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Clinical Potential of Effective Noninvasive Exclusion of *KEL1*-Positive Fetuses in *KEL1*-Negative Pregnant Women

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ies that penetrate through the placenta during incompatible pregnancy cause suppression of erythropoiesis. The 'K' antigen is expressed by erythroid precursor cells, in contrast to the Rh proteins that are expressed on the surface of mature erythrocytes. In this case, the level of maternal anti-K antibodies is not a good indicator of hemolytic disease of the fetus and newborn [2, 3]. Noninvasive assessment of the fetal *KEL* genotype is not yet available in the Czech Republic.

The *KEL* gene has two major co-dominant alleles: *KEL1* and *KEL2*. The difference in the gene sequence between the *KEL1* and *KEL2* alleles is caused by a single nucleotide substitution in exon 6 at position 578 (c.578C>T, rs8176058); the presence of T characterizes the *KEL1* allele and the presence of C characterizes the *KEL2* allele [4, 5]. The single nucleotide change 578C>T results in the substitution of threonine ('k') to methionine ('K') at position 193 (p.Thr193Met) [6, 7]. For the detection of particular alleles, allele-specific polymerase chain reaction (AS-PCR) or PCR with subsequent cleavage by restriction enzyme *Bsm*I (PCR-RFLP) may be used [5, 8–11]. It is also possible to use more sensitive and faster detection methods, such as allelic discrimination using TaqMan PCR, minisequencing using the SNaPshot system (Applied Biosystems, USA) or a DNA microarray platform [7, 10]. Briefly, minisequencing is composed of PCR amplification for the formation of PCR products containing the target SNP and a single base extension (SBE) of the oligonucleotide primer using a fluorescently labeled dideoxynucleotide base at the 3′ end of each primer located proximal to the target SNP (minisequencing PCR). The length and type of fluorescence dye of the single base extended primer (for a particular SNP) is detected by capillary electrophoresis. For noninvasive prenatal *KEL1/KEL2* genotyping, SABER-based MALDI-TOF

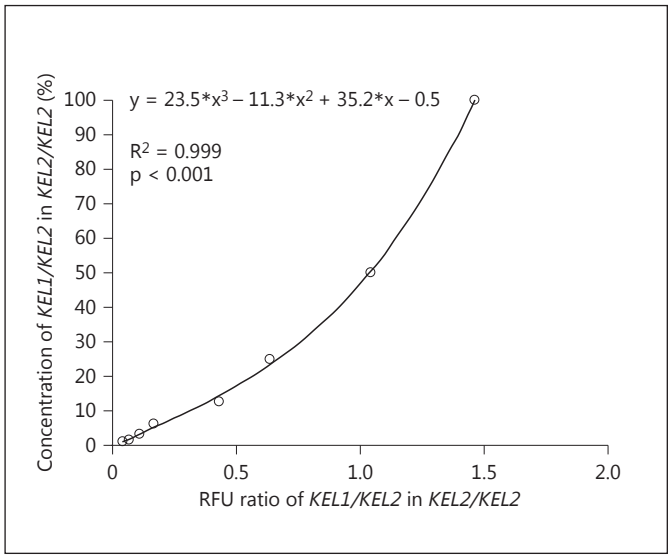


Fig. 1. The calibration curve of 0.78–100% artificial plasma cf DNA mixtures of heterozygous *KEL1/KEL2* in a *KEL2/KEL2* homozygote sample. The x axis shows the RFU ratio of *KEL1/KEL2* in *KEL2/KEL2* measured by peak height. The y axis shows the percentage of the *KEL1/KEL2* DNA concentration in *KEL2/KEL2* samples.

er (10 pmol/l) (Sigma-Aldrich, USA) and 11.5 μl of plasma cff DNA samples or plasma cf DNA samples from the dilution series. PCR amplification with DNA samples isolated from control buccal swabs of newborns was performed in a final volume of 12.5 μl. The PCR premix for the buccal swab DNA contained 6.25 μl of Combi PPP Master Mix, 5.25 μl of PCR water (Top-Bio, USA), 0.25 μl of forward primer (10 pmol/l) (Sigma-Aldrich, USA), 0.25 μl of reverse primer (10 pmol/l) (Sigma-Aldrich, USA) and 1 μl of DNA. PCR for all samples was performed in a Thermocycler C1000 (Bio-Rad, USA) under the following conditions: 95°C for 10 min (95°C for 30 s, 59.5°C for 1 min, 72°C for 1 min) 35 times, and 72°C for 10 min. Following purification of the PCR products, enzymatic purification using exonuclease (Exonuclease I; Thermo Scientific, USA) and alkaline phosphatase (FastAP Thermosensitive Alkaline Phosphatase; Thermo Scientific, USA) was performed according to the manufacturer's instructions. A minisequencing extension reaction was performed using the SBE primer: 24T-TGG TAA ATG GAC TTC CTT AAA CTT TAA CCG AA [10]. Amplification was performed using the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems, USA) in a final volume of 8 μl. The plasma sample amplification mix contained 4 μl of SNaPshot Multiplex Ready Reaction Mix, 1 μl of SBE primer (10 pmol/l) (Sigma-Aldrich, USA) and 3.5 μl of purified PCR product from plasma cff DNA samples or plasma cf DNA samples from the dilution series. The SNaPshot premix for buccal swab DNA contained 4 μl of SNaPshot Multiplex Ready Reaction Mix, 0.5 μl of SBE primer (10 pmol/l) (Sigma-Aldrich, USA), 1.5 μl of PCR water (Top-Bio, USA) and 2 μl of purified PCR product from buccal swabs. Purification of the products used for minisequencing was performed with an alkaline phosphatase enzyme (FastAP Thermosensitive Alkaline Phosphatase) according to the ABI PRISM SNaPshot Multiplex Kit protocol. Fluorescence intensity and size of the minisequencing products were determined by capillary electrophoresis on a Genetic Analyzer ABI PRISM 3130 (Applied Biosystems, USA) using polymer NanoPOP-7 (MCLAB, USA) and a capillary 36 cm in length. One microliter of purified minisequencing product was mixed with 8.5 μl of Hi-Di formamide (Applied Biosystems, USA) and 0.5 μl of GeneScan-120 LIZ Size Standard (Applied Biosystems, USA). Each sample of plasma cff DNA was assessed in two capillary electrophoresis conditions. The first injection was for 5 s at 1.2 kV with electrophoresis for 27 min at 15 kV, and the second injection was for 16 s at 1.2 kV with electrophoresis for 27 min at 15 kV. The capillary conditions for the buccal swab samples were injection for 5 s at 1.2 kV with electrophoresis for 27 min at 15 kV. The data were analyzed by GeneMapper v 4.1. Quantification was assessed using the *KEL1* peak relative fluorescence unit (RFU) and the *KEL1/KEL2* peak RFU ratio.

For statistical analysis, calibration curves were computed using a nonlinear regression polynomial model of the third order with the best fit. The discrimination ability of signals for *KEL1* was analyzed using receiver operating characteristic (ROC) curves and described by the area under curve with corresponding 95% confidence intervals and statistical significance. Cut-off values were derived according to the sum of the sensitivity and specificity values. Statistical analysis was performed using SPSS 22 (IBM Corporation, 2013).

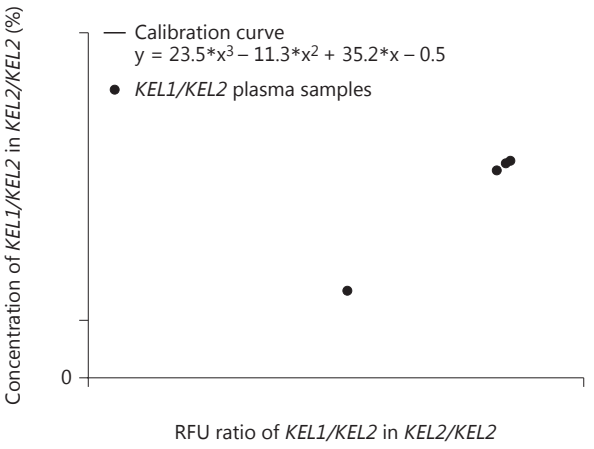
Results

The minisequencing calibration analyses showed only C/T nucleotide-specific peaks that were visible at concentrations of at least 0.78% (equivalent to 0.039 ng/μl) of *KEL1/KEL2* in the *KEL2/KEL2* admixture. The ratio of the RFU *(KEL1/KEL2)* values was 0.041 in 0.78% of the admixture (fig. 1).

The analysis repeatedly failed in 3/138 plasma samples. The signal intensity of PCR products (both maternal and fetal) was undetectable. The failure was probably caused by very low concentration of DNA in plasma due to DNA degradation during sample handling (transport, plasma acquisition, DNA isolation, etc.). Seven of 135 analyzable plasma samples could not be genotyped due to maternal heterozygosity. The heterozygosity *KEL1/KEL2* is very clearly visible and detectable from maternal plasma as the maternal fluorescent signal from *KEL1* and *KEL2* alleles is quantitatively comparable (see online supplement 1; for all online suppl. material, see www.karger.com/doi/10.1159/000441296). The genotype frequencies were 94.8% for *KEL2/KEL2* and 5.2% for *KEL1/KEL2*. The *KEL1* RFU measurements were performed in 128 plasma *KEL2/KEL2* samples, of which four were truly *KEL1*-positive (fig. 2, online supplement 2). Plasma cff DNA concentration was evaluated from the calibration curve (fig. 1) with the assumption that PCR efficiency and

RFU ratio of artificial mixtures in plasma cf DNA are similar to the admixture of plasma cff DNA in maternal plasma cf DNA.

The results were confirmed with newborn DNA (table 1). About one order weaker false-positive *KEL1* RFU signals were observed in 22 samples in one or two of four measurements, corresponding to approximately 0.01–0.02% of *KEL1* signals (table 1). This very low signal (RFU about 20) could be caused by the very sensitive fluorescent baseline. The four true *KEL1* signals (RFU from 200 to 500) were detected in all four measurements for each sample (0.4–0.9%) and postnatally confirmed (*KEL1/KEL2* samples). Gestational week of the lower signal-positive sample (0.4%) was 10, the remaining three samples (about 0.9%) were gestational weeks 12, 12 and 13. Using aggregated measurements the tested sensitivity and specificity reached 100% (table 2, fig. 3). No false *KEL2/KEL2* sample was detected.



Discussion

The frequency of the *KEL1* allele is population-specific and ranges from 2.2 to 4.6% in European populations [15–17]. The *KEL1* allelic frequency in our sample collection of pregnant women was close to the lower border of this range (2.6%) and corresponds with data from National Center for Biotechnology Information [17]. The *KEL1/KEL2* heterozygous father genotype demonstrates a theoretical 50% probability that the fetus of a *KEL2/KEL2* woman inherits the paternal *KEL1* allele. We found a 3.1% *KEL1/KEL2* prevalence of heterozygous fetuses in *KEL2/KEL2* homozygous pregnant women, which correlates with 2.6% of the theoretical probability of incidence of the fetal *KEL1* allele in the general population.

We also tested the TaqMan real-time PCR method for the noninvasive determination of *KEL1* that is suitable for SNP discrimination. We determined that real-time PCR using TaqMan probes was unsuitable for distinguishing *KEL1*-positive and *KEL1*-negative pregnancies, probably due to the nonspecific binding of the *KEL1* probe to the *KEL2* allele [18]. More specific SNP detection can be achieved using locked nucleic acid probe-based real-time PCR. Locked nucleic acid-based *KEL1* genotyping showed increased specificity of the complementary DNA strand. It is thermally stable and has excellent mismatch discrimination. Finning et al. [12] used this variant of real-time PCR for noninvasive *KEL1* genotyping. The method increased the detection specificity of the *KEL1* allele, although its sensitivity was reduced.

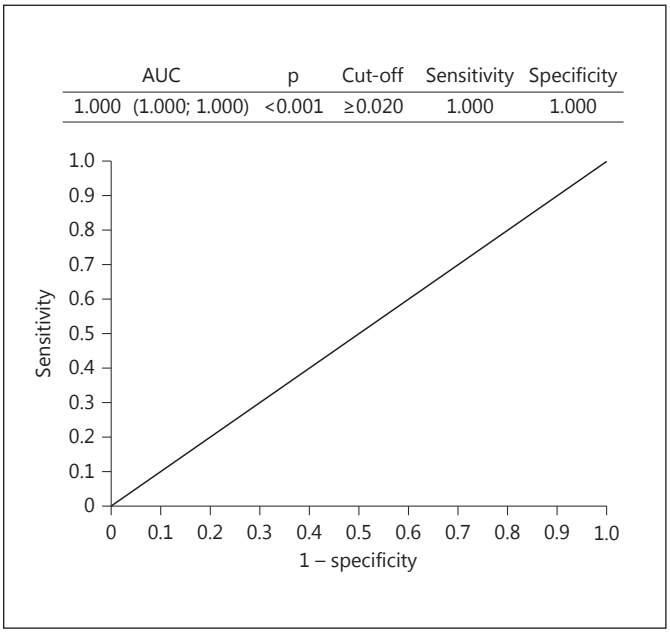


Table 2. Discrimination power of the *KEL1* signal for positivity by a RFU measurement of the *KEL1* signal and on the basis of the *KEL1/KEL2* RFU ratio (sample size n = 128)

Capillary condition[1]	Sample A 1st injection	Sample B 1st injection	Sample A 2nd injection	Sample B 2nd injection	Aggregated measurements[2] 1st and 2nd injection
Cut-off (RFU of *KEL1* signal)	≥12.5	≥19	≥126	≥122.5	–
Cut-off (*KEL1/KEL2* RFU ratio)	≥0.012	≥0.014	≥0.022	≥0.023	≥0.020
Sensitivity (RFU of *KEL1* signal)	1.000	1.000	1.000	1.000	–
Sensitivity (*KEL1/KEL2* RFU ratio)	1.000	1.000	1.000	1.000	1.000
Specificity (RFU of *KEL1* signal)	0.984	1.000	1.000	0.992	–
Specificity (*KEL1/KEL2* RFU ratio)	0.984	1.000	1.000	0.992	1.000
AUC[3] (RFU of *KEL1* signal)	0.996	1.000	1.000	0.992	–
AUC[3] (*KEL1/KEL2* RFU ratio)	0.990	1.000	1.000	0.998	1.000
p[4] (RFU of *KEL1* signal)	<0.001	<0.001	<0.001	<0.001	–
p[4] (*KEL1/KEL2* RFU ratio)	<0.001	<0.001	<0.001	<0.001	<0.001

[1] Capillary condition of the 1st injection was 5 s, 1.2 kV and of the 2nd injection 16 s, 1.2 kV, 15 kV. [2] Aggregated measurements were performed using the average of all measurements on the basis of *KEL1/KEL2* RFU ratio. [3] AUC (area under the curve) is related to the maximum and gives a measure of the quality of the test. [4] The p value was calculated with 95% confidence interval.

PNAClamp technology is another modification that increases the specificity of a reaction. Peptide nucleic acid (PNA) probes are bound to a complementary strand of DNA with high affinity, and the resulting PNA-DNA duplexes are more thermally stable. The PNA-DNA duplex is destabilized in the presence of the SNP and occurs in the amplification of the mutant allele [19]. Scheffer et al. [13] proposed clamping the PNA to the *KEL2* allele to prevent nonspecific amplification of the *KEL2* allele. This method increased the detection of fetal *KEL1* and also decreased the test sensitivity.

To assess minisequencing for noninvasive *KEL1/KEL2* genotyping, we first replicated the technique described by Di Cristofaro et al. [10] on samples isolated from the leukocytes of peripheral blood. Based on the confirmation of minisequencing specificity, we adapted the methodology for plasma cff DNA genotyping. The plasma cff DNA molecules are largely degraded into shorter fragments under 200 bp [20, 21]. Therefore, we reduced the length of the PCR products from 350 to 124 bp. The plasma cff DNA yield of the *KEL1* allele appeared to be <1% (fig. 2). The amount of plasma cff DNA could be increased using isolation kits that were designed for plasma cff DNA or for smaller DNA molecules. Nevertheless, the kit that we used to isolate the plasma cff DNA is quite commonly used for noninvasive diagnostic purposes [12, 13].

The final low yield of plasma cff DNA can be due to the preferential amplification of maternal DNA during minisequencing. The RFU quantity of positive samples indicates lower cff DNA concentration of the sample from the 10th gestational week compared to three samples from the 12th and 13th gestational week. Despite the absence of false-negative results in our study, the inclusion of a fetal control marker might have been considered. However, in the case of the most commonly used marker *RASSF1A*, the restriction site of *Bst*UI is close to the *KEL1/KEL2* polymorphism site; thus, this marker is not suitable as a fetal DNA control.

The false-positive (background) *KEL1* signal was very low; the detectable RFU values and their percentages were an order of magnitude lower than those of the true-positive *KEL1* plasma samples. Based on the results of ROC discrimination analysis, minisequencing is most accurate in the sample duplicate method and under two different electrophoresis conditions (fig. 3). The method is sufficiently sensitive and specific despite the relatively lower gain of plasma cff DNA.

The minisequencing method is mainly designed for the detection of the *KEL1* polymorphism (c.578C>T). There are rare SNPs at codon 193 (c.578C>G, c.577A>T). Our method should be able to detect the SNP variant c.578C>G as a different fluorescent signal (blue channel). The variant was not observed in our set of samples. It is also possible to detect the 577A>T variant using specific SBE primer (one nucleotide shorter) in separate minisequencing PCR. Both SNP variants result in the 'K' antigen, but the alloimmune response in this case was weaker and not as clinically significant [3, 7].

In conclusion, we described an effective, sufficiently specific and sensitive minisequencing method that is potentially suitable for the routine diagnosis of fetuses with *KEL1/KEL2* genotypes in pregnant women with *KEL2/KEL2* genotypes.

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Disclosure Statement

There is no known conflict of interest in this paper.

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