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Review

Noninvasive fetal blood group antigen genotyping

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Noninvasive fetal blood group antigen genotyping serves as a diagnostic tool to predict the risk of hemolytic disease of the fetus and newborn in pregnancies of immunized women. In addition, fetal *RHD* genotyping is used as an antenatal screening to guide targeted use of immunoglobulin prophylaxis in non-immunized RhD negative, pregnant women. Based on testing of cell-free DNA extracted from maternal plasma, these noninvasive assays demonstrate high performance accuracies. Consequently, noninvasive fetal blood group antigen genotyping has become standard care in transfusion medicine.

Keywords: *blood group genotyping, immunization, HDFN, cell-free DNA, prophylaxis.*

INTRODUCTION

Pregnancy is a significant aspect of human life, but sometimes it can pose challenges for both the woman and her fetus. One of these challenges is the natural variation in human blood groups[1,2]. If the fetus has inherited a certain blood group that the pregnant woman does not have, there is a risk that the woman may react against that unknown antigen by producing alloantibodies and thus become immunized[3]. A woman can also become immunized due to other reasons, including blood transfusion. When immunized, there is a risk that in a subsequent pregnancy the woman may attack her fetus causing hemolytic disease of the fetus and newborn (HDFN)[3]. HDFN is in utero characterized by fetal hemolytic anemia, which may lead, if untreated, to hydrops fetalis and intrauterine death; postnatally, if not timely recognized, the newborn might develop jaundice, kernicterus, and neonatal death[4]. The leading cause of HDFN is mediated by RhD (formerly known as Rhesus D), which has led to the implementation of prophylaxis programs, predominantly in high-income countries, to decrease the risk of immunization as well as screening programs to timely treat the few cases in which prophylaxis has failed. Although less frequent, antibodies against other blood group antigens, in particular antibodies such as anti-c, anti-E, or anti-K, can also cause severe HDFN[5,6]. Notably, this clinical situation is not related to any a priori fetal or maternal disorder. It is merely a healthy fetus undergoing a transient dangerous situation which can be alleviated or treated allowing a safe start to life for the implicated newborns. The care for these women has long been a central part of Transfusion Medicine and Clinical Immunology[7,8]. One of the recent tools to assist this pregnancy care is predicting the fetal blood group antigen on the basis of

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a noninvasive DNA analysis of a standard blood sample from the pregnant woman[5,7-11]. In 1997, the presence of fetal DNA was discovered in the maternal blood circulation[12]. Apparently, fetal DNA is released from the syncytiotrophoblast layer of the placenta and ends up in the maternal blood circulation as so-called cell-free DNA (cfDNA)[13]. In 1998, it was shown that the *RHD* gene could be found in the plasma of RhD negative women carrying an RhD positive fetus[14,15]. It became clear that this simple, yet reliable DNA analysis could predict the fetal RhD type during pregnancy and thus potentially function as a noninvasive guide for monitoring and treatment of RhD negative pregnant women[15,16]. Twenty-five years later, fetal *RHD* genotyping serves as a standard clinical service in many countries worldwide for assessing the risk of HDFN in immunized RhD negative women[17-19]. In addition, many countries, especially in Europe, have implemented a screening setup to guide antenatal anti-D prophylaxis for non-immunized RhD negative pregnant women[5,9,20-22]. And recently, several diagnostic assays have been developed for other fetal antigen targets[5,11]. This review provides a brief overview of the current state-of-the-art of noninvasive cell-free fetal DNA testing for fetal blood group antigen genotyping, covering antenatal *RHD* screening to guide targeted anti-D prophylaxis for non-immunized RhD negative pregnant women, and noninvasive fetal blood group antigen genotyping in immunized women.

Antenatal *RHD* screening to guide targeted anti-D prophylaxis in non-immunized, RhD negative pregnant women

Since the late 1960s, the use of prophylactic polyclonal human anti-D immunoglobulin has markedly decreased the risk of becoming immunized when carrying an RhD positive fetus. Although dependent on the ABO compatibility, the average risk of an RhD negative woman to become immunized when pregnant with an RhD positive fetus decreased dramatically from up to 17 to 0.6-1.5%[23-26]. Traditionally, postnatal prophylaxis has been administered after birth if indicated by an RhD positive test of cord blood from the newborn. Later, some countries implemented antenatal prophylaxis which in combination with postnatal prophylaxis further minimizes the RhD immunization risk to 0.2-0.4%[23,27-30]. Combined prophylaxis has thus been shown to reduce the immunization risk approximately by half[26,31,32], with a parallel 50% reduction in severe HDFN cases[26]. Traditionally, antenatal prophylaxis was offered in a universal manner to all non-immunized RhD negative pregnant women because the fetal RhD type was unknown during pregnancy and despite having no intended benefit in women carrying an RhD negative fetus[8]. Depending on the Rh genetics of a given population, a substantial group of women were then given unnecessary prophylaxis, in Europe around 40% of the RhD negative women[33], amounting to approximately 6% of all pregnant women. Thus, a strong ethical case exists to avoid treating pregnant women unnecessarily with a human blood product[34,35]. In addition, worldwide there is a shortage of anti-D and for e.g., Europe is dependent on US plasma for the provision of anti-D. During the COVID-19 pandemic the vulnerability of this dependency was shown, further advocating for a rational use only in cases with assumed effect. Furthermore, due to the success of the prophylaxis there is a strong decline in naturally immunized anti-D donors and the production of anti-D is mainly derived from plasma of immunized volunteers, who after becoming immunized are rendered with less options in the case of needing an emergency transfusion, especially in Asian countries with limited availability of RhD negative donor blood. With the new possibility for noninvasive testing, it seemed feasible to set up a program for targeted prophylaxis targeting the antenatal prophylaxis only for women carrying an RhD positive fetus. Consequently, noninvasive testing of cell-free fetal DNA was pursued in three trials in 2006 and 2008 as antenatal screening for non-immunized RhD negative pregnant women to assess assay reliability, robustness, and performance[36-38]. The results were highly accurate with assay sensitivities of 99.6-99.7%[36-38]. After these promising trials, clinical implementation occurred in several European countries[20]. Current reported performances reflect high assay sensitivity of 99.9%[9,22]. **Table I** provides an overview of antenatal *RHD* screening performances of routine testing programs. It is important to note that the sensitivities and specificities of fetal RhD predictions using noninvasive fetal *RHD* genotyping are always calculated using the results from postnatal cord blood RhD typing as reference and thereby assuming the postnatally determined RhD phenotype as the true RhD phenotype.

Table I - *Results from clinical antenatal RHD screening programs*

Country	Reference	Samples	*RHD* exon targets	GW	Sensitivity	Specificity	FN	INC
Sweden*	Uzunel *et al.*, 2022[39]	4,337	4	10-12	99.93%	99.56%	1/2,169	3.5%
Denmark	Clausen *et al.*, 2014[40]	12,688	5,7; 5,10; 7/10	24-26	99.86%	99.3%	1/1,153	2.2%
the Netherlands	de Haas *et al.*, 2016[41]	25,789	5,7	27-29	99.94%	97.74%	1/2,865	0%
Finland	Haimila *et al.*, 2017[42]	10,814	5,7	24-26	99.99%	99.81%	1/10,814	0.8%
Norway	Stensrud *et al.*, 2023[43]	16,378	7/10; 5,7;10	24	99.93%	99.24%	1/2,340	1.3%
Switzerland	Schimanski *et al.*, 2023[44]	7,072	5,7	18-24	100%	99.96%	1/>7,072	1.7%
England	Soothill *et al.*, 2015[45]	502	5,7	15-17	100%	100%	1/>502	12.4%
Belgium*	Blomme *et al.*, 2022[46]	127	5,7	from 11	100%	100%	1/>127	5.5%
Italy*	Londero *et al.*, 2022[47]	116	5,7;10	22-24	100%	97.9%	1/>116	1.4%
TOTAL		77,823		10-29	99.94%	98.92%		1.2%

*Regional data. The total values of sensitivity, specificity, and inconclusive results were calculated using weighted averages.
GW: gestational week; FN: false negative result; INC: inconclusive result.

However, on several occasions, the fetal *RHD* genotyping has been shown to detect fetal cases which were missed by standard postnatal serology[37,41,46], thus rendering the fetal *RHD* genotyping overall more accurate than postnatal serology. The methodology of antenatal *RHD* screening is almost invariably based on DNA amplification using real-time PCR and using a combination of reagents targeting either one or more exons of the *RHD* gene[48-50]. In its simplest interpretation, an *RHD* positive PCR result will indicate the presence of an RhD positive fetus, especially when the amplification of *RHD* comprises only a fraction of the total DNA amplified. For RhD positive predictions of the fetus and for inconclusive results, the woman is recommended to receive prophylaxis. For RhD negative predictions of the fetus, it is recommended that the woman should not receive prophylaxis. Predominantly, automated equipment is used for extracting the DNA from plasma[49,50], providing high reproducibility and less errors than using manual extraction. Assay sensitivity, which is the most important parameter for the antenatal *RHD* screening, can be affected by the low levels of fetal cfDNA in plasma[9]. In addition to several pre-analytical issues[51-53], one important factor is the gestational age, as the levels of fetal cfDNA steadily increase over the course of pregnancy[54]. Thus, the risk of false-negative results is higher when testing in early pregnancy[55], although several studies have shown sufficient sensitivities from 10-11 weeks of gestation[39,55-58]. Specificity can be affected by the presence of *RHD* variants. The Rh blood group system is famous for its many variants[59-61], and several variants can complicate a straightforward prediction of the fetal RhD type[5,19]. For example, a pregnant woman may carry an *RHD* variant which does not express the RhD protein at all or a variant RhD protein missing immunogenic epitopes. Consequently, this woman is treated as RhD negative in serology, but is *RHD* positive genetically, and the amplification of her non-functional or variant *RHD* gene may mask the amplification of fetal *RHD*. In certain cases, however, it is possible to design an assay which enables amplification of fetal *RHD* and not certain maternal *RHD* variants[5]. It can also be a necessary solution to supplement a simple PCR assay with additional and more advanced tools or include a specially designed solution for the most frequent and most relevant variants present in the targeted population. Such strategies are exemplified by an elaborate setup in an Argentinian setting[62], a selective testing for a common variant in the Chinese population[63], or application of amplicon sequencing in a Japanese setting[64].

In general, a fetal *RHD* detection strategy should adapt to the target population to provide all women access to an equal level of care, and various strategic and technological options may be relevant to consider when designing a setup suitable for a population with highly mixed ethnicities. In addition, a robust screening program requires a good health care organization and strong collaboration among the different parties involved. Additional causes of discrepant results have been investigated comprehensively, including rare cases of handling mistakes, sample mix-up, vanishing twins, stem cell transplantation, or false-negative serology[65-68]. As a consequence of the high performance of the antenatal *RHD* screening, postnatal cord blood testing has been terminated in The Netherlands[41], Denmark[40], Finland[42], Sweden[39], and Norway[43]. Reported consequence of antenatal *RHD* screening is avoiding unnecessary antenatal prophylaxis in 97.3-99.6% of the RhD negative women who carry an RhD negative fetus[20]. In addition, four CE-IVDR kits are now available on the market in Europe[69,70]. Recommendations for assay validation and quality assurance have been published by a large expert group formulated and endorsed in collaboration with the cfDNA subgroup of the working party of Red Cell Immunogenetics and Blood Group Terminology at the International Society of Blood Transfusion (ISBT)[71]. Overall, antenatal *RHD* screening is now an established, reliable clinical tool which can be applied to avoid unnecessary prophylaxis in RhD negative, pregnant women.

Noninvasive fetal blood group antigen genotyping in immunized women

For women who have become immunized, noninvasive fetal blood group antigen genotyping is used to assess the risk of HDFN as part of pregnancy monitoring. The test reveals if the fetus is positive or negative for the antigen in question. If positive, the monitoring may be intensified; if negative, the monitoring may be lowered or even stopped[71]. In contrast to non-immunized RhD negative pregnant women, the analysis of immunized women is often done in early pregnancy. This allows for early intervention which for some immunizations, such as with anti-K, is absolutely essential[72]. In immunized women, noninvasive prediction of fetal RhD is mostly done using real-time PCR[49,50]. For other targets, standard allele-specific real-time PCR is not optimal and additional modifications or other techniques are required. Specifically, when an antigen is genetically determined only by one or a few single nucleotide variations (SNVs), potential, unspecific amplification of the maternal DNA can affect the amplification of fetal DNA, rendering false results. Alternative techniques circumventing this issue include DNA-sequencing[73,74] and droplet digital PCR (ddPCR)[75]. Another important advantage of these latter techniques is that they allow a more accurate determination of the total fetal DNA concentration. Preferably, a fetal control should be used to verify the presence of fetal DNA for negative results, or the test may be repeated on a sample drawn later in pregnancy to make a negative blood group prediction based on at least two independent samples[71]. For noninvasive fetal *RHD* genotyping, high diagnostic accuracy has been demonstrated repeatedly, and the service has been implemented in several countries worldwide[18,19,76]. For other blood group antigens than RhD, an overview of different setup and their test accuracies is provided in **Table II**. Overall, these results demonstrate high prediction accuracies for these antigen targets (with 100% accuracies for KEL1 using either NGS or ddPCR), thus demonstrating the potential of noninvasive fetal antigen blood group as a clinical tool in monitoring immunized pregnant women. **Table II** also demonstrates a shift in preferred technique from qPCR in earlier studies to NGS and ddPCR in recent studies. In contrast to fetal *RHD* testing, reports on other blood group antigen targets are often based on small cohorts simply because the cases are much rarer. It does affect the level of assay validation when implemented into clinical routine[71]. The use of spiked samples for validation has been reported recently[96], although real samples must be considered mandatory for a validation. In addition to fetal blood group antigens, human platelet antigens (HPA) are becoming targets of increasing interest, as antibodies against HPA can cause fetal and neonatal alloimmune thrombocytopenia (FNAIT). So, similar to predictions of blood group antigens, noninvasive prediction of fetal HPA may help in the management of women with fetuses at risk of FNAIT[5,69,89,97]. Although that immunizations against antigens other than RhD are rare, they represent clinical incidents, in which the risk against the fetus is possible to predict and manage, and, therefore, at least the clinically most

Table II - *Performance of noninvasive testing for non-RhD blood group antigen targets*

References	*RHC*		*RHc*		*RHE*		*KEL1*		*ABO*		Methods
	Samples (No.)	Accuracy (%)	Samples (No.)	Accuracy (%)	Samples (No.)	Accuracy (%)	Samples (No.)	Accuracy (%)	Samples (No.)	Accuracy (%)	
Early studies (2002-2013)											
Legler, 2002[77]	23	100	1	100	35	100					qPCR
Hromadnikova, 2005[78,79]			41	100	45	100					qPCR
Finning, 2007[80]	13	100	44	100	46	100	70	98.6			qPCR
Li, 2008[81]							32	93.8			Maldi-TOF
Orzinska, 2008[82]			11	100							qPCR
Gutensohn, 2010[83]	46	100	87	100	100	100					qPCR
Scheffer, 2011[84]			19	100	21	100	33	100			qPCR
Rieneck, 2013[85]							2	100			NGS
Recent studies (2015-2023)											
Orzinska, 2015[86]	64	100	24	100	26	100	43	95.5			qPCR
Böhmova, 2016[87]							128	100			minisequencing
Cro', 2016[88]							2	100			qPCR
Orzinska, 2019[89]*							4	100			NGS
Rieneck, 2019[90]									19	100	NGS
O'Brien, 2020[91]**			8	100	21	100	46	100			ddPCR
Durdova, 2020[92]							309	99.7***			minisequencing
Rieneck, 2021[93]	5	100	17	100	8	100					NGS
Vodicka, 2021[94]	6	100	11	100	16	100	10	100			ddPCR
Rieneck, 2022[72]							34	100			NGS
Orzinska, 2022[95]							49	100			ddPCR

This table was expanded using Table 4 from van der Schoot *et al.*[5]. *This study demonstrated 100% accuracy for additional targets, including Fy^a, Fy^b, Jk^a, Jk^b, S, but unsuccessful detection of MN. **This study also demonstrated 100% accuracy for the detection of Fy^a and Fy^b. ***Sensitivity was 92.86%

relevant cases (c, E and K) should be included where possible as part of a monitoring strategy in Transfusion Medicine.

Conclusion and future directions

Noninvasive fetal blood group antigen genotyping is characterized by high assay performance. Monitoring of RhD immunized women is widely offered across the world. Antenatal *RHD* screening of non-immunized women has been implemented mainly in European countries. Noninvasive prenatal tests for other fetal antigens are used in few labs and require advanced equipment.

Expanded use of noninvasive fetal blood group antigen genotyping is anticipated. Future challenges are effective use of fetal RhD genotyping in mixed ethnic populations and the need for improved care in low-income countries across the world. However, in the low-income countries, the first challenges to overcome are the identification of which pregnant women are at risk as they are RhD negative, as well as the wider availability of RhD immunoprophylaxis.

AUTHORS' CONTRIBUTIONS

Both Authors have contributed to writing the manuscript, and both Authors have approved the final version.

The Authors declare no conflicts of interest.

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