Clinical investigation of posttransfusion Kidd blood group typing using a rapid normalized quantitative polymerase chain reaction

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BACKGROUND: Accurate typing of a patient’s RBCs in the setting of prior transfusion or a hemolytic transfusion reaction is crucial in the selection of compatible blood but is time consuming, technically difficult, and sometimes impossible. To address this problem, a simple, rapid, and inexpensive quantitative PCR method was developed to identify the single nucleotide polymorphism (SNP) of the Kidd blood group. We applied this method in a clinical investigation of 54 multiple-transfusion patients.

STUDY DESIGN AND METHODS: Patients were eligible if they had received at least one RBC transfusion within 30 days and had a sample referred to our regional reference lab for assistance with compatibility testing requiring reticulocyte separation, hypotonic saline treatment, or chemical modification to remove IgG. We compared serologic result to the normalized quantitative PCR. For discrepant cases, or when no serologic type could be assigned, DNA sequencing characterized the patient’s Kidd SNP.

RESULTS: Of the 54 patients, the reference lab could assign a serologic Kidd type for 33. Quantitative PCR assigned a Kidd type for 53 of the 54. In three cases, where serology and PCR were discrepant, and for all cases where serology could not assign a Kidd type, DNA sequencing verified the Kidd typing assigned by PCR.

CONCLUSION: A simple, rapid, and accurate technique has been developed. The assay performs well in the clinical setting. With further study, and inclusion of other blood group systems, this may become an important supplemental technique for selected patients in the immunohematology reference laboratory.

ANTIBODIES TO THE KIDD BLOOD GROUP ANTIGENS ARE AMONG THE MOST PROBLEMATIC IN CLINICAL TRANSFUSION PRACTICE. MOLECULAR GENOTYPING OF BLOOD GROUPS, INCLUDING KIDD, USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM AND SEQUENCE-SPECIFIC PCR, HAS BEEN EMPLOYED TO ADVANCE CURRENT TECHNIQUES. THESE TECHNIQUES HAVE BEEN DEMONSTRATED TO BE SUCCESSFUL IN PREDICTING THE PHENOTYPE OF PATIENTS WHO HAVE RECEIVED RECENT TRANSFUSIONS OR HAVE BEEN MASSIVELY TRANSFUSED. RAPID REAL-TIME PCR METHODOLOGY HAS ALSO BEEN APPLIED TO GENOTYPING BLOOD GROUP POLYMORPHISMS. THE CLINICAL APPLICATION OF MOLECULAR METHODOLOGY HAS BEEN SLOWED, HOWEVER, BY THE COST, COMPLEXITY, TIME REQUIRED FOR THE ANALYSIS, THE NEED TO DISTINGUISH SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) RELIABLY, AND THE TIMES COMPLEX RELATIONSHIPS BETWEEN GENOTYPE AND PHENOTYPE.

The Kidd protein is a 391 amino acid multipass integral membrane protein that functions as the constitutive urea transporter in the RBC membrane, where it is thought to preserve osmotic stability. The gene that codes for the antithetical alleles, $\text{Jk}^a$ and $\text{Jk}^b$, is located on chromosome 18q11-q12. These alleles result from an SNP.
guanine or adenine, leading to amino acid substitution Asp280Asn.\textsuperscript{13} The inheritance of \(Jk^a\) and \(Jk^b\) is codominant. A third Kidd antigen, JK3, is found in more than 99 percent of Caucasians. There is also a null phenotype of Kidd, derived from several different genetic alterations, which is resistant to lysis in 2-mol/L urea.

Allosensitization to Kidd is responsible for at least 30 percent of all delayed hemolytic transfusion reactions (DHTR) and an even higher percentage of severe DHTRs.\textsuperscript{34} These IgG or IgM antibodies are sometimes difficult to detect in pretransfusion testing because Kidd antibodies frequently coexist with other alloantibodies. They can display a waning titer and may fail to react with cells that are heterozygous for the antigen. Also, upon repeat exposure through transfusion, a strong anamnestic response with efficient complement fixation can lead to severe delayed hemolysis. Finally, significant racial differences in the Kidd gene frequencies can lead to antigenic exposure when donor and recipient populations are racially disparate.

This combination of circumstances frequently leads to situations where compatible blood is needed urgently for a patient who is experiencing a severe DHTR and whose Kidd phenotype may be very challenging to determine accurately by serologic methods. To address this problem, we have developed a unique real-time allele-specific PCR assay for the rapid differentiation of the Kidd blood group SNP. Of note, the typing strategy we have developed may be completed in hours with “walk-away” simplicity utilizing a closed-reaction system, and it requires no hybridization probe or expensive proprietary reagents. In this pilot clinical investigation, we examined the assay’s performance by genotyping Kidd (\(Jk^a\), \(Jk^b\)) alleles both in reference samples of known Kidd phenotype and in defined clinical samples submitted to the immunohematology reference laboratory from multiple-transfusion individuals. For discrepant samples and for samples whose Kidd type could not be determined serologically, we validated the PCR genotype by direct sequencing and compared all genetic results to available serologic data.

MATERIALS AND METHODS

Study design and eligibility

Patients were eligible if they had received at least one RBC transfusion within 30 days and had a sample referred to the regional reference laboratory at Blood Centers of the Pacific for assistance with compatibility testing, which required reticulocyte separation, hypotonic saline treatment, or chemical modification to remove IgG. Fifty-four consecutive eligible subjects were enrolled. The primary outcome variable was the ability to assign an accurate Kidd typing. Using leftover post-transfusion patient samples supplied to the reference laboratory, we compared serologic results to the Kidd type assigned by PCR. In cases where results were discrepant, or where no serologic type could be assigned, direct DNA sequencing was performed to characterize the Kidd SNP. Residual samples from the immunohematology reference laboratory at Blood Centers of the Pacific (San Francisco, CA), from which all patient identification information was removed, were used for all genetic analyses. The use of anonymized samples was reviewed and approved by the Committee on Human Research at the University of California San Francisco.

Control samples

Four control samples, two Jka+Jkb− and two Jka−Jkb+, collected from healthy blood donors at Blood Centers of the Pacific were characterized serologically using standard methods for use as controls. Amplification of DNA replicates from these controls was used to determine linearity range and reproducibility.

Serologic phenotyping of clinical RBC samples

When patients are recently transfused, the presence of transfused donor RBCs can produce mixed-field phenotyping results that are difficult to interpret. To increase the number of available autologous erythroid cells for typing, reticulocytes were separated before typing by differential centrifugation.\textsuperscript{15} Reticulocytes from patients with sickle cell disease, however, fail to separate by differential centrifugation, and a hypotonic saline technique is needed to separate autologous sickle cells from transfused cells.\textsuperscript{15} When RBCs were coated with IgG (DAT+), it was necessary to remove the IgG coating before the cells could be typed by the IAT. Chemicals such as EDTA-glycine\textsuperscript{15} and chloroquine\textsuperscript{15} were used to remove the IgG coating before serologic phenotyping. In some rare cases, the chemicals failed to remove the IgG coating, and serologic phenotyping was impossible.

DNA extraction from MNCs for PCR genotyping

Cells were extracted from a 0.5-mL frozen whole blood for which ACD or EDTA had been used as anticoagulant. The samples were kept at \(-80^\circ\text{C}\) until processing. The blood was thawed and microcentrifuged at 2000 × g for 5 minutes. The supernatant was aspirated and residual RBCs were lysed by the addition of 1 mL Solution A (0.1 mol/L KCl, 0.01 mol/L Tris Base, 0.0025 mol/L MgCl\(_2\)·6H\(_2\)O, pH 8.3) with 200 μL of Saponin lysis solution (0.4% Saponin in 0.5% NaCl, pH 7.4). The preparation was microcentrifuged at 7000 × g for 5 minutes. The supernatant was aspirated and the pellet washed with 1 mL of Solution A. A crude DNA lysate was prepared from this pellet by the addition of 100 μL of solution containing equal volume of Solution A.
A and Solution B (10 mmol/L Tris [pH 8.3], 2.5 mmol/L MgCl₂, 1% Tween-20, 1% NP40), and 12.5 μg proteinase K (GibCo BRL, Carlsbad, CA). The same protocol for preparing DNA was used for controls and clinical samples.

Optimization of PCR genotyping
The assay was optimized using DNA from blood donor samples of known serologic type, Jk(a+) or Jk(b+). The Jk⁺ and Jk⁻ primers were designed with the allele-differentiating base at the 3’ end of one primer of the primer pair. No mismatches were introduced in the primers. A variety of conditions including different primer pairs, magnesium concentrations, and primer concentrations were tested. Annealing temperatures of 56°C, 60°C, and 64°C were evaluated. The results were assessed based on the cycle threshold (Ct) difference between positive and negative controls. A Ct difference of 10 was designated as the acceptable distance between positive and negative controls. A Ct difference assessed based on the cycle threshold (Ct) difference between Jk(a+) and Jk(b+) samples. Amplification runs were carried out to the 45th cycle to catch all delayed reactions.

Amplification of Kidd polymorphism
Five microliters of DNA lysate was added to 10 μL of PCR buffer containing 3.75X SyBr Green (BioWhittaker Molecular Applications, Rockland, ME), 0.7 U of Amplitaq Gold (Applied BioSystems, Foster City, CA), and 1 μmol/L dNTPs (Roche Diagnostics GmbH, Mannheim, Germany). For Kidd, the following primers were used:

- Jk⁺: 0.5 μmol/L JK781F316 (CAT GCT GCC ATA GGA TCA TGG C)[Forward], JkR116 (CCA GAG TCC AAA GTA GAT GTC)[Reverse]; 84 bp product.
- Jk⁻: 0.3 μmol/L JkB117 (CTC AGT CTT TCA GCC CCA TTT GAG A)[Forward]; Jk943R316 (GAG CCA GGA GGT GGG TTG GC)[Reverse]; 124 bp product.

The polymerase was activated at 95°C for 10 minutes and followed by 45 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds. Amplification was performed using the GeneAmp5700 system (Applied Biosystems).

Quantification of genomic DNA
The HLA DQ-alpha (HLA DQA) region of DNA in control and clinical samples was amplified in parallel from 5 μL of DNA lysate using 0.5 pmol of primers, GH26 (GTG CTG CAG GTG TAA ACT TGT ACC AG) and GH27 (CAC GGA TCC GTG AGC AGC GGT AGA GTT G). One-in-ten dilutions of DNA from cultured cells of A3.01 (National Institute of Health, Bethesda, MD) were used as a quantitative standard. The range of the quantitative standard was 10⁰ to 10⁶ per reaction.16

PCR analysis of Kidd and HLA DQ-alpha data, normalized quantitative kPCR results
An essential element in our PCR analysis was to standardize the threshold from run to run to a fixed cycle such that the quantitative level of amplified DNA could be normalized. In real-time PCR, the threshold can be set along a wide range of values, provided it is set in the area where amplification is linear. Generally, protocols allow for setting the threshold arbitrarily, provided the correlation of the standard curve (target input vs. Ct) is acceptable (>0.98000).16 Our protocol uses one of the HLA-DQA quantitative standard controls used for determining the genomic input to derive a reference point for setting a normalization threshold for both alleles and for all runs. We assume that all aliquots of HLA-DQA controls have the same value and thereby can provide a reference point for separate runs. For each run, the threshold was set so that cycle 23 would correspond to the Ct for the 1000 copies per 5 μL DNA input (HLA-DQA assay) control. This threshold was called the Reference Ct (RCt), which served as a reference threshold for interpretation of quantitative results for all HLA DQ-alpha and qualitative (by Ct) Kidd alleles in clinical samples. The undiluted quantitative control, A3.01, determined the RCt because manual or mechanical errors (i.e., from pipetting or dilutions) were reflected in diluted samples. Such errors would appear as variances in the slope or correlation of the standard curve. In this method, the positive or negative Kidd interpretation was derived from allele Ct. Because Kidd Ct may vary by as much as 10 cycles depending on where the threshold is set, normalization of threshold setting through a quantitative standard will yield a consistent interrun analysis.

Sequencing protocol
The region flanking the Kidd polymorphism was amplified and then the amplicon was sequenced. We used forward primer JK781F316: CAT GCT GCC ATA GGA TCA TGG C and reverse primer JK943R316: GAG CCA GGA GGT GGG TTG GC. The product length was 163 bases. The PCR product was run in 6-percent polyacrylamide gel to check for purity and product size. The specific band was excised and the DNA purified using the Qiagen gel extraction kit (Qiagen Corporation, Valencia, CA). Sequencing of the purified amplicon was performed at the University of California San Francisco Cancer Center Core Facilities (San Francisco, CA). Five picomoles of forward or reverse sequencing primer were added to the amplified product. DNA sequencing was performed using Big Dye (version 3.0) chemistry according to manufacturer’s recommendations (Applied Biosystems) and run on an ABI3700 capillary sequencing instrument. Data were extracted and analyzed with software (Sequencer Analysis Software version 3.7, Applied Biosystems).
RESULTS

Clinical results: complex serology, normalized quantitative PCR, and DNA sequencing

Table 1 presents a comparison of complex serologic results obtained by the blood center reference lab with results obtained using normalized quantitative PCR. Both Jk\textsuperscript{a} and Jk\textsuperscript{b} alleles were analyzed for all samples using the delta Ct approach (described in detail below). Despite prior transfusion and extensive variability in age and DNA content of clinical samples, the Kidd SNP was unambiguously resolved by normalized quantitative PCR in every case but one. Twenty-one serologic tests could not be completed for technical reasons. For all of these samples, DNA sequencing was performed to compare results with the PCR results; in every case the sequencing verified the PCR type. Three patient samples produced discordant results between serology and PCR. The first was serologically Jk(a–b+), whereas PCR determined it to be Jk\textsuperscript{a}/Jk\textsuperscript{b}; sequencing result for this discordant sample was positive for both adenine and guanine, confirming the genotype assigned by PCR. The second discordant sample was serologically Jk(a+b+) and PCR determined it to be Jk\textsuperscript{b}/Jk\textsuperscript{b}; sequencing again confirmed the PCR result. The third discordant sample tested serologically as Jk(b–), whereas the type Jk\textsuperscript{a}/Jk\textsuperscript{b} was assigned by PCR and confirmed by sequencing. For both missing and discordant, sequence data uniformly corroborated the PCR typing. One clinical sample displayed an abnormal amplification curve for Jk\textsuperscript{b}. This amplification curve was easily identifiable due to its unusual shape. Multiple repeat testing showed this to be a reproducible phenomena though the reason for this could not be readily determined.

Assay calibration

Comparison of three amplification graphs from 1000 copies of the HLA-DQA control is shown in Fig. 1. Each graph is generated through amplification of a 1000-copy standard using a different machine or a different reagent lot. The reaction intensity, Rn shown on the y-axis, varies depending on run. These small variations in fluorescence efficiency are normalized by assigning cycle 23 as Ct for the 1000-copy standard in all graphs.

Normalized quantitative PCR analysis

To characterize the relationship between DNA input and amplification Ct of specific gene segments, two positive control samples for each allele were selected and dilutions (original concentration, 1/10, 1/100) were amplified, on four separate runs with one set of dilution amplified per run. The results were plotted as number of cells (HLA-DQA) on the x-axis and the Ct of specific gene amplification on the y-axis (not shown). Plotted on Fig. 2 are the mean allele Ct values for 10,000, 1000, 100, and 10 HLA DQA copies for each allele based on the regression line determined from the dilutions. The regression line derived from the mean values is shown in Fig. 2 (y = –3.2686x + 33.582). The SD of the Cts was also determined, and the

<table>
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<th>TABLE 1. Comparison of PCR and serologic results</th>
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<td>Total clinical samples</td>
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<td>------------------------</td>
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<tr>
<td>Jk\textsuperscript{a}</td>
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<td>Jk\textsuperscript{b}</td>
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* Reference lab unable to assign a probable Kidd typing from complex serology.
† One sample yielded an abnormal amplification profile.
line representing mean ± 3 SD was plotted. Fig. 2(A) shows the result for \( J_k^a \) positive controls, and Fig. 2(B) shows the result for \( J_k^b \) positive controls. Barring any statistical bias from control selection, this should detect 99.7 percent of tested samples.

Delta Ct analysis

The second methodology used to analyze the same data was the Delta Ct, which is the difference in Ct between the reference amplification and the specific allele amplification. The reference amplification in our system was HLA-DQA, a pan-WBC genetic element present at two copies per cell. Therefore, for each specific allele, Delta Ct was calculated by subtracting the Ct of the HLA-DQA from the Ct of the specific allele. The Delta Ct of the two Kidd alleles is summarized in Table 2. For \( J_k^a \), the Delta Ct range was -0.99 to 3.53 for positive controls and 10.13 to 14.27 for negative controls. For \( J_k^b \), the positive Delta Ct range was 0.41 to 3.88, and the negative was 10.97 to 13.86. There was no overlapping value between positives and negatives for both alleles.

Sequencing chromatograms

For samples with discrepant serologic and PCR typing, direct analysis of the Kidd SNP was undertaken by DNA sequencing. Fig. 5 shows three examples of Kidd chromatograms from this sequencing. The top panel illustrates heterozygosity, \( J_k^a/J_k^b \), with both adenine and guanine detected at position n pointed to by the arrow. The middle panel illustrates homozygosity for \( J_k^a/J_k^a \) and the lower figure homozygosity for \( J_k^b/J_k^b \), with each having only a guanine or an adenine detected.
Zygosity of Kidd

Amplification of both Jka and Jkb alleles for all samples allowed the determination of zygosity. Of the 53 samples tested (one sample generated an inconclusive result for Jkb), 30 (56.6%) were determined to be heterozygous, 12 (22.6%) were homozygous for Jka, and 11 (20.8%) were homozygous for Jkb.

DISCUSSION

We have developed a unique, simple, and powerful system for rapid PCR genotyping of the Kidd blood group polymorphism and applied the assay in a clinical investigation of 54 patients. Our method has allowed correct assignment of genotype not only for laboratory controls but also for actual clinical samples. Where discrepancies occurred, the data suggest that PCR is likely to be more reliable than traditional complex serologic methods used commonly by blood center reference labs. Further, the PCR analysis may be completed in a favorable time frame (4-6 hr), which can make results potentially useful in the selection of compatible blood for transfusion.

The genotyping method we have developed is not probe-based, does not depend on proprietary reagents, and is readily generalizable to include other clinically relevant blood groups. Our validation data using donor samples of known Kidd phenotypes indicate that the method is robust in its ability to resolve the Kidd SNP both accurately and rapidly. Further, the data presented also indicate that zygosity can be determined for the Kidd SNP. Our preliminary work with other blood groups systems suggests that a uniform annealing temperature should allow a complete set of relevant blood group polymorphism analyses to be completed with a single amplification plate, thus greatly reducing complexity and cost.

When applied to posttransfusion clinical samples whose historical (pretransfusion) Kidd phenotype was not known at the time of testing, PCR results from posttransfusion samples agreed with serologic results in all but three cases. For all three of these discrepant samples, DNA sequence analysis agreed with the genotype assigned by PCR, not the genotype suggested by phenotypic analysis of the posttransfusion samples. These three discrepant results could indicate a Kidd genotype that is not predictive of phenotype. However, it is more likely that these discrepancies are caused by either the suppressed expression of blood group antigens in the reticulocytes or by inherent limitations of the complex serologic methods used to analyze posttransfusion samples. Although chemical treatment of RBCs to remove IgG is an accepted technique, EDTA-glycine is known to destroy certain blood group antigens, including the antigens of the Kell blood group, and prolonged exposure to chloroquine is known to alter the Rh antigens. Monoclonal antisera are frequently used to type IgG-coated cells by direct agglutination, but con-

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<th>TABLE 2. Analysis of clinical samples using delta Ct*</th>
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<td>Samples</td>
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<tr>
<td>Jka* Pos</td>
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<tr>
<td>Jkb* Pos</td>
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* Delta Ct is the difference between the Cts of DQA and the respective alleles. Positives samples have lower delta Cts than negatives samples. There is no overlap between the delta Cts of positive and negative samples.
controls must be performed carefully to avoid false-negative results caused by prozone effect. Finally, cell separation techniques also have inherent limitations. When patients are not producing reticulocytes or when patients have received massive transfusion, cell separation techniques may be unreliable. For example, when a hypotonic wash technique is used to separate sickle cells, it is essential to remove the stroma from the lysed donor cells. If the stroma remains with these patient’s RBCs, the stroma may adsorb typing serum and cause false-negative typing results. For all of these reasons, an assay to determine a reliable genotype rapidly in certain complex posttransfusion settings has the potential to increase transfusion safety. Comparison of our PCR and sequence results with Kidd serology performed in the absence of transfusion would be very valuable, but these data are not available in the current study design, which relied upon unlinked samples.

In this study, we have not addressed directly the Jk null phenotype. The null phenotype is rare but clinically important. Homozygosity for five different mutations is responsible for the null Jk(a–b–) phenotype and more may exist yet undiscovered. Jk null phenotype has been reported in Polynesians, Filipinos, Indonesians, Chinese, Japanese, Asian Indians, native Brazilians, an African American, a Tunisian, and in Europeans, including Finns, French, Swiss, and English. The Polynesian mutation is a G-to-A transition in the invariant 3’ acceptor splice site of intron 5 of a Jkb allele, causing loss of exon 6 from mRNA transcripts. The Finnish mutation is a T871C substitution in a Jkb allele, encoding the amino acid change Ser291Pro. In one Jk(a–b–) sample from a white French person, a G-to-T transversion in the donor splice site of intron 7 of a Jkb allele led to skipping of exon 7 from the mRNA transcript. A deletion of about 1.6 kb encompassing exons 4 and 5 of a Jka allele in two English sisters and a Tunisian produced the Jk(a–b–) phenotype. Finally, a nonsense mutation in exon 7 of a Jka allele converted the codon for Tyr194 to a stop codon in three Jk(a–b–) Swiss sisters. Although it is rare, the null phenotype is very important to consider in the clinical setting. It may not be practical to apply molecular analyses such as we describe to the systematic detection of all known null genotypes of Kidd. Instead, an alternate screen, such as the urea lysis test, might be used to exclude the null phenotype for selected patients. However, reliance on such a strategy would require careful validation of the urea lysis test for this purpose, something which has not been included in our current study.

Because many clinically relevant blood group polymorphisms are SNPs, the technical ability of a genotyping method to resolve SNPs is crucial to expanded application of molecular methods in blood bank reference labs. Discrimination of SNPs using real-time PCR is highly dependent on primers binding to specific sequences and on the extension efficiency. Generally, primer specific PCRs, which distinguish a single base difference, have primer designs that differ at the 3’ end, mismatching at the last nucleotide. This technique introduces inefficient annealing of the primer to the target and in theory prevents extension by the polymerase. Were a misextension of the primers to occur, because the sequence in this site is similar except for one base, the nonspecific product generated would have the same sequence and melting temperature as the specific product. In practice, we have found that misextensions do occur, rendering analyses by dissociation curves of amplified products useless and real-time PCR using specific primer detection of SNPs difficult to optimize. It should be noted, however, that a recent publication by Araujo et al. used the LightCycler (Roche Molecular Systems, Alameda, CA) to genotype Kel.

Fig. 5. Chromatograms from sequencing data. This figure consists of three chromatograms for Kidd, showing a (A) heterozygous, Jkα/Jkβ chromatogram. Both adenine and guanine are detected at position n. (B) Homozygous Jkα/Jkα. (C) Homozygous for Jkβ/Jkβ, with each having either a guanine or adenine solely at position n pointed to by the arrow.
and JK using dissociation curves. In that study, the polymorphism discrimination was incorporated into the probe.

A second important tenet in quantitative real-time PCR is that the amplification curve of the specimen with the highest DNA content is detected first. Misextensions, when they occur, are much less efficient, and the product generated delays the appearance of product by approximately 10 cycles. A 10-cycle difference, such as that exhibited between a misextension and a specific extension, translates to a difference of \(2^{10}\) or 1024 times the DNA input between two positive samples. Therefore, this system must be standardized to a reference genomic DNA input. In this paper, we utilized an amplification of the HLA-DQ alpha region and quantitative standards consisting of \(10^4\), \(10^3\), \(10^2\), \(10^1\), and \(10^0\) per reaction to quantify the genomic input. Alleles for Kidd and HLA-DQA were amplified at the same annealing temperature so that the amplification could be accomplished in one plate. The use of two primer pairs to detect separately the two antithetical forms of the gene is meant to reduce incorrect genotyping and facilitate determination of the zygosity.

The range of genomic DNA input into a quantitative PCR genotyping assay is an important variable by which to validate assay performance. For our clinical samples, 500 to 100,000 copies were present, a much wider variation than the range encountered with control samples, which ranged from 1000 to 10,000 copies. We attribute this variation to the nature of the clinical samples; conditions such as hematopoietic malignancy or chemotherapy cause the observed order of magnitude variations in WBC count, which are not found in healthy blood donors. The clinical samples tested were also older than control samples and were residual volume from serologic tests. The storage conditions of these samples could also have contributed to differences in DNA content. Finally, data plotted from amplifications of positive samples are linear and have a high correlation value compared to data from negative samples. Negative samples express a more random amplification pattern when they occur, are much less efficient, and the product generated delays the appearance of product by approximately 10 cycles. A 10-cycle difference, such as that exhibited between a misextension and a specific extension, translates to a difference of \(2^{10}\) or 1024 times the DNA input between two positive samples. Therefore, this system must be standardized to a reference genomic DNA input. In this paper, we utilized an amplification of the HLA-DQ alpha region and quantitative standards consisting of \(10^4\), \(10^3\), \(10^2\), \(10^1\), and \(10^0\) per reaction to quantify the genomic input. Alleles for Kidd and HLA-DQA were amplified at the same annealing temperature so that the amplification could be accomplished in one plate. The use of two primer pairs to detect separately the two antithetical forms of the gene is meant to reduce incorrect genotyping and facilitate determination of the zygosity.

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We based our assignment of genotype on quantitative analysis of the data based on Delta Ct. The Delta Ct analysis measures the relative difference between the two amplifications as a basis for determining the allelic polymorphism. In general, the Delta Ct analysis does not use a standard curve, only the relative distance between the Cts of the two amplifications, the reference (DQ-alpha) and the target (\(J^a\) and \(J^b\)). An advantage to this system is that it is robust and less demanding than some forms of quantitative PCR. In using this analysis, however, one must demonstrate that certain conditions are intrinsically satisfied because there is no calibration curve in this assay. The analysis of Delta Ct assumes that the amplification conditions are correct and consistent, that the amplification is efficient, and that the dynamic range is acceptable. The validation process in our method verifies that these conditions are met.

One clinical sample yielded an abnormal amplification curve for \(J^b\), which could not be interpreted. This sample yielded the same result on a repeat testing but the cause of this phenomenon remains unexplained and may represent another limitation to consider were this methodology to be applied clinically in the future.

In summary, we have developed a rapid, reliable, and precise technique to determine the common Kidd blood group genotypes, which allows correct assignment of the genotype even for highly variable posttransfusion samples from patients requiring complex serologic analysis by a blood center reference lab. Despite the limitations we have described, this protocol appears robust in many critically important respects and, with further study and development, may prove to have clinical utility in the selection of compatible blood for transfusion of the most ill patients when serologic methods are less than optimal.

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