, In(b–) phenotype, Indian blood group system

The Indian blood group system comprises six antigens, of which In^a (IN1) occurs in low prevalence among the people of Indian origin.[1] Its antithetical antigen, In^b (IN2), and four other antigens—namely, INFI (IN3), INJA (IN4), INRA (IN5), and INSL (IN6)—are high-prevalence antigens (HPAs).[2–5] Whereas most patients lacking these HPAs were found among people with ancestry in the Indian subcontinent, individuals lacking the INFI (IN3) antigen were found among Moroccans.[3] A transfusion recipient lacking an HPA with corresponding alloantibody in plasma may pose a problem in finding compatible blood units for transfusion.[4,6] Keeping this in mind, we initiated a screening program to identify prospective donors who lack the HPA In^b for future transfusion needs.

Materials and Methods

Research Design

As the In^b antigen is antithetical to In^a,[2] we hypothesized that an In(b–) donor would be found among In(a+) individuals as the homozygote *IN*01/IN*01 (IN*A/A)*. We strategized our approach to test donors initially with anti-In^a, and individuals found to be In(a+) were further tested serologically with anti-In^b to look for absence of In^b. The In(a+) donors were then genotyped using a DNA-based molecular assay for both In^a and In^b to confirm our serologic findings.

Serology Workup

Blood samples for serologic testing were obtained from the blood donors' pool at the Lok Samarpan Regional Blood Center, Surat, India. Only group O donors were included to meet our aim to find rare In(b–) donors whose red blood cell (RBC) units could be transfused across ABO blood groups. Consent was obtained in the pre-donation form completed by the donors at the time of donation.

Method of Testing

Reagent Antisera

Because a commercial supply of reagent anti-In^a and anti-In^b was not available, we used locally identified antibodies obtained from alloimmunized patients. Both anti-In^a and anti-In^b were standardized for their reactivity and specificity using a reagent RBC panel with known antigen profile. A control RBC, previously identified as In(a+b+), was tested in every batch of testing to validate the results obtained.

Recycling of Anti-In^a

We recycled anti-In^a to make economical use of available antiserum. The donors' RBCs were initially tested serologically with anti-In^a by the conventional tube method in the saline phase using low-ionic-strength solution (LISS), followed by the indirect antiglobulin test (IAT) using antihuman globulin

reagent. In brief, equal volumes of RBC suspension (5% concentration in saline), anti-In^a, and LISS were mixed and incubated at 37°C for 15 minutes. The test was centrifuged, and the test supernatant (TS) was aspirated from each tube and pooled for the subsequent screening test. The IAT steps used for the sensitized RBCs were followed to record the final results of the test. More donors were screened using the saved TS following the same steps as for the original antiserum, although no more LISS was added, since it was already present in the TS from previous testing. The TS showed good reactivity in three consecutive collections, as depicted in Table 1. The antibody reactivity in TS-4 was considerably reduced, so we made it more concentrated by absorbing out the water content from the TS using Carbo-wax (polyethylene glycol; Dow, Midland, MI), an hygroscopic substance, as outlined here.

Table 1. Agglutination reactivity of anti-In^a with different test supernatants (TS) saved after the tests

Cells	Original antiserum	TS-1	TS-2	TS-3
In(a+b+)	3–4+	3+	1–2+	1+

The dialysis tube (Carolina Biological Supply Company, Burlington, NC) was filled with TS and placed on a tray. Carbo-wax was sprinkled over the tube as shown in Figure 1A, and the tube was left at room temperature. Note that it takes about 15 hours to remove water content from 200 mL of TS to bring its volume down to 8 mL (original volume of serum used). The tube was manually stripped by holding it between the thumb and finger and tying it with thread and then dialyzing it overnight using phosphate-buffered saline to equilibrate the salinity (Fig. 1B). The content was saved in a clean glass tube and used in the same way as the original serum.

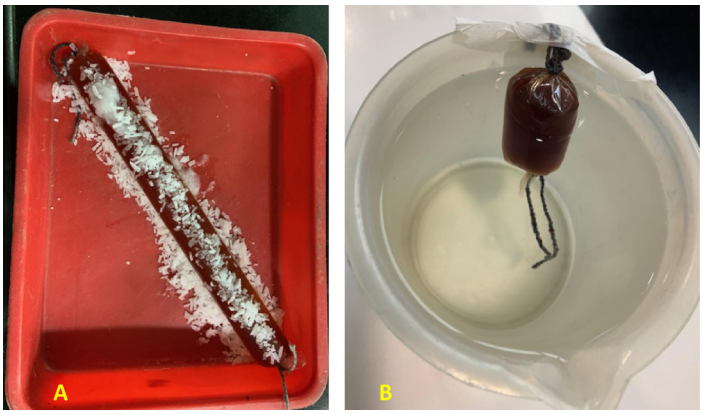

Fig. 1 The first step of concentration of test supernatant (TS) using Carbo-wax (A) and the next step of dialysis of the TS-concentrate using phosphate-buffered saline (B).

Anti-In^b was used as native serum to test RBCs of those donors who typed as In(a+).

Molecular Testing

DNA was extracted, from buffy coat augmented off the whole blood sample collected in EDTA, using the FlexiGene DNA Kit (QIAGEN, Hilden, Germany). Genotyping for *IN*01* and *IN*02* was carried out by sequence-specific primer polymerase chain reaction (PCR-SSP) as previously described.[9] Briefly, the *IN*01*-specific sense primer 5′-GTCGCTACAGCATCTCTCC-3′ and the *IN*02*-specific forward primer 5′-TCGCTACAGCATCTCTCG-3′ were used along with their common reverse primer 5′-CCATTCAGCTGTGGGAAAGGAGC-3′ under PCR conditions: initial denaturing at 94°C for 5 minutes followed by 6 cycles of 30 seconds at 94°C, 40 seconds at 67°C (touchdown for 0.5°C per cycle), and 72°C for 50 seconds; 27 cycles at 94°C for 30 seconds, 64°C for 40 seconds, and 72°C for 50 seconds; and 1 cycle of 72°C for 2 minutes in two separate reactions. The PCR mixture (25 µL) consisted of 50–100 ng genomic DNA, 10 µmol/L of each deoxynucleotide triphosphate (Bioron Diagnostics, Römerberg, Germany), 2.5 µL 10× complete buffer containing $MgCl_2$, 1.0 µmol/L forward and reverse primers, and 1.0 U Taq DNA polymerase (GeNei, Bangalore, India). Human growth hormone control was co-amplified as internal control (forward primer 5′-GCCTTCCCAACCATTCCCTT-3′ and reverse primer 5′-TAGACGTTGCTGTCAGAGGC-3′; Sigma-Aldrich, Darmstadt, Germany). PCR products were analyzed by electrophoretic separation on a 1.5 percent agarose/tris acetate-EDTA (TAE) gel containing 0.2 µg/mL ethidium bromide. Presence of the 211-bp single nucleotide polymorphism (SNP)-specific band indicated the presence of the *IN*01* allele, and the 209-bp SNP indicated the presence of the *IN*02* allele.

Statistical Analysis

From the values obtained on the three observed phenotypes, the genotypes and gene frequencies of the two alleles (namely, *IN*01* and *IN*02*) were derived using an online calculator.[10] The *p* value for statistical significance was derived online.[11]

Results

Of the 6300 donors tested with anti-In^a, 196 (3.1%) were found to be In(a+). The In(a+) donors were tested with anti-In^b, and all were found to be In(b+), showing their phenotype

as In(a+b+). Table 2 shows the distribution of the three phenotypes—namely, In(a+b+), In(a–b+), and In(a+b–)—and their computed expected prevalence together with the gene frequencies of *IN*01* and *IN*02* of the Indian blood group system among the population under study. The rare In(a+b–) phenotype was not found among the donors tested, suggesting that its observed prevalence would be less than 1 in 6300. However, its expected prevalence of 0.02 percent derived through the statistical analysis suggests that the phenotype might have been missed among the large population tested. The results of the molecular tests were in accordance with the serologic results, and the genotype was found to be heterozygous *IN*01/IN*02* among all the In(a+) donors.

Table 2. Distribution of the three phenotypes and frequency of the two alleles of the Indian blood group system among the 6300 blood donors screened

Phenotype	*n* Observed	*n* Expected	Statistical significance	Allele frequency
In(a+b+)	196	192.95		
In(a–b+)	6104	6105.52	χ^2 = 1.573 with 2 df	*IN*01:* 0.0156
In(a+b–)	0	1.52	p = 0.455436 (not significant at $p < 0.05$)	*IN*02:* 0.9844

df = degrees of freedom.

Discussion

The Indian blood group system (ISBT IN 023) has several HPAs.[2–5] Transfusion recipients lacking some of these antigens on their RBCs and having alloantibody in their plasma have posed enormous difficulty in finding compatible blood for transfusion.[4,6] There is no rare donor registry in place even though there are quite a few rare individuals in India,[13] mostly among the patients who once required blood transfusions. The list of such individuals is scattered, and the information is being maintained at individual blood banks in the country. Emergently procuring blood at the time of need becomes a herculean task. The In(b–) phenotype is rare in any given population. Anti-In^b reagent is not available in a quantity that may allow screening a large number of donors. In^b is antithetical to In^a,[2] and therefore testing the donors initially with anti-In^a and then testing In(a+) donors with anti-In^b to find In(b–) donors was a reasonable strategy adopted to conserve the scarce antisera. Any donor who typed as In(b–) could be confirmed through molecular testing as homozygous *IN*01/IN*01 (IN*A/A)*. In the present study, none of the donors was found to be In(b–), presumably because of its extremely rare occurrence in the Indian South-Asian population.[12]

The limited amount of anti-In^a at our disposal encouraged us to conserve the reagent by recycling the antibody to screen a large number of donors. This approach proved to be an economical and realistic way to carry out a project of this magnitude.[7,8] Recently, the diluted anti-In^b serum was used elsewhere in microtechniques to conserve resources.[14] Nevertheless, the use of this microtechnique may still have limitations in screening a large number of donors for the In(b–) phenotype.

The molecular basis for the Indian blood group system has been elucidated. *IN*01* and *IN*02* differ by a single nucleotide change at position 137; c.137G encodes p.Arg46 (In^a) and c.137C encodes p.46Pro (In^b). This information was used to predict the phenotype for In^a (*IN*01*) as well as In^b (*IN*02*) using simple DNA-based assays such as PCR-SSP. The DNA-based assay in the present study helped to confirm our results obtained through serologic testing. However, many blood banks may not have the required resources in terms of infrastructure and expertise to carry out such molecular testing.

The observed prevalence of In(a+) donors, 3.1 percent in the present study, is comparable to earlier reports on the Indian population.[1] There are few reports available in the literature to show the prevalence of the In(b–) phenotype among the population in India. Previously, one author (SRJ), observed a prevalence of the In(b–) phenotype to be 0.14 percent (1:700) among donors in Mumbai tested during the serologic compatibility testing for a patient with anti-In^b.[6] More recently, a genomic study carried out in Mumbai yielded no donor with the *IN*01/IN*01* homozygote [i.e., In(b–) phenotype] because of its rarity in the Indian population.[9] The prevalence of the In(a+b–) is rare among several other populations studied.[12] In the present study, screening a large number of homogeneous Gujarati-speaking donors, suggests that the In(b–) phenotype is rare among the population in the city of Surat in western India. The three cases of In(b–) reported earlier from Mumbai[6] were from different linguistic ethnicities in Marathi-speaking and Telugu-speaking regions. The other reason for missing the rare entity might be due to a selection bias of the samples, being that we only included group O donors, rather than donors of all ABO groups, and thus the phenotype of interest might have been missed among the other blood groups in the large pool of donors who were not tested. Group O donors were selected mainly because of the universal use of such donors for

recipients of any ABO blood group and the rarity of the In(b–) phenotype.

Genotyping by PCR-SSP on In(a+) donors revealed that all individuals serologically identified as In(a+) were heterozygotes (*IN*01/02*). The statistical analysis, based on the observed findings of In(a+) donors, suggests an expected prevalence for the *IN*01/01* homozygote [i.e., In(b–) phenotype] to be 0.02 percent among the 6300 donors tested—indicating its very low prevalence. To the best of our knowledge, this study reports the largest single-center attempt to screen for In(b–) donors with a strategic design involving the conservative use of available resources. The present study also emphasizes putting sustained efforts into screening for In(b–) donors to meet the eventual need. Anti-In^b has been reported to cause severe hemolytic transfusion reactions signifying its clinical significance.[6] Recently, an anti-In^b in the plasma of a 2-year-old child in the United States posed great difficulty in finding blood for her transfusion, requiring an international effort, including the help of social media, to procure appropriate blood.[14]

Conclusion

The present study reports an economical approach used in screening blood donors for the rare In(b–) phenotype of the Indian blood group system. Because In^b is antithetical to the In^a antigen, there is a greater possibility to find the *IN*01/IN*01* homozygote [i.e., In(b–) phenotype] among In(a+) donors. The attempt to find the In(b–) phenotype via screening for In^a and confirming serologically using anti-In^b and molecularly by genomic testing could help in the search for such rare blood in the most resource-conservative manner. Although none of the donors in this sample were found to be In(b–), there is an indication that the phenotype is expected to be present, albeit of rare occurrence. More efforts are needed to identify In(b–) donors not only by increasing the number of donors to be tested but also by including donors from various population pools.

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Authorship Contributions

SRJ designed the research concept. SRJ, SBS, and PS collected samples, standardized and performed the serological testing, analyzed the data, and interpreted the results. HDM, PDK, and SSK performed the molecular testing and analyzed and interpreted the data. SRJ, HDM, and SSK drafted the manuscript and revised it critically. All authors approved the final version of the manuscript.

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