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Molecular typing for blood group antigens within 40 minutes by direct PCR from plasma or serum

Franz Friedrich Wagner¹, Willy Albert Flegel², Rita Bittner¹, and Andrea Döscher³

¹DRK Blutspendedienst NSTOB, Institute Springe, Springe, Germany

²Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, USA

³DRK Blutspendedienst NSTOB, Institute Bremen-Oldenburg, Oldenburg, Germany

Abstract

Determining blood group antigens by serological methods may be unreliable in certain situations, such as in patients after chronic or massive transfusion. Red cell genotyping offers a complementary approach, but current methods may take much longer than conventional serological typing, limiting their utility in urgent situations. To narrow this gap, we devised a rapid method using direct polymerase chain reaction (PCR) amplification while avoiding the DNA extraction step. DNA was amplified by PCR directly from plasma or serum of blood donors followed by a melting curve analysis in a capillary rapid-cycle PCR assay. We evaluated the single nucleotide polymorphisms underlying the clinically relevant Fy^a, Fy^b, Jk^a and Jk^b antigens, with our analysis being completed within 40 min of receiving a plasma or serum sample. The positive predictive value was 100% and the negative predictive value at least 84%. Direct PCR with melting point analysis allowed faster red cell genotyping to predict blood group antigens than any previous molecular method. Our assay may be used as a screening tool with subsequent confirmatory testing, within the limitations of the false-negative rate. With fast turnaround times, the rapid-cycle PCR assay may eventually be developed and applied to red cell genotyping in the hospital setting.

Keywords

immunohematology; blood groups; red cell genotyping; direct PCR; transfusion medicine

Address for correspondence: Willy A Flegel MD, Department of Transfusion Medicine, NIH Clinical Center, National Institutes of Health, 10 Center Drive, Bethesda MD 20892-1184, USA; waf@nih.gov, Phone 301-594-7401, FAX 301-496-9990.

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Introduction

Molecular immunohematology methods are increasingly used to determine blood group antigens in routine patient care. Established first line indications for red cell genotyping include prenatal diagnosis, such as the prediction of the fetus' phenotype from the mother's peripheral blood; blood group phenotypes that cannot be discriminated by serology; and blood group typing in recipients of massive or chronic transfusions (Denomme and Flegel 2008, Wagner 2010). Direct polymerase chain reaction (PCR) has been applied to blood donor typing using heat-treated plasma samples without DNA isolation (Silvy *et al*, 2015), a simple but not rapid procedure. Direct PCR with inhibitor-resistant polymerases (Trombley Hall *et al*, 2013) and real-time PCR (Chen *et al*, 2015), widely applied in microbiology, has also enabled fast turnaround times, but has not been applied to red cell genotyping so far.

Since they were introduced in 1992 (Higuchi *et al*, 1992) and applied to quantitative PCR using dual-labelled fluorogenic probes in 1996 (Heid *et al*, 1996), real-time PCR platforms generally offer the fastest turnaround times. Instrumentation for rapid thermocycling in capillary tubes by a stream of hot air was designed in 1989 (Wittwer *et al*, 1989) and by 1997, this capillary rapid-cycle PCR, when combined with resonance energy transfer probes and fluorescence analysis (Lay and Wittwer 1997), enabled allele determination, known as genotyping, in patients. Araújo *et al* (2001, 2002) were the first to apply capillary rapid-cycle PCR to red cell genotyping. This approach has repeatedly been used for blood group genotyping by Polin and colleagues since 2007 (Polin *et al*, 2009, Polin *et al*, 2007, Polin *et al*, 2008).

The original implementation of red cell genotyping required agarose gel electrophoresis (Gassner *et al*, 1996, Flegel *et al*, 1998, Prager 2007) and covered a relevant number of all known alleles at the time (Flegel 2007). The first advanced platforms, including BloodGen (Avent *et al*, 2009) and BeadChip (Hashmi *et al*, 2005) focused on the highly accurate determination of most clinically relevant alleles to limit the rate of incorrect antigen predictions (Flegel 2007). While this approach was conceptually critical to foster the adoption of red cell genotyping, it required rather elaborate assays and the acceptance of higher costs and longer turnaround-times. An ideal assay for red cell genotyping would be highly accurate, cheaper than conventional serology and at least as fast.

For many years, the perceived slowness of red cell genotyping impeded its wide implementation (Flegel *et al*, 2015a): Most serological antigen determinations are completed within a few minutes to half an hour. These serological results are generally very reliable in donors and patients, if not the recently transfused (Rujirojindakul and Flegel 2014). In contrast, current molecular methods (Wagner 2010) usually take several hours if the time required for DNA isolation and data analysis is properly considered. Often samples have to be transferred to a different laboratory, incurring additional delays until the results become available. In the case of imminent blood need, the results of red cell genotyping are never available when blood units have to be released. Overcoming this gap in turnaround time (Flegel *et al*, 2015a) may enable the wider use of molecular methods in patients who are known to benefit from red cell genotyping.

Currently the fastest available or published turnaround times, such as the conventional capillary cycling approaches (Polin *et al*, 2008) and the Flougene platform (Honger *et al*, 2015), approximate 90 min after DNA isolation. A turnaround time of 1 h is considered acceptable for red cell genotyping in the case of urgent blood need (Flegel *et al*, 2015a).

Possible targets for increasing the turnaround time for PCR assays include the times needed for DNA extraction, PCR thermocycling and post-PCR analysis. We developed a method that eliminated the need for DNA extraction, applied a very rapid thermocycling scheme and enabled PCR product detection in the PCR tubes. Our method combined direct PCR from plasma or serum with melting curve analysis. We issued screening results of red cell genotyping within 40 min of receiving a plasma or serum sample.

Materials and Methods

Blood donors

We collected 2 sets of 100 blood samples at random from routine blood donations in Northern Germany (cohort 1 and 2), collected at different times and representative of Caucasian individuals. Written informed consent for use in research of blood donor samples was obtained from all subjects as part of the routine blood donation procedure.

Blood samples

Whole blood samples were collected, either with EDTA as anticoagulant (plasma for Cohort 1) or without anticoagulation (serum for Cohort 2), and centrifuged at 1140 g for 4 or 5 min to obtain plasma or serum, respectively. No DNA was extracted. Serum was older than 1 day at the time of testing (Lee *et al*, 2001).

DNA amplification with melting point analysis

The assay was completed in one tube. Either plasma or serum was used; we did not make use of any extracted DNA. Our capillary rapid-cycle PCR was an adaption of previously used methods (Polin *et al*, 2008) with the thermocycling conditions pushed to an extreme short time limit. We added 1 µl of serum or plasma to 9 µl of a master mix. The final volume of 10 µl contained 5 µl of PCR mix (Finnzyme Phusion Blood Direct PCR kit; Biozym, Hessisch-Oldendorf, Germany), 4 µl of a solution of primer and probe oligonucleotides (Table I) and 1 µl of sample. Where necessary, we designed longer PCR primers allowing higher annealing temperatures (Table I), thus reducing the time lapse for cooling to reach the required annealing temperature. The total reaction mixture was transferred to a glass capillary (Light cycler capillaries 20 µl; Roche Diagnostics, Mannheim, Germany). The DNA amplification started at 98 °C for 60 s; followed by 50 rounds of thermocycling, each consisting of 98 °C for 0 s (no hold time), 63 °C for 2 s, and 72 °C for 8 s, finishing with 98 °C for 30 s. The temperature controlled by the instrument (LightCycler 1.2; Roche Diagnostics) indicates the actual air temperature around the glass capillaries adjusted to induce the, somewhat lower, actual reaction temperature of the PCR in the capillaries, selected by the operator for thermocycling (Fig. 1).

Melting curve analysis was performed between 45 °C and 80 °C with a temperature increment of 0.1 °C/s. The melting curves were sketched using the instrument's data analysis software (Light cycler software, version 3.5; Roche Diagnostics) and evaluated visually (Fig. 2). Total time of the procedure was clocked from the first pipetting step touching the plasma or serum until the melting curve analysis was interpreted and the results released by the operator.

Serological typing

The Fy^a, Fy^b, Jk^a, and Jk^b antigens were typed by routine serology with licensed polyclonal antisera (Biolith, Hannoversch-Münden, Germany).

Resolution of discordant results

Indeterminate and discrepant results were evaluated by repeat testing, which confirmed the molecular and serological results. The discrepant results in 2 donor samples were resolved by additional molecular testing (RBC-Ready Gene KKD; Inno-train, Kronberg, Germany) by using PCR-single specific primer (SSP) for the *FY* (also termed *ACKR1*, *DARC*) gene including the *FY^x* allele. (Gassner *et al*, 2000) Hence, there was no need to employ alternate sources of antisera to exclude serological variants.

Statistics

We determined the value of the genotyping result to predict the serological typing. The positive predictive value (PPV) was calculated by the sum of the true positive (positive in both serological and molecular methods) divided by the sum of the true positive and false positive cases (positive molecular result but negative serological result); (Gilchrist 2009) the negative predictive value (NPV) was calculated accordingly.

Results

To enable a fast turnaround time, we developed a direct PCR with melting curve analysis in glass capillaries using plasma or serum without DNA isolation.

Rapid-cycle PCR

The thermocycling times of our assay were minimized compared to thermocycling with standard temperature profiles, such that 50 cycles were completed in less than 23 min for 3 samples (Fig. 1, upper panel). Immediately following the thermocycling, the tube was cooled to 45 °C. Then, the temperature was steadily increased by 0.1 °C/s from 45 °C to 80 °C; this melting curve analysis was completed in less than 7 min. The fluorescence detected during the DNA amplification is a function of the cycle number (Fig. 1, lower panel). The whole process of direct PCR with melting curve analysis in glass capillaries was finished within 30 min for 3 samples (Fig. 1).

Melting curve analysis

The absolute fluorescence, detected during the melting process, was used to calculate the fluorescence change (dF/dT) as a function of the temperature (Fig. 2). The melting curve analysis (Fig. 2, upper panels) showed a rapid decrease of fluorescence occurring at 63 °C in

carriers of *FY*B* alleles (69°C for *FY*A*, 63 °C for *JK*B* [also termed *SLC14A1*B*], 66°C for *JK*A*). The position of the peak is consistent among all samples. Even in *FY* heterozygous samples, the peaks for *FY*A* and *FY*B*, being separated by 6 °C, can unambiguously be distinguished (Fig. 2, lower left panel). For *JK* homozygous samples, the peaks for *JK*A* and *JK*B*, being separated by 3 °C only, overlapped considerably (Fig. 2, lower right panel), which caused a broad peak at 64 °C for *JK* heterozygous samples.

Turnaround time

Total time for the analysis was determined from the first pipetting step to the final interpretation of the melting point analysis, when the results were released (Table II). This time depended on the numbers of samples but was less than 45 min for up to 10 samples. The major time block was thermocycling and melting curve analysis, with approximately 9 min represented by hands-on time including administrative work.

Red cell genotyping

We tested 100 plasma samples in a blinded fashion and obtained the serological results only after red cell genotyping had been completed (Table III). For the two major antigens in the *FY* and *JK* blood group systems, the concordance of molecular and serological results was great; in only two samples the presence of one *Fy^a* and one *Jk^a* antigen was missed, respectively. When our direct PCR assay was applied to serum samples from an independent cohort of 100 random donors, the results were comparable (Table IV); the assay was indeterminate or missed the presence of one antigen in only 6 samples.

PPV and NPV

The PPVs were 100% for *Fy^a*, *Fy^b*, *Jk^a* and *Jk^b* antigens in plasma and serum (Table V). With one exception, the NPVs were well above 90%; our rapid-cycle PCR assay seemed to perform better with plasma than serum.

Discussion

We developed an assay for red cell genotyping of the major *FY* and *JK* antigens with a rapid turnaround time. The assay was completed within 40 min from receiving a plasma or serum sample. This screening assay may be useful in clinical settings requiring *FY* and *JK* antigen information, while definitive red cell genotyping is pending. Our assay utilized a DNA polymerase mix that allowed direct amplification from plasma or serum in conjunction with rapid thermocycling and melting curve analysis.

The optimal assays suited for distinct clinical applications may differ, because high accuracy, low cost and fast turnaround times are often mutually exclusive and unlikely to be featured by a single assay. Highly accurate platforms are the best fit for red cell genotyping of patients in elective situations; they can be licensed as tests of record but tend to be elaborate and low throughput (Avent *et al*, 2009, Hashmi *et al*, 2005). For mass scale donor typing, the largest datasets were established using methods optimized for low cost and high throughput screening, correcting for inaccuracies by targeted serological checks (Flegel *et al*, 2015b, Wagner 2010, Perreault *et al*, 2009). For patients with urgent transfusion needs,

we reasoned that the decisive feature was turnaround time (Flegel *et al*, 2015a). The current study demonstrated a turnaround time that compared favourably with that of standard serology, particularly if serological typing is difficult to perform following multiple transfusions.

Conventional PCR assays typically require DNA isolation. The various commercial DNA extractions methods (Tan and Yiap 2009) take between 15 min and 1 h before isolated genomic DNA becomes available. Simplified DNA isolation methods allowed us to save time and cost (Wagner *et al*, 2008). The logical next step was bypassing DNA isolation altogether by using DNA polymerase mixes enabling direct amplification from blood. This approach has been established for some conventional agarose-based PCR (Fuehrer *et al*, 2011, Awad and El-Tarras 2011) and in infectious disease testing (Fuehrer *et al*, 2011, Taylor *et al*, 2011). Direct PCR from unprocessed whole blood had also been combined with a rapid detection method by Manage *et al* (2010) utilizing a special microfluidic device. The discrimination of alleles remained a challenge in the presence of haemoglobin. Hence, we switched to using plasma and serum as the source of DNA.

DNA analysis in the plasma of a pregnant mother is routine for determining the fetal genotype (Doescher *et al*, 2013, Scheffer *et al*, 2011). Even during pregnancy the majority of the DNA in plasma represents the mother's genotype and only up to 10% derives from the fetus (Bauer *et al*, 2006). Hence, plasma should be an acceptable source for patient red cell genotyping. Our assay, with amplicons of less than 170 bp, adjusted well to the highly fragmented DNA found in plasma (Chan *et al*, 2004). Serum may be an even more efficient source of human DNA as the DNA concentration in serum may greatly increase over its concentration in plasma after several days of storage (Lee *et al*, 2001) as nucleated cells release their DNA while disintegrating during clotting and storage. Serum also contains fewer components that could interfere with PCR.

We evaluated both plasma (Table III) and serum (Table IV) as sources for DNA. Our results, however, did not suggest improved accuracy when using serum. In an urgent situation, plasma may be slightly faster to obtain and better defined than serum because the clotting process can intentionally be disregarded. Neither serum nor plasma has been widely applied for red cell genotyping in patients. Although, reliability after massive plasma transfusion, in pregnant patients and in aplastic patients is yet to be established, clinical experiences with red cell genotyping are generally very promising (Rujirojindakul and Flegel 2014). The false-negative results (Tables III and IV) may be explained by the small sample input of 1 μ l plasma or serum, fairly devoid of nucleated cells. The low DNA concentration, representing a few gene copies per tube, may impose a bottleneck, as observed before in nano-volume assays (Flegel *et al*, 2015b). The current NPV (Table V) precluded use of our assay as a test of record. It may be utilized in clinical situations when a result is rapidly needed for a patient. False-negative results have limited impact, because they will lead to an overly conservative transfusion strategy rather than cause harm to any patient.

Our thermocycling instrument was limited to the detection of 2 colours, not allowing us to multiplex more than 2 reactions at this time. For our proof of principle, we chose the 2 main polymorphisms of the FY and JK blood group systems. These antigens are clinically

important and often difficult to determine after recent transfusions (Rujirojindakul and Flegel 2014) or in the presence of a strongly positive direct agglutination test (Reid 2007). Additional alleles, such as *FY*02N.01* (Storry *et al*, 2014) with a *GATA box* mutation (Tournamille *et al*, 1995, Schmid *et al*, 2012) should be added in the future along with other clinically relevant blood group systems. While we do not expect limitations by moving the technique to newer platforms that allow multiplexing of 4 or more reactions in a single capillary or tube, adaption to each new platform has to be carefully evaluated. The first increase in fluorescence occurred at cycles 30 to 32 and all positive samples yielded positive signals at cycle 40 (Fig. 1). Hence, fewer than 50 cycles may suffice, decreasing the thermocycling time to less than 22 min (Table II).

Our assay is a promising novel approach. We adapted an almost 20-year-old technique, hybridization probes in conjunction with rapid-cycle PCR. Elimination of a DNA extraction step further shortened the time to result and also much reduced the hands-on time (Table II). Red cell genotyping was thus accomplished within 40 min, a turnaround time generally accepted in case of urgent blood need (Flegel *et al*, 2015a). In addition, because the assay required only 2 pipetting steps, it was mostly walk-away, and could be developed for automatic visual interpretation, making molecular analysis easier to integrate into the routine serological workflow. Further development may eventually facilitate red cell genotyping with fast turnaround times in routine hospital settings.

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Key point

Direct PCR amplification from plasma or serum allows red cell genotyping within 40 minutes, enabling use of red cell genotyping in urgent situations.

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Fig. 1. Thermocycling profile and DNA amplification

The thermocycling instrument controls the thermocycling temperatures for 50 cycles (temperature profile of the thermocycling instrument, upper panel). Immediately following the thermocycling, the tubes are cooled to 45 °C; to measure the melting pattern, the temperature is steadily increased to 80 °C. The fluorescence detected during the DNA amplification is a function of the cycle number (lower left panel). The fluorescence of the melting pattern (lower right panel) is used for the melting curve analysis, shown separately in greater detail (see Fig. 2). Note: Original figures have been rescaled and axes were relabelled in order to show fluorescence intensity in parallel to temperature.

Fig. 2. Melting curve analysis

The absolute fluorescence in the tubes is measured dependent on the temperature (upper panels). The fluorescence change (dF/dT) is calculated by the instrument as a function of the temperature (lower panels). Results are shown for FY (left panels) and JK (right panels): 3 Fy(a+b-) or 3 Jk(a+b-) samples (green), 3 Fy(a+b+) or 3 Jk(a+b+) samples (blue) and 3 Fy(a-b+) or 3 Jk(a-b+) samples (red). Note: Colours of the original figures were recoded and axes were relabelled in order to group similar results and enhance readability.

Table 1

Primers and probes

Oligonucleotide	Nucleotide sequence *	Length (bp)	Melting temperature (°C)	Final concentration (µM)	Use	Reference
FyF4	AGCTCTCCGCCCTCAACTGAGAAC	23 bp	64.2 °C	0.3 µM	FY amplification (sense) [‡]	this study
FyR2	GCAGAGTCATCCAGCAGGTTA	21 bp	59.8 °C	0.3 µM	FY amplification (antisense) [‡]	(Polin <i>et al.</i> , 2008)
Fyanchor	LC705-CTGGGAAGGAATTCATTCACACCATAGGAAGAA-PH	32 bp	66.9 °C	0.3 µM	FY melting point anchor	(Polin <i>et al.</i> , 2008)
Fysens	TTCCAGGTTGGCACCATAGTCTCCAT-FLU	26 bp	64.8 °C	0.3 µM	FY melting point detector	this study
Jks	ATCCCACCCCTCAGTTTCCT	19 bp	56.7 °C	0.24 µM	JK amplification (sense) [‡]	(Polin <i>et al.</i> , 2008)
JKas2	GCCATGAACATTCCTCCCATTCG	23 bp	62.4 °C	0.66 µM	JK amplification (antisense) [‡]	this study
JKanchor	LC640-ACTCTGGGGTTTCAACAGCTCTCTGGCCT-PH	29 bp	69.5 °C	0.36 µM	JK melting point anchor	(Polin <i>et al.</i> , 2008)
JKsens 1a	TCAGCCCCCAATTGAGGACATCTACTTg-FLU	28 bp	65.1 °C	0.12 µM	JK melting point detector	(Polin <i>et al.</i> , 2008)

* Small letters indicate phosphorothioate nucleotides.

[‡]The amplified products correspond to NG_011626 for FY (157 bp amplicon size; nucleotide positions 5746 to 5902) and to NG_011775 for JK (168 bp; positions 20331 to 20498).

FLU – Fluorescein; LC640 – LightCycler Red 640; LC705 – LightCycler Red 705; PH – phosphate

n.a. – not applicable

Table II

Time schedule

Step	Workload	Time (min)			Hands-on time
		1 sample	10 samples	25 samples	
1	Take PCR mix and controls from freezer and defrost, add 1 µl plasma or serum	2 min	4 min	10 min	Yes
2	Spin down in centrifuge	1 min	1 min	1 min	Yes
3	Load glass capillaries and prepare thermocycling instrument	1 min	1 min	2 min	Yes
4	Thermocycling	22 min	24 min	27 min	No
5	Melting curve analysis	7 min	7 min	8 min	No
6	Visual interpretation	3 min	8 min	12 min	Yes
Total turnaround time		36 min	45 min	60 min	

PCR, polymerase chain reaction.

Table III

Red cell genotyping by direct PCR from plasma in 100 random blood donors (Cohort 1)

Serological phenotype	Red cell genotyping results*			
	positive	negative	positive	negative
Duffy system	FY*A	FY*A	FY*B	FY*B
Fy(a+b-)	21	0	0	21
Fy(a+b+)	45	1 [†]	46	0
Fy(a-b+)	0	33	33	0
Kidd system	JK*A	JK*A	JK*B	JK*B
Jk(a+b-)	20	0	0	20
Jk(a+b+)	53	1 [‡]	54	0
Jk(a-b+)	0	26	26	0

* The shaded cells indicate concordances between serological reactivity and red cell genotyping (predicted phenotype) results. All results outside of the shaded cells represent discordances.

[†] The molecular result was indeterminate. The Fy^a phenotype could not be predicted by the molecular assay, but was noted as Fy^a, a conservative assumption for transfusion purposes.

[‡] A false-negative result for the Jk^a antigen occurred in a sample different from the sample with the Fy^a indeterminate result.

Table IV

Red cell genotyping by direct PCR from serum in 100 random blood donors (Cohort 2)

Serological phenotype	Red cell genotyping results*			
	positive	negative	positive	negative
Duffy system	FY*A	FY*A	FY*B	FY*B
Fy(a+b-)	19	0	0	19
Fy(a+b+)	49	2 [‡]	51 [‡]	0
Fy(a-b+)	0	30	30	0
Kidd system	JK*A	JK*A	JK*B	JK*B
Jk(a+b-)	27	0	0	27
Jk(a+b+)	54	3 [‡]	56	1
Jk(a-b+)	0	16	16	0

* The shaded cells indicate concordances between serological reactivity and red cell genotyping (predicted phenotype) results. All results outside of the shaded cells represent discordances.

[‡] The Fy^b antigen was weakly expressed in 2 samples due to the *FY^X* allele, determined by PCR-SSP. For transfusion purposes, the patients can be considered as Fy^b positive.

[‡] Discrepant results occurred in 6 samples: The red cell genotyping results were indeterminate in 1 Fy^a and 3 Jk^a samples. False negative results occurred in 1 Fy^a and 1 Jk^a sample each.

Table V

Predictive values for the capillary rapid-cycle PCR without DNA extraction

Antigen	Plasma		Serum	
	PPV	NPV	PPV	NPV
Fy ^a	100%	97%	100%	94%
Fy ^b	100%	96%	100%	100%
Jk ^a	100%	100%	100%	84%
Jk ^b	100%	100%	100%	96%

PPV – positive predictive value, NPV – negative predictive value (numbers rounded to integers)