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

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\textbf{Key Words}
Noninvasive fetal genotyping \cdot KEL1 \cdot Minisequencing \cdot Kell blood group system \cdot Red blood cell alloimmunization \cdot Hemolytic disease of the fetus and newborn

\textbf{Abstract}

\textbf{Background:} The clinical importance of assessing the fetal KEL genotype is to exclude ‘K’-positive fetuses (genotype KEL1/KEL2) in ‘K’-alloimmunized pregnant women (genotype KEL2/KEL2). Noninvasive assessment of the fetal KEL genotype is not yet available in the Czech Republic. \textbf{Objective:} The aim of this study was to assess the fetal KEL1/KEL2 genotype from cell-free fetal DNA in the plasma of KEL2/KEL2 pregnant women. \textbf{Methods:} The fetal genotype was assessed by minisequencing (a dilution series including control samples). A total of 138 pregnant women (between the 8th and 23rd gestational week) were tested by minisequencing. The fetal genotype was further verified by analysis of a buccal swab from the newborn. \textbf{Results:} Minisequencing proved to be a reliable method. In 2.2\% (3/138) of the examined women, plasma sample testing failed; 94.8\% (128/135) had the KEL2/KEL2 genotype, and a total of 3.1\% of fetuses (4/128) had the KEL1/KEL2 genotype. Sensitivity and specificity reached 100\% (p < 0.0001). \textbf{Conclusion:} Minisequencing is a reliable method for the assessment of the fetal KEL1 allele from the plasma of KEL2/KEL2 pregnant women.

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\textbf{Introduction}

In pregnant women diagnosed with red blood cell alloantibody anti-K (‘K’-negative, genotype KEL2/KEL2), the fetuses are at risk of hemolytic disease only if antigen ‘K’ (‘K’-positive, genotype KEL1/KEL2) is present on their erythrocytes. In reality, however, approximately 95\% of fetuses are ‘K’-negative (genotype KEL2/KEL2) and thus are not at risk of hemolytic disease [1]. The clinical importance of assessing the fetal KEL genotype is to exclude ‘K’-negative fetuses (genotype KEL2/KEL2) in ‘K’-negative pregnant women. Maternal anti-K antibod-
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 Bsm1 (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 mass spectrometry and TaqMan PCR are used [12–14].

The aim of this work was to utilize the minisequencing method for noninvasive KEL1/KEL2 (c.578C>T) genotyping to design and test a reliable diagnostic strategy that is applicable to routine practice.

### Methods

Peripheral blood samples of pregnant women, confirmation samples of newborn buccal swabs and control plasma samples for calibration and optimization were collected in collaboration with the Department of Medical Genetics, the Department of Obstetrics and Gynecology and the Department of Transfusion Medicine of the University Hospital Olomouc. All of the women enrolled in the study signed an informed consent form approved by the Ethics Committee of the University Hospital Olomouc. The number of samples analyzed using minisequencing was as follows: the analysis was performed in 138 randomly selected plasma samples taken from pregnant women >18 years with a singleton pregnancy between 8 and 23 gestational weeks and 128 control newborn buccal swabs (table 1). The mean and median gestational age was 13 and 12 weeks, respectively.

All of the blood samples (samples and control) were collected into two parallel 9-ml tubes (‘A’ and ‘B’) containing ethylenediaminetetraacetic acid. Anticoagulated blood was placed on ice immediately after collection and was processed up to 4 h after sampling. Plasma was separated from the cellular fraction of blood using double centrifugation (2,700 g for 10 min and 3,500 g for 20 min). The plasma samples were frozen until further processing at −28°C. Plasma cell-free (cf) DNA was isolated in each of two parallel tubes (‘A’ and ‘B’). The DNA isolation of 1 ml of plasma was performed using the QIAamp DNA Mini Kit (Qiagen, USA). The incubation step for the isolation took place at 56 °C, with an elution volume of 65 μl. Plasma cf DNA for the dilution series was obtained using a QIAamp DNA Mini Kit from 5 ml of plasma eluted in a 325-μl total volume under the conditions described above. The isolation of the control DNA from newborn buccal swabs was performed using a QIAamp DNA Mini Kit according to the manufacturer’s instructions.

The dilution series was prepared to determine the sensitivity threshold of capillary electrophoresis minisequencing and to simulate the KEL1 heterozygous admixture genotype of the fetus in a KEL2/KEL2 mother. The dilution series were created using an artificial DNA mixture of diluted genotypes of serologically proven KEL1/KEL2 heterozygous and KEL2/KEL2 homozygous samples from an initial concentration of 5 ng/μl (measured using the Quantifiler Human DNA Quantification Kit, Applied Biosystems, USA). The calibration range for minisequencing was 0.78–100%.

The SNaPshot system was used for minisequencing KEL1/KEL2 detection and quantification. The first PCR amplification was performed with the primers used by Poole et al. [7] as originally determined for real-time PCR. The PCR primer sequences were GGA GGC TGG CGC ATC TG, forward, and GCA GGA TGA GGT CCT A, reverse. The samples were analyzed in two parallel reactions (‘A’ and ‘B’). The PCR plasma cell-free fetal (cf) DNA and plasma cf DNA samples in the dilution series were amplified in a final 25-μl volume. The PCR premix contained 12.5 μl of Combi PPP Master Mix (Top-Bio, USA), 0.5 μl of forward primer (10 pmol/l) (Sigma-Aldrich, USA), 0.5 μl of reverse prim-

### Table 1. Summary of the analyzed samples

<table>
<thead>
<tr>
<th>Type of primary sample</th>
<th>Plasma samples (cf DNA)</th>
<th>Newborn DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of analyzed samples</td>
<td>138</td>
<td>128</td>
</tr>
<tr>
<td>Non-analyzable samples</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>KEL1/KEL2 heterozygous pregnant women</td>
<td>102</td>
<td>124</td>
</tr>
<tr>
<td>Negative KEL2/KEL2 pregnant women</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>KEL1/KEL2 samples</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>KEL2/KEL2 samples</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>False-positive KEL1/KEL2 samples</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>False-negative KEL2/KEL2 samples</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Fig. 1. The calibration curve of 0.78–100% artificial plasma cf DNA mixtures of heterozygous KEL1/KEL2 in a KEL2/KEL2 homoyzogate 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.

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 2). Plasma cff DNA concentration was evaluated from the calibration curve (fig. 1) with the assumption that PCR efficiency and

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

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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.
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 BstUI 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.

### 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)

<table>
<thead>
<tr>
<th>Capillary condition</th>
<th>Sample A 1st injection</th>
<th>Sample B 1st injection</th>
<th>Sample A 2nd injection</th>
<th>Sample B 2nd injection</th>
<th>Aggregated measurements 2nd injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cut-off (RFU of KEL1 signal)</td>
<td>≥12.5</td>
<td>≥19</td>
<td>≥126</td>
<td>≥122.5</td>
<td>–</td>
</tr>
<tr>
<td>Cut-off (KEL1/KEL2 RFU ratio)</td>
<td>≥0.012</td>
<td>≥0.014</td>
<td>≥0.022</td>
<td>≥0.023</td>
<td>≥0.020</td>
</tr>
<tr>
<td>Sensitivity (RFU of KEL1 signal)</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Specificity (RFU of KEL1 signal)</td>
<td>0.984</td>
<td>1.000</td>
<td>1.000</td>
<td>0.992</td>
<td>–</td>
</tr>
<tr>
<td>AUC3 (RFU of KEL1 signal)</td>
<td>0.996</td>
<td>1.000</td>
<td>1.000</td>
<td>0.992</td>
<td>1.000</td>
</tr>
<tr>
<td>AUC3 (KEL1/KEL2 RFU ratio)</td>
<td>0.990</td>
<td>1.000</td>
<td>1.000</td>
<td>0.998</td>
<td>1.000</td>
</tr>
<tr>
<td>p4 (RFU of KEL1 signal)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>–</td>
</tr>
<tr>
<td>p4 (KEL1/KEL2 RFU ratio)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

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.
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Acknowledgment

Funding for this study and its publication was provided by grant agency IGA MZ CR: NT12225.

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Disclosure Statement

There is no known conflict of interest in this paper.