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Arbeit unter der Leitung von

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Noninvasive prenatal diagnosis of fetal RhD status using cell-free fetal DNA in maternal plasma with TaqMan® real-time PCR assay

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1. Introduction

Prenatal diagnosis is now part of established obstetric practice in many countries. However, conventional methods of prenatal diagnosis of obtaining fetal tissues for genetic analysis, including amniocentesis and chorionic villus sampling, are invasive and constitute a finite risk to the unborn fetus[1]. At present, it is widely accepted that both intact fetal cells as well as cell-free fetal DNA are present in the maternal circulation and can be recovered for non-invasive prenatal genetic diagnosis[2,96,97]. However, the rarity of circulating fetal cells has limited the development and practical use of this approach. Fetal DNA is present in maternal plasma in much higher fractional concentration than fetal DNA in the cellular fraction of maternal blood. This important feature has made the robust detection of fetal DNA possible, even without special enrichment procedures.

As a result of these developments, fetal DNA in maternal plasma has been used for the noninvasive prenatal diagnosis of sex-linked disorders and single gene disorders such as beta-thalassemia, congenital adrenal hyperplasia and achondroplasia[3-5]. In addition, quantitative aberrations of cell-free fetal DNA have also been found in various pregnancy-associated disorders, including preeclampsia, preterm labor and fetal trisomy 21[6,7].

Maternal plasma DNA analysis is also useful for the noninvasive prenatal determination of fetal RhD blood group status in RhD - negative pregnant women[8]. For the management of Hemolytic disease of the fetus and newborn (HDFN) a reliable non-invasive approach to determine fetal RhD genotype would be of great clinical value. RhD genotyping would

also eliminate the routine administration of Rh-immunoglobulin in RhD-negative patients.

This approach has been shown by many groups to be highly accurate (See Table 1), and has been introduced as a routine service in several centers in Europa[49,53].

In this thesis, the detail protocol of noninvasive detection of fetal RhD status using TaqMan® real-time polymerase chain reaction (PCR) technique is going to be described.

2. Rh status in Hemolytic disease of the fetus and newborn (HDFN).

Hemolytic disease of the fetus and newborn (HDFN) represents one of the most significant examples in medicine of successful management of a disease and adequate prophylaxis. Nowadays, HDFN accounts for 5% of all perinatal deaths[12]. HDFN is a potentially fatal condition caused by maternal alloantibodies directed against paternally inherited antigens on fetal red cells. Rh system`s anti-D antibodies are responsible for the majority of clinically detectable HDFN cases. This situation is observed in Rh-negative mothers whose husbands are Rh-positive, and whose immunization occurred during pregnancy, abortion, postpartum or incompatible transfusion.

The initial antibodies are of immunoglobin M (IgM) nature, with a high molecular weight and are unlikely to cross the placenta. As a result, they do not produce fetal hemolysis labely in pregnancy, the IgG antibodies cross the placenta and produce hemolysis. Antigen D has already developed by the 35^{th} to 45^{th} day of gestation, which

explains why 4-5% of posabortion patients may become sensitized. Intravenous drug abuse can also lead to isoimmunization[9]. There are certain obstetrics events that can increase the risk, such as placenta preavia, ruptured placental membranes, external version, cesarean section, manual removal of placenta and in the early stages of pregnancy-abortion, ectopic pregnancy and all invasive procedures[10,12]. For example, amniocentesis is associated with a 0,5-1% risk of spontaneous abortion and, moreover, is associated with a 17% risk of transplacental haemorrhage, which, if the fetus were RhD-positive, could boost the maternal anti-D, enhancing the risk of severe HDFN. Chorionic villus sampling (CVS) is associated with a similar risk of spontaneous abortion, but a reduced risk of transplacental haemorrhage[1,10,12,].

On the other hand ABO incompatibility in an Rh-negative patient provides partial protection against primary anti Rh-isoimmunization, but not against a secondary immunologic response[10]. There is also evidence that male fetuses have more severe HDFN than female fetuses[11].

According to the severity of hemolysis, HDFN will be anemic, icteroanemic, or hydropic. In hydropic HDFN, the hepatic parenchyma is replaced partially with secondary erythropoiesis tissue, which causes aportal and umbilical venous hypertension syndrom, as well as alterations in the metabolism of proteins, and decreased albumin. Both clinical conditions cause edema and ascitis, which are characteristic of hydrops. Frequently, fetal cardiac failure secondary to severe anemia is observed. The other two forms of HDFN, anemic and icteroanemic, are the result of a less severe hemolysis that does not compromise either the cardiocirculatory system or the protein metabolism.

When RhD sensitization occurs, careful follow-up of these mothers and judicious intervention can result in good outcomes for most pregnancies. At present, Doppler measurement of middle cerebral martery peak systolic velocity constitutes the follow-up method of choice for fetal anemia with 100% sensitivity and 88%specificity[12]. Spectral analysis of amniotic fluid (ΔOD50) and ultrasound markers, such as polyhydramnion, cardiomegalia, splenohepatomegalia, ascitis, soft tissue edema are useful in the diagnosis of fetal anemia[13]. Intrauterine fetal transfusion is currently the therapy of choice in cases of severe anti-D isoimmunization. However, it has been reported, that transfusiol therapy before 32 weeks of gestation is associated with a higher fetal mortality rate[14]. Several studies have shown, that high-dose ϒ -globulin therapy followed by intrauterine fetal transfusions (IUTs) improves fetal survival[15].

Since 1960s Rhesus (Rh) D immunoglobulin (anti-D) has been used in women who are Rh D-negative to prevent Rh D immunisation after giving birth to a baby who is Rh-D positive. Anti-D immunoglobulin is a human blood product provided by a small group of immunised volunteer donors. Prevention of Rh D immunisation has been a major medical achievement, as Rh D immunisation was a significant cause of perinatal morbidity and mortality in subsequent pregnancies of affected women.

In practice, the combination of antenatal and postnatal prophylaxis will prevent immunization in 96% of the high-risk cases. The remaining 4% corresponds to the absence or inappropriate administration of immunoglobulin when it is indicated. However administration of this human blood product carries a small but real risk of associated blood-borne infection and moreover worldwide supplies of RhD immunoglobulin are limited. Furthemore, about 40% of Caucasian population of the Rh-

negative women will receive unnecessary administration of antenatal anti-D as they carrying a D-negative child. Haemolytic disease of the fetus and newborn, despite Rh-immune prophylaxis, has not been eradicated, but the risk of alloimmunization has indeed decreased from 13%to 0,16% [16,17,21].

2.1. Fetal RhD gene diagnosis

2.1.1. Invasive prenatal diagnosis

If the laboratory parameters such as titers or tests (an indirect Coombs test) on the biological activity are indicative of possible fetal hemolysis in RhD-negative patients, it is important to know the phenotype of the fetus. Before the molecular basis of the Rh-antigens was known, the phenotype had to be determined by serological testing of fetal red cells, which could be obtained by cordocentesis or chorionic villus sampling.

The elucidation of the molecular basis of the blood group systems allowed the development of PCR-based assays for blood group typing. These assays can be performed with fetal DNA obtained via invasive means, such as amniocentesis or chorionic villus sampling. The first application of fetal RhD genotyping was prenatal testing for fetal RhD status to indicate the requirement for anti-D prophylaxis and clinical management of pregnant women with anti-D[18]. Several methods for PCR-based RhD phenotyping have been published[18,19]. However all these methods were found to be expensive, invasive and present a risk to the mother and fetus[1].

2.1.2. Noninvasive prenatal diagnosis

2.1.2.1. Fetal cells in maternal circulation

As early as 1893, Schmorl described the deportation of trophoblast sprouts into the pulmonary circulation of pregnant women[20]. At present, it is widely accepted that fetal cells are present in maternal blood[96]. Lo et al. were the first to describe that fetal sex can be determined by examing Ychromosome-specific PCR in fetal-cell DNA isolated from maternal samples[97]. Later this group showed that fetal RhD status can be determined by amplification of the RhD gene sequence in maternal DNA extracted from peripheral blood of pregnant women[24]. Meanwhile, several groups have performed the fetal RhD genotyping from maternal plasma[25,26].

However, the reported accuracy of these tests was low, with low specificity and sensitivity. This may be due to the scarcity of fetal cells (about1, 2 cell/ml maternal blood)[27]. Moreover, it has been shown that the presence of fetal cells can persist post partum. Bianchi et al. reported the persistence of male CD34+ progenitor cells in maternal blood for as long as 27 years after delivery[28]. It is evident that these cells from previous pregnancies can lead to false-positive fetal blood group typing during a new pregnancy.

In a large multi-centre trial investigating the approach of recovering fetal cells from the maternal circulation, the NIFTY study (national Institute of Child Health and Development Fetal Cell Isolation Study), various enrichment techniques using fluorescent-activated cell sorting (FACS) or magnetic activated cell sorting (MACS) and micromanipulation coupled to FISH analysis have been evaluated for clinical use[22]. Also, attempts at selectively culturing viable fetal cells from maternal peripheral blood have

been made[23]. This approach has proven to be too labor intensive, with discouragingly low detection and high false positive rates. Although the biologic availability of fetal cells has been demonstrated, the development of practical technology still requires further developments.

In summary, despite the development of many ingenious methods for fetal cell isolation, no method has been described which is both sufficiently accurate and economical enough to be used on a large scale. This difficulty has been impeding the actual clinical utilization of non-invasive prenatal testing for fetal blood group status.

2.1.2.3. Cell - free fetal DNA in maternal plasma

The existence of cell free fetal DNA in the maternal circulation has opened new possibilities of non-invasive prenatal diagnosis. Cell-free fetal DNA in maternal circulation was first described by Lo et al. who demonstrated the presence of the Y sequences in maternal blood of women carrying male fetuses[2]. Subsequently this group have used quatitative real-time PCR analysis on SRY and β-globin gene sequences in women pregnant with a male fetus to show that fetal DNA represents ≈ 3%(mean 25 genome equivalents/ml; range: 3-69) of cell-free DNA in maternal plasma during the first trimester of pregnancy, rising to about 6%(mean 292 genome equivalents/ml; range: 77-769) in the third trimester[29].

Further studies shown that cell-free fetal DNA can be detected in maternal plasma already at the 5th week of gestation[30], and is cleared rapidly from the maternal plasma

following delivery, with a mean half-life of 16 min (range4-30min) following Caesarien section[31], but generally between 10 and 100 h following vaginal delivery[32].

Shortly after the discovery of circulating cell-free fetal DNA[2] in maternal blood in 1998 Lo et al. and Fass et al. demonstrated almost simultaneously that the RhD sequence could be reliably amplified from the plasma of pregnant women with high sensitivity and specificity[8,35]. Since then, many groups have replicated these findings and therefore many studies have been published on non-invasive prenatal RhD genotyping in relatively large patient series (See Table 1).

The largest clinical experience in the noninvasive fetal RhD genotyping resides with the International Blood group Reference Laboratory (IBGRL) in Bristol, United Kingdom[98]. This group has been providing fetal blood group genotyping to obstetricians as part of the National Blood Service since 1995. In 2001, they implemented a clinical service for noninvasive diagnosis of RHD genotype based on initial work that showed 100% accuracy[43].

Similar large-scale reports have been published reflecting multi-year experience in France[44,45]. Gautier et al. studied 283 RhD negative pregnant women with no false positive or negative results[44]. Rouillac- Le Sciellour et al. performed lange-scale pre-diagnosis study and shown 99,5% diagnostic accuracy of multiplex fetal RhD genotyping using conventional and real-time PCR. The results showed that real-time PCR was more convenient for a diagnostic purpose however it hasn`t been found more sensitive than conventional PCR for the detection of plasma DNA in early pregnancy[45].

The group from Netherlands tested 2,359 plasma samples from D-negative pregnant women for the presence of RhD sequences at 30 weeks of gestation using an automated approach with the diagnostic accuracy of 99,4%[46].

In 2006 Geifman-Holtzman et al. published „A meta-analysis „ of diagnostic accuracy of noninvasive fetal Rh genotyping from maternal blood[61]. The results of this meta-analysis demonstrated that the diagnostic test of determining fetal RhD type using free-fetal DNA in maternal blood is 94,8%. The various strategies developed to target the RhD gene by range from uniplex to multiplex PCR system. However the various groups using the same protocol have shown the different diagnostic accuracies of this test (See Table 1). Several group have used more than 1 protocol to determine fetal status from materal blood[36-39]. Interestingly the best diagnostic accuracies were demonstrated in the first trimester, while it is supposed that false negative cases of this test are mainly due to a lack of fetal DNA in the maternal sample due to early gestation[62].

The meta-analysis shows that the accuracy of fetal RhD determination decreased with the use of maternal whole blood, RNA, or fetal cell from maternal blood and maternal serum and plasma were found to be the best source for accurate diagnosis of fetal RhD type.

Table 1 Published studies on RhD genotyping from fetal plasma in maternal plasma

References	Methods	No.tested	Gestation (weeks)	RhD	Accuracy	Controls
Lo et al,1998[8]	Real-time PCR	57	7-41	exon10	96%	HBB
Fass et al.1998[35]	PCR	31	16-17	exon 7	100%	None
Bischoff et al.1999[36]	Nested PCR Fluerescence PCR;(serum)	20	15-36	exon 7	70%	RHCE
Zhong et al.2000[40]	Nested RCR	22	10-21	exon 7	95%	SRY, HBB
Zhang et al.2000[46]	Real-timePCR	58	All three trimesters	exon 7	98%	None
Nelson et al.2001[47]	PCR	26	9-34	exon 10	100%	None
Finning et al.2002[48]	Real-time PCR	137	8-42	exons 4,5,6,10	100%	SRY
Costa et al.2002[42]	Real-time PCR	102	8-14	exon 10	100%	Mouse galt
Legler et al.2002[41]	Real-time PCR	27	11-38	exons 4,7	96%	E.coli plasmid DNA,RHCE,C,c,E
Turner et al.2003[48]	Real-time PCR	31	< 20	exon 10	90%	ACTB
Johnson et al.2003[37]	PCR	47	18-40	exons 4,5,10	91%	None
Siva et al.2003[50]	PCR	26	15-17	exons 10, 7	98%	SRY,β-globin
Randen et al.2004[49]	Real-time PCR	114	6-38	exon 7	92%	SRY
Rijinders et al.2004[38]	Real-time PCR	72	11-19	exon 7	99%	SRY,ALB
Rouillac et al.2004[45]	Real-time PCR	893	All three trimestres	exons 7,10	98,9%	None
Guz et al. 2004[58]	Real-time PCR	45	All three trimesters	exons 7,10	100%	SRY, GSTM1, RHCE
Finning et al.2004[53]	Real-time PCR	283	All three trimesters	exons 4,5,10	98%	SRY, CCR5
Harper et al.2004[56]	Real-time PCR	2	18	exons 4,5,10	100%	SRY
Clausen et al.2005[51]	Real-time PCR	56	15-16	exons 7,10	100%	None
Hromadnikova et al. 2005[39]	Real-time PCR	45	11-40	exons 7,10	100%	RHCE,SRY
Zhou et al. 2005[52]	Real-time PCR	98	14-42	exons 4,5 10	94%	SRY,GAPD,IDPs
Gautier et al. 2005[44]	Real-time PCR	274	All three trimesters	exon 10	100%	None
Hromadnikova et al. 2005[78]	Real-time PCR	45	11-40	exons 7,10	100%	RHCE, SRY, GLO
Hromadnikova et al. 2005[77]	Real-time PCR	23	11-37	exons 7,10	100%	RHCE, GLO
Gonzalez et al. 2005[54]	Real-time PCR	20	11-16	exon 7	100%	β-globin
Brojer et al. 2005[55]	Real-time PCR	255	All three trimesters	exons 7,10 intron 4	99%	SRY, β-actin
Minon et al. 2005[92]	Real-time PCR	218	All three trimesters	exons 4,5, 10	100%	None
Lazar et al. 2007[57]	Real-time PCR	30	11-22	Exon 7	95%	None

The meta-analysis results show that the test meets gold standard criteria: it is accurate, precise, and has high sensitivity and low false-positive and low false-negative results. A false-positive result is probably the „preferred“ mistake of this test as it does not carry the same risk as a false-negative result. In the studies that were reviewed, the explanation for false-positive were gene variation, presence of atypical mutation, or gene deletion. The studies show that the diacnostic accuracy of fetal RhD typing using cell-free fetal DNA in maternal plasma is more sensitive than using fetal cells. This is due to high concentration of cell-free fetal DNA in plasma and unlike fetal cells in maternal blood, plasma DNA analysis is not complicated by the effects of persistence from prior pregnancies. Moreover this approach was found to be rapid and not too labor intensive.

In some cases, where there is a high risk that the fetus may be affected by HDFN, it is great value to know whether the woman`s RhD-positive partner is hetero-or homozygous (56% in white populations) for the RhD gene. If the father is heterozygous for the D-antigen, the fetus has a 50% chance of being RhD-negative and therefore only in cases of paternal RhD heterozygosity would be important to perform the fetal noninvasive RhD typing in maternal plasma for the detection of foetal RhD status. In small pilot studies, different approaches have been suggested for the determination of the zygosity of the RhD gene[47,59]. In 2000, Pertl et al. have schown that quantitative PCR assays, in particular real-time TaqMan PCR can be used to determine the RhD zygosity. The results have shown that the fluorescent PCR-based DNA test allows accurate and rapid determination of zygosity for the RhD gene[60].

Since the success with detecting plasma DNA, a number of investigators have turned their attention to plasma RNA. In 2000 Poon et al. were the first to show that fetal RNA

is present in the plasma of pregnant woman[33], subsequently Ng et al. have shown that mRNA expressed by the placenta is readily detectable in maternal plasma[34]. It is supposed that mRNA measurement in maternal plasma may be a useful tool for non-invasive prenatal gene expressing profiling and it is expected that plasma RNA markers will be developed over the next few years. But in 2005 Chiu et al. demonstrated that fetal RhD mRNA is not detectable in maternal plasma and therefore this approach couldn`t be used for the prenatat fetal RhD genotyping[89].

3. Genetic aspects of RhD gene

The Rh blood group system is the most polymorphic of the human blood groups, consisting of at least 45 independent antigens and, next to ABO, is the most clinically significant in transfusion medicine.

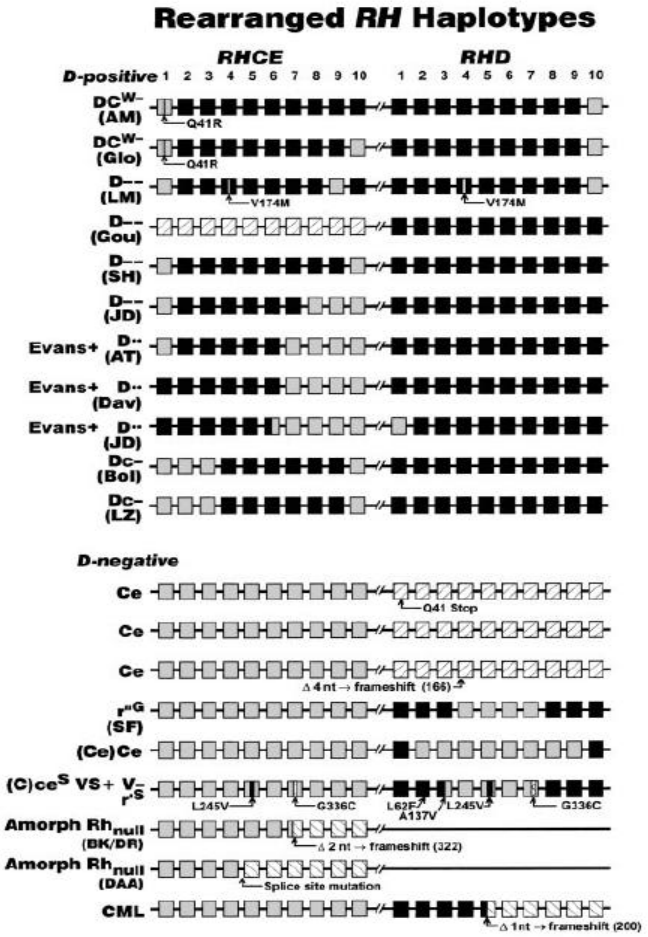
The history of the Rh system began in 1941 with its discovery as the cause of severe jaundice and fetal demise, i.e. erythroblastosis fetalis[64].

The antigens of the Rh system are located on the surface of the erythrocyte, although they are thought to be part of the trophoblast[65]. The genetic basis of the Rh blood group proteins has been intensely investigated in the past decade, and the polymorphisms responsible for most of the antigens have now been determined[94].

Two genes (RhD, RhCE) in close proximity on chromosome 1 encode the erythrocyte Rh proteins, RhD and RhCE; one carries the D antigen, and the other carries CE antigen in various combinations (ce, ce, cE or CE)[66]. The genes each have ten exons, are 97%

identical, and arose via gene duplication. RhD and RhCE proteins differ by 32-35 of 416 amino acids. This is in contrast to most blood group antigens, which are encoded by single genes with alleles that differ by only one or a few amino acids. Individuals who lack RhD protein, "Rh or D negative", most often have a complete deletion of the RhD gene (See Figure 1). Exceptions to this rule occur when the gene is present but not translated, not expressed (RhD pseudogene -RhDΨ) or when the epitopes of the antigen are composed of weak D (epitopes weakly expressed) or partial D (epitopes are absent) phenotypes[67].

The D antigen comprises numerous epitopes on the six extracellular loops of the polytopic RhD protein. There are many variants of D, which fall, in general into two types: weak D, in which all D epitopes are expressed weakly; and partial D, where some or many D epitopes are absent. Weak D usually arises from missense mutations in RhD, encoding amino acid substitutions in non-extracellular domains of the RhD protein[68], whereas most types of partial D arise from hybrid genes in which part of RhD is replaced by the equivalent region of RhCE, changing amino acid sequences in the extracellular domains[69]. Some partial D phenotypes are also caused by simple amino acid substitutions in extracellular domains. The complete absence of some D epitopes in partial D makes it possible for those D-positive individuals to produce a D-like antibody, following immunization with normal D-positive red cells expressing all D epitopes.



Avent and Reid

Blood,15 January 2000; Volume 95, Number 2

Figure 1. Rearrangements at the Rh locus giving rise to D-negative and Rh deletion haplotypes.

The structures of the RH locus (located at 1p34-36) that have been defined in various D-negative phenotypes and rare Rh antigen deletion phenotypes are depicted. Each RH gene is represented as 10 boxes, each box representing an exon, where RhCE is shown as gray, RhD as black. Crosshatched boxes depict silent RhD alleles (eg, RhD Q41X). The position of microinsertions or deletions of DNA that cause or are indicative of D-negative phenotypes are shown as triagles. Because exon 8 of RhCE and RhD are of identical sequence and their origins are not possible to define, they are shaded according to the gene loci position.

In 2000 Singleton et al. identified an inactive RhD gene, so-called pseudogene (RhDψ) in Rh D-negative Africans. RhDΨ contains all 10 exons, but is inactive because of two inactivating mutations, a 37-bp duplication in exon 4 and a nonsense mutation (Y269X) in exon 6, there are also four characteristic single nucleotide polymorphism (SNPs) in exons 4 and 5; another gene that is relatively common in Africans and produces no RhD antigen, despite the presence of some RhD exons, is RhD-CE-D^{s}, this hybrid gene comprises exons 1 and 2, the 5' end of exon 3, and exons 9 and 10 from RhD, and the 3'end of exon 3 and exons 4-8 from RhCE[70].

In general D-negative is prevalent in Caucasians (15-17%), but less common in Black Africans (5%) and Asians (3%). The D-negative phenotype has arisen numerous times, and multiple genetic events are responsible for loss of RhD expression in different populations. Caucasians have a deletion of the entire RhD gene[71], but Africans and Asians often have an inactive or silent RhD. Approximately 66% of South African, D-negative, Black persons have RhDΨ. In Asians, 10-30% of D-negative phenotype are Del and have very low levels of D antigen not detectable by standard typing but these adsorb and elute anti-D, hence the name. Del often result from a mutation causing reduced synthesis of the 3`region of RhD. Del are less frequently in Europeans (0,027%) and result from an amino acid change, M2951[72].

After RhD, the two Rh antibodies that most commonly cause HDFN are anti-Rhc and anti-RhE. Anti-K of the kell blood group system is also a major cause of HDFN. Many other blood group polymorphisms, including S/s, E/e, Kp/KP, Js/Js, Fy/Fy, Jk/JK, Di/Di and Co/Co, result from SNPs and are associated with HDFN, but only on rare occasions[69]. It would be feasible to develop non-invasive methods for all these

polymorphisms, but the demand would be extremely low owing to the rarity of severe disease[93].

4. RhD test with TaqMan® real-time PCR

4.1. Summary

Determination of fetal RhD status is extremely important for prenatal RhD prophylaxis and for the treatment of RhD-sensitized pregnant women whose partners are heterozygous for the RhD gene. The discovery of cell-free fetal DNA in maternal plasma provides the possibility for non-invasive detection of fetal RhD status. In the present study, we examined a relatively large number of maternal plasma samples at risk for HDFN caused by RhD-alloimmunization with TaqMan® real-time-PCR assay. The aim was to establish a rapid and accurate method for the detection of the fetal RhD in Rh-negative pregnant women and to further analyse the realibility of clinical application.

We studied 233 non-sensitized RhD-negative pregnant women and their fetuses (Gestation age: 6-40, mean: 26,24). DNA was extracted using automated Magna Pure TM LC Instrument. Fetal RhD status was detected using uniplex exon 7 TaqMan® real-time PCR assay and were comfirmed by the serological analysis on cord blood after delivery in all RhD-negative pregnant women. One sample was excluded due to controversial results from two examinations. Among 232 RhD-negative women, the results from 230

samples were concordant with the results of serologic analysis. Two were scored as false negative. We achieved a 98% of sensitivity and 100% of specificity. The presence or absence of the fetal RhD gene could be correctly determined in 99,4% of cases. Our results have shown that non-invasive fetal RhD genotyping can be performed rapidly and reliably using cell-free fetal DNA in maternal plasma with TaqMan® real-time-PCR assay.

4.2. Materials and methods

4.2.1. Sample collection

Between spring 2006 and autumn 2007 the multi-centre study was evaluated at the Department of Transfusion Medicine, University of Goettingen, Germany. The study was approved by the Ethics Committee of the local institution Goettingen and informed consent was obtained from all study patients. 15ml EDTA- blood samples were collected from 1000 RhD negative pregnant woman. All blood samples were centrifuged at 3500rpm for 10 minutes. The plasma was transfered without disturbing the buffy coat and recentrifuged again at 14.000rpm for 45 minutes, afterwords plasma was collected in a new 2ml tubes and stored at -80°C and buffy coat was stored at -80°C as well.

233 plasma samples from non-sensitized RhD-negative pregnant women in the 6th to 40th weeks (mean: 26,24) of pregnancy were transported in Basel (University Women`s Hospital) from Goettingen in dryice and were stored at -70°C until further proccesing.

4.2.2. DNA isolation

DNA was isolated with the automated Magna Pure TM LC Instrument (Roche Applied Science). It is an automated system employing robotics, precision pipettors and glass magnetic particals to purify nucleic acids from a variety of sample materials (whole blood, blood cells, culture cells)(See Figure 2). To isolate cell-free fetal DNA from maternal plasma we used''Magna Pure LC Total Nucleic Acid Isolatoin Kit –Large Volume,'' the extraction protocol with a specimen volume of 1000 µl plasma; purified nucleic acid was deluted in 200 µl of low salt elution buffer. Other details, such as reagent volumes and number of reaction tips needed for the run were automatically calculated by the software. DNA of 200 µl volume was frozen and kept at -20°C before to perform the real-time PCR.

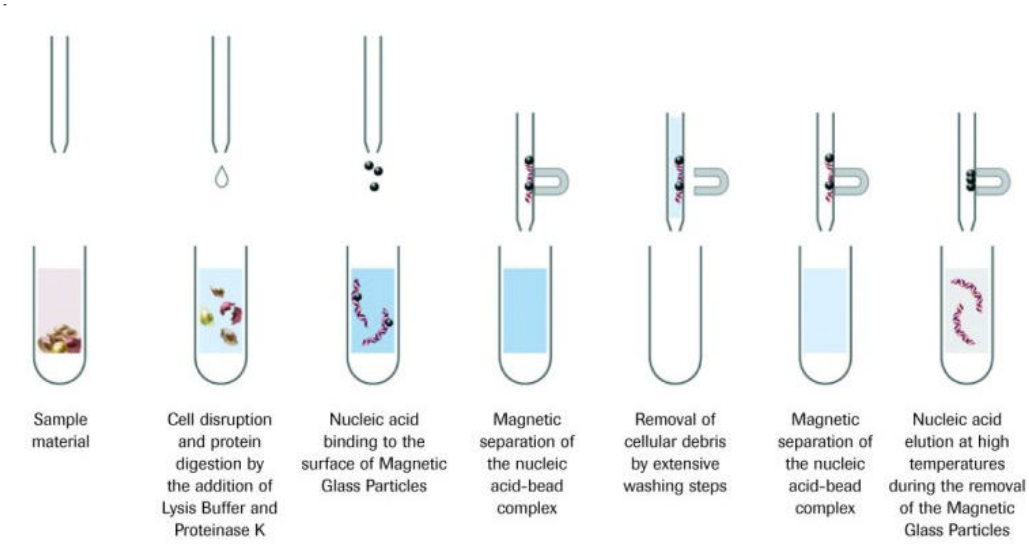


Figure 2. The Stepps of a Magna Pure LC DNA isolation procedure

The figure shows samples being dissolved and simultaneously stabilized by incubation with a buffer containing denaturing agents and unbound substances are removed by several washing steps; the purified nucleic acids are then eluted in alow salt buffer.

4.2.3. TaqMan â real-time PCR analysis

The non-invasive fetal Rh exon 7 genotyping was performed by the use of TaqMan® real-time PCR (Model 7000, Applied Biosystems), which is a combined thermal cycler and fluorescence detector with the ability to monitor the progress of individual PCR reactions optically.

In TaqMan chemistry[73], a hybridization probe included in the PCR is cleaved by the 5' nuclease activity of Taq DNA polymerase only if the probe target is being amplified. Using a fluorogenetic probe, first synthesized by Lee et al.[74,75], enables cleavage of the probe to be detected without post-PCR processing (See Figure 3).

It is already evident that the real-time PCR has a number of advantages in comparison to conventional PCR[37,45]. The advantages of TaqMan Chemistry are as follows; specific hybridization between probe and target is required to generate fluorescent signal, probes can be labeled with different, distinguishable reporter dyes, which allows amplification of two distinct sequences in one reaction tube, post-PCR processing is eliminated, which reduces assay labor and material costs. The primary disadvantage of TaqMan Chemistry is that the synthesis of different probes is required for different sequences.

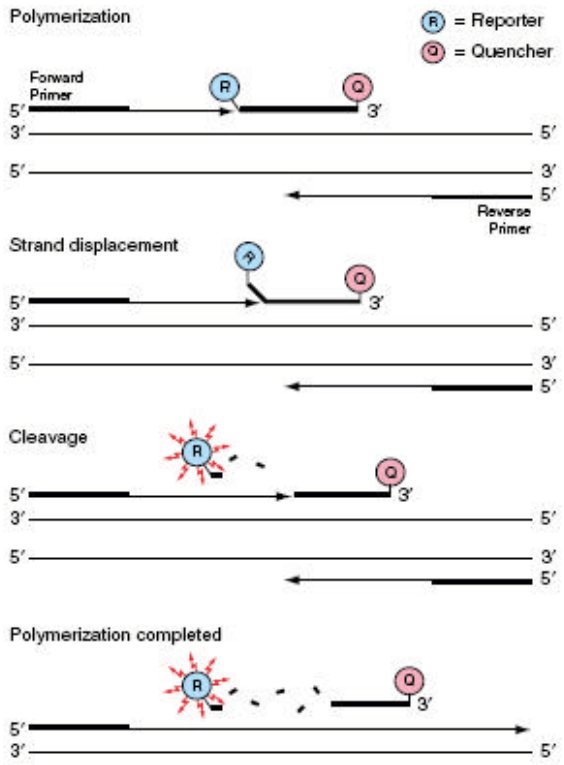


Figure 3. 5'Nuclease Assay

The figure shows that Taqman Probe and Primers anneal and extension begins. When the enzyme reaches the probe, the 5`nuclease activity of the enzyme begins to displace the probe. Cleavage of the probe begins and Reporter starts to fluoresce as it is separated from Quencher.

For the detection of fetal RhD status we used uniplex RhD exon 7 genotyping TaqMan system, which consisted of amplification forward primer, reverse primer and dual-labeled fluorescent probe, FAM [6 carboxyfluorescein] and TAMRA [6 carboxytetramethylrhodamine] were the fluorescent reporter dye and quencher dye (See Table 2). The primers and probe were targeted toward the 3'untranslated region (exon 7)

of the RhD gene. Taqman amplification reaction were set up in a reaction volume of 25 µl containing 2x Taqman Universal PCR master Mix (Perkin-Elmer) corresponding to 3,5 mmol/L MgCl2, 100µmol/1 dNTPs, 0,025 U/µL AmpliTaq Gold and 0,01 U/µL AmpErase; 300nM of nM of each primers, 100 nM of probe and 8µl of plasma DNA.

Thermal cycling was initiated with a two-minute period of incubation at 50°C to allow time for the enzyme UNG (Uracil N-glycosyle), which destroys any contamination PCR amplicons (elimination of carryover dUTP labeled PCR products), to act.

This Step was follwed by initial denaturation for 10 minutes at 95°C, required for activation of AmpliTaq Gold DNA polimarase; 40 cycles was used for the denaturation at 95°C for 15 seconds and finelly reannealing and extantion for 1 minute at 60°C. Amplification data were collected and analyzed by Sequence Detection System software (Applied Biosystems).

Table 2. Primers and probe used in the detection of the RhD gene

Gene	Sequence	Direction
RhD Exon 7	5`-ggg TgT TgT AAC CgA gTg CTg-3	Forward
RhD Exon 7	5`-CCggCTCCgACggTATC-3`	Reverse
RhD Exon 7	5`FAM-CCC-ACA-gCT-CCA-TCA-Tgg-gCT-ACA-A-TAMRA3	Reverse

4.2.4. Quality control

Plasma DNA isolation, PCR reaction mixtures and TaqMan real-time PCR reaction were performed in the separated aereas. Aerosol-resistent pipette tipps (Molecular BioProducts, San Diego, California. U.S.) were used throughout. All equipment and work areas were swabbed with DNA Zap^{TM}, PCR DNA Degradation Solution (Ambion, The RNA Company, An Applied Biosystems Business). The TaqMan® real-time PCR reagents including 2x mixture, primers and probes were prealiquoted into small volumes. During TaqMan® real-time PCR amplification, carry-over contamination was prevented by the use of uracil N-glycosylase, which destroyed uracil-containing PCR products. Negative controls using distilled sterile water were used to check for positive signals, which if present would indicate contamination occuring during the process. A serial dilution of gDNA has been used as a positive control as well as monitoring the sensitivity of the reaction in each individual amplification run.

4.2.5. Lab aquapments, consumables

Sterile Serological Pipets 5-, 10-, 25ml (BD Sciences, Basel, Switzerland), Pipetboy (Integra Biosciences, Switzerland); Pipets 0,5-10µl, 0,1-2,5µl, 2-20µl, 20-200µl, 10-100µl, 100-1000µl (Eppendorf Research, Germany), Pipet Tips 10, 20, 100, 200, 1000 (Eppendorf, Germany).

Laminair flow (Q-BIO gene, Germany), Laminair flow (LaminAir® Hb 2460, Heraeus Instruments, Germany).

Benchtop centrifuge for 15-and 50 ml Falcon-tubes (MultlFUGE 3s, Heraeus, Germany), Benchtop centrifuge for 1,5- and 2ml eppendorf tubes (Centrifuge 5415 D, Eppendorf, Germany), Benchtop centrifuge for 1,5-and 2ml(Qualitron, INC, Korea)

Vortex Juergens (Heidolph No. 54 119, Germany).

Magna Pure LC (LCPG 0289 Roche Diagnostics GmbH), Magna Pure LC DNA Isolation Kit (No.03 310 515 001,Roche Diagnostics GmbH), MagNa Pure LC Cartridge Seal (03 118 827 001,Roche Diagnostics GmbH). Magna Pure LC Reagent Tubs;Tip Stannds,Tub Lids,Reaction Tips (Roche diagnostics GmbH).

ABI Prism®7000 Sequence detection System (Applied Biosystems, USA), PCR detection plate (Abgene, AB-1100,UK), Asolute QPCR Seal (Abgene,AB-1170,UK).

4.3. Results

4.3.1. Validation of the assay

To determine the sensitivity of RhD TaqMan® real-time-PCR analysis, genomic DNA from an RhD-positive subject was diluted serially in water. Subsequently the standard curve was constructed according 10-fold dilution series with known concentrations - 33ng, 3,3ng, 0,33ng, 0,033ng of gDNA. The software with the PCR instrument calculates the standard curve for each run based on the Threshold cycle (Ct) for each standard (See

Figure 4). Threshold cycles were measured in duplicates and were plotted against the known copy number of the standard sample. The generated standard curve covered a linear range of four orders of magnitude and showed linearity over the entire quantitation range (slope = -3,685). Based on these values, a linear regression line is plotted. The coefficient of linear regression (R^2) was equal to 0,9915 intercept equal to 40,525.

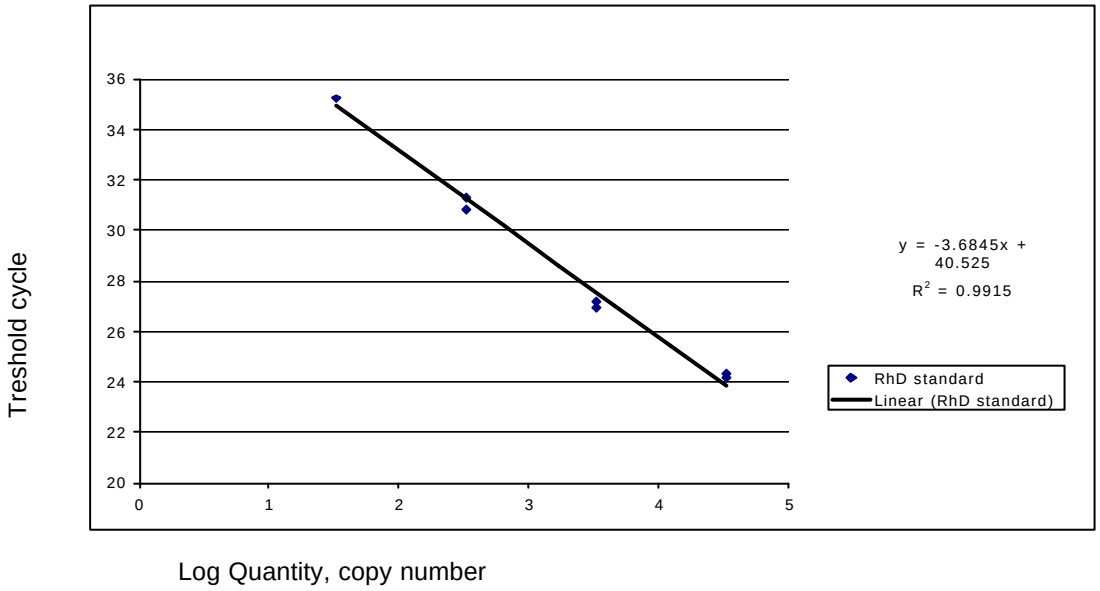


Figure 4. Standard curve for the real-time PCR assay for fetal RhD typing

Each dot represents the result of duplicate amplification of each dilution. Tenfold dilutions of standard DNA prior to amplification were used and are presented on the x-axis, whereas the corresponding Ct values are presented on the y-axis. The coefficient of determination (R^2), the intercept and the slope value of the regression curve were calculated and are indicated.

4.3.2. Prediction of fetal RhD in maternal plasma

We examined 233 plasma samples from non-sensitized RhD-negative pregnant women with TaqMan® real-time PCR assay. A Ct value of less than 40 was taken as a positive amplification. The presence or absence of the fetal RhD gene was arbitrarily determined as follows: first the samples were examined in duplicate. If the both reactions presented as positive, we considered the fetus to be carrying the paternal RhD gene. If the both reactions were negative or one of the two was positive, the sample was repeated in another duplicate. In total, if the fetal RhD sequence was detectable in 2 or more of the four reactions, the result was scored as positive. Otherwise, it was considered to be negative (See Figure 5). Our results were confirmed by the analysis of serological test on cord blood after delivary, which have been performed by the clinical diagnostic laboratories in Germany.

One sample (Gestation age: 25 weeks) was excluded due to controversial results from two examinations. Among 232 plasma samples, 3 were in their first trimester of pregnancy, 82 were from the second trimester and 147 were from the third trimester. 2 samples were scored as false negative and no false positive results were obtained (See Table 3). Our results indicated that the uniplex-TaqMan® real-time PCR assay achieved a sensitivity of 98% (158/156), and the specificity was 100% (74/74). As a whole, the fetal D-Status was correctly determined in 230 of 232 cases with the diagnostic accuracy of 99,4%. The false negative results were obtained at the 25^{th} and 30^{th} weeks of gestation.

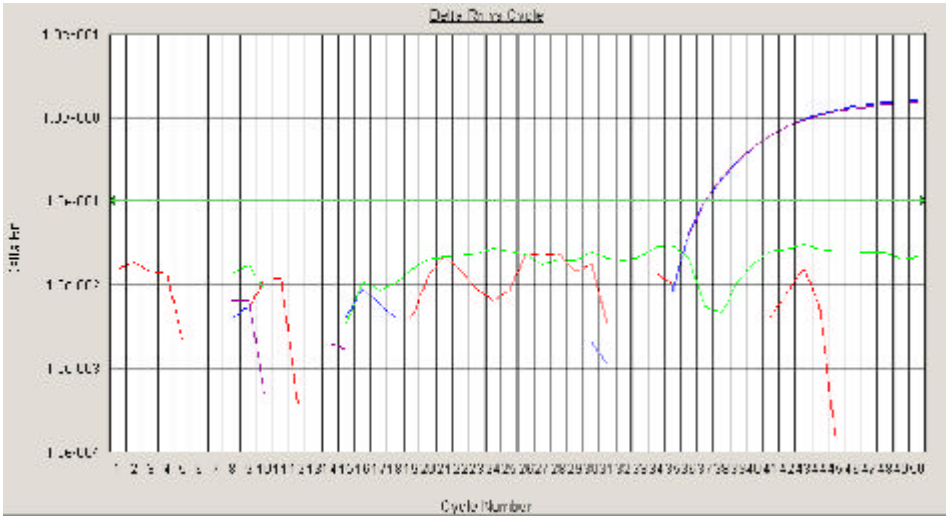


Figure 5. Amplification curve

The figure shows the two replicates of the detected and undetected foetal RhD sequences.
The treshold line shows the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The threshold line is set in the exponentioal phase of the amplification for the most accurate reading; The cycle at which the sample reaches this level is called the cycle Threshold, Ct and Baseline shows the point of initial cycles of PCR, in which there is the little change the fluorescence signal

Table 3. Prediction of fetal RhD type in maternal plasma from RhD-negative pregnant women

Gestation age	Predicted RhD type/ Serological RhD type		Concordance (%)
	Positives	Negatives	
1st trimester	3/3	0/0	100
2nd trimester	60/60	22/22	100
3rd trimester	93/95	52/52	98

4.4. Discussion

To assist in the management of pregnancy when an RhD negative woman is pregnant with an RhD positive fetus, reliable and accurate non-invasive diagnostic methods to predict the fetal RhD type will be of great clinical value. It can be offered to the general RhD-negative obstetric population whose partners are RhD-positive, whether or not fetal tissue sampling is performed. Furthermore, this approach would be especially advantageous during the first trimester of pregnancy to plan for further investigation or treatment in obstetrical situations such as miscarriage, antenatal haemorrhage or pregnancy termination. Moreover, within the next few years, it is likely that fetal RhD screening of all D-pregnant women will become routine practice in countries where rouine antenatal anti-D prophylaxis is employed. A simple, non-invasive, fully automated method of predicting fetal RhD phenotype from maternal plasma would reduce wastage of anti-RhD Ig, making the test cost-effective. It has been supposed that the total cost of this assay could be around one half of the cost of antenatal immunoprophylaxis[76]. As mentioned above, administration of the anti-D immuniglobulin carries a small but real risk of associated blood-borne infection and moreover worldwide supplies of RhD immunoglobulin are limited.

Several studies appeared during recent years on non-invasive prenatal RhD genotyping in relatively large patient series (See Table 1.). These results have shown that fetal RhD genotype could be determined with a high level of accuracy (close to 100%), by the analysis of cell-free fetal DNA in maternal plasma with real-time PCR. Several studies have shown that the best diagnostic accuracy of the RhD typing is in the first trimester[42]or at the beginning of the second trimester[63]. Thus the early availability of

such reliable results gives clinicians sufficient time to plan for further tests or treatment of sensitized RhD-negative patients such as fetal-blood sampling and fetal transfusion, which are usually performed at the beginning or in the middle of second trimester.

In this study, we examined a large number of RhD-negative maternal plamsa samples from non-sensitized RhD-negative pregnant women with TaqMan real time PCR assay. Our results have indicated that an analysis of 233 maternal plasma samples (6 to 40 weeks of gestation) resulted in a sensitivity of 98% and a specificity of 100%. One case was excluded due to controversial results from two examinations. Our first analysis for this sample confidently showed that the fetus inherited the paternal RhD gene. The CT numbers were 35, 36, 37, 38 in quadruplicate. However, the clinical outcome confirmed that the fetal RhD gene was negative. We re-aquired another 1ml aliquot of plasma from this sample in the University Women's Hospital, Goettingen Germany. We re-performed the analysis with the same procedure for plasma DNA isolation as well as TaqMan real time PCR assay. However, the results showed that no fetal RhD gene could be detected in maternal plasma in the second analysis. The causes could be due to the sample mishandling or contamination when the plasma was processed.

The false-negative results were scored from two samples taken at 25 and 30 weeks of gestation age respectively. In our study, we have used an automated Method- "Magna Pure TM LC Instrument" for DNA isolation. Magna Pure has been proved by different groups to be more efficient and has been considered as a quick nucleic acid extraction system in comparison of manual isolation of cell-free DNA involving spin columns[79,80]. 1 ml of plasma was used for DNA isolation and the DNA was eluted into 200 µl of elution buffer. 8 µl of the eluted plasma DNA was used for TaqMan real time PCR assay. Thus,

the amount of the template DNA might have been too small for achieving a higher sensitivity. In addition, the different individuals contain variable concentration of cell-free fetal DNA in plasma. In future, the use of large volume of plasma for DNA isolation as well as the increase of the template DNA alone with the PCR total reaction volume might improve the sensitivity.

To avoid false negative results caused by insufficient cell-free fetal DNA, the possible need for an internal positive control to demonstrate whether a particular maternal plasma sample actually contains a detectable concentration of cell-free fetal DNA has been repeatedly discussed. Researchers have been using the Y chromosome-specific sequences from male fetuses as an internal positive control[40]. This approach is obviously not applicable for the 50% of pregnancies involving female fetuses. Other potentials for internal controls that detect DNA sequences in fetal DNA inherited from the father and not present in the mother are insertion and deletion polymorphisms[81] and short tandem repeat sequences[82]. The recent successful development of fetal epigenic markers: maspin gene[83] and RASSF1A sequences[84], hold promise as a universal way to confirm that fetal (placental) DNA is present in maternal plasma. When applied to prenatal RhD genotyping, these markers allowed the detection of false-negative results caused by low fetal DNA concentrations in maternal plasma. These developments hold promise to allow the eventual widespread utilization of maternal plasma DNA analysis for the non-invasive prenatal diagnosis of blood group mismatches between the mother and fetus.

We did not include in our study the real-time PCR with primers for RhDΨ present in most D-individuals of African descent, because there were no such persons in our group.

In general, there are very few such individuals in our population, but we may consider RhDΨ typing in the future, because of increasing migration of people.

In general the diagnostical accuracy of uniplex RhD detection using RhD exon 7 TaqMan system was as high as that of multiplex RhD PCR system. However several groups have achieved 100% diagnostic accuracies using uniplex exon 7 and multiplex RhD typing (See Table 1). The prenatal laboratory of Women`s Hospital Basel, where this work has been performed, has run a series of studies for RhD typing. This group has used several protocols including uniplex and multiplex PCR using several internal controls; determination of paternal zygosity has been also performed [40,90,91,95]. These studies show that at present, it is impossible to achieve 100% diagnostic accuracy in this test. But in general we supposed that using either multiplex RhD genotyping or the increased concentration of primers and probes might improve the sensitivity of this test.

It is important to note that this testing strategy has been already introduced as a routine clinical service in a number of European centers[43,93]. A number of authorities have called for an even wider application of this technology[85]. The major ethical issues that will need to be addressed arise from the inevitable small number of incorrect results. “False positives” will be of minimal importance, as the mother will receive anti-D prophylaxis that is not required, but “false negatives” will mean that therapy will not be given to a D-negative woman carrying a D-positive fetus.

Understanding and overcoming the limitations of prenatal diagnosis of fetal RhD determination from maternal plasma has the potential to make this test the gold standard test for fetal Rh genotyping because it is accurate, precise and has high sensitivity and specificity and low false-negative and positive results.

4.5. Future perspectives

Cell-free fetal DNA presents only about 3% of total maternal plasma DNA. Due to the predominance of maternal DNA background, only the fetal DNA sequences, which are completely absent from maternal genome, such as Y-chromosome specific gene, or RhD gene in RhD-negative pregnant women, has been reliably detected with real-time PCR assay. However, those fetal genes, which are slightly different from maternal genome, such as fetal single gene mutations, are difficult to detect just by conventional real- time PCR-based methods. For other blood group genotyping, e.g. fetal KEL1 gene, RhEC gene, new technologies have to be developed.

Matrix-assisted laser-desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS)[87] has emerged as a new platform for fetal genotyping using cell-free fetal DNA in maternal plasma. Our group has examined a relatively large number of maternal plasma samples from pregnancies at risk for HFND caused by KEL1-alloimmunization with MALDI-TOF MS assay (manuscript accepted). This technology is also potentially applicable to the prenatal diagnosis of fetal RhD genotyping[88]. Compared to real-time PCR, the major disadvantage of this technology is the requirement of expensive instrument as well as the costy comsumable reagents.

As molecular methods for predicting blood group phenotypes are becoming more widely used in the clinical laboratory, it is important that they are properly regulated. One form of regulation must be an external quality assurance schema, a standard requirement for national clinical laboratory accreditation schemes. A European network

of excellence on special non-invasive advances in fetal and neonatal evaluation, funded by the European Union, has been established. This provides money for research, meetings and workshops[86], to enhance communication among workers in the field.

In the future it would be very useful to have a single standardized assay among the laboratories for the non-invasive fetal RhD detection with the diagnostic accuracy of 100%.

4.6. Conclusions

The "Holy Grail" within prenatal diagnosis has been non-invasive access to fetal genetic material. Many years have been spent trying to isolate fetal cells for this purpose, with only very limited success. The molecular detection of RhD blood group using cell-free fetal DNA with real time PCR technology is the first example in which non-invasive prenatal diagnosis has been successfully taken from research into clinical practice and promises the prospect of many important applications and developments over the next few years.

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