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Changing practice: red blood cell typing by molecular methods for patients with sickle cell disease

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Abstract

BACKGROUND: Extended red blood cell (RBC) antigen matching is recommended to limit alloimmunization in patients with sickle cell disease (SCD). DNA-based testing to predict blood group phenotypes has enhanced availability of antigen-negative donor units and improved typing of transfused patients, but replacement of routine serologic typing for non-ABO antigens with molecular typing for patients has not been reported.

STUDY DESIGNS AND METHODS: This study compared the historical RBC antigen phenotypes obtained by hemagglutination methods with genotype predictions in 494 patients with SCD. For discrepant results, repeat serologic testing was performed and/or investigated by gene sequencing for silent or variant alleles.

RESULTS: Seventy-one typing discrepancies were identified among 6360 antigen comparisons (1.1%). New specimens for repeat serologic testing were obtained for 66 discrepancies and retyping agreed with the genotype in 64 cases. One repeat Jk(b−) serologic phenotype, predicted Jk(b+) by genotype, was found by direct sequencing of *JK* to be a silenced allele, and one N typing discrepancy remains under investigation. Fifteen false-negative serologic results were associated with alleles encoding weak antigens or single-dose Fy^b expression.

CONCLUSIONS: DNA-based RBC typing provided improved accuracy and expanded information on RBC antigens compared to hemagglutination methods, leading to its implementation as the primary method for extended RBC typing for patients with SCD at our institution.

Red blood cell (RBC) transfusions are an essential treatment for patients with sickle cell disease (SCD) but alloimmunization to RBC antigens remains a major complication

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CONFLICT OF INTEREST
The authors have disclosed no conflicts of interest.

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(reviewed in Chou et al.[1] and Yazdanbaksh et al.[2]). Differences in antigen prevalence among patients of African descent and donors of European background provide one major explanation for high alloimmunization rates in patients with SCD in Europe and the United States. *RH* heterogeneity resulting in altered Rh antigen expression on patient RBCs is an additional risk factor.[3–5] Pretransfusion determination of extended Rh (CcEe) and K antigen phenotypes is necessary for prophylactic matching strategies that have been implemented to reduce alloimmunization for chronically transfused patients. Transfusion with donor units negative for C, E, and K for patients who lack these antigens is effective in reducing alloimmunization.[6,7] More than 80% of comprehensive sickle cell centers obtain a pretransfusion extended RBC phenotype (Rh, Kell, Kidd, Duffy, Lewis, and MNS systems) to provide C-, E-, and K-matched RBCs and to guide new antibody evaluations,[8] but this practice is not universal standard of care.[9]

RBC phenotyping by hemagglutination has been the gold standard, but is labor-intensive and hampered by subjectivity in interpreting agglutination reactions and transcription errors when manually transcribing results. Moreover, patients who have been recently transfused or have a positive direct antiglobulin test may not be accurately typed. Testing is also limited by lack of availability of reagents for a number of clinically significant antigens.[10]

DNA-based assays targeting single-nucleotide polymorphisms (SNPs) associated with blood group antigen expression offer an alternative.[11–14] Genotyping methods provide information on RBC antigens for which standardized serologic typing reagents are not available and are amenable to high-throughput testing with automated computerized interpretation. Here we compared RBC antigen phenotypes determined by single-nucleotide polymorphism analysis with serologic testing for 13 routinely tested RBC antigens and report the prevalence of 35 antigens predicted by DNA in 494 patients with SCD.The aim of this study was to determine the accuracy and any potential benefits of RBC typing with DNA methods to replace extended serologic antigen typing for patients with SCD.

MATERIALS AND METHODS

This is a retrospective study of RBC antigen phenotypes and genotypes of patients with SCD at The Children's Hospital of Philadelphia performed under a protocol approved by the institutional review board. RBC genotyping was performed for 494 subjects between 2008 and 2014 by human erythrocyte antigen (HEA) BeadChip DNA array (Bioarray/Immucor, Warren, NJ) to determine polymorphisms associated with 35 antigens in 11 blood group systems (Rh, Kell, Kidd, Duffy, MNS, Dombrock, Lutheran, Landsteiner-Wiener, Diego, Colton, and Scianna). DNA-based typing results were electronically imported into a study database (Filemaker, Inc., Santa Clara, CA) and compared to serologic data.

Historic serologic RBC antigen phenotypes, performed by standard manual tube hemagglutination methods, were ascertained from blood bank records. Per institution protocol, the phenotype is performed once on a pretransfusion sample and includes C, c, E, e, K, Fy^a, Fy^b, Jk^a, Jk^b, Le^a, Le^b, M, N, S, s, and P1 antigens. The RBC phenotype is typically obtained at age 1 year or at the first clinic visit upon transfer of care from another institution. The historic serologic phenotype was compared to the genotype result, with the

exception of Lea, Leb, and P1, which are not included on the HEA BeadChip. Twenty-four patients had no serologic phenotype recorded for one or more antigens with a total of 62 antigens missing serologic data; thus, a total of 6360 serologic and genotype antigen results were compared.

To resolve discordant results between antigen type predicted by DNA and historical serologic phenotype, repeat serologic typing was performed on a new sample as discrepancies were identified. For patients transfused in the preceding three months, autologous RBCs for serologic retype were isolated by a hypotonic saline wash procedure (n = 25).[15] Samples remaining discordant after repeat serologic typing were tested with multiple commercial reagents and gene sequencing was performed to investigate for a silent or variant allele. Statistical analysis of antigen frequency was performed using bivariate comparisons of categorical variables using a two-tailed chi-square test with Yates' correction or a Fisher's exact test.

RESULTS

The 494 subjects included hemoglobin (Hb)SS (67.2%), HbSC (22.4%), HbSβ thalassemia (9.5%), or HbSβ^{variant} (0.9%) genotypes. A total of 135 patients received chronic transfusions (27.3%), 190 episodic transfusions (38.5%), and 169 (34.2%) had not been transfused. A total of 236 patients (47.8%) were female and 258 (52.2%) were male. The median patient age was 13.6 years (range, 1–43 years). The prevalence of 35 RBC antigens predicted from the genotype in this cohort of patients was similar to historical values determined by serology for blacks[16] (Fig. 1A, Table 1, and Table S1 [available as supporting information in the online version of this paper]). As expected, the prevalence of C, E, and K antigens in patients with SCD was low and there were significant differences in the prevalence of Jk^b ($p < 0.0001$), Fy^a ($p < 0.0001$), Fy^b ($p < 0.0001$), and S ($p < 0.0001$) compared to Caucasians. Twenty-five percent of patients expressed V, 28% VS, and 17% Js^a, which are low-frequency antigens in all but African ethnic groups.

Historical serologic RBC antigen phenotypes were compared to DNA-based types. We identified 71 typing discrepancies among 6360 antigen comparisons (1.1%) in 57 patients (11.5%). Of these 71 discordant antigen types, 34 serologic types were performed before 2008, and 37 were obtained between 2008 and 2014. Serologic typing was performed by manual tube method and there have been no notable changes in serologic typing reagents during this time frame. One antigen typing discrepancy was found in 47 patients, two discrepancies in seven, three in two, and four in one patient. Antigen results most often discrepant included 16 Fy^b, 13 Jk^b, 10 M, 10 N, and seven S (Fig. 1B). New specimens for repeat serologic typing were obtained for 66 of 71 discrepancies. Two patients were unavailable to obtain a repeat specimen. Serologic retyping was not performed for three discrepancies: two Fy(b−) historic serologic results were explained by inheritance of *FY*X* (265C>T missense mutation)[17] encoding very weak antigen expression often not detected by serologic reagents and one weak e antigen expression associated with *RHCE*ceJAL* in trans to *RHCE*cE*[18] revealed by high resolution *RH* genotyping (Table S2, available as supporting information in the online version of this paper). Retyping agreed with the genotype in 64 cases (Table S2). Many discrepancies were associated with samples with

single-dose Fy^b expression that were not originally detected as positive; 86% of Fy^b discrepancies reacted weak+ to 2 + on the repeat serologic testing (Table S2). Direct sequencing revealed one Jk^a discrepancy explained by a *JK*A* nucleotide 130G>A change[19] associated with weak antigen expression. Overall, discrepant results were equally associated with false-positive (n = 34) and false-negative (n = 33) historical serologic types.

Only two repeat serology results were consistent with historical serologic records (Table S2). One Jk(b−) serologic phenotype, predicted to be Jk(b+) by genotype, was found by direct sequencing of *JK* to be a silenced allele (*JK*B* 191G>A).[20] A serologic N+ type predicted N− by genotype is under investigation. Thus, the concordance rate between DNA-based testing and serology confirmation was 0.9997, with two true genotype-phenotype discrepancies in 6360 total antigens.

DNA-based testing predicted expression for 35 antigens that can guide antibody evaluations and choice of donor units. In these 494 subjects, 66 lack or have altered high prevalence antigens: 54 hr^B–, five U–, three U variant, three Jo(a−), and one Hy– (Table 1). The assay also identifies the T>C substitution in the *FY* erythroid promoter that disrupts binding of the GATA1 erythroid transcription factor and results in RBC-specific loss of Fy^b antigen expression.[21] The RBC genotype of 410 patients (83%) was predicted to be Fy^b–, but 404 (98.5%) were positive for the GATA mutation and, thus, not at risk for producing anti-Fy^b.

Patients with SCD commonly inherit *RH* variant alleles that result in RBCs that lack common or carry novel Rh antigenic epitopes.[3–5] DNA-based testing identified 122 V+ (24.6%) and 139 VS+ (28.1%) individuals (Table 1). Fifty-four patients (11.0%) were homozygous for the 733C>G change (predicting V/VS+) associated with loss of the high-prevalence hr^B antigen. The presence of markers for a V–VS+ phenotype identified the potential for a hybrid *RHD*DIIIa-CE(4–7)-D* gene that does not encode D but encodes partial C antigen in 30 patients. High-resolution *RH* genotyping confirmed *RHD*DIIIa-CE(4–7)-D* alleles in 23 of the 30 individuals (77%). The RBCs serologically type as C+ but these patients can develop anti-C if exposed to conventional C antigen.[22] Providing C– RBCs to these individuals minimizes anti-C alloimmunization.

DISCUSSION

The field of transfusion medicine has over a decade of experience with RBC genotyping. However, elimination of serologic testing and implementation of DNA-based typing for non-ABO blood groups, without confirmatory serologic testing, requires acceptance of practice change. Many studies in blood donors demonstrate that genotyping is reliable and correlates with serologic typing.[10,23] Of the 71 serologic antigen types that were discordant with the genotype, 34 were performed before 2008 and 37 were typed between 2008 and 2014. Serologic typing was performed by manual tube method for all samples, and the authors are not aware of any major changes in commercial serologic typing reagents during this time period. The erroneous serologic results revealed in this study likely have multiple etiologies inherent to all manual test methods. These include recording and/or processing errors with manual typing, testing performed inadvertently on a posttransfusion specimen, or an antigen that was expressed weakly and not detected by serologic reagents.

In this study, the concordance rate between DNA-based testing and serologic confirmation was 0.9997, similar to a 0.9995 concordance rate observed in a comparison of 356 reagent donor RBCs analyzed by hemagglutination and genotype using the same high-throughput DNA platform used here.[24] Fifty-seven patients (11.5%) had at least one serologic discrepancy noted, compared to studies in multiply transfused patients with thalassemia and SCD that reported serologic and DNA-predicted phenotype discrepancies in 36% to 51% of individuals.[25,26] The lower rate of discordant results in this study likely reflects the policy of obtaining an extended RBC phenotype by age 1 year before any transfusion and systematically obtaining the RBC phenotype after a hypotonic saline wash if a patient was recently transfused.

Based on the data reported here, RBC genotyping has replaced serologic typing for blood groups other than ABO, RhD, and Lewis at our institution. This change of practice does require recognition of some limitations of DNA-based testing. The genotype predicts phenotype and does not directly detect antigen expression. High-throughput genotyping platforms identify the most common polymorphisms and do not detect all variants or rare silenced alleles that result in false-positive types. For the Rh system, genotype prediction of common C/c and E/e antigens correlated well with the patients' serologic results, but high-resolution *RH* genotyping is required to distinguish most Rh variants.3–5 From a cost perspective, an extended RBC antigen genotype (35 antigens) costs approximately 15% less than a serologic phenotype (15 antigens) when performed by our reference laboratories.

Efforts to prevent alloimmunization in patients with SCD have focused on prophylactic antigen matching. Antigen matching programs for Rh and K have decreased alloimmunization in patients with SCD, but these strategies have not been as effective as predicted.[3,5,6,27] Individuals with variant Rh antigens continue to form antibodies against the Rh blood group system despite receiving Rh-matched transfusions.[3,5] Extended matching for Kidd, Duffy, and S antigens can further minimize alloantibody formation, but is limited by donor availability and cost. RBC genotyping of both patients and donors may have the potential to improve RBC antigen matching. DNA-based typing provides data on significantly more antigens than routine serologic typing, including the clinically relevant U, Do^a, Do^b, partial C, and hr^B antigens. Providing antigen-negative units as a prophylactic prevention strategy for patients who lack high-prevalence antigens (hr^B, U, Jo^a, Hy) could improve transfusion safety but would require a large increase in minority donations and donor genotyping. DNA-based screening of donors can help expand antigen-negative unit inventories, particularly with strategies to increase donations from minority populations.

Major challenges for mass scale genotyping of donors and patients are data handling and integration of information technology systems between reference molecular laboratories, blood donor centers, and hospital transfusion services. Electronic importing of DNA-predicted RBC phenotypes from the molecular laboratories to hospital transfusion services would be ideal. A data clearinghouse is also necessary to efficiently match donors and patients using RBC genotype information along with ABO status that currently remains dependent on serologic methods. Numerous blood bank, reference laboratory, and blood donor center information systems currently exist. Thus, a concerted effort among providers

to build a shared information system is crucial and necessitates uniform standards for antigen nomenclature, data representation, and interfacing.

Implementation of RBC genotyping for patient care requires acceptance of molecular typing without serologic confirmation, which represents a major practice change for transfusion services. This large cohort study demonstrates that use of a DNA-based array to predict RBC antigen phenotype is highly reliable in patients with SCD, including those who are chronically transfused. High alloimmunization rates despite prophylactic Rh and K matching in this patient population suggest that more precise matching is necessary.[3,7,27] Based on these findings, our institution has adopted molecular typing as the primary method to predict RBC antigen phenotypes outside the ABO, RhD, and Lewis systems. RBC genotyping can provide antigen status on many clinically significant antigens for which serologic reagents are limited or not available, which can expedite new antibody evaluations and potentially improve RBC matching. Blood donation centers are readily adapting DNA-based screening of donors to efficiently manage their inventories. Now, the field of transfusion medicine is poised to integrate RBC genotyping of both patients and donors into routine clinical practice, which can particularly benefit individuals with SCD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS:

HEA	human erythrocyte antigen
SCD	sickle cell disease

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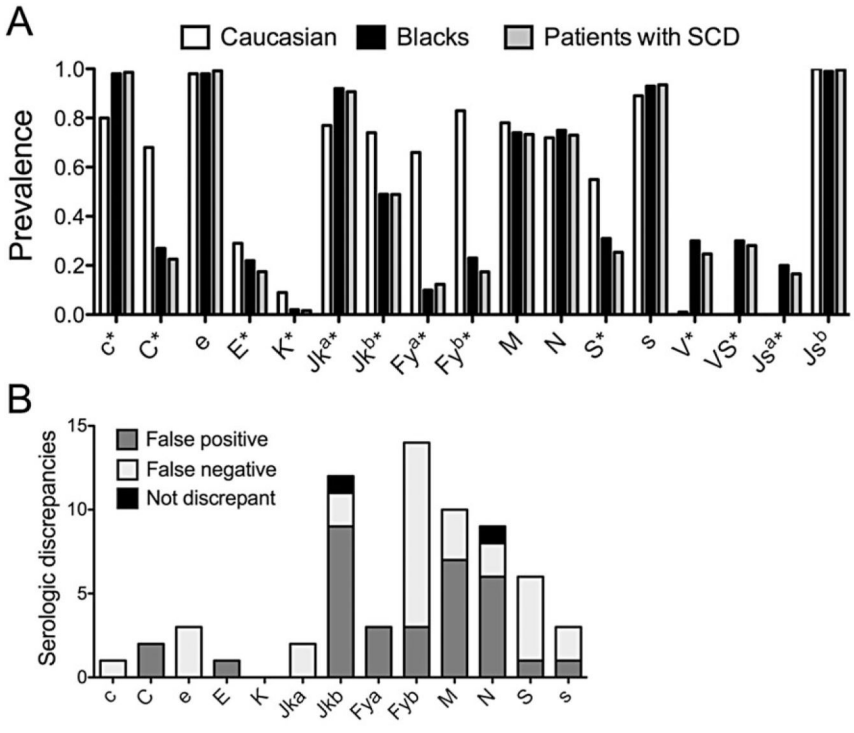

Fig. 1.

Comparison of DNA-based RBC typing with serologic typing. (A) Prevalence of RBC antigens predicted by genotyping in a cohort of patients with SCD (n = 494) compared to reported prevalence determined by serologic typing for black and Caucasian populations in Reid et al.[16] *Antigens for which the frequency between Caucasians and patients with SCD had a p value of less than 0.0001. (B) Seventy-one total discrepancies out of 6360 antigen result comparisons between serologic and genotype predicted results. Bars indicate number of serologic discrepancies per antigen for which repeat serologic testing confirmed the genotype result in all but two cases. Not discrepant indicates the two repeat serology results that were consistent with the historical serologic record.

TABLE 1.

Prevalence of 23 antigens not routinely available by serologic testing*

	Patients with SCD		Prevalence		
Antigen	**Positive**	**Negative**	**Patients with SCD**	**Blacks**	**Caucasian**
hr^{B}†	436	54‡	0.883	0.960	1.000
U	489§	5	0.990	0.990	0.999
Joa	491	3	0.994	0.990	1.000
Hy	493	1	0.998	0.990	1.000
V†	122	372	0.247	0.300	0.010
VS†	139	355	0.281	0.300	0.0001
Js^{a}†	82	412	0.166	0.200	0.0001
Js^{b}	492	2	0.996	0.990	1.000
k	494	0	1.000	1.000	0.998
Kp^{a}	0	494	0.000	0.0001	0.020
Kp^{b}	494	0	1.000	1.000	1.000
Lu^{a}‖	18	464	0.037	0.050	0.080
Lu^{b}‖	485	0	1.000	0.998	0.998
Di^{a}‖	0	493	0.000	0.0001	0.0001
Di^{b}‖	493	0	1.000	1.000	1.000
Co^{a}	494	0	1.000	0.999	0.999
Co^{b}†	8	486	0.016	0.100	0.100
Do^{a}†	214	280	0.433	0.550	0.670
Do^{b}†	460	34	0.931	0.890	0.820
LW^{a}	494	0	1.000	1.000	1.000
LW^{b}	0	494	0.000	NA	0.010
Sc1	494	0	1.000	0.990	0.990
Sc2	0	494	0.000	NA	0.010

*Number of patients with SCD (n = 494) who genotyped positive or negative for each RBC antigen. Antigen prevalence among this cohort was compared to reported prevalence for blacks and Caucasians determined by serological typing in Reid et al.[16] with the exception of hr^{B} (authors' experience).

†Antigens for which the frequency between Caucasians and patients with SCD had a p value of less than 0.0001.

‡Does not include four individuals predicted to be hr^{B}– associated with DcE/DcE (R2R2).

§Includes three U variants.

‖No results were determined for 12 Lu^{a}, nine Lu^{b}, one Di^{a}, and one Di^{b} due to low signal intensity on the HEA BeadChip.

NA = not available.