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Genomic analyses of RH alleles to improve transfusion therapy in patients with sickle cell disease

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Abstract

Background: Red cell (RBC) blood group alloimmunization remains a major problem in transfusion medicine. Patients with sickle cell disease (SCD) are at particularly high risk for developing alloantibodies to RBC antigens compared to other multiply transfused patient populations. Hemagglutination is the classical method used to test for blood group antigens, but depending on the typing methods and reagents used may result in discrepancies that preclude interpretation based on serologic reactivity alone. Molecular methods, including customized DNA microarrays, are increasingly used to complement serologic methods in predicting blood type. The purpose of this study was to determine the diversity and frequency of *RH* alleles in African Americans and to assess the performance of a DNA microarray for *RH* allele determination.

Material and methods: Two sets of samples were tested: (i) individuals with known variant Rh types and (ii) randomly selected African American donors and patients with SCD. Standard hemagglutination tests were used to establish the Rh phenotype, and cDNA- and gDNA-based analyses (sequencing, PCR-RFLP, and customized RHD and RHCE microarrays were used to predict the genotype.

Results: In a total of 829 samples (1,658 alleles), 72 different alleles (40 *RHD* and 32 *RHCE*) were identified, 22 of which are novel. DNA microarrays detected all nucleotides probed, allowing for characterization of over 900 alleles.

Conclusions: High-throughput DNA testing platforms provide a means to test a relatively large number of donors and potentially prevent immunization by changing the way antigen-negative

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Authorship

M.E.R. designed the study, provided oversight, analyzed the data, and wrote the manuscript. C.H.H. managed the project, designed and performed experiments, analyzed the data and edited the manuscript. K.H-R. performed experiments, analyzed the data, and edited the manuscript. C.H. provided SCD patient blood samples, contributed to the study design, and edited the manuscript. All authors have read and approve the final manuscript.

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blood is provided to patients. Because of the high RH allelic diversity found in the African American population, determination of an accurate Rh phenotype often requires DNA testing, in conjunction with serologic testing. Allele-specific microarrays offer a means to perform high-throughput donor Rh typing and serve as a valuable adjunct to serologic methods to predict Rh type. Because DNA microarrays test for only a fixed panel of allelic polymorphisms and cannot determine haplotype phase, alternative methods such as Next Generation Sequencing hold the greatest potential to accurately characterize blood group phenotypes and ameliorate the clinical course of multiply-transfused patients with sickle cell disease.

Keywords

Blood groups; compatibility testing in sickle cell disease; DNA testing to predict blood groups; RH blood group system; RH genotype matching

Introduction

Red blood cell (RBC) blood group alloimmunization, elicited by antigens on donor RBCs that are absent from the recipient's RBCs, remains a major problem in transfusion medicine. This is particularly true for multiply transfused patients with sickle cell disease (SCD), who notoriously produce more alloantibodies to RBC antigens than any other patient population. Indeed, alloimmunization incidence rates of up to 36% have been reported in patients with SCD, compared to 5% in transfusion-dependent patients with thalassemia, and approximately 2% in other regularly transfused patient groups[1-7]

Red blood cell transfusion is a major therapeutic option in sickle cell disease (SCD). There is strong evidence for its efficacy, in the prevention and treatment of SCD-associated complications particularly primary and secondary stroke in children and acute chest syndrome[8,9]. The need for indefinite transfusions to prevent primary stroke in SCD was confirmed by findings from the STOP 2 (Optimizing Stroke Prevention in Sickle Cell Anemia) showing that discontinuation of transfusion after at least 30 months in subjects who had converted to normal transcranial Doppler (TCD) velocities was associated with high risk of reversion to abnormal and chance of stroke[10,11]. Discontinuation of prophylactic transfusions was also associated with an increased risk of silent brain infarction in these children[12]. In the phase 2 Study of Neuropsychological Dysfunction and Neuroimaging Abnormalities in Neurologically Intact Adult Patients with Sickle Cell Disease, adult SCD patients receiving chronic transfusions had fewer adverse events and hospitalizations than patients randomized to the non-transfusion arm[13]. The efficacy of preoperative transfusion in SCD patients undergoing low- or moderate-risk surgical procedures was recently demonstrated in a randomized controlled study showing a significantly decreased rate of post-operative complications in patients receiving transfusion prior to surgery compared to those who had not received pre-operative transfusion[14]. Evidence supporting the use of transfusion for other complications of SCD, such as prolonged vaso-occlusive pain episodes, recurrent acute chest syndrome, and pulmonary hypertension, is based on review of literature, consensus or expert opinion[15-17].

With improvements in the management of SCD, patients are now living beyond their 6th decade and are likely to be exposed to several or many transfusion episodes that potentiate the risk of alloimmunization. With the availability of oral iron chelators and erythrocytapheresis, an increasing number of patients are now receiving prolonged or lifelong chronic transfusions for a variety of clinical indications. It is anticipated that children currently receiving chronic transfusion for stroke prevention will make up a growing population of adults requiring lifelong transfusion[18].

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Despite efforts to minimize the risk of alloimmunization in SCD, an estimated 4% to 11% of patients with SCD who receive transfusions develop overt delayed hemolytic transfusion reactions (DHTRs). Moreover, the clinical picture and laboratory abnormalities associated with less severe DHTRs may be misinterpreted as a vaso-occlusive pain event and go unreported[19,20]. Ranging between 18 and 36%, the increased rates of alloimmunization observed in SCD patients in the USA is primarily related to the disparity in RBC antigens between predominantly African American (AA) recipients and donors of largely European background[2,21].

In contrast, the rate of RBC alloantibody formation in Uganda, where donor and recipient populations are ethnically similar, is 6%[22]. Similarly, Olujohungbe *et al*. reported the development of antibodies in only 2.6% of 190 transfused SCD patients in Jamaica compared to 76% of 37 patients in England[23]. Given the disparity between donor and recipient antigen profiles among various ethnic groups, phenotype-matched blood for SCD patients in the USA is most likely to be found among AA donors. For example, the C–, E–, S–, K–, Fy(a–), Jk(b–) blood type is found in 1 in 6 AA donors, but in only 1 in 131 Caucasian donors[24]. In an effort to increase the number of AA donors, many blood donor centers have instituted targeted donor recruitment.

Transfusion with RBC components matched for highly immunogenic Rh (D, C, E, c, e and K) antigens was found to decrease the rate of RBC alloimmunization from 35% to 0% [25] and from 3% to 0.5% per RBC component transfused[26] . However, even with limited phenotype matching, patients may still become immunized to multiple blood group antigens, or to an antigen of high-prevalence, underscoring the need to transfuse even more precisely matched blood to prevent alloimmunization and DHTR[27-29].

Blood donor centers face additional challenges in providing fully phenotype-matched blood for transfusion. While serologic testing is simple and requires little in the way of equipment, it is labor-intensive, requires trained technologists, as well as manual entry of results. In addition, routine testing frequently will not detect antigens, such as partial Rh antigens, that are more likely to be expressed in AA donors or for which antisera are scarce, e.g., to detect V/VS, Go^a, and DAK antigens. These Rh antigens are immunogenic and while they occur infrequently in Northern European populations, they are found in up to 20% of the AA population[30]. Furthermore, if RBCs used for antibody screening do not express these antigens, the corresponding antibodies will not be detected unless an anti-globulin cross-match is performed. For patients with a negative antibody screen, it is now common practice to generate a computer match rather than a physical testing match[31].

Prevention of alloimmunization in SCD has thus been hampered by an incomplete knowledge of the blood group phenotypes within the AA population, inadequate supplies of reliable antisera, and insufficient inventories of antigen-negative blood. An improved understanding of the molecular basis of blood group antigens, as well as technologic advances have permitted prediction of RBC phenotypes using genomic approaches[30,32]. However, DNA analyses for the prediction of Rh antigens are particularly complex because of the extreme homology between the RH genes, *RHD* encoding the D antigen, and *RHCE* encoding the C/c and E/e antigens. These two genes segregate as a single haplotype. As a result of the homology between the *RHD* and *RHCE* and their opposite orientation within the RH locus, rearrangements are not uncommon and many have been reported[30,33]. Although some variant RH alleles are due to single nucleotide changes, the majority are due to gene rearrangements that result in hybrid alleles. Various laboratory developed tests (LDTs), including PCR-RFLP, AS-PCR, sequencing of cDNA RHD and RHCE (exons 1-4 and exons 5-10), sequencing specific exons from gDNA, and cloning as well as DNA microarrays have been used to predict blood group phenotypes for antibody identification, to

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reveal the molecular changes of novel blood groups, and to select appropriate blood donors. Novel high-throughput genotyping methods are now being applied to enable rapid selection of units matched at multiple blood group loci.

The purpose of this study was to determine the diversity and frequency of *RH* alleles in the AA population and better define RBC phenotype-genotype associations in transfused SCD patients. Genotyping results for RHD and RHCE alleles using LDTs were compared to Rh phenotypes determined by hemagglutination. Samples were also tested on prototype RHD and RHCE BeadChips™ to assess the utility of DNA microarrays to predict Rh phenotypes. Such data are needed to determine the feasibility of matching the predicted *RH* type of a donor RBC component to a transfusion-dependent patient of African ancestry.

Material and methods

Subjects

Between January 2008 and October 2011, blood samples were obtained following IRB protocols and were assigned a unique reference code. Samples were collected from two source populations: (i) archived samples from AA individuals with known variant Rh phenotypes (n = 200) selected to test for allelic diversity, especially RHCE. These samples were obtained from individuals whose RBCs showed discrepant D, c, e, C, or E antigen typing, and/or expressed a low-prevalence antigen, or whose plasma contained alloanti-D, -C, -c, -E, or -e in an antigen-positive patient, (ii) random samples from self-identified AA donors at the NYBC (n = 482) and a cohort of transfused SCD patients from Children's Hospital Oakland (CHO) (n = 147) to estimate the frequency of variant alleles.

Whole blood samples were processed as follows: plasma was separated, DNA was extracted, RNA was extracted and cDNA was prepared by RT-PCR, and an aliquot of blood was placed in TRIzol. Aliquots of each of these were frozen at appropriate temperatures. The remaining red cell fraction was washed in a sucrose solution and frozen in droplets in liquid nitrogen. A subset of samples was tested to validate prototype RHD and RHCE microarrays.

Laboratory Developed Tests

Rh cDNA cloning and sequencing—RNA was isolated from reticulocytes with TRIzol (PureLink Micro-to-Midi Total RNA Purification System, Invitrogen, Carlsbad, CA). Reverse transcription was carried out with gene-specific *RHD* and *RHCE* primers (Life Technologies, Inc., Gaithersburg, MD) and Superscript III according to manufacturer's instructions (Superscript III First Strand Synthesis SuperMix, Invitrogen, Carlsbad, CA) (Table 1a). Polymerase chain reaction (PCR) amplification was carried out for 35 cycles with primers cRHx1F and cRHx5R to amplify exons 1 to 4, and cRHx4F and cRHx10R to amplify exons 5 to 10 of *RHD* and of *RHCE*. PCR products were checked for purity on agarose gels and sequenced (GENEWIZ, Inc., South Plainfield, NJ). Sequences were aligned and analyzed using Sequencher 4.9 software (GeneCodes, Ann Arbor, MI). Cloning was performed by GENEWIZ.

Genomic DNA sequencing—Genomic DNA was extracted from peripheral blood (QIAamp or QIAcube; Qiagen, Valencia, CA) and amplified by PCR using RHD exon-specific and RHCE exon-specific primers located in the flanking introns (Table 1b). The PCR products were directly sequenced (GENEWIZ), and compared with *RHD* (GenBank accession no. L08429) and *RHCE* (GenBank accession no. DQ322275) sequences.

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Other assays—A PCR multiplex assay was used to simultaneously detect *RHD* exons 4 and 7, the inactivating *RHD* pseudogene and to determine the *RHCE*C/c* status[34]. Two assays were used to determine *RHD* zygosity[33,35]. PCR-RFLP assays were used on a subset of samples to validate sequencing results[36].

DNA microarray

Samples were tested using prototype RHD and RHCE BeadChip microarrays as per manufacturer's instructions (BioArray Solutions-Immucor, Warren, NJ). The RHD and RHCE BeadChips probe for 36 and 26 nucleotide changes, respectively[37]. The RHD and RHCE BeadChip results were analyzed by BAS using a customized software program incorporating proprietary algorithms that, along with predicted allele assignments and haplotypes, yield confidence scores[38,39]. Interpretations of the RHD and RHCE BeadChip analyses were compared to LDT and serologic testing results.

Serologic testing

Serological testing for specific Rh antigens was performed on selected samples using standard hemagglutination methods. The samples had also been previously tested, to varying degrees by numerous technologists over many years, using standard methods appropriate for the reagent used. The majority of serotypes were obtained from historical records; however, when appropriate, additional hemagglutination in test tubes or on gel cards was performed.

Results

Diversity RHD and RHCE alleles

An enormous quantity of data was generated and only a summarized overview is presented here. Using the high-resolution LDT sequencing methods described, 72 variant alleles (40 *RHD* and 32 *RHCE*) were identified among all 829 samples (1658 alleles). Of these, 22 (31%) were novel variants (Table 2). Not unexpectedly, most variant alleles were found in the archived samples from individuals who had complex anti-Rh identified in the plasma or demonstrated apparently discrepant antigen types.

Frequency of RHD and RHCE alleles

The RHD and RHCE alleles found in our study population of randomly selected AA donors and patients with SCD are summarized in Tables 3 and 4. Given the limited sample size, combined frequencies are also given (n = 620; 1240 alleles).

DNA BeadChip testing

The prototype RHD and RHCE BeadChip assays were validated in a subset of the study cohort. Of the 470 samples (940 alleles) analyzed with the RHD BeadChip, 764 alleles (81%) were in agreement with the LDT results and 176 alleles were in apparent disagreement (Table 5). Of the 482 samples (964 alleles) analyzed with the RHCE BeadChip, 893 alleles (94%) were concordant with the LDT results and 71 were in apparent disagreement (Table 6). For both BeadChips, the apparent discrepancies were due to the absence of nucleotide probes necessary to discriminate specific alleles. Thus, to the extent that the nucleotide probes necessary to detect specific RHD and RHCE alleles were included on the BeadChips, there were no actual discrepancies.

The comparison between predicted antigens by DNA BeadChip testing and hemagglutination results showed complete agreement for D, C, E, c, e, VS/V, hr^S, and hr^B antigens. In one sample showing agreement with LDTs, but not with hemagglutination, a rare silenced RHCE*cE allele was identified[40].

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Discussion

Alloimmunization remains a major complication of transfusion therapy in SCD and while matching for simple antigens (D, C, E, c, e, K, and maybe Fy^a, Jk^a, and Jk^b) is successful in preventing alloimmunization in the vast majority of patients, there are occasions when provision of more precisely matched blood is required to prevent life-threatening DHTR. Such alloantibodies in multiply transfused SCD patients are often against unusual antigens in the Rh system and are poorly defined. Once sensitized, these patients may develop complex antibody specificities and present a challenge in finding compatible blood products. Many Rh variants can be defined by a panel of monoclonal anti-D or anti-e, however, these reagents are not fool-proof because they can give different reactions depending on the formulation of the reagent, the condition of the RBCs, and haplotypes *in trans*. DNA testing serves as a complementary tool to predict the presence or absence of a variant antigen. Indeed, molecular techniques may be useful for identification of donor RBCs that are predicted to lack certain combinations of antigens and/or a high-prevalence antigen and thereby increase the inventory of antigen-negative blood.

While DNA microarrays have been used for several years to predict antigen types in several non-Rh blood groups systems[41-47], such analysis of *RHD* and *RHCE* is complicated by the numerous rearrangements between these homologous alleles. Nevertheless, DNA microarrays are increasingly being used to analyze blood samples for RH alleles, albeit using complex algorithms[48]. Due to the complexity of serological testing in patients with SCD, more precise matching may only be possible at the DNA level and superior to hemagglutination. Our findings build on ongoing efforts to determine the diversity of alleles encoding Rh phenotypes in the AA population. Once information regarding the clinical relevance of variant RH alleles has been clearly established, appropriate probes should be included in DNA microarrays that are used to screen patients and donors.

Despite the limited sample size, the data generated can be used to assess the feasibility of supplying DNA-matched blood to chronically-transfused patients. Although the molecular bases of many antigens in the Rh system have been reported, it is clear that more alleles exist. Indeed, of the 72 alleles we found, 22 were novel. These results show a remarkable and greater than predicted diversity of RH alleles in AAs. Also our results show that variant RH alleles are not uncommon in this population. For example, the single nucleotide change, *RHCE*733C>G*, was found in 279 of the random samples [ce^S, (C)ce^S, ce 733G with 941C, ce^S(340), see Table 4], which represents, somewhat surprisingly, 42.9% of samples with this nucleotide change. Of the 279 samples, 38 were homozygous *RHCE*733G/G*, whereas 16 were heterozygous *RHCE*733G in trans* to RHCE*cE, a genotype that could result in production of an anti-e-like antibody. Furthermore, in this study the frequency of RH alleles in the random AA patients from Oakland and donors from New York was similar, suggesting that the pattern of variation in RH alleles does not differ across the US (Tables 3 and 4).

Based on a combination of test results (sequencing, BeadChip, *RHD* pseudoexon, *RHD* zygosity, hemagglutination) and known frequency data, likely haplotypes were deduced in as many subjects as possible and are summarized in Table 7. Among the SCD patients, 4 were homozygous for a variant RHCE allele, 2 were heterozygous RHCE*(C)ceS/ceS alleles, and 8 had a variant RHCE allele *in trans* to an RHCE*cE allele. Two patients had a variant RHD allele *in trans* to a silenced *RHD*. Thus, 14 [antigen-positive] patients are at risk of making alloantibodies to the partial antigens present (e.g., C/c and e) and 2 of making anti-D. The proportion of the variant alleles among the AA donors was similar (22 were homozygous for a variant RHCE allele, 2 were heterozygous RHCE*(C)ceS/ceS alleles, and 14 had a variant RHCE allele *in trans* to an RHCE*cE allele), and could reasonably be

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assumed to be able to support this cohort of SCD patients. The effect of different combinations of the Rh protein complex within the RBC membrane on antigen expression is largely unknown. However, the majority of samples was heterozygous for various combinations of alleles (almost 82% of the patients, Table 7) and thus, would not be expected to be at risk of mounting an antibody response to an unusual Rh antigen. This explains why matching of simple Rh antigens (i.e., D, C, E, c, e) is successful in preventing alloimmunization to Rh antigens in the majority of patients.

Comparison of BeadChip and LDT results showed an 81% concordance in RHD allele calls and a 94% in RHCE allele calls. The apparent discrepancies (9% for *RHD* and 6% for *RHCE*) were due to the absence of nucleotide probes necessary to discriminate particular alleles using the BeadChip. For example, the *RHD* probes used to detect the nucleotide change associated with DAU0 (*RHD*1136C>T*) are not included on the BeadChip. Similarly, the BeadChip lacked the RHCE probes necessary to detect nucleotides 254, 941 and 105. To the extent that nucleotide probes (necessary to detect specific RHD and RHCE alleles) were included on the BeadChip, there were no true discrepancies.

DNA microarray technology is a powerful tool; however, it can only predict a blood type. For example, in an Rh_{null} phenotype, an allele may be present but its expression silenced by one of a large number of reasons, including nucleotides changes in a distinct but related gene (*RHAG*). This makes it impractical to predict using a set panel of probes included on a DNA microarray. This approach does not reveal nucleotide changes in other parts of the allele and to minimize the chance of misinterpreting results obtained by DNA testing, it is important to include assays for additional nucleotide changes that are known to weaken or silence the expression of encoded antigens, especially those that are relatively frequent, e.g., *RHD*ψD*[34].

Determination of which nucleotide changes are present on which allele, i.e., *in cis* vs. *in trans* haplotype configuration, is not practical by DNA microarrays. For example, with a *RHCE*ce 733C/G, 1025C/T* result, the 733G could equally well travel with the 1025C (*RHCE*ce^S*) or with 1025T (*RHCE*ceTI*). Thus, the resulting phenotype could be ceTI/ceS or ceTI Type2/ce. In general terms, this is relevant when it affects the antibodies that could be made by a person with one versus the other phenotype. Fortunately, in most cases the presence of a variant protein encoded by homozygous alleles, a rare event, is a prerequisite for antibody production. Other scenarios include heterozygosity for a variant *RHD in trans* to a silenced *RHD*, or heterozygosity for a variant *RHCE in trans* to a silenced *RHCE* or *RHCE*cE*, where the person could make an anti-e-like antibody. This is consistent with the data we obtained in testing the 200 archived samples from patients or donors with plasma containing antibodies to Rh antigens (other than anti-D, -C, -E, -c, or -e) or with RBCs with unusual Rh antigen typings.

In the situation of a heterozygote variant allele *in trans* to a null allele, the patient can make an antibody to the absent antigen. In such cases, on testing DNA, a valuable donor would go undetected, and a patient would be predicted to have RBCs that express antigens and, thus not be identified as a candidate to make alloantibody(ies). To further complicate matters, different combinations of a more than one RHCE variant have been described in patients who made anti-Rh-18, an antibody that has caused fatal transfusion reactions[49]. The variant proteins ceAR, ceMO, ceEK, ceBI, and ceMI all lack a related high-prevalence antigen, hr^S. However, antibody made by a patient with one of these variants is not necessarily compatible with RBCs from a donor with a different molecular basis for the hr^S-negativity. For example, serum from a ceEK/ceEK person is compatible with ceAR/ceAR RBCs, but serum from a ceAR/ceAR person is not compatible with ceEK/ceEK RBCs.

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Yet another level of complexity occurs when the variant *RHCE* is inherited in tandem with an altered *RHD* and, thus, in addition to making anti-hr^S or anti-hr^B, the patient also can make anti-D. On initial presentation, such a case would appear serologically to be D+, e+ with alloanti-D and alloanti-e, and most likely with co-existing alloantibodies to other blood groups systems. Such cases are difficult to resolve and it is extremely difficult to find compatible blood[50]. It is currently a major problem to supply D+, let alone D–, RBCs with certain RHCE phenotypes that are C–, E–, hr^S–, or C–, E– hr^B–. Theoretically, any *RHD* alleles may occur *in cis* with any *RHCE* alleles. Some combinations of variants are more common than others (e.g., *DIVa* with *RHCE*ceTI* [51]; DAR with *RHCE*ceAR* or *RHCE*ceEK* [52]; *DIIIa*, *DIIIa-CE(4-7)-D*, or *D-CE(4-7)-D* with *RHCE*ceS* [40].

A rearranged RHD allele can encode an altered D antigen, the so-called partial D antigen, and an individual homozygous for the rearranged RHD allele or compound heterozygous for an *in trans* silenced RHD allele, can produce anti-D. Rarely is an attempt made to classify the anti-D, because it has been common practice to provide D-negative RBC components rather than opting for the more complicated process of trying to match the partial D type. Most partial D phenotypes are usually only recognized after production of anti-D or by detection of a low-prevalence antigen that can serve as a marker (e.g., DAK, Go^a, D^W), although certain partial D phenotypes are typed as D– with some examples of anti-D. Similarly, a rearranged RHCE allele can encode an altered C, c, E, or e antigen and individuals homozygous for the rearranged RHCE allele or heterozygous with an *in trans* silenced RHCE allele can make an alloantibody corresponding to the epitopes missing from the altered (partial) antigen. These individuals are also typically only identified either after production of anti-C, anti-c, anti-E, or anti-e or by detection of a marker antigen (e.g., V/VS, Rh32, Crawford, JAL). Antibodies to a high-prevalence antigen on a variant Rhce protein appear as anti-Rh17. In contrast to the partial D/anti-D scenario, provision of rare Rh17– (D-- phenotype or Rh_{null} phenotype) RBC components is not feasible, therefore it is the current practice to name the Rhce high-prevalence antigens, thereby providing a means of communicating the needs regarding location of compatible blood. Examples of this are RH46, which is antithetical to RH32; CELO, which is antithetical to Crawford, and CEST, which is antithetical to JAL[30].

DNA microarrays are ideally suited to screen a large number of donors (albeit at a cost), thereby increasing the inventory of antigen-negative RBC components. The larger the antigen-negative inventory, the more likely appropriate blood can be supplied to a patient. Given the diversity of the RH alleles, DNA testing serves as an important complement to serologic testing, allowing for superior matching of patient and donor.

Conclusions

Although DNA testing is unlikely to completely replace hemagglutination, it serves as a valuable adjunct. Moreover, the increasing cost and diminishing supplies of source material of classical antisera needed for hemagglutination tests make DNA testing an attractive complement to existing hemagglutination methods.

The prediction of Rh phenotypes has proven difficult due to the complexity and homology of RH genes and the high number of rare, yet clinically relevant, alleles identified in individuals of African ancestry. Although some variant RH alleles are due to a single nucleotide change that is readily detectable by a DNA microarray, the majority are due to gene rearrangements that result in hybrid alleles. Moreover, accurate typing relies on the detection of silencing nucleotide changes.

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Our results provide data regarding the diversity and frequency of alleles that can be applied to fine-tune the DNA microarray platforms currently on the market. This will allow prediction of the absence of a high-prevalence antigen in the Rh blood group system. Furthermore, knowing the identity of alleles underlying the phenotype of RBCs used in antibody identification panels will aid in the identification of some Rh alloantibodies in AAs, which currently are poorly defined.

Rapid advances in technological and bioinformatics methods hold promise for development of cost-efficient DNA microarrays as a complementary strategy to prevent alloimmunization in multiply transfused patients. Furthermore, it is only a matter of time before next generation sequencing (full sequencing of *RHD* and *RHCE*) will supplant DNA microarrays, not only in terms of completeness, but also efficiency and cost[53-55] . Once an individual's blood group genotype has been defined, a simple unique profile could be used on subsequent occasions. This should not only reduce costs but also speed the matching process.

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Abbreviations used

AA	African American
BAS	BioArray Solutions-Immucor
CHO	Children's Hospital Oakland
DHTR	Delayed hemolytic transfusion reaction
DNA	deoxyribose nucleic acid
LDT	Laboratory developed test
NYBC	New York Blood Center
PCR	Polymerase chain reaction
RBC	Red blood cell
RCT	Randomized control study
RFLP	Restriction fragment length polymorphism
RT	Reverse transcription
SCD	Sickle cell disease
STOP	Stroke prevention trial in sickle cell anemia
TAPS	Transfusion alternative preoperatives in sickle cell disease
TCD	Transcranial Doppler

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Table 1

Primer sequences, locations, and amplicon sizes

a. cDNA primers

Primer Name	Primer Sequence (5' – 3')	Assay (Amplicon size, where appropriate)
cD×10Rchen	gtattctacagtgcataataaatggtg	*RHD* gene-specific RT-PCR
cCE×10Rchen	ctgtctctgaccttgtttcattatac	*RHCE* gene-specific RT-PCR
cRH×1Fchen	agctctaagtacccgcggtctgtcc	Exons 1 to 4 on gene-specific cDNA (660bp)
cRH×5Rchen	tggccagaacatccacaagaagag	
cRH×4Fchen	acgatacccagtttgtctgccatg	Exons 5 to 10 on gene-specific cDNA (681 bp)
cRH×10Rchen	tgaacaggccttgtttttcttggatgc	
cRH×2Rseq	gaagaggttgaaggccacac	Sequencing primer
cRH×6Fseq	acctgatcccttctccgtg	Sequencing primer
cRH×6Rseq	ggagaagggatcaggtgaca	Sequencing primer

b. *RHD* gDNA primers

Primer Name	Primer Sequence (5' – 3')	Location	Assay (Amplicon size)
RHD5-re01F	atagagaggccagcacaa	5'UTR of exon1	RHD Exon 1 (~450bp)
RH3-re11dR	agaagatgggggaatctttttcct	Intron1	
nRHD-I1-1405F	catttcccctatttaacagacaagaacaag	Intron1	RHD Exon 2 (1702bp)
RHDI2+61R	ggcaatatcccagatcttctggaacc	Intron2	
Rh-I2F	gcccaacagtgtttgttgaa	Intron2	RHD Exon3 (575bp)
RhD-Intron3R	atgttgcccagctcggtcc	Intron3	
RHDI3S	ggcttgccccgggcagagg	Intron3	RHD Exon4 (321bp)
RHDI4AS	gcttcagacacccaggggaac	Intron4	
RhDI4F	taagcacttcacagagcagg	Intron4	RHD Exon 5 (476bp)
RHDCEINTR5	gtgtgctagtcctgttagacc	Intron 5	
RHI5-F	agtgtgatgggtgcctaggatgctgagcacct	Intron 5	RHD Exon 6 (478bp)
RHDI6-R	cctgctggccttcagccaaagcagaggagg	Intron 6	
RhD-I6F	cttcatttcaacaaactccccga	Intron6	RHD Exon 7 (500bp)
DNB-R	gtgataaatccatccaaggtaggggccggccagaat	Intron7	
RhD-I7F	ctggaggctctgagaggttgag	Intron 7	RHD Exon 8 (516bp)
Rh-I8-R	catagacatccagccacacggca	Intron 8	
RHD I8-67F	tgagatactgtcgttttgacacacaatacttc	Intron 8	RHD Exon 9 (268bp)
RHD I9+62R	gttttactcataaacagcaagtcaacatatatcct	Intron 9	
RH-10-F	caagagatcaagccaaaatcagt	Intron 10	RHD Exon 10 (382bp)
RhD-10-R	agcttactggatgaccacca	3' exon10	

c. RHCE gDNA primers

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Primer Name	Primer Sequence (5' – 3')	Location	Assay (Amplicon Size)
CEex1S	gcacacaggATGAGCTCTAA	5'UT/Ex 1	CE Exon 1 (283bp)
CEInt1R	agatgggggaatcttttcctc	Intron 1	
RHCEI1F	gcttcccctcctccttctcac	Intron 1	RHC−c+ Exon 2 (313bp)
RHDI2+61R	ggcaatatcccagatcttctggaacc (non-specific – RHD/RHCE)	Intron 2	
RHCEI1-1432F	gtgcgaaaacagttggtgattattgataag	Intron 1	RHC+c− or C+c+ Exon 2 (1705bp)
RHDI2+61R	ggcaatatcccagatcttctggaacc (non-specific – RHD/RHCE)	Intron 2	
RHIn1-165F	cagttgagaacattgaggctca	Intron 1	sequencing primer for exon 2
CEInt2F	gtgtttgttgaatgaatgaatgaatg	Intron 2	CE Exon 3 (266bp)
CEInt3R	cggaagccccaccaaatggag	Intron 3	
CEInt3F	ggctttcccctgggcagagg	Intron 3	CE Exon 4 (321bp)
CEInt4R	gcttcagacacccaggggaag	Intron 4	
CEI4	ggcaacagagcaagagtcca	Intron 4	CE Exon 5 (531bp)
RHDCEIn5R	gtgtgctagtcctgttagacc	Intron 5	
RH-I5-F	gagtgtgatgggtgcctaggatgctgagcacct	Intron 5	CE Exon 6 (478bp)
RHCE-I6-R	cctgctggccttcagccaaagcagagagca	Intron 6	
CEInt6F	ccattgatgtgagtacacattc	Intron 6	CE Exon 7 (456bp)
CEInt7R	ctggacataatttctgaataaatc	Intron 7	
RhCE-I7-F	ctggaggctctgagaggttaaagg	Intron 7	CE Exon 8 (516bp)
Rh-I8-R	catagacatccagccacacggca	Intron 8	
RH-10-F	caagagatcaagccaaaatcagt	Intron 9	CE Exon 10
RhCE-10-R	ccaaaactttaataatgtgtctgtaacc	3' UTR exon 10	(362bp)

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Table 2

RHD and *RHCE* alleles identified in total sample population (n= 829 samples)

RHD alleles (n = 40)		**RHCE alleles (n =32)**	
D	*DAR*	*ce(48G or 48C)*	*ce 48C, 733G, 1006T*
DIIIa	Weak *D Type 4.0*	*Ce*	*ceAR*
ΨD	*D-CE-D (r'^S)*	*cE*	*ceBI*
D deleted	*D 1097A*	*CCWe*	*ceCF*
DIVa Type 1	Weak *D Type 40*	*CCXe*	*ceEK*
DIVa Type 2	*DOL*	*Ce 48G*	*ceMO*
DAU-0.1	*DOL-2*	*ce 48C*	*ceTI*
DAU-0	*Weak RHD(165C>T)*	*ce 733G (48G or 48C)*	*ce 48G, 733G, 1025T*
DAU-3	*D-CE(4-7)-D*	D^{HAR}	*RN*
DAU-4	*DIIIa-CE(4-7)-D*	*ce 48C, 733G, 916A*	*ce 48C, 193G, 733G*
DAU-5	Weak *D Type 4.2.2*	*ceSL*	***ce 340T, 733G****
DIII type 4	Weak *D Type 4.2.3*	***ceSM***	***ce 48G, 733G, 340T****
DFV	*D-CE(3)-D*	***ce 254G***	***ce 48C, 733G, 941C***
***DIIIa* 819A**	*D-CE(2-7)-D*	***ce 733G, 941C***	***CeTI***
D ex7 del (or altered)	*D-ceEK(2-7)-D*	***ce 48C, 105T***	***ceTI 48C,733G, 744C, 1025T***
D 384C	*D-CE(3-7)-D*	***ce 285T***	***ce 48C,193G, 733G***
D 541T	***D 963A***		
D 569T	***D 186T, 364A, 873A***		
D 841C	***DIIIa 819A, 384C***		
D 941T	***DAU 667G, 1136T***		

Bold font = previously published by us as a result of this study or not yet published

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Table 3

Frequency of *RHD* alleles in random AA donor and SCD patient samples (n= 620)

Allele name *RHD*	**From both sets of samples**		**Samples from SCD patients**		**Samples from NYBC blood donors of African descent**	
	Total # alleles	**Allele frequency**	**# alleles**	**Allele frequency**	**# alleles**	**Allele frequency**
D	783	0.631	170	0.607	613	0.639
DAU-0	200	0.161	48	0.171	152	0.158
ΨD	42	0.034	12	0.043	30	0.031
complete deletion	38	0.031	4	0.014	34	0.035
DIIIa-CE(4-7)-D	36	0.029	9	0.032	27	0.028
DAU-3	24	0.019	7	0.025	17	0.018
weak D type 4.0	23	0.019	7	0.025	16	0.017
DIIIa 819A	17	0.014	6	0.021	11	0.011
DIVa.2	16	0.013	4	0.014	12	0.013
DAU-5	15	0.012	4	0.014	11	0.011
Novel allele	11	0.009	2	0.007	9	0.009
DAU-0.1	9	0.007	1	0.004	8	0.008
RHD-CE(3-7)-D	7	0.006	0	0.000	7	0.007
weak D type 4.2.2	5	0.004	4	0.014	1	0.001
DAU-4	3	0.002	0	0.000	3	0.003
DOL	2	0.002	0	0.000	2	0.002
D-CE(4-7)-D	2	0.002	2	0.007	0	0.000
D (ex7altered or deleted)	1	0.001	0	0.000	1	0.001
DAU-6	1	0.001	0	0.000	1	0.001
DIII type 4	1	0.001	0	0.000	1	0.001
DOL-2	1	0.001	0	0.000	1	0.001
weak D type 4.2.3	1	0.001	0	0.000	1	0.001
DFV	1	0.001	0	0.000	1	0.001
weak RHD(165C>T)	1	0.001	0	0.000	1	0.001
#Alleles:	**1240**		**280**		**960**	
#Samples: # Samples:	**620 9 ND**		**140**		**480**	
			7 ND		**2 ND**	

ND = not determined

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Table 4

Frequency of *RHCE* alleles in random AA donor and SCD patient samples (n= 620)

Allele name *RHCE**	**From both sets of samples**		**Samples from SCD patients**		**Samples from NYBC blood donors of African descent**	
	Total # alleles	**Allele frequency**	**# alleles**	**Allele frequency**	**# alleles**	**Allele frequency**
ce (48G or 48C)	537	0.428	123	0.418	414	0.431
ce 733G (48G or 48C)	216	0.172	53	0.180	163	0.170
Ce	173	0.138	38	0.129	135	0.141
cE	129	0.103	27	0.092	102	0.106
ce 254G	56	0.045	9	0.031	47	0.049
(C)ceS	44	0.035	14	0.048	30	0.031
ceTI (Ce TI)	24	0.019	7	0.024	17	0.018
ceMO	20	0.016	5	0.017	15	0.016
ce 733G 941C (48C)	17	0.014	2	0.007	15	0.016
ce 105T (48C)	14	0.011	5	0.017	9	0.009
ceEK	7	0.006	3	0.010	4	0.004
ceAR	4	0.003	3	0.010	1	0.001
ceBI	3	0.002	0	0.000	3	0.003
Novel allele	3	0.002	2	0.007	1	0.001
CCWe	2	0.002	2	0.007	0	0.000
ceS(340)	2	0.002	0	0.000	2	0.002
ceCF	1	0.001	0	0.000	1	0.001
ceTI type 2	1	0.001	0	0.000	1	0.001
RN	1	0.001	1	0.003	0	0.000
#Alleles:	**1254**		**294**		**960**	
#Samples: **# Samples:**	**627** **2 ND**		**147**		**480** **2 ND**	

ND = not determined

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Table 5

RHD alleles identified by BAS RHD BeadChip versus LDT

Alleles in agreement		Alleles in apparent disagreement[a]		
Allele	Number of alleles	Allele by LDT	Allele called by BAS	Number of alleles
D	600	*DAU-0*	*D*	149
ΨD	32	*DAU-5*	*DV type1*	11
DIIIa-CE(4-7)-D	27	*DAU-0.1*	*D*	3
DIVa.2	12	*DAU-6*	*D*	1
Weak D type 4.0	16	*DIII type 4*	*DIIIa-CE(4-7)-D*	1
DAU-3	17	*WT D*	*ex9 del*	1
RHD deletion	34	*DOL-2*	*DOL*	1
RHD-CE(3-7)-D	5	*Weak D type 4.2.3*	*DAR*	2
DIIIa 819A	13	*DFV*	*D*	2
DAU-4	3	*Novel allele*	*D*	4
D (ex7 altered or deleted)	3	*Weak RHD (165C>T)*	*D*	1
DOL	2			
Total:	**764**	**Total**		**176**

LDT: Laboratory developed tests, including cDNA cloning and sequencing, and gDNA sequencing.

[a] probes necessary to detect these alleles were not included in RHD BeadChip panel.

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Table 6

RHCE alleles identified by RHCE BAS BeadChip versus LDT

Alleles in agreement		Alleles in apparent disagreement[a]		
RHCE alleles	**Number of alleles**	**Allele by LDT**	**Allele called by BAS**	**Number of alleles**
ce (48G or 48C)	409	*ce 254G*	*ce*	47
ce 733G (48G or 48C)	164	*ce 733G 941C (48C)*	*ceS*	15
Ce	136	*ce 105T (48C)*	*ce (48C)*	9
cE	106	**Total**		**71**
(C)ceS	32			
ceTI	20			
ceMO	15			
ceBI	3			
ceEK	4			
ceAR	1			
ceCF	1			
ceS(340)	2			
Total:	**893**			

LDT: Laboratory developed tests, including cDNA cloning and sequencing, and gDNA sequencing.

[a]probes necessary to detect these alleles were not included in RHD BeadChip panel.

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Table 7

Summary of *RHD/RHCE* haplotype combinations found in random AA sample populations tested

Haplotype	Blood donors	Patients with SCD
*RHD*D-RHCE*ceS* homozygous[a]	15	4
RHD hetero/*ceS* homozygous[a]	4	0
RHD hetero/*(C)ceS* homozygous[a]	1	0
RHD hetero/*RHCE*(C)ceS/ceS*[a]	2	2
RHCE variant/RHCE*cE[a]	14	8
*RHCE*ce254G* homozygous[a]	2	0
*RHD variant /RHD*ΨD* [a]	51	2
Homozygous 'common'[a]	4[b]	8[c]
Heterozygote	315 (69.2%)	120 (81.6%)
Total number of samples	**455**	**147**

[a]patients with these haplotypes can produce alloantibodies to antigens absent from, and to partial Rh antigens on, their RBCs

[b]*RHD*D-RHCE*Ce* (n = 15), *RHD*D-RHCE*ce* (n = 21), and *RHD*D-RHCE*cE* (n = 5)

[c]*RHD*D-RHCE*Ce* (n = 5), *RHD*D-RHCE*ce* (n = 2), and *RHD*D-RHCE*cE* (n = 1)