High-throughput Kell, Kidd, and Duffy matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry–based blood group genotyping of 4000 donors shows close to full concordance with serotyping and detects new alleles

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BACKGROUND: After the ABO (*ABO*) and Rh (*RHD* and *RHCE*) blood group systems, Kell (*KEL*), Kidd (*SLC14A1*), and Duffy (*DARC*) represent the second most important clinically relevant antigens.

STUDY DESIGN AND METHODS: Samples from 4000 Swiss blood donors, with serologic prevalues for K/k, $Kp^{a/b}$, $Jk^{a/b}$, and $Fy^{a/b}$, and 48 additional samples of presumptive black African origin were genotyped using high-throughput matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry, applying one single-multiplex polymerase chain reaction/primer-extension reaction simultaneously detecting 15 single-nucleotide polymorphisms.

RESULTS: Genotype/phenotype concordance for K/k, $Kp^{a/b}$, $Jk^{a/b}$, and all $Fy^{a/b}$ specificities were 100, 99.98, 99.93, and 99.20%, respectively. Discrepancies were caused by erroneous serologic profiles (n = 33), mainly attributed to weakly expressed Fy^x (n = 28). Only three discrepancies had a genetic basis. They could all be explained by newly observed silenced alleles: one *KEL*02N.34* and one *FY*02N.03* with predicted R700Q and G261R amino acid exchanges, respectively, and one *JK*B*, with an as-yet-unidentified silencing cause. According to NCBI SNP database entry for rs8176034, another new allele, *KEL*02.38*, had been expected, and we formally demonstrated its presence. We furthermore identified individuals with rare phenotypes, such as $Js^{a/b}$ heterozygotes among Caucasians, rare alleles, the "Swiss" *JK*01N.03*, and rare genotypes, such as one Fy^x homozygote.

CONCLUSION: Genotyping proved its practicability in the daily routine setting and qualitatively outperformed serology. Technology is ideal for time-insensitive donor genotyping and allows for a broad range of throughput needs. Consequently, from a technologic point of view, serotyping should be replaced by genotyping for donors' blood groups encoded by *KEL*, *SLC14A1*, and *DARC*.

In clinical terms, after the ABO (*ABO*) and Rh (*RHD* and *RHCE*) blood group systems Kell (*KEL*), Kidd (*SLC14A1*), and Duffy (*DARC*) can be considered as the next most important blood group antigens, as observed by the frequency of alloantibody occurrence during pregnancy. This is especially true for the K antigen, as maternal anti-K alloimmunization is observed in approximately one-quarter to two-thirds of all non-Rh red blood cell (RBC) antibodies.[1-3]

ABBREVIATIONS: ENA = European Nucleotide Archive; MALDI-TOF MS = matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry; SNP(s) = single-nucleotide polymorphism(s).

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TRANSFUSION **;**:**-**.

However, the frequency and specificity of alloimmunizations through transfusions are different. While the prevalence of RBC alloantibodies in an average population is approximately 1%,[1,3] a 2% to 9% immunization rate of patients can be expected after transfusion of 1 or more units of RBCs.[4,5] Among predominantly male military veterans, RBC alloantibody prevalence was 2.4%, and the 10 most frequent alloantibodies, as a percentage of total male antibodies, were K (21.9%), E (19.4%), D (9.1%), Le^a (7.4%), Fy^a (5.4%), c (4.8%), C (4.6%), P1 (3.9%), Jk^a (3.7%), and Le^b (3.5%).[6]

Therefore, to avoid potentially fatal complications,[7,8] the next logical and effective step seems to be donor typing of the Kell, Kidd, and Duffy blood group antigens and considering appropriate matched transfusions whenever possible. The replacement of conventional serologic methods with genotyping, for pretransfusion blood group testing, has been discussed for several years and is already widely accepted to provide optimally matched donations for patients with existing antibodies or patients with a known predisposition to alloimmunization, such as those with sickle cell disease.[9]

Genotyping for *KEL* (Kell), *SLC14A1* (Kidd), and *DARC* (Duffy) is probably the prime example, since their respective antigens are almost exclusively a result of single-nucleotide polymorphisms (SNPs).[10,11] This is in strong contrast to the RhD and RhCE or MNSs blood group systems, where additional phenotypic variation is caused by hybrid alleles, which are the molecular results of gene conversion events between the two (or three, respectively) highly homologous genes, thereby drastically complicating genetic testing.[12,13]

Current technologic platforms allow for massively parallel, high-throughput, SNP genotyping for far more than a single biallelic or triallelic variation, as for the genes encoding K/k, $Jk^{a/b}$, and $Fy^{a/b}$, with only moderate additional costs.[14-17] Furthermore, other variants of the above mentioned blood groups, such as barely expressed "mod" and "el" alleles, and unexpressed "null" alleles can be interrogated for a precise analysis. This is already exemplified by current commercially available blood group genotyping platforms.[18,19]

The aim of this study was the implementation and validation of a high-throughput blood group genotyping approach, based on matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS). A single multiplex reaction amplified both common and selected rare alleles and was named the *KEL-JK-FY* module.[14] We compared the genotypes of 4000 donors to their serologically determined phenotypes for Kell, Kp, Kidd, and Duffy. We observed an extremely high concordance rate between serology and the MALDI-TOF MS–based blood group genotyping results in all three RBC systems.

MATERIALS AND METHODS

Samples

All original 4080 samples were sequentially taken from repetitive RBC donors below the age of 60 years, with a minimum of four donations in total or three consecutive donations within 1 year and historic phenotype data for K/k, $Kp^{a/b}$, $Jk^{a/b}$, and $Fy^{a/b}$. Donor samples were obtained from the two regional blood transfusion services Basel (total n = 198, dropout n = 13, valid n = 185) and Zurich (total n = 3882, dropout n = 67, valid n = 3815).

According to the ethical commission of Zurich, there was no need for ethical approval of the study. However, all donors explicitly permitted genetic laboratory investigations of their sample material by written consent. For an interlaboratory reproducibility study, two 384-well plates, representing 760 of the 4080 samples from Switzerland, were shared with the laboratory in Hamburg, Germany, in a blinded way.

A panel of 48 DNA samples of presumptive black African ancestry was collected from excess routine diagnostic sample material. Swiss national legislation allows for blood group genotyping for the sole purpose of blood group typing, without prior informed consent. Evidence for ethnicity was only deduced by existing positive genotyping results with for at least one of the following alleles: *RHD*04N.01*, *RHD*01N.06*, *RHD*DAU1-7*, or *FY*02N.01*. Since only partial serology was available for these samples, they were excluded from the serotype versus genotype concordance study.

Serologic blood group typing in routine and handling of discrepant results

Routine K blood group typing was performed on an automated system (Galileo, Immucor Medizinische Diagnostik GmbH, Rödermark, Germany) using human anti-K (Kell) testing (ImmuClone, Immucor Medizinische Diagnostik GmbH). ID-system gelcards for K (Rh-Subgroups+Cw+K); k, $Kp^{a/b}$, and $Jk^{a/b}$ (antigen Profile II); and MN, Ss, and $Fy^{a/b}$ (antigen Profile III, DiaMed-ID Microtyping System, Bio-Rad, Cressier, Switzerland) were used according to the manufacturer's instructions. All serotypes may be considered as "historical," since determined over years. In cases of observed discrepancies between geno- and phenotype, we performed confirmatory retyping on freshly drawn blood as described above.

Serotyping for $Js^{a/b}$ was done retrospectively on genetically identified selected *KEL*02.06*-positive and -negative individuals. Antibodies were of human origin and samples were tested with the tube-agglutination technique. An in-house anti-Js^a was used, and anti-Js^b was provided by Ortwin Walla (Antitoxin GmbH, Sinsheim-Hoffenheim, Germany).

DNA extraction

For manual and automated DNA preparation, magnetic bead technology (Nucleon BACC 3, Gen-Probe Life Sciences Ltd, Manchester, UK; and Chemagen, Perkin Elmer, Baesweiler, Germany, respectively) was used.[14]

SNPs included in the *KEL-JK-FY* module

SNPs for the common alleles of the *KEL*, *SLC14A1*, and *DARC* blood group genes and additional point mutations known to eliminate or reduce allelic expression are described in Table 1.[11,20] Assay design software (TYPER 4.0, Sequenom, Hamburg, Germany) and online assay design tools (MySequenom, Sequenom) were used to select polymerase chain reaction (PCR) primer sets, which uniquely amplified only the genomic region of interest, and to validate the primers within a multiplex for undesired cross-reactions with homologous regions of the genome.

All SNPs were genotyped following the standard Sequenom MassARRAY iPLEX Pro genotyping procedure and as described previously.[14] Data analysis was performed using software (TYPER 4.0, Sequenom). A computer spreadsheet program (Microsoft Excel, Microsoft, Redmond, WA) was used to translate genotyping data into predicted phenotypes.

Before the project samples were typed, all batches of amplification and extension primers were regularly verified and validated using a panel of 180 DNA samples with known serotypes and genotypes for selected specificities. For specificities in case no natural control DNA was available, for example, *KEL*02N.02*, *KEL*02M.05*, and *JK*01N.03*, artificial controls were generated by PCR, using primers containing the respective mutations (data not shown) and used in equimolar concentrations compared to natural DNA.

Confirmatory blood group (geno)typing and sequencing of *SLC14A1* (Kidd) and *DARC* (Duffy) on genomic DNA

Confirmatory blood group genotyping and serotyping was performed for all discrepancies, except for samples that had a *FY*02M* (Fy^x)-positive genotype. Genotyping was performed on a new material, using manual DNA extraction and commercially available genotyping kits (RBC-Ready Gene KKD, RBC-Ready Gene KELplus, and RBC-Ready Gene JKplusFy, Inno-Train GmbH, Kronberg im Taunus, Germany).

Sequencing was done on samples with confirmed discrepancies between phenotype and genotype and for two samples with an indicated new synonymous substitution 1546C>A *KEL*02* allele. For *KEL* sequencing, amplification and sequencing primers were the same as described previously, and for *SLC14A1* and *DARC*, all amplifications were performed with 300 nmol/L primer (Table 2) con-

TABLE 1. Specificities of the *KEL-JK-FY* module*

SNP	Number of alleles	Blood group	Gene	Chromosome	Allele name 1	Allele name 2	Amino acid	Position+	nt 1	nt 2	rs
1	2	K/k	*KEL*	7q34	*KEL*01.1*	*KEL*02 (wt)*	M193T	578	T	C	rs8176058
	1	K_{mod}	*KEL*	7q34	*KEL*02 (wt)*	*KEL*01M.01*	T193R	578	C	G	rs8176058
2	1	Kp^a/Kp^b	*KEL*	7q34	*KEL*02.03*	*KEL*02 (wt)*	W281R	841	T	C	rs8176059
3	1	Js^a/Js^b	*KEL*	7q34	*KEL*02.06*	*KEL*02 (wt)*	P597L	1790	C	T	rs8176038
4	1	K_0	*KEL*	7q34	*KEL*02 (wt)*	*KEL*02N.06*		IVS3+1g>a	G	A	No rs
	1	K_0	*KEL*	7q34	*KEL*02 (wt)*	*KEL*02N.01*		IVS3+1g>c	G	C	No rs
5	1	K_0	*KEL*	7q34	*KEL*02 (wt)*	*KEL*02N.04*	Q348X	1042	C	T	rs61729054
6	1	K_0	*KEL*	7q34	*KEL*02 (wt)*	*KEL*02N.02*	R128X	382	C	T	rs61729053
7	1	K_0	*KEL*	7q34	*KEL*02 (wt)*	*KEL*02N.12*		IVS8+1g>a	G	A	No rs
	1	K_0	*KEL*	7q34	*KEL*02 (wt)*	*KEL*02N.13*		IVS8+1g>t	G	T	No rs
8	1	K_0	*KEL*	7q34	*KEL*02 (wt)*	*KEL*02N.17*	R516X	1546	C	T	rs8176034
	1	Open	*KEL*	7q34	*KEL*02 (wt)*	*KEL*02.38*	R516R	1546	C	A	rs8176034
9	1	K_{mod}	*KEL*	7q34	*KEL*02 (wt)*	*KEL*02M.05*	G573G	1719	C	T	No rs
10	2	Jk^a/Jk^b	*SLC14A1*	18q11-q12	*JK*01, or JK*A*	*JK*02, or JK*B*	D280N	838	G	A	rs1058396
11	1	Jk null	*SLC14A1*	18q11-q12	*JK*wt*	*JK*02N.01*		IVS5-1g>a	G	A	rs78937798
		Jk null	*SLC14A1*	18q11-q12	*JK*wt*	*JK*01N.06*		IVS5-1g>a	G	A	rs78937798
	1	Jk null	*SLC14A1*	18q11-q12	*JK*wt*	*JK*02N.02*	Y194X	582	C	G	rs34756616
12	1	Jk null	*SLC14A1*	18q11-q12	*JK*wt*	*JK*01N.03*		IVS5-1g>c	G	A	rs12075
13	2	Fy^a/Fy^b	*DARC*	1q23.2	*FY*01, or FY*A*	*FY*02, or FY*B*	G42D	125	G	A	
14	1	Fy null erythroid	*DARC*	1q23.2	*FY*wt*	*FY*02N.01*		P-67T>c	T	C	No rs
15	1	Fy^b/Fy^x	*DARC*	1q23.2	*FY*wt*	*FY*02M*	R89C	265	C	T	No rs
15	23	Total									

* wt = wild-type allele; nt = nucleotide; rs = reference SNP(ID number); P (in P-67T>c) = promoter; IVS = intervening sequence (introns); null or N = unexpressed alleles; mod or M = modified alleles. +coding relative to ATG or respective IVS.

TABLE 2. Oligonucleotides for amplification and sequencing *SLC14A1* (Kidd) and *DARC* (Duffy)

SLC14A1 exons	Amplification primer name	Sequence 5′>3′	Length (bp)	Sequencing primer name	Sequence 5′>3′
1, 2	Jk–514–F	GCAGCAGAGTATGTCCAAGAATTCTCAC	2359	Jk–296–F	CTTAGCTTCCGAGTCCAC
	Jk–i2+910–R	CTGGCACCGGCCTTTCCTCTAC		Jk–38–R	CTCCCACTTCAGGCATC
				Jk–i1–278–F	CGAGGCGAGTGTCTACAAG
				Jk–i2+351–F	CTCTTCAGGTGCAGCTTCCAG
3	Jk–i2–211–F	CTTGTGAATGCTTACAAAGCGTGGC	1060	Jk–i2–164–F	CCACATCATGCATCCTTCTTC
	Jk–i3+337–R	GCAGCCAGGACCTTCTGCTAATG		Jk–i3+151–R	GCCTCTAAACCAAGGTGAGC
4, 5	Jk–i3–436–F	CAAGTAGCTGATCAGCCTGCATTGG	1603	Jk–i3–174–F	GACACTGATGGCAAAGGTACTG
	Jk–i5+212–R	GTTAGCACGTAATGCCACAAGAATCTG		Jk–i5+148–R	GAGACTGTAACTGATTCCCTGAC
6	Jk–i5–388–F	GCATTAGCTTCTCATAAGAGTGCGAAC	1159	Jk–i5–366–F	CCATTATGAACTGGGCATGTG
	Jk–i6+594–R	CCATGGAAGTGTCAATGTGCAGC		Jk–i6+563–R	GAGAGGTATTAGTGGATGTG
7	Jk–i6–338–F	GGAGGGACATAAGAGCAAGTGGAGG	819	Jk–i6–226–F	CTGAGCAACCCCAGAGTAG
	Jk–i7+240–R	CAGTGGAACTGTACTCTGGGGTTAG			
8, 9	Jk–i7–289–F	GGCACCAAGCTGGGAGCTTAAC	1033	Jk–i7–191–F	GAAGGGCTGGTGTATCTCTG
	Jk–i9+198–R	CTCAATAGGCTCCTGCCTTCACAATC			
10, 11	Jk–i9–159–F	CAGAGCCCATGGAGCTCCTAAGTG	1959	Jk–i9–139–F	GGAGCCAGGATTTGAACC
	Jk–i11+176–R	CACCTGGATGTGACTCTCATATGCTC		Jk–i11+106–R	GATTACAGACGCGTCAGTATG
DARC	Amplification			Sequencing	
1, 2	Fy–363–F	GTGCTTGAAGAATCTCTCCTTGCTGG	2117	Fy–214–F	CTGAGTGTAGTCCCAACCAGC
	Fy+217–R	CTTCTCCCTTGAGATGGCCGTG		Fy+194–R	CCACACCTCAAGAGAACATC
				Fy–i1–126–F	CACTGTCCGCACTGCATCTG

centration, under cycling and sequencing conditions, exactly as previously described for *RHD* sequencing.[20,21] Complete coding plus exon-flanking sequences of the new alleles were submitted to the European Nucleotide Archive (ENA).

Allele frequency calculation according to Hardy-Weinberg

In contrast to allele frequency determination by counting of specifically detected alleles (Table 3), Hardy-Weinberg proportions were used for frequency estimates of the newly discovered alleles.[22] For example, the correct frequency for *KEL*02N.34*: $2*FRQ_{KEL*02}*FRQ_{KEL*02N.34} = 1/4000$. Since $FRQ_{KEL*02} = 0.95763$, $FRQ_{KEL*02N.34} = 0.00013$.

Software for prediction of transmembrane domains and posttranslational modifications

The presence of transmembrane (TM) domains was predicted using five different software analysis tools, HMM TOP, Phobius, SOSUI, TMPred, and TopPred.[23-27] Prediction of palmitoylation, N-gylcosylation, O-gylcosylation, and disulfide bond connectivity prediction was done using CSS-Palm, NetNGlyc, NetOGlyc, and DiANNA, respectively.[28-31]

RESULTS

The *KEL-JK-FY* module: a single multiplex comprising 15 SNPs

The blood group typing *KEL-JK-FY* module is a single multiplex reaction, comprising a total of 15 biallelic or triallelic SNP assays for the simultaneous analysis of the antigens K, k, Kp^a, Kp^b, Js^a, Js^b, Jk^a, Jk^b, Fy^a, and Fy^b and additionally enables identification of seven different K_0 alleles, *KEL*01M.01* and *KEL*02 M.05*, four different Jk null alleles, *FY*02M* (Fy^x), and *FY*02N.01* and *FY*01N.01* (both with the promoter mutation –67t>c; Table 1).[11,20]

High-throughput MALDI-TOF MS genotyping of the originally 4080 Swiss donors, all with serologic prevalues for K/k, $Kp^{a/b}$, $Jk^{a/b}$, and $Fy^{a/b}$, plus 48 presumptive black African individuals, was performed in six independent typing runs in 6 days, excluding DNA extraction and analysis of discrepancies between genotypes and serotypes.

Calling failures were caused by samples with either negative results for all SNPs (total DNA dropouts n = 67), or for only single SNP assay failures in a sample (n = 13), and were all excluded from the final 4000 donor data set further presented here. Total calling failure rate was 1.94% (80 of 4080 plus 48 samples). Excluding one particular run with technical issues affecting 53 samples, the mean call failure rate would have resulted in only 0.65% (27 of 4080 plus 48 samples).

TABLE 3. Allele frequencies for all alleles observed among 4000 Swiss blood donors and 48 individuals with presumptive African ancestry

Causing phenotype	Allele	Number of alleles in homozygous individuals	Number of alleles in heterozygous individuals	Allele % Zurich (n total = 8000)	Number of alleles in homozygous individuals	Number of alleles in heterozygous individuals	Allele % black African (n = 48)
K	*KEL*01.1*	18	196	0.02675	0	2	0.02083
k	*KEL*02*	7350	311	0.95763	76	9	0.88542
Kp^a	*KEL*02.03*	2	106	0.01350	0	0	0.0
Js^a	*KEL*02.06*	0	9	0.00113	2	6	0.08333
K_{mod}	*KEL*02M.05*	0	2	0.00025	0	0	0.0
k (assumed)	*KEL*02.38*	0	5	0.00063	0	1	0.01042
K_0	*KEL*02N.34*	0	1	0.00013*	0	0	0.0
Sum		7370	630	1.00001	78	18	1.00000
Jk^a	*JK*01, or JK*A*	2102	2003	0.51313	38	19	0.59375
Jk^b	*JK*02, or JK*B*	1892	2000	0.48650	20	19	0.40625
Jk null	*JK*01N.03*	0	2	0.00025	0	0	0.0
Jk null	*JK*B* null, cause unclear	0	1	0.00024*	0	0	0.0
Sum		3994	4006	1.00012	58	38	1.00000
Fy^a	*FY*01, or FY*A*	1406	1914	0.41500	2	8	0.10417
Fy^b	*FY*02, or FY*B*	2572	1931	0.56288	0	12	0.12500
Fy null	*FY*02N*	4	47	0.00638	58	14	0.75000
Fy^x	*FY*02M*	2	123	0.01563	0	2	0.02083
Fy null	*FY*02N.03*	0	1	0.00030*	0	0	0.0
Sum		3984	4016	1.00018	60	36	1.00000

* Allele frequency calculated from heterozygous individuals according to Hardy-Weinberg.[22]

Performance comparison in two independent laboratories in Hamburg and Zurich

A random set of 760 samples was chosen to assess the reproducibility of this MALDI-TOF MS–based blood group genotyping on two independent Sequenom mass spectrometers at the collaborating centers. All process steps, including oligonucleotide order and generation of primer mixes, were performed at both centers autonomously and independently.

Assay performance comparison resulted in total raw data call rates of 98.9 and 98.6%, in Hamburg and Zurich, respectively. Each 384-well plate included four nontemplated control samples, which were all negative except for a single false-positive nonsense artifact call out of 136 possible calls in Zurich. After automatic calling and manual postprocessing analysis, we obtained final call rates of 99.0 and 98.5% in Hamburg and Zurich, respectively. Comparison of the final results showed 100% genotyping concordance between the sites.

KEL (Kell) genotyping, $Js^{a/b}$ heterozygous individuals among Caucasians, and a new silent *KEL*02.03* (Kp^a) allele

All genotype and phenotype results with respect to the *KEL* (Kell) antigens K, k, Kp^a, and Kp^b were fully concordant for all 4000 samples investigated, except a single discrepancy, resulting in an overall concordance rate of 99.98% (Fig. 1, Table 3). The discrepancy was found in an individual sample that had a genotype of *KEL*02/02.03*, which was indicative of a $Kp^{a/b}$ predicted phenotype, but with negative standard and adsorption-elution serology for Kp^a. Subsequent sequencing of all exons, including at least 50-bp intron flanking regions, revealed a new 2099G>A mutation resulting in an Arg700Gln amino acid exchange. This is the first report of a silenced *KEL*02.03* (Kp^a) allele. According to the ISBT terminology committee, the allele was named *KEL*02N.34* and deposited at the ENA (Accession HG512886).

As expected among the Swiss study cohort, *KEL*02.06* positives were only observed very rarely and only as $Js^{a/b}$ heterozygous individuals (allele frequency *KEL*02.06* = total 0.00113; Table 3). Retrospective serotyping for $Js^{a/b}$ of genotypically identified samples showed full concordance between the two methods but, due to the limited availability of anti-Js^a antibody, only 10 *KEL*02.06*-negative ($Js^{b/b}$ homozygous) and 11 *KEL*02/02.06* ($Js^{a/b}$)-heterozygous samples were analyzed. In addition, two samples that carried *KEL*02M.05*, but no other *KEL*02N* alleles were detected (Table 3).

SLC14A1 (Kidd) genotyping, a presumptive null allele and confirmation of the "Swiss" *JK*01N.03* allele in the Zurich area

The 4000 samples investigated for *SLC14A1* (Kidd) showed 99.93% concordance between genotyping and serology

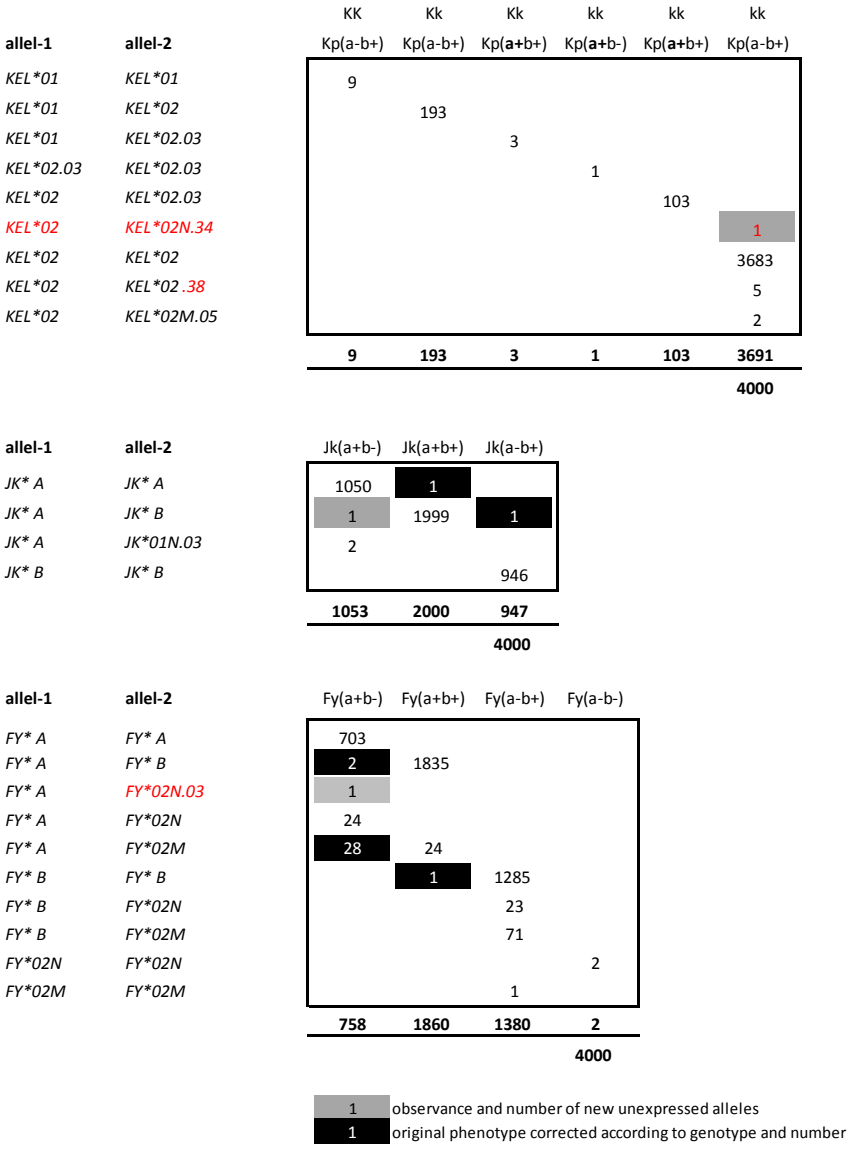

Fig. 1. Panels Kell, Kidd, and Duffy. Comparison of observed genotypes and phenotypes among 4000 Swiss donors.

with three discrepancies (Fig. 1, Table 3). Of these, two serologic results were found to be incorrect, the third sample was found to carry a silenced *JK*B* allele in a genetically *JK*A/B* heterozygous individual. This was subsequently demonstrated by consequent negative standard and adsorption-elution serology for Jk^b. However, we were unable to identify a causative mutation in this sample. In detail, we detected no genetic variation from the *JK*B* wild-type consensus sequence in the exons, at least 50 bp of intron sequence flanking the respective exons and 514 bp upstream of Exon 1 (Table 2). A large deletion within the genomic region of the presumptive new silenced *JK*B* allele can be excluded, since every genomic *JK* region was amplified separately, and showed at least one heterozygous position, indicative of the presence of both parental alleles.

A presumably region-specific observation was made in two Jk(a+b–) individuals, identifying a 582C>G SNP, coding for *JK*01N.03*, an allele originally observed in Switzerland for the first time in 2002.[32]

DARC (Duffy) genotyping, a new null allele, and *FY*02M* (Fy^x) homo- and heterozygous individuals

With respect to *DARC* (Duffy), we observed a concordance of 99.20% between genotyping and serotyping and, as reported previously, 28 of a total of 52 (54%) *FY*A/02M* (*FY**A/X) heterozygous individuals had been serologically mistyped as Fy(a+b–) (Fig. 1, Table 3).[33] Three other discrepancies were found to be due to different errors in Fy serology, while one represented a sample with *FY*A/B* heterozygous genotype, but showed negative standard and adsorption-elution serology for Fy^b, suggestive of an unspecified silenced *FY*B* allele. The sample showed a 781G>A mutation, resulting in a predicted Gly261Arg amino acid exchange in the primary structure of the protein and was named *FY*02N.03* according to the ISBT terminology committee and deposited at ENA (Accession HG512885).

Among all 4000 donors investigated, allele frequencies specific for *FY*02M (Fy^x)* and *FY*02N.01* (and potentially for the same mutation in *FY*01N.01*, although unexpected in Switzerland) were 0.0156 and 0.0064 (Table 3), respectively.[34] A remarkably rare observation was the identification of a *FY*02M* (Fy^x)-homozygous individual, with phenotype records for Fy(a–b+).

Genotyping 48 samples with presumptive African ancestry and the first observation of a hypothetically expected *KEL*02(R516R)*

Genotyping the 48 samples of individuals with presumptive black African ancestry delivered data as expected for this ethnicity (Tables 3 and 4). In addition, detection for *KEL*02N.17* at SNP rs8176034 was found to be specific for a third allele, mentioned in the NCBI SNP database.[35] The presence of A at this position was reported to have a minor allele frequency of 0.042 in the PGA-African-Panel, but approximated zero in other populations, encoding an

TABLE 4. Observed *KEL* (Kell), *SCLC14A1* (Kidd), and *DARC* (Duffy) genotypes among 48 individuals with presumptive black African ancestry

KEL genotypes	Number	*SLC14A1* genotypes	Number	*DARC* genotypes	Number
*KEL*01/02*	2	*JK*A/A*	19	*FY*A/A*	1
*KEL*02/02*	38	*JK*A/B*	19	*FY*A/B*	3
*KEL*02/02.06*	6	*JK*B/B*	10	*FY*A/02N*	5
*KEL*02.06/02.06*	1			*FY*B/02N*	8
*KEL*02/02.38*	1			*FY*B/02M*	1
				*FY*02N/02N*	29
				*FY*02N/02M*	1
Total genotypes	48		48		48

until now unreported and, therefore hypothetical 1546C>A variant of *KEL*02*, not affecting codon for Amino Acid R516.[35] As expected, one sample from the African panel, and surprisingly, also five samples from the 4000 Swiss donors were heterozygous for this allele (Tables 3 and 4). Sequencing of two cases showed identical sequences and an additional silent mutation in Exon 8, 846G>C. According to the ISBT terminology committee, the allele was named *KEL*02.38* and deposited at ENA (Accession HG512887).

Prediction of TM domains and posttranslational modification of the two new alleles *KEL*02.03* and *FY*B* with amino acid exchanges R700G and G261R, respectively

In an attempt to explain the lack of antigen presence, TM domain and posttranslational modification prediction software analysis tools were used. For *KEL* we compared *KEL*01*, *KEL*02*, *KEL*02.03*, *KEL*02.06*, *KEL*02M.02*, and *KEL*02M.04* and the new *KEL*02.03* with R700G, and for *DARC*, we compared *FY*A*, *FY*B*, and *FY*B* with amino acid exchange A100T, *FY*02M*, and the new *FY*B* with a predicted amino acid exchange G261R. For the new alleles, the software predicted no differences in palmitoylation and N- or O-glycosylation to the standard alleles. However, with respect to TM domains, *FY*B* with amino acid exchange G261R showed shifting of TM domain six by up to seven amino acids upstream (all five predictions) and up to three amino acids downstream for TM7 (two of five predictions) or even the loss of a TM domain (one of five predictions), whereas TM prediction for *KEL*02.03* with R700G remained unchanged (data not shown). Despite the predicted TM differences observed for *FY*B* with amino acid exchange G261R, all predictions were in line with previous observations.[11] Extracellular disulfide bond predictions for *DARC* were incongruent to its accepted TM model and therefore not considered for further analysis.[11] With respect to *KEL*, seven disulfide bonds were predicted for all alleles mentioned above and as commonly accepted and therefore failed to characterize the new variant *KEL*02.03* with R700G as unique.[11]

DISCUSSION

Applying the MALDI-TOF MS–based genotyping method on 4000 blood donor samples, DNA-typing accuracy was 100% for K/k and 99.988% (1 "error" in 8000 alleles investigated each) for all three blood group systems, Kp, Jk, and Fy, respectively. Full fidelity for all blood group genotypes was not achieved due to the presence of three new alleles: new *KEL*02N.34* and *FY*02N.03* with predicted R700Q and G261R amino acid exchanges, respectively, and one *JK*B*, with an as-yet-unidentified silencing mutation. All alleles were in fact correctly recognized as *KEL*02.03*, *JK*B*, and *FY*B*, respectively, but may be translated into the wrong phenotypes if unrecognized as specific null alleles. Fidelity of the presented genotyping approach is highly remarkable, since there is no piece of evidence for one single erroneous genotype call out of the 60,000 analyzed in total (15 SNPs of 4000 samples).

In blood group genetics, null alleles are an ever-present and prevailing topic.[36-38] In genotyping blood groups, there are different requirements for analytic accuracy between donor- and recipient-specific genotyping with respect to the necessary resolution level.[14] For instance, in donor typing the risk of unidentified null alleles is negligible, since such heterozygous "pretender results" would phenotypically behave as homozygous and are therefore unlikely to harm the recipient, if being transfused according to the recipients' heterozygous phenotype. However, due to the high-resolution power of genotyping by MALDI-TOF MS including assays for null alleles, for example, a total of seven different K_0 alleles, *KEL*01M.01* and *KEL*02M.05*, four different Jk null alleles, *FY*02M* (Fy^x), and *FY*02N.01* and *FY*01N.01* (both with the promoter mutation –67t>c; Table 1), and other variant alleles in the blood group systems investigated, genotype misinterpretation can be strongly reduced if not fully avoided. In addition, donor demographics such as zip code could be used to maximize the search for antigen-negative donors, once heterozygous individuals were identified.[17]

However, against the theoretical expectation of 8.4 individuals, as calculated using frequency data from

neighboring Austria, no haploid carriers of a K_0 allele, and only two of the K_{mod} type (*KEL*02M.05*) were identified among all 4000 Swiss donors investigated.[20] Still, shortly after end of sample entry into this study, a *KEL*02N.13* sample, with an IVS8+11g>t splice site mutation, was observed in a consecutive *KEL-JK-FY* typing run in the ongoing project.

Due to limitations in the amount of the anti-Js^a, validation of genotyping results specific for $Js^{a/b}$ was only done on a total of 11 (five samples from this report and six from a parallel ongoing project, respectively) genetically heterozygous *KEL*02/02.06* ($Js^{a/b}$), and 10 *KEL*02.06* negative blood donor samples, resulting in full concordance. Since the statistical chance of correctly picking 11 $Js^{a/b}$ heterozygous individuals from the investigated Caucasians is as low as 7.75×10^{-30}, correct genotyping for Js^a may be implied, although only validated in a few samples. As expected, allele frequency of *KEL*02.06* was much higher among the 48 individual with presumed African ancestry, compared to Caucasians (Table 3).[39,40]

The null allele typing of *SLC14A1* (Kidd) resulted in the detection of two individuals with a haploid presence of *JK*01N.03*. Respective detection did not change phenotype interpretation since we observed cosegregation with a *JK*A* allele in both samples. In contrast, in *JK*B/01N.03* heterozygous cases, only the specific identification of *JK*01N.03* would have resulted in a correct Jk^b-"homozygous" phenotype prediction. A presumably new silenced *JK*B* allele was observed in this study; however, the causative genetic background is still unknown. This has also been observed for other genes with strong evidence for their inactivation, concomitant with an apparent lack of causative mutation(s) as reported very recently in individuals with a haploid presence of the rare *KEL* alleles specific for KUCI and KETI.[41] However, in the case reported here, the presence of both parental alleles during the whole analytical process excludes large gene deletions as causative mutational events, since they would result in failure of amplification and could not be detected.

In addition to the new unexpressed *FY*B* reported here, only five other silent *FY*01* or *FY*02* alleles, plus the prevalent promoter mutant *FY*02N.01* (and *FY*01N.01*), have been reported so far.[42,43] This low prevalence of alleles causative of Fy-null phenotypes may be due to the relatively small size of the *DARC* gene with respect to its coding length and the presence of only two exons.[44]

With respect to the observed discrepancies between genotypes and phenotypes, serology was found to be much more erroneous for *DARC* (Duffy) typing. In most cases this was due to the inability of serology to identify Fy(a+x+) phenotypes. This weakly expressed Fy^b (also known as Fy^x) antigen was correctly identified in only 46% (e.g., 24 of 52). In contrast, genotyping identified all samples heterozygous for *FY*A/02M*, which is in line with previous reports.[33,45] In addition, two false Jk and three false Fy serologic determinations were encountered in the 4000 serologic records. This showed that genotyping is an ideal tool for quality control of serotyping results. The power of genotyping in defining reference RBCs used for antibody identification in patients sera has been addressed accordingly.[46]

Results of the interlaboratory reproducibility study highlight the stability and robustness of both the assay biochemistry and the MassARRAY MALDI-TOF MS technology. A very small difference was observed in the quality of spectra and genotyping calls as well as to the number of call failures between the two collaborating centers, which resulted in a marginal call rate difference of 0.5% (99.0% vs. 98.5%). Given that apart from DNA preparation, all laboratory processes and procedures were performed independently, including pipetting, setup and adjustment of primer mixes, and nanodispensing of analytes, and given all the different robots, thermocyclers, nanodispenser, and mass spectrometer that were used, these results emphasize the high robustness and reproducibility of blood group genotyping by means of MALDI-TOF MS.

The assay module investigated includes 15 blood group–specific SNP assays (specificities see Table 1), multiplexed into a single PCR well. One technician is able to process 760 DNA samples with 2.5 hours of hands-on time (not including DNA extraction and data processing). Data are available 8 hours after amplification setup was started. The here presented high-throughput technology is ideal for time-insensitive donor genotyping and allows for a broad range of throughput needs, for example, from genotyping a few 1000 up to several 10,000 donors per year.

With respect to cost, the technology requires special and quite expensive pieces of post-PCR equipment. However, beside these one-time costs for the required instruments, the MALDI-TOF method is far more cost-effective to run than other high-throughput genotyping methods, and costs are comparable to, or even lower than, conventional serology. Nevertheless, a full cost-effectiveness study, comparing MALDI-TOF with several other platforms and also with serologic testing, was not part of this study.

OUTLOOK AND CONCLUSION

The *KEL-JK-FY* module described here is part of a larger project including a planned total of 117,000 single-multiplex reactions including 1) specificities for *GYPA* (MN and others) and *GYPB* (Ss and others), 2) extensive typing for *RHD* and low resolution for *RHCE*, 3) one multiplex specific for HPA and certain HNA alleles, and 4) two multiplexes for typing high-frequency antigens and their rare counterparts on a total of 36,000 DNA samples.[14] Specificity, capability (throughput), quality, and costs of the here described module are highly competitive in

comparison to other published high-throughput methods.[47-49] Consequently, from a technologic point of view, serotyping should be replaced by genotyping for donors' blood groups encoded by *KEL*, *SLC14A1*, and *DARC*.

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CONFLICT OF INTEREST

SM, NT, CB, JG, and BMF are employees of the Blood Transfusion Service Zurich, SRC, Switzerland, and have disclosed no conflicts of interest. AB is employee of the Blood Transfusion Center, Basel, and has disclosed no conflicts of interest. CG is an employee of the Blood Transfusion Service Zurich, SRC, Switzerland, and acts as a consultant for Inno-Train GmbH, Kronberg im Taunus, Germany. CV is employed at Sequenom GmbH, Hamburg, Germany.

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