The Indian blood group system (ISBT 023) comprises one low-prevalence antigen, In⁴ (IN:1), and five high-prevalence antigens: In⁵ (IN:2), INFI (IN:3), INJA (IN:4), INRA (IN:5), and INSL (IN:6). The antigens are located on the single-pass trans-membrane glycoprotein encoded by the CD44 gene. The present study was designed to identify the prevalence of the INRA– (IN:−5) phenotype and the frequency of its associated allele (IN*02.–05) to inform us of the probability of finding antigen-negative donors and to assess the risk of antibody formation in transfusion recipients. Buffy coats were extracted from EDTA-anticoagulated whole blood samples, collected with consent from 5261 random blood donors in Surat, Gujarat, India. Standard serologic methods were performed with a modification allowing the use of antiserum generated by recycling the antibody augmented from the test already performed. A real-time polymerase chain reaction–based assay was devised to genotype c.449G>A (p.Arg150His) single nucleotide variation in exon 5 of the CD44 gene. None of the 411 donors tested by serology or the 5261 donors tested molecularly were positive for the IN:−5 phenotype or the allele (IN*02.–05), respectively. The allele frequency estimate ranged from less than 1 in 10,522 (0.01%) to 1 in 3203 alleles (0.03%) in the study cohort (95% confidence interval, Poisson distribution). The absence of this rare allele in the present survey could be due to an ethnic difference, since the donors mostly came from the Hindu community, and the only case of the IN:−5 phenotype was found in the Muslim community. The p.150His variant may be either restricted to the index case family or only found in the Muslim community. Further studies in local subpopulations may provide more information on the frequency of p.150His and its immunogenicity in transfusion recipients if occurring among blood donors.

**Materials and Methods**

**Human Research Subjects**

Blood samples from 5261 healthy volunteers, aged 18 years and older, were collected with informed consent at the Lok Samarpan Raktadan Kendra Blood Bank in Surat, Gujarat, India. Buffy coats were extracted from EDTA-anticoagulated whole blood samples. Theuffy coats were pooled in batches of 20 and shipped to the Department of Transfusion Medicine at the U.S. National Institutes of Health (NIH) Clinical Center for DNA extraction (EZ1 DNA blood kit on a BioRobot EZ1 Workstation; Qiagen, Valencia, CA) and molecular analysis.

**Serologic Testing**

Red blood cells (RBCs) from the healthy volunteers were tested with the serum from an original patient having an antibody to IN:5. Because the antibody agglutinated RBCs in low-ionic-strength saline (LISS) medium, serologic testing was performed by the LISS tube method. However, for any donor RBCs showing weak reactivity, particularly in the test supernatant (TS), the test was repeated by the indirect antiglobulin test (IAT) using original serum. No
dosage effect was observed, since the RBCs from the children of the proband (i.e., heterozygous for IN:5) reacted with the same agglutination strength as that of their father or other individuals outside the family (i.e., homozygous for IN:5). Because the antibody was in extremely short supply, we used the resource-conserving Nirantara approach, as reported earlier, by recycling the antibody. In brief, the test was initially performed as usual by mixing equal volumes of antiserum, LISS, and the donor’s RBC suspension and incubating for 15 minutes at 37°C; the results were read after centrifugation. The test was centrifuged, and the TS was saved. In a separate procedure, the sensitized RBCs from the test were pooled, and an eluate was prepared by the ether elution method. The eluate antibody was mixed with the saved TS and used in screening further donors. While using TS in screening, no LISS was added, since it was already present from previous testing. If a donor’s RBCs showed weak agglutination in the test with TS, then the test was repeated using original antiserum and by the IAT. The test was validated for serologic activity and specificity by running the appropriate control in parallel with every batch of testing.

**Molecular Screening**

A real-time polymerase chain reaction (PCR)-based assay was devised to identify the frequency of the allele (IN*02.–05; p.150His) encoding the INRA– phenotype (IN:−5). Primers 5’-GCGGGGCTCTTCCAGCTATTGTTAACCA-3’ and 5’-ATTCTCCTTTCTGGACATAGCGGG-3’ were designed to genotype c.449G>A (p.Arg150His; rs771323886) single nucleotide variation (SNV) in exon 5 of the CD44 gene and applied to genomic DNA (gDNA). Controls included in each plate were heterozygous for c.449G>A from the children of the proband (INRA, IN:−5), homozygous for c.449G from a random specimen from an individual outside the family of the proband, and homozygous for c.449A from the proband herself.

An equal aliquot of buffy coat from 20 individuals was pooled after collection. gDNA was extracted and quantified for each pool. Analytical sensitivity was evaluated by spiking c.449A gDNA in pooled gDNA samples (heterozygous c.449G>A sample: homozygous c.449G sample; 1:0, 1:1, 1:5, 1:10, 1:20, 1:30, and 0:1). Sensitivity of 100 percent was achieved in pools of size 20. Briefly, 20 ng of pooled gDNA was added to the real-time PCR assay. The pools were tested twice to confirm sensitivity and to ensure that individuals harboring c.449A variant are not missed.

**Statistical Analysis**

Ninety-five percent confidence intervals (CIs) for allele frequencies were calculated using the Poisson distribution.

**Results**

The blood donor profile at the blood center where this study was carried out consisted mainly of the Hindu community; only a few sporadic donors came from the proband’s Muslim community.

**Serologic Screen**

Blood donor units were chosen to screen for the IN:−5 phenotype, since there was a clear need to meet the possible requirement for transfusion. We had tested 411 donors by serology using the available antiserum obtained from the proband. None of these donors typed as IN:−5 by serologic testing. Because antiserum was not available to test more donors, further testing was carried out to directly screen for the variant allele coding the p.150His (IN*02.–05) allele using the molecular method.

**Molecular Screen**

We did not find a signal for the SNV indicative of the p.150His (IN*02.–05) among 5261 random blood donors in 264 pools. The c.449G allele frequency was 100 percent in this set of donors. We show an example of a positive real-time genotyping result with a sample homozygous for c.449A in Figure 1. The data revealed that there was no discrepancy observed in the testing based on serology and by DNA-based molecular method among the samples tested.

**Population Frequency**

The allele frequency estimate, based on the data derived by the molecular screen, ranged from less than 1 in 10,522 (0.01%) to 1 in 3203 alleles (0.03%) in the population (95% CI, Poisson distribution).

**Discussion**

The Indian blood group system comprises six antigens thus far, of which In a is a low-prevalence antigen and In b is an HPA, which together are an antithetical pair. Additionally, four other HPAs have been recognized, some of which were found to be clinically significant as to illicit immune response and to cause hemolytic transfusion reaction. Although an antibody to a low-prevalence antigen in a transfusion recipient may not
Blood donors: serology and molecular screen

pose a problem in finding units of antigen-negative blood for transfusion, an antibody to an HPA typically presents an enormous challenge to provide units of blood for transfusion.\textsuperscript{4,8}

The aim of the present study was to screen for blood donors should a patient (the proband) require appropriate compatible units of blood for transfusion. The study also aimed to find the prevalence of the variant allele (\textit{IN*02.–05}; p.150His) encoding the IN:−5 phenotype and to assess the risk of antibody formation in transfusion recipients.

None of the donors screened in the present study by serologic and molecular methods showed the IN:−5 phenotype. The Genome Aggregation Database (gnomAD) lists c.449A allele frequency as less than 0.01 percent among 122,365 individuals, with c.449A (p.150His)-positive individuals found in East Asian, South Asian, and Finnish populations.\textsuperscript{13}

A limitation of our study is that the vast majority of the tested donors (99.9%) came from the Hindu Gujarati community. The IN:−5 proband has been identified in the Rayeen community, an endogamous caste group of the Sunni Muslim sect. The parents of the proband denied consanguinity in their marriage. Although the Rayeen community is mostly settled in Uttar Pradesh, a northern state of India, some of the families have migrated to Gujarat state to earn their livelihood through their ancestral business as greengrocers. If efforts were made to screen the members of the proband’s Rayeen community, the chance of finding this rare phenotype or allele of interest would have been greater.

A large-scale screening program requires material resources including antiserum and other reagents, and this need made it difficult for us to meet some of the requirements.

The use of a resource-conservative strategy in serologic testing by the Nirantara method and the use of pooled buffy coats for DNA study (as was earlier used on blood specimens) may prove not only economical but also feasible to accomplish such a screening program in resource-scarce areas.\textsuperscript{9,14}

Conclusions

We did not detect this SNV among 5261 random blood donors in the state of Gujarat in western India. Because we originally observed the INRA− phenotype with the p.150His variant allele in a Muslim patient, it is conceivable that the trait is either restricted to the index case family or only common in the Muslim community to which the patient belongs. Further studies in the local subpopulations may provide more information on the frequency of the \textit{IN*02.–05} allele and the immunogenicity of IN:5 in transfusion recipients, if occurring among blood donors.

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References


For information concerning *Immunohematology* contact us by e-mail at immuno@redcross.org.