

# 14 Years of Polish Experience in Non-Invasive Prenatal Blood Group Diagnosis

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## Keywords

Non-invasive prenatal diagnostics · Alloimmunization · Real-time PCR

## Summary

**Background:** Blood cell antigens may cause maternal alloimmunization leading to fetal/newborn disorders. Non-invasive prenatal diagnostics (NIPD) of the blood group permits the determination of fetomaternal incompatibility. **Aim:** To evaluate 14 years of blood group NIPD at the Institute of Hematology and Transfusion Medicine (IHTM) in Warsaw. **Methods:** Plasma DNA from 536 RhD-negative, 24 Rhc-negative, 26 RhE-negative, 43 K-negative, and 42 HPA-1a-negative pregnant women was examined by real-time PCR to detect *RHD*, *RHCE\*<sub>c</sub>*, *RHCE\*<sub>E</sub>*, *RHCE\*<sub>C</sub>*, *KEL\*01* and *HPA\*1A*, respectively. We tested for *CCR5*, *SRY* or bi-allelic polymorphisms and quantified the total or fetal DNA. **Results:** The results of fetal antigen status prediction by NIPD in all but one case (false-positive result of *KEL\*01*) were correct taking neonate serology as a reference. It was confirmed that all negative results of NIPD contained fetal DNA except for four cases where there was no difference between the parents' polymorphisms. **Conclusions:** A fetal genotype compatible with the mother was determined in 25% of all pregnancies tested at the IHTM for the fetal blood group. These cases were not at risk of disease, so it was possible to avoid invasive procedures.

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## Introduction

Several blood cell antigens are of clinical significance and may lead to the immunization of antigen-negative women during pregnancy, if the fetus inherits the antigen from the father. Maternal alloantibodies cross the placenta and opsonize fetal blood cells, which are then destroyed in the spleen. If these are directed against red blood cell antigens, this leads to hemolytic disease of the fetus and newborn (HDFN). If they are directed against platelet or granulocyte antigens, this causes fetoneonatal alloimmune thrombocytopenia (FNAIT) or neutropenia (NAIN). However, if the fetus is antigen-negative, the maternal antibodies are not harmful to the child. Determining the fetal antigen status during pregnancy, especially when the father is known to be heterozygous, helps to plan the monitoring and future care of the mother. According to data collected by the Institute of Hematology and Transfusion Medicine (IHTM) together with Polish Regional Blood Transfusion Centers (RBTC) in 2007–2008, anti-D antibodies are detected in 1:1,000 pregnant women per year in Poland [1, 2], in spite of the fact that all women are subjected to anti-D immunoprophylaxis after delivery. Non-D antibodies against red cell antigens are found in about 1:2,000 pregnant women annually, and in this group the most frequent are anti-K or anti-c antibodies. The frequency of anti-HPA-1a antibodies during pregnancy, the most common cause of FNAIT, is the same as for anti-D [3].

For many years, invasive methods of collecting fetal cells have been used in order to predict the fetal antigen phenotype. However, such methods may be dangerous for the pregnancy and stimulate further maternal immunization. In 1997, Lo et al. [4] described that fetal genes can be detected in maternal plasma, and this discovery started a new era in NIPD. Fetal DNA circulates in

maternal plasma as cell-free DNA (cffDNA) [5]. Its amount increases during pregnancy but after delivery cffDNA is cleared rapidly from the maternal circulation within a few hours [6, 7], making it an excellent source of information about the current pregnancy. Since 2000, fetal *RHD* in the plasma of RhD-negative pregnant women have been analyzed at Warsaw's IHTM (the reference laboratory for pregnant Polish women with detected alloantibodies) using real-time PCR (qPCR) [8]. Over the years, we have modified the *RHD* protocol in subsequent projects to adjust the test to the Polish population of RhD-negative individuals, to cut costs, or to adapt it to new equipment [9, 10]. As in many laboratories across Europe, we provide *RHD* NIPD as a service for RhD-negative mothers with anti-D antibodies. We have also prepared protocols for NIPD of fetal Rhc, RhC, and RhE antigens, which are recommended for immunized pregnant women [11]. Due to the low specificity of qPCR in genotyping a single nucleotide polymorphism (SNP), determination of the *KEL\*01* or *HPA\*1A* status of the fetus is still performed in a research setting, although the results are passed on to clinicians [12]. The aim of this study was to summarize and evaluate blood group NIPD from maternal plasma carried out at the IHTM over 14 years.

## Material and Methods

Between 2000 and 2014 fetal noninvasive blood group genotyping was performed using the plasma of 658 pregnant women (from week 5–39 of pregnancy). We recommended week 15 as the minimum stage of pregnancy for NIPD from maternal plasma. Table 1 shows the full characteristics of the tested group. The data included the sex as well as the phenotype or genotype of neonates obtained from gynecologists, Regional Blood Transfusion Centers or hospital records to confirm the specificity of the tests.

A quantity of 10–20 ml of blood was collected from each pregnant woman into two EDTA vacutainer tubes with a gel barrier. Blood was centrifuged within 4 h to separate plasma over the gel barrier according to the manufacturer's instructions. The tubes were sent to our institute at 4 °C or room temperature within 48 h. In the case of *KEL\*01* genotyping the plasma was centrifuged after reaching our laboratory at 16,000 × g for 10 min, and the supernatant was kept in a separate tube. Samples were stored at 4 °C or kept at –20 °C for up to 1 week.

Data on fetal phenotype routinely obtained in hospital and were transmitted by email or phone, but if they were not available, 0.5 ml of cord blood or material from swabs was sent to us for testing.

Maternal plasma DNA was extracted automatically from 3–4 ml plasma using easyMag Nuclisens (Biomerieux, Boxtel, the Netherlands) and 25 µl DNA was obtained from each 1 ml. All preamplification steps were performed in a separate room. Genomic DNA from the blood of donors, parents, and neonates was isolated manually using the NucleoSpin DX Blood Kit (Marcheney Nagle GmbH, Düren, Germany).

The target gene/allele was detected using qPCR with Taqman primers and probes on ABI Prism7700 (Applied Biosystems, Branchburg, NJ, USA) up to 2013 and later on LightCycler II 480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland). The primers and probes used up to 2012 for fetal *RHD* typing have been described in a previous article [8]. In 2012, we developed a new protocol with a multiplex reaction for *RHD* exon 7 and exon 5 (designed by Finning et al. [13]). The primers and probes for fetal *RHCE\*c* (except for 11 cases described in a previous article), *RHCE\*E* and *KEL\*01* typing were used as described by Finning et al. [13] and those for *RHCE\*C* typing as specified by Legler et al. [11, 14, 15]. *HPA\*1A* detection was performed after pre-PCR digestion with MspI enzyme using primers and probes as described by Scheffer et al. [16].

Before fetal genotyping, each reaction was standardized with plasma DNA from antigen (or polymorphism)-positive and -negative donors. To determine the sensitivity of the reaction, a standard curve was constructed with plasma DNA from an antigen(or polymorphism)-positive donor (diluted from 2,500 to 4 genome equivalents (geq) per reaction) in plasma DNA from an antigen(or polymorphism)-negative donor (at a concentration of 5,000 geq per reaction as this is close to maternal plasma).

All maternal samples were tested as follows: in triplicate for gene/allele encoding significant antigen, in duplicate for *SRY* or bi-allelic polymorphism inherited from the father, and once only for *CCR5* [8, 13].

The fetus was considered to be antigen-positive if all PCR results for gene/allele encoding antigen were positive (Ct<40) but was predicted to be antigen-negative when there was no signal of amplification after up to 45 cycles in any of the replicates. If there were any discrepancies in the results, the test was repeated from the second tube or additional blood collection at a later stage of pregnancy was recommended.

*CCR5* amplification was performed to estimate the concentration of plasma DNA in the reaction [13, 16]. In the case of *RHD* diagnosis the Ct value for *CCR5* was compared with the Ct value for *RHD* in order to exclude patients with *RHD* variants in their genome.

To confirm the presence of fetal DNA in cases with an antigen-negative result, *SRY* or another marker inherited from the father (from a panel of 21 bi-allelic polymorphisms) was tested in each setting in two separate tubes as described previously [8]. Analysis of *RHD* variants was performed with RBC-Ready Gene tests (Innotrain, Kronberg, Germany) using genomic DNA.

## Results

Between 2000 and 2014 fetal noninvasive blood group genotyping was performed in the plasma of 658 pregnant women at a median gestational age of 19 weeks. The detailed characteristics of antibodies detected in this group are shown in table 1.

In 7/536 RhD-negative pregnant women (including the 230 cases described previously) comparison of *CCR5* Ct with *RHD* Ct indicated that the *RHD* gene was present in the maternal genome. Further analysis of the genomic DNA revealed the presence of the following variants: *RHD\*01W.1* (n = 3); *RHD\*01W.2*; *RHD\*01W.3*; *RHD\*DEL8*; *RHD\*R*. It was impossible to test the fetal *RHD* genotype in these cases, except for the mother with *RHD\*R*. Amplification of *RHD* exon 5 detects the sequence encoding normally ex-

**Table 1.** Number and characterization of antigen-negative pregnant women undergoing NIPD and their neonates (if available)

NIPD of	Number of tested antigen-negative women	Number of neonates with known phenotype/genotype
<i>RHD</i>	282 (85*) anti-D 160 (145*) without anti-D	174 (85*) 155 (145*)
<i>RHD+RHCE*C</i>	76 anti-D+C	59
<i>RHD+RHCE*c</i>	5 anti-G	5
<i>RHD+RHCE*E</i>	13 anti-D+E	13
<i>RHCE*E</i>	13 anti-E	13
<i>RHCE*c</i>	24 (11*) anti-c	24
<i>KEL*01</i>	43 anti-K	43
<i>HPA*1A</i>	42 (11 anti-HPA-1a)	42
*Cases previously described [8, 11].		

**Table 2.** NIPD results for *RHD*, *RHCE*, *KEL\*01*, *HPA\*1A* genotyping verified by the phenotype or genotype of neonates

NIPD of	Number of tested pregnant women	Number of fetal/neonate antigen results	
		positive	negative
<i>RHD</i>	407*	308/308	99/99
<i>RHCE*c</i>	24**	17/17	7/7
<i>RHCE*E</i>	26	15/15	11/11
<i>RHCE*C</i>	64***	39/39	25/25
<i>KEL*01</i>	43	23/22	20/21
<i>HPA*1A</i>	42	33/33	9/9

\*230 cases previously described [8].  
\*\*11 cases previously described [11].  
\*\*\*All pregnant women were tested for *RHD* and *RHCE\*C* since they had anti-D+C (59) or anti-G (5) antibodies detected.

pressed RhD antigen, and it allowed us to predict the *RHD*-positive genotype of this fetus.

In 407 cases (including the woman with *RHD\*R*), we were able to collect the data on the RhD phenotype or *RHD* genotype of neonates (table 2). There was full concordance between fetal *RHD* genotyping and the results in the neonate. In about one quarter of cases (99/407), children were predicted to be RhD-negative, and almost half of them (n = 45) were carried by women with anti-D antibodies.

We examined 76 pregnant women with anti-D+C and 5 with anti-G antibodies in order to determine the presence of *RHCE\*C* together with *RHD*. For the first group we obtained four types of NIPD results: 37 *RHD+RHCE\*C+*, 11 *RHD+RHCE\*C-*, 2 *RHD-RHCE\*C+* and 9 *RHD-RHCE\*C-*, and this agreed fully with the data on the RhD and RhC status of 59 neonates. Testing women with anti-G antibodies revealed that in 4 cases the fetus was *RHD+* and *RHCE\*C+*, and in one case the fetus was compatible with its mother, which was confirmed by serological results after delivery.

In cases with a *RHD*-negative result, the presence of fetal DNA was confirmed by finding the marker inherited from the father and absent in the maternal genome. In two cases there was no difference between the parents' DNA, and the *RHD*-negative results of the fetus were not confirmed but NIPD was repeated at a later stage of pregnancy with the same result.

Table 2 presents the NIPD results of *RHCE\*c*, *RHCE\*E* or *KEL\*01* typing of 93 pregnant women with non-D antibodies against other red cell antigens. In 24 Rhc-negative and in 26 RhE-negative pregnant women, the results of fetal *RHCE\*c* or *RHCE\*E* typing were the same as the phenotypes of the neonate. All 18 antigen-negative results of the fetus were confirmed in the same run by the detection of the paternal marker in maternal plasma DNA.

In 43 cases of pregnant women with anti-K antibodies the results of fetal *KEL\*01* genotyping were in agreement with the results of the neonate's phenotype, except for 1 patient where a false-positive *KEL\*01* result was obtained. In that case detailed analysis of the *CCR5* result (Ct value = 27) indicated that the total amount of DNA was highly above that recommended by Finning et al. [13]

causing non-specific amplification from the maternal *KEL\*02* allele. The result was not interpreted correctly since the test should have been repeated. Contrary to protocols of fetal *RHD* or *RHCE* genotyping, the confirmation of *KEL\*01*-negative results through the amplification of another paternal marker could not be performed in the same run due to the higher temperature of the *KEL\*01* reaction. For cases with fetal *KEL\*01*-negative results, a second run was performed to amplify the polymorphism inherited from the father in maternal plasma DNA. The paternal marker was found in the sample of maternal DNA, except for two cases where there were no differences between the parents.

In all 42 cases *HPA\*1A* detection combined with pre-PCR digestion of the maternal *HPA\*1B* allele genotyped the fetal status correctly, with 33 *HPA\*1A*-positive results and 9 *HPA\*1A*-negative results taking the serological results of neonates as reference. It was impossible to confirm fetal *HPA\*1A*-negative results using the digested plasma DNA. In the same run, we checked the amplification of the paternal marker using the remaining part of the undigested DNA. Whenever plasma was available, fetal *HPA\*1A*-negative results were confirmed from undigested maternal DNA.

## Discussion

In our study we assessed and summarized 14 years of blood group NIPD from maternal plasma performed in our reference laboratory, offered to alloimmunized pregnant women from throughout Poland. Comparison of the results obtained from the fetal *RHD*, *RHCE\*c*, *RHCE\*C*, *RHCE\*E*, *KEL\*01* or *HPA\*1A* typing of 653 pregnant women with data of the neonate's status showed that our methods of blood group NIPD are a highly reliable diagnostic tool. In 146 out of 542 pregnancies tested at the IHTM, using qrtPCR to detect the fetus antigen genotype, we found that the fetus was compatible with the mother and invasive diagnostic procedures could be avoided. In the remaining cases where the fetus was predicted to be antigen-positive and thus at risk of HDFN or FNAIT, it was possible to further manage the pregnancy accordingly.

In total we authorized 647 results of fetal antigen typing and sent them to clinicians. This included 6 cases with *RHD* in the mother's genome making it impossible to carry out fetal *RHD* typing. In four cases (of 542 verified by the neonate's status), there was no appropriate marker of the presence of fetal DNA in *RHD*-negative samples, and one sample gave a false-positive *KEL\*01* result.

Although cfDNA testing is now highly reliable and is becoming widely implemented in antenatal care, there are still some basic complications in diagnosis from this material. The main limit is that cfDNA is present in maternal plasma in extremely low concentrations (we detected from 5 to 15,000 geq/ml in plasma); thus, fetal diagnosis at an early gestational age is subject to false-negative results. It is possible to test fetal DNA at the 5–6th week of pregnancy in a scientific setting, but for diagnostic purposes the high prevalence of false-negative results below the 11th week of pregnancy is not acceptable in the case of immunized women [7, 17, 18].

In our opinion and from a clinical point of view, it is important to obtain fetal results in the second trimester. Thus, we recommend not to type the fetal target antigen before the 14–15th week of pregnancy [14, 19, 20].

The second complication concerning fetal typing is associated with the mixing of fetal DNA with the huge amount of maternal plasma DNA. The correct proportions of cfDNA and plasma DNA are crucial for the reliable assessment of fetal status regardless of the method used for typing [21]. Different scientific teams report different cfDNA quantities depending on the method of determination. They vary from 3–6% using qrtPCR to 10% using digital PCR [6, 22]. In our protocols, it is obligatory to estimate the level of plasma DNA by amplification of the *CCR5* gene in order to exclude samples with an excessive background of genomic DNA released from blood cells. However, we observed that this problem was eliminated by using tubes with a gel barrier and plasma centrifugation prior to sending the sample.

Summarizing our results of non-invasive diagnosis performed over 14 years, we found that in 25% of all pregnancies tested at the IHTM using qrtPCR, the fetus was compatible with the immunized mother, and invasive diagnostic procedures could be avoided. In future, our reference laboratory plans to expand non-invasive diag-

nosis to include fetomaternal incompatibilities that are less common but equally severe to other red blood cell, platelet or granulocyte antigens based on SNP. Taking into account the huge number of accurate results of fetal *RHD* typing before antenatal prophylaxis collected over the last few years, the real-time technique has proved highly reliable for NIPD of RhD antigen, but alternative methods seem to be more promising for detecting SNP and internal control. However, more clinical data is required before these become routine interventions [17, 23–25].

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## Disclosure Statement

The authors declare that they have no conflicts of interest relevant to this publication.

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## Erratum

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In the article by

Orzińska A, Guz K, Dębska M, Uhrynowska M, Celewicz Z, Wielgo M, Brojer E, entitled '14 Years of Polish Experience in Non-Invasive Prenatal Blood Group Diagnosis' [Transfus Med Hemother 2015;42:361–364, DOI:10.1159/000440821], the following changes in the author names need to be added:

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