ORIGINAL REPORT

Assessment of common red blood cell pretreatments to yield an accurate serologic antigen phenotype compared with genotype-predicted phenotype

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For patients requiring multiple transfusions and patients with positive direct antiglobulin tests (DATs), an extended red blood cell (RBC) phenotype can provide valuable information and help to determine the risk of forming alloantibodies. In some instances, the phenotype may be used for prophylactic matching. Phenotyping in this patient population is often hindered by the presence of circulating donor cells and/or by a positive DAT. Several methods, such as EDTA glycine acid (EGA) treatment to remove IgG, hypotonic saline wash to separate autologous RBCs, or reticulocyte separation, are often used in these situations to isolate patient RBCs for serologic phenotyping. This study aimed to determine the accuracy of each RBC pretreatment method by comparing serologically determined antigen types with those predicted by RBC genotyping. Forty-eight peripheral blood samples from recently transfused patients were phenotyped for selected antigens in the Rh, Kell, MNS, Duffy, and Kidd systems. Treatment methods for the sample sets were reticulocyte separation (N = 12), EGA (N = 16), and hypotonic saline wash (N = 20). DNA was extracted using standard methods, and genotyping was performed using the HEA BeadChip panel. In addition, 21 samples positive for RBC-bound IgG were EGA-treated up to two times. These samples were analyzed pre- and post-EGA treatment for RBC-bound IgG by tube DAT and by flow cytometry with fluorescein isothiocyanate–labeled anti-human IgG. After reticulocyte separation, 3 of the 12 samples had discordant types with one antigen each: Fy^b, N, and K; serologic results were negative compared with genotype-predicted positive phenotype results. The EGA-treated sample set showed one discordant type: Fy^b; serologic results were negative compared with genotype-predicted positive phenotype results. Four of the 20 samples had discordant types involving the following antigens: Fy^b, N, e, and M; serologic results were negative compared with genotype-predicted positive phenotype results. After EGA treatment of 21 samples, 14 (67%) were negative for RBC-bound IgG by tube DAT, and 7 remained positive. Using flow cytometry, EGA treatment rendered only 4 samples negative, and 17 remained positive. In the antigen testing sample set of 48 samples, 10 of 511 total antigen types tested were discordant. Discordant types were most frequent in the hypotonic saline wash sample set (N = 6). In the flow cytometry sample set, 48 percent of the samples negative by tube DAT after EGA elution had detectable RBC-bound IgG by flow cytometry. These findings suggest that caution should be taken when using phenotype results from all pretreated RBCs and support the use of RBC genotyping to predict RBC antigen expression in samples from recently transfused patients.
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Key Words: antigen phenotype, microhematocrit separation, EDTA glycine acid (EGA), hypotonic saline wash, RBC genotyping

Red blood cell (RBC) phenotyping is valuable for transfusion management of multiply transfused patients, including the determination of the risk of forming alloantibodies. Extended phenotype matching is most commonly used in situations where there is a need to avoid sensitization in a nontransfused patient[1] or to avoid further alloimmunization in a patient who has already been sensitized.[2–5] Phenotyping of patients in these situations is often hindered by the presence of circulating donor cells or by a positive direct antiglobulin test (DAT). Because a donor RBC can survive in circulation for up to 120 days, it is not recommended to phenotype individuals who have been transfused in the past 3 months.[6] In addition, phenotyping with certain types of antisera may be hindered in patients with autoantibodies causing a positive DAT. In these scenarios, methods—such as EDTA glycine acid (EGA) treatment to remove IgG, hypotonic wash to separate autologous cells from patients with sickle cell disease, or microhematocrit centrifugation to isolate reticulocytes—are often used in an attempt to obtain a phenotype.[6] The effectiveness of removing IgG from RBCs to obtain DAT-negative RBCs can vary between methods.[7] With the increasing availability of RBC genotyping, more blood banks are using this testing to obtain a predicted RBC phenotype as an alternative to RBC pretreatments followed by serologic antigen typing.[8] A RBC genotyping panel such as the U.S. Food and Drug Administration (FDA)-approved PreciseType Molecular BeadChip[9] (Immucor, Norcross, GA) can predict antigen status for many of the major clinically significant blood group systems (Table 1). Especially in patients with hemoglobinopathies, genotyping is a routine approach to obtaining an extended RBC phenotype.[3,5,10] It has been previously documented that genotyping can provide more accurate RBC phenotype results than routine serology.[11]

Table 1. Blood group antigens predicted by the HEA BeadChip genotyping panel

Blood group system	Antigens
RH	C, c, E, e, V, VS
FY	Fy^a, Fy^b, Fy^b silenced
DO	Do^a, Do^b, Hy, Jo^a
SC	Sc1, Sc2
DI	Di^a, Di^b
LW	LW^a, LW^b
CO	Co^a, Co^b
JK	Jk^a, Jk^b
KEL	K, k, Kp^a, Kp^b, Js^a, Js^b
LU	Lu^a, Lu^b
MNS	M, N, S, s, U, U^{VAR}*

*Includes U^{VAR} (P2) and U^{VAR} (NY).

A small study was performed to measure, by tube and flow cytometry, the effectiveness of EGA treatment in removing IgG from RBCs. This study aimed to compare the accuracy of the results obtained from the commonly used RBC pretreatment methods with the results from a genotype-predicted phenotype using the BeadChip platform.

Materials and Methods

This study was approved by the American Red Cross institutional review board. A total of 48 peripheral blood samples from recently transfused patients were phenotyped for RH, KEL, MNS, FY, and JK antigens (Table 2). RBC pretreatment methods included reticulocyte separation (N = 12), EGA treatment (N = 16), and hypotonic wash (N = 20). Serologic antigen typing was performed by the tube method with licensed antisera from various sources (American Red Cross, Gaithersburg, MD; Immucor). Genomic DNA was extracted from peripheral blood mononuclear cells using DNA Blood Mini Kits (Qiagen, Carlsbad, CA), and genotyping was performed per the manufacturer's directions using the HEA BeadChip (Immucor) with a 96-well Veriti thermal cycler (Applied BioSystems, Foster City, CA), InSlideOut oven (Boekel Scientific, Feasterville, PA), and Array Imaging System (Immucor). The blood group antigens predicted by the genotyping panel are listed in Table 1. For the samples studied by flow cytometry and tube-DAT testing, 21 additional samples positive for RBC-bound IgG were EGA-treated (Gamma EGA kit, Immucor) until a negative tube DAT was obtained (up to two times). The samples were analyzed before and after EGA treatment for RBC-bound IgG by tube DAT (Immucor) and by flow cytometry (Becton Dickinson FACScalibur, San Jose, CA) with fluorescein isothiocyanate–labeled anti-human IgG (Life Technologies, Carlsbad, CA).

Results

Among the 147 antigen typing results in 12 samples tested after reticulocyte separation, 3 (2.0%) were discordant with 1 antigen each; 1 sample (R-1) phenotyped Fy(b–) and was predicted to be $Fy(b+^w)$ by genotyping, 1 sample (R-4) phenotyped N– and was predicted to be N+ by genotyping, and 1 sample (R-7) phenotyped K– and was predicted to be K+ by genotyping. Among the 116 antigen typing results in the 16 EGA-treated samples, 1 was discordant (0.8%); sample (E-12) phenotyped Fy(b–) and was predicted to be $Fy(b+^w)$ by genotyping. Among the 248 antigen typing results in the 20 hypotonic wash samples, 6 (2.4%) were discordant; 2 samples (H-10, H-15) phenotyped Fy(b–) and were predicted to be Fy(b+) by genotyping, 2 samples (H-11, H-16) phenotyped N– and were predicted to be N+ by genotyping, 1 sample (H-18) phenotyped M– and was predicted to be M+ by genotyping, and 1 sample (H-16) phenotyped e– and was predicted to be e+ by genotyping (ruling out common e variants interrogated by the HEA BeadChip). The antigen typing results for all samples are shown in Table 2. For comparison of effectiveness of EGA treatment by tube-DAT and flow cytometry, 21 samples were tested. Fourteen (67%) samples were negative for RBC-bound IgG by tube DAT and 7 were positive. When tested by flow cytometry, 4 (19%) samples were negative after EGA treatment, and 17 remained positive. Interestingly, of the 17 samples that were positive by flow cytometry, 10 were negative by tube DAT after EGA treatment.

Discussion

This study aimed to compare antigen types obtained after commonly used RBC pretreatments to RBC genotyping using an FDA-approved test. A total of 10 discordant results were discovered in 48 samples, with discordant types identified in each of the three treatment sets and with each discordant result being a false negative by phenotyping after RBC treatment. Discordant types (N = 6) were most frequently identified in the hypotonic wash sample set (N = 20), with 25 percent of samples being discordant with one or more antigens. The total number of antigens that were tested on these samples was 248, 2.4 percent of which were found to be discordant.

Table 2. Antigen typing results [positive (+), negative (0), or weak (+w)] for reticulocyte separation samples (R-1 through R-12), EDTA glycine acid (EGA)-treated samples (E-1 through E-16), and hypotonic saline wash samples (H-1 through H-20).

Sample	C	E	c	e	M	N	S	s	K	Fy^a	Fy^b	Jk^a	Jk^b
R-1	+	0	+	+	0	+	0	+	0	0	0/+w	0	+
R-2	+	0	0	+	NT	NT	0	+	0	+	+	+	+
R-3	0	0	+	+	+	+	NT	NT	0	NT	NT	0	+
R-4	+	0	+	+	+	0/+	+	+	NT	+	0	+	+
R-5	0	0	+	+	+	+	0	+	0	+	0	+	+
R-6	0	0	+	+	+	+	0	+	NT	0	0	+	0
R-7	0	+	+	0	+	+	+	0	0/+	0	+	+	+
R-8	0	0	+	+	0	+	0	+	0	0	0	+	+
R-9	0	0	+	+	+	+	+	+	0	0	0	+	+
R-10	0	+	+	0	0	+	+	+	0	+	0	+	+
R-11	0	0	+	+	+	0	+	+	0	0	0	+	0
R-12	0	+	+	+	+	+	+	+	NT	+	+	0	+
E-1	NT	NT	NT	NT	NT	NT	+	+	NT	0	+	+	0
E-2	NT	NT	NT	NT	NT	NT	0	+	NT	+	+	+	NT
E-3	NT	NT	NT	NT	NT	NT	0	+	NT	0	+	+	+
E-4	+	0	0	+	0	+	0	+	NT	+	+	0	+
E-5	NT	NT	NT	NT	NT	NT	+	0	NT	+	+	NT	NT
E-6	NT	NT	NT	NT	NT	NT	0	+	NT	0	+	NT	NT
E-7	+	+	+	+	+	+	0	+	NT	+	+	+	+
E-8	0	0	+	+	+	0	+	+	NT	+	+	+	0
E-9	NT	NT	NT	NT	NT	NT	+	0	NT	+	+	NT	NT
E-10	+	0	+	+	+	0	+	+	NT	0	+	0	+
E-11	+	0	+	NT	0	+	+	0	NT	+	0	0	+
E-12	NT	NT	NT	NT	NT	NT	0	+	NT	+	0/+w	+	+
E-13	NT	NT	NT	NT	NT	NT	+	+	NT	+	0	+	+
E-14	NT	NT	NT	NT	NT	NT	0	+	NT	+	+	+	0
E-15	NT	NT	NT	NT	NT	NT	0	+	NT	0	+	+	0
E-16	NT	NT	NT	NT	NT	NT	0	+	NT	0	+	NT	NT
H-1	+	0	+	NT	0	+	0	+	0	0	+	+	+
H-2	+	0	+	+	0	+	0	+	0	0	0	0	+
H-3	0	0	+	NT	NT	NT	0	+	0	0	0	+	0
H-4	0	0	+	NT	+	0	+	+	0	0	0	+	0
H-5	0	0	+	+	+	NT	0	+	NT	0	0	+	+
H-6	+	0	0	NT	+	+	0	+	0	0	0	+	0
H-7	0	0	+	NT	+	+	+	+	0	0	0	+	0
H-8	0	0	+	+	+	+	+	+	0	0	0	+	+
H-9	+	0	+	+	+	+	0	+	0	0	0	+	+
H-10	0	0	+	+	+	0	0	+	0	0	0/+	+	+
H-11	0	0	+	+	NT	0/+	0	+	0	0	0	+	0
H-12	+	0	+	+	+	+	0	+	0	0	0	+	+
H-13	0	+	+	+	+	+	+	0	0	+	0	+	0
H-14	+	0	+	+	+	+	0	+	0	0	+	+	+
H-15	0	0	+	NT	0	+	+	+	0	0	0/+	+	0
H-16	0	+	+	0/+	+	0/+	0	+	0	+	+	+	+
H-17	+	0	+	+	+	+	0	+	0	0	0	+	0
H-18	0	0	+	NT	0/+	+	0	+	0	0	0	+	0
H-19	0	0	+	+	+	+	0	+	0	0	0	+	0
H-20	+	0	+	+	0	+	0	+	0	0	0	0	+

Discordant antigens are highlighted in gray with serologic result presented first and genotype-predicted phenotype after the slash. Antigens not tested by serology are indicated by NT.

One discordant type was noted in the EGA-treated sample set (*N* = 16), with 6 percent of samples being discordant with one or more antigens and 0.8 percent of antigens tested (*N* = 116) being discordant. Three discordant types were noted in the reticulocyte-separated sample set (*N* = 12), with 25 percent of samples being discordant with one or more antigens and 2 percent of total antigens tested (*N* = 147) being discordant (Figs. 1 and 2).

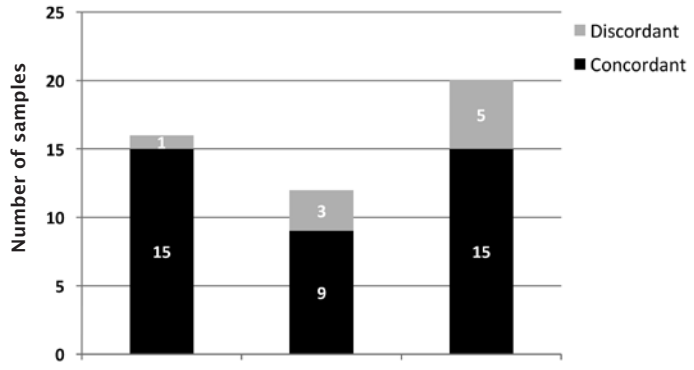

Fig. 1. Concordance of samples tested by treatment. EGA = EDTA glycine acid–treated sample set; Retic = reticulocyte-separated sample set; Hypo = hypotonic saline wash sample set.

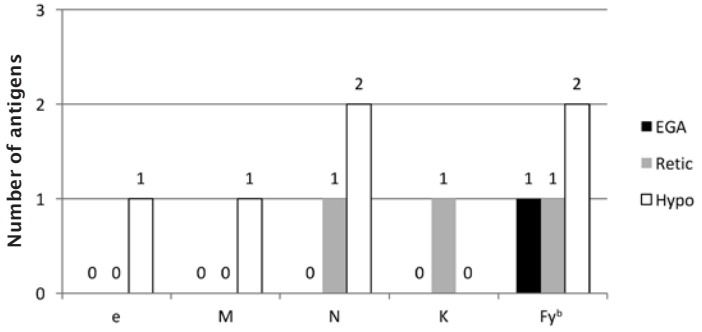

Fig. 2. Total discordant antigens per treatment. EGA = EDTA glycine acid–treated sample set; Retic = reticulocyte-separated sample set; Hypo = hypotonic saline wash sample set.

Two of the 10 total discordant samples (1 reticulocyte separation, 1 EGA treatment) carried the missense variant c.265C>T in the *ACKR1* gene, which encodes the FY antigens. This variant causes marked weakening of the Fy^b antigen (Fy^x phenotype), which can be weakly agglutinated by some commercial antisera but may be missed by others[12] and is a common cause of Fy^b typing discrepancies. This scenario may result in interpreting a patient with Fy^x as Fy(b–) and may complicate provision of antigen-matched RBCs for transfusion-matching protocols requiring FY status. More recently, a similar variant was found that can cause weakening of Fy^a.[13]

Furthermore, flow cytometry of EGA-treated RBCs suggests that samples negative by tube DAT after EGA elution may still have trace amounts of RBC-bound IgG detectable only by flow cytometry.[14] It is therefore critical that controls for residual IgG coating be performed with each sample treated with EGA (if the treatment is intended to remove RBC-bound IgG for the purpose of antigen testing), since the effectiveness of the treatment on removal of IgG may be different for individual patient samples.

A recent survey of current practices for providing blood to patients with warm-reactive autoantibodies (WAA) showed that 75 percent of laboratories surveyed provided phenotype-matched or genotype-matched RBCs for transfusion, with 80 percent of laboratories using RBC genotyping as part of an antibody workup in patients with WAA.[15] These findings suggest that caution should be taken when using phenotype results from treated RBCs to confirm suspected antibody specificities or to provide extended matching for future transfusions. This study did not rule out the presence of uncommon variants in the 10 samples with discordant antigen types. The discrepancies described here were associated with false-negative typings after cell treatment. False-negative results may cause the laboratory to misidentify an antibody specificity and distract the tester from identifying the true specificity, especially in patients with variant antigens when the phenotype is being used to help rule in or out certain antibodies.

For example, in our study, one sample was discrepant for e (serologic E+e–, genotype predicted E+e+); if the patient was receiving blood matched for RH antigens, e– blood would have been sourced, increasing the complexity because of the lower incidence of the e– phenotype. If this testing were to be used to provide antigen-matched blood, it could potentially cause delays in blood selection because of the perceived need for more antigens to be negative than is needed.

This study shows the advantages of using genotyping to predict RBC antigen expression and confirms that it is preferable in difficult patient samples. Our results show that RBC manipulation can result in serologic/genotypic antigen discrepancies and suggest that if extrapolated to general clinical use, additional antigen discrepancies—some with significant clinical impact—could be recognized.

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