Peer reviewed article

Risk free simultaneous prenatal identification of fetal Rhesus D status and sex by multiplex real-time PCR using cell free fetal DNA in maternal plasma

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Summary

Questions under study: Pregnancies with a Rhesus constellation still present a considerable obstetric problem. In addition, pregnancies with male Rhesus D fetuses are more severely affected by haemolytic disease of the newborn, requiring more transfusions *in utero* and having a three fold higher mortality than female Rhesus D fetuses. Furthermore, almost 150 X-linked genetic deficiencies have now been characterised, increasing the need for prenatal sex determination in pregnancies at risk for such a disorder. In order examine these two important fetal loci in a risk free manner, we have developed a novel multiplex real-time PCR assay for the analysis of extracellular fetal DNA in maternal plasma.

Methods: Cell free DNA was isolated from 34 maternal plasma samples and examined by a multiplex real-time PCR assay for the Rhesus D gene and the SRY locus on the Y chromosome.

Results: Our study showed that we were able to genotype 12/13 Rhesus D males correctly. All 5 Rhesus d males were correctly identified. In addition a 100% concordance was found in the 16 samples obtained from pregnancies with female Rhesus D or Rhesus d fetuses.

Conclusions: By developing a novel multiplex real-time PCR assay we present the first report describing the determination of multiple fetal loci from cell free DNA in maternal plasma by these means. As this assay is suitable for automation, our data, therefore, suggest that such assays provide a good basis for the clinical examination of multiple fetal loci, in particular Rhesus D status or fetal sex, and can be performed effectively using real-time multiplex PCR assays.

Keywords: Rhesus incompatibility; X-linked non-invasive prenatal diagnosis; fetal DNA; maternal plasma

Introduction

Approximately 15% of Caucasian pregnancies are potentially at risk for severe haemolytic disease of the fetus and newborn due to Rhesus D incompatibility [1]. The effect of this disorder is more pronounced in pregnancies if the Rhesus D fetus is male, as it has been shown that these generally require more in utero transfusions than Rhesus D female fetuses [2, 3]. In addition, at time of the initial transfusion, male fetuses have a lower gestational age, cord blood haemoglobin content and haematocrit. The resulting lethality is also threefold higher in male Rhesus D fetuses [2, 3].

The need to know the fetal sex prenatally has also gained in importance due to the large number of X-linked disorders, over 150, which have now been characterised [4].

In order to obtain fetal tissue for the diagnosis of these and other fetal genetic traits, invasive means, such as amniocentesis of chorion villus sampling, are currently required. A further important point to consider when dealing with a pregnancy at risk of Rhesus dependent haemolytic disease is that the invasive procedure used may actually lead to a boosting of the maternal anti-Rhesus D titre, thereby contributing to the disorder and compromising the pregnancy outcome. For this reason, it is imperative that non-invasive alternatives be sought, developed and transferred to the clinic [5].

For this pressing reason several groups, including at least one major international clinical study [6, 7], are actively pursuing the development of new non-invasive risk free methods for prenatal diagnosis [8, 9].

From these studies two methods by which fetal material can be obtained for a genetic analysis in a non-invasive manner have emerged: (1) by the enrichment of fetal cells from the blood of pregnant women [10] and (2) by isolating cell free fetal DNA in the maternal plasma [11].

Previous studies both from our laboratory and others have indicated that both fetal aneuploidies and single gene disorders can be detected by using fetal erythroblasts which have been enriched from the maternal circulation (reviewed in [8, 9, 12]). We have recently examined the efficacy of such an approach for the determination of fetal Rhesus D status and fetal sex [13]. In this study, for which we recruited 19 Rhesus D pregnant women, single erythroblasts isolated by micromanipulation were examined for these two fetal loci using a highly sensitive nested multiplex PCR assay. Our results showed that we were able to correctly determine the fetal genotype for both loci interrogated in 74% of the cases examined. In the remaining 5 cases, we were not able to perform a diagnosis as we no erythroblasts were detectable following enrichment. This study, which is the largest published to date concerning the non-invasive diagnosis of fetal genetic loci by these means, hence, indicated that the technique is currently not sufficiently sensitive for diagnostic applications in a clinical setting [13].

For this reason we turned to another approach we have been investigating, the analysis of cell free fetal DNA from maternal plasma, which has recently been shown to be a promising technique for the identification of certain fetal genetic loci, including fetal sex [11] and fetal Rhesus D status [14, 15]. A caveat of this approach is that since cell free DNA derived from the mother is also present in maternal plasma, only those fetal genes not present in the maternal genome can be detected (reviewed in [16]). For this reason most researchers have focused on fetal genes such as Rhesus D in pregnancies with a Rhesus constellation, or the Y chromosome or on paternally inherited short tandem repeats (STRs) [16].

In our initial studies [17] we used the highly sensitive multiplex PCR assay we had developed for the single cell analysis of fetal RhD and sex [13]. This study showed that we were able to correctly determine these two fetal genetic loci with a great degree of accuracy. The major problem with this approach was that the PCR assay, although sensitive and accurate, was not suitable for the routine analysis of numerous samples [18]. As it is our aim to develop techniques which could be withstand the rigours of daily life in a clinical diagnostic laboratory [5], we set out to develop a new assay using the new development of real-time quantitative PCR [19].

The reason for choosing this approach is that real-time quantitative PCR offers several further advantages over normal PCR procedures. For instance, it is less prone to contamination, as the results are analysed automatically without having to open the PCR reaction vessel. It also permits the rapid analysis of numerous samples, up to 96, in one analytic run and is therefore much better suited to automation. A further reason is that independent studies both in our laboratory and that of Dennis Lo in Hong Kong have shown that free fetal DNA levels can be elevated in pregnancies with aneuploid fetuses [20, 21] or in pregnancies affected by preeclampsia [22, 23], the onset of preterm labour [24] or polyhydramnios [25]. Thus, by also being able to quantitate the amount of this fetal material one may be able to obtain some additional predictive knowledge regarding a possibly adverse pregnancy outcome.

In this paper, we present the first report of a novel multiplex real-time assay which permits the simultaneous examination of these two clinically important fetal loci on cell free fetal DNA in maternal plasma and which provides the basis for an assay suited to a clinical diagnostic setting.

Materials and methods

Patient samples: 34 peripheral blood samples (15 ml on average) were obtained from Rhesus d pregnant women in the second trimester about to undergo an invasive procedure. The average gestational age at the time of blood sampling was 14 + 4 weeks (range 13–17 weeks). Informed consent was obtained in all instances. The samples were processed within 24 hours. Approval for the study was provided by the ethical review committee of our institution. All experimental results were confirmed by comparison with those obtained from the examination of the invasive procedure.

DNA extraction and PCR analysis: Plasma was separated by centrifugation at 1800 g for 8 minutes. 1.5 ml plasma aliquots were removed and stored at –80 °C. DNA was extracted from 400 µl plasma using the QIAamp Blood Kit (Qiagen, Basel, Switzerland) as described previously [17]. To minimise the possibility of contamination aerosol resistant tips (ART, Molecular Bio-Products, San Diego) were used throughout all the experimental procedures. Multiple negative controls were included in each analysis. The DNA was eluted in 50 µl elution buffer (10 mM Tris HCl pH 7.4: 1 mM EDTA), of which 2 µl was used as template for the PCR reaction.

TaqMan PCR analysis: For the TaqMan real-time PCR analysis we used a Perkin Elmer Applied Biosystems 7700 Sequence Detector. For the simultaneous detection of Rhesus D and male fetal DNA we used the following primers and dual labelled probes:

Rhesus D:	(forward):	5' CCT CTC ACT GTT GCC TGC ATT 3'
	(reverse):	5' AGT GCC TGC GCG AAC ATT 3'
Rhesus D probe		5' (VIC) TAC GTG AGA AAC GCT CAT GAC AGC AAA GTC T(TAMRA) 3'
SRY:	(forward):	5' TCC TCA AAA GAA ACC GTG CAT 3'
	(reverse):	5' AGA TTA ATG GTT GCT AAG GAC TGG AT 3'
SRY probe		5' (FAM)CAC CAG CAG TAA CTC CCC ACA ACC TCT TT (TAMRA) 3'

As the Rhesus D probe has a different fluorescent label (VIC) than that of the SRY probe (FAM), these two reactions can be performed simultaneously in the same well. The use of these primers and probes in a non-multiplex setting has been deseribed previously [14, 21, 25, 26]. The multiplex PCR assay was designed in such a manner that identical thermal profiles were used for both the SRY and Rhesus D TaqMan assays thereby permitting the analysis of both these two markers on the same plate in the same analytic run. The Taqman real-time PCR was carried out as described previously [25].

To confirm that DNA was indeed present in each sample analysed, we also performed a real-time PCR analysis for the ubiquitous GAPDH (glyceraldehyde 3 phosphate dehydrogenase) gene [25]. In this manner we could ascertain that no erroneous data could be attributed to technical drop-outs occurring due to the lack of DNA in the sample examined.

Figure 1

Correlation of amplification efficiency for the Rhesus D and SRY loci on a standard curve using single Rhesus D or SRY- specific real-time PCR as well as multiplex Rhesus D / SRY real-time PCR.

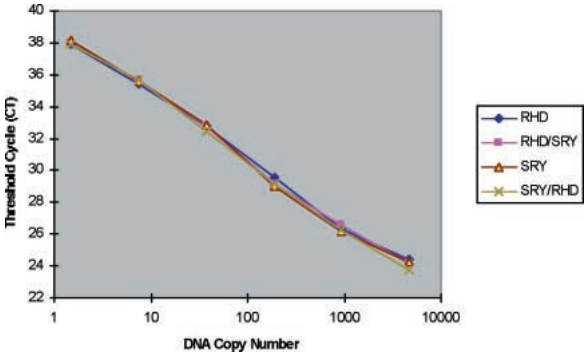

Table 1

Accuracy of real-time multiplex PCR in determining sex and Rhesus D status on cell free DNA isolated from the plasma of healthy blood donors.

PCR result/ donor genotype	SRY+/RhD+	SRY+/RhD–	SRY–/RhD+	SRY–/RhD–
Male/ RhD	17/17	0/17	0/17	0/17
Male/Rhd	0/16	16/16	0/16	0/16
Female/RhD	0/10	0/10	10/10	0/10
Female/Rhd	0/8	0/8	0/8	8/8

Table 2

Accuracy of real-time multiplex PCR in determining sex and Rhesus D status on cell free DNA isolated from the plasma of pregnant women.

PCR result/ fetal genotype	SRY+/RhD+	SRY+/RhD–	SRY–/RhD+	SRY–/RhD–
Male/ RhD	12/13	1/13*	0/13	0/13
Male/Rhd	0/5	5/5	0/5	0/5
Female/RhD	0/14	0/14	14/14	0/14
Female/Rhd	0/2	0/2	0/2	2/2

* This sample proved to be Rhesus D positive upon repeat analysis with a larger volume of plasma.

Results

Although we and other have reported on the use of single real-time PCR assays [14, 21, 25–27], as we were now dealing with a multiplex PCR assay, one concern was that both templates are not amplified with equal efficacy. This could mean that only one genetic locus would be detected effectively, whereas the sensitivity for the detection of the other fetal locus would be much lower. For this purpose we examined the amplification efficacy for both loci on experimental serial dilutions, where we observed a very good correlation between for the two loci (Figure 1). This means that both loci are amplified with the same efficacy. A further concern was that since there is a predominance of ma-

ternal DNA in the maternal plasma samples used [27], that this abundance of maternal free DNA would hinder the effective detection of the few copies of free fetal DNA present in the sample examined. In our experiments we determined that this was not the instance, in that we were still able to detected single copy levels of by male Rhesus D DNA which had been diluted into a vast excess of female Rhesus d DNA.

Next we had to test the specificity of the assay. For this purpose we examined 51 plasma samples obtained from healthy blood donors, where no discordant results were obtained (Table 1). Now that we were confident of the accuracy and sensitivity of our assay we next examined 34 samples obtained from Rhesus D pregnant women (Table 2). Here we obtained only one discordant result out of all 34 cases examined (2.9%), namely for a male Rhesus D fetus who was incorrectly identified as being Rhesus d. In this solitary case we were able to obtain a correct result, by doubling the volume of plasma used for the initial DNA preparation.

Discussion

Pregnancies with a Rhesus constellation, especially those with a male fetus, still present a considerable obstetrical problem [1–3]). Currently, the diagnosis of these two fetal genetic traits still relies on an invasive procedure, which is associated with a risk of fetal loss, but which may actually lead to a boosting of the maternal anti-Rhesus D titre. For this reason, it is imperative that non-invasive alternatives be sought, developed and transferred to the clinic [5].

In this study, we report the first use of a multiplex real-time assay for the simultaneous detection of multiple fetal loci using cell free fetal DNA in maternal plasma. Of the 34 patient samples examined only one discordant result was obtained (2.9%) which was correctly determined upon re-examination using a larger amount of input template. These encouraging results indicate that this type of analysis provides a good basis for the routine examination of these clinically important fetal loci by a non-invasive risk free method.

Although we have previously shown that these two fetal genetic loci can be reliably identified using a conventional nested PCR approach [17], we have chosen to establish a real-time PCR assay, since it is more amenable to the rigorous criteria required for routine diagnostic applications [18]. The reasons for this decision are that the real-time PCR system is less prone to contamination and that it readily facilitates the automatic analysis of up to 96 samples. A further advantage of this quantitative assay is that by permitting an assessment of the concentration of free fetal DNA it may additionally help to identify pregnancies with an adverse outcome, as elevations in fetal DNA have been observed in pregnancies with aneuploid fetuses [20, 21] and in those affected by preeclampsia [22, 23], preterm labour [24] or polyhydramnios [25].

The contribution to obstetrical healthcare of the ability to prenatally diagnose the fetal Rhesus D status and sex by a non-invasive method would be very useful in the treatment of Rhesus D-sensitised pregnant women, as one would no longer have to resort to risk associated invasive procedures. Furthermore, since there is a 50% chance that the fetus in a pregnancy with a Rhesus constellation has a Rhesus D genotype, the described assay can help prevent the unnecessary use of expensive prophylactic treatments [1]. In addition, since there is generally a more problematic outcome in such pregnancies if the fetus is male, it is a real advantage of also knowing the fetal sex. In addition, the described test can also be of great benefit in assisting the genetic counselling of pregnancies at risk for one of numerous X-linked disorders [4], as this knowledge can again help to reduce the number of necessary invasive procedure by 50%.

Hence, the ability to reliably determine these two clinically important fetal loci in a non-invasive manner using real-time PCR on free fetal DNA in maternal blood presents a significant achievement in the transition of a research technique to routine clinical use.

Acknowledgements: This study was supported by SNF grant number: 3200-059275.99/1 and NIH contract number: N01-HD-4-3202-09.

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References

1 Mackenzie IZ, Bowell P, Gregory H, Pratt G, Guest C, Entwistle CC. Routine antenatal Rhesus D immunoglobulin prophylaxis: the results of a prospective 10 year study. Br J Obstet Gynaecol 1999;106:492–7.

2 Ulm B, Ulm MR, Panzer S. Fetal sex and hemolytic disease from maternal red-cell alloimmunization. N Engl J Med 1998;338: 1699–700.

3 Ulm B, Svolba G, Ulm MR, Bernaschek G, Panzer S. Male fetuses are particularly affected by maternal alloimmunization to D antigen. Transfusion 1999;39:169–73.

4 Handyside AH. Clinical evaluation of preimplantation genetic diagnosis. Prenat Diagn 1998;18:1345–8.

5 Holzgreve W. Will ultrasound-screening and ultrasound-guided procedures be replaced by non-invasive techniques for the diagnosis of fetal chromosome anomalies? [Editorial.] Ultrasound Obstet Gynecol 1997;9:217–9.

6 de la Cruz F, Shifrin H, Elias S, Bianchi DW, Jackson L, Evans MI, et al. Low false-positive rate of aneuploidy detection using fetal cells isolated from maternal blood. Fetal Diagn Ther 1998;13:380.

7 Bianchi DW, Simpson JL, Jackson LG, Evans MI, Elias S, Holzgreve W, et al. Fetal cells in maternal blood: NIFTY clinical trial interim analysis. Prenat Diagn 1999;19:994–5.

8 Holzgreve W, Hahn S. Fetal cells in cervical mucus and maternal blood. Baillieres Obstet Gynaecol 2000;14:709–22.

9 Bianchi DW. Fetal cells in the maternal circulation: feasibility for prenatal diagnosis. Br J Haematol 1999;105:574–83.

10 Holzgreve W, Garritsen HS, Ganshirt Ahlert D. Fetal cells in the maternal circulation. J Reprod Med 1992;37:410–8.

11 Lo YMD, Corbetta N, Chamberlain PF, Sargent JL. Presence of fetal DNA in maternal plasma and serum. Lancet 1997; 350:485–7.

12 Hahn S, Sant R, Holzgreve W. Fetal cells in maternal blood: current and future perspectives. Mol Hum Repro 1998;4: 515–21.

13 Troeger C, Zhong XY, Burgemeister R, Tercanli S, Minderer S, Holzgreve W, Hahn S. Approximately half the erythroblasts in maternal blood are of fetal origin. Mol Hum Reprod 1999; 5:1162–5.

14 Lo YM, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF, et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. N Engl J Med 1998; 339:1734–8.

15 Faas BH, Beuling EA, Christiaens GC, von dem Borne AE, van der Schoot CE. Detection of fetal RHD-specific sequences in maternal plasma. Lancet 1998;352:1196.

16 Lo YM. Fetal DNA in maternal plasma. Ann N Y Acad Sci 2000;906:141–7.

17 Zhong XY, Holzgreve W, Hahn S. Detection of fetal Rhesus D and sex using fetal DNA from maternal plasma by multiplex polymerase chain reaction. BJOG 2000:107:766–9.

18 Zhong XY, Hahn S, Holzgreve W. Prenatal diagnosis using free DNA in maternal plasma: safe enough for routine use? Lancet 2001; *in press.*

19 Heid C, Stevens J, Livak K, Williams P. Real-time quantitative PCR. Genome Res 1996;6:986–94.

20 Lo YM, Lau TK, Zhang J, Leung TN, Chang AM, Hjelm NM, Elmes RS, Bianchi DW. Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. Clin Chem 1999;45:1747–51.

21 Zhong XY, Bürk MR, Troeger C, Jackson LR, Holzgreve W, Hahn S. Fetal DNA in aneuploid fetuses is elevated in pregnancies with aneuploid fetuses. Prenatal Diag 2000;20:795–8.

22 Lo YM, Leung TN, Tein MS, Sargent IL, Zhang J, Lau TK, Haines CJ, Redman CW. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. Clin Chem 1999; 45:184–8.

23 Zhong XY, Laivouri H, Livingston JC, Ylikorkala O, Sibai BM, Holzgreve W, Hahn S. Both maternal and fetal extracellular circulatory DNA are elevated in the plasma of pregnant women with preeclampsia. Am J Obst Gynecol 2001; *in press.*

24 Leung TN, Zhang J, Lau TK, Hjelm NM, Lo YM. Maternal plasma fetal DNA as a marker for preterm labour. Lancet 1998;352:1904–5.

25 Zhong XY, Holzgreve W, Li JC, Aydinli K, Hahn S. High levels of fetal erythroblasts and fetal extracellular DNA in the peripheral blood of a pregnant woman with idiopathic polyhydramnios: case report. Prenatal Diagnosis 2000;20:838–41.

26 Zhong XY, Burk MR, Troeger C, Kang A, Holzgreve W, Hahn S. Fluctuation of maternal and fetal free extracellular circulatory DNA in maternal plasma. Obstet Gynecol 2000:96:991–6.

27 Lo DYM, Tein MSC, Lau TK, Haines CJ, Leung TN, Poon PMK, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998;62:768–75.

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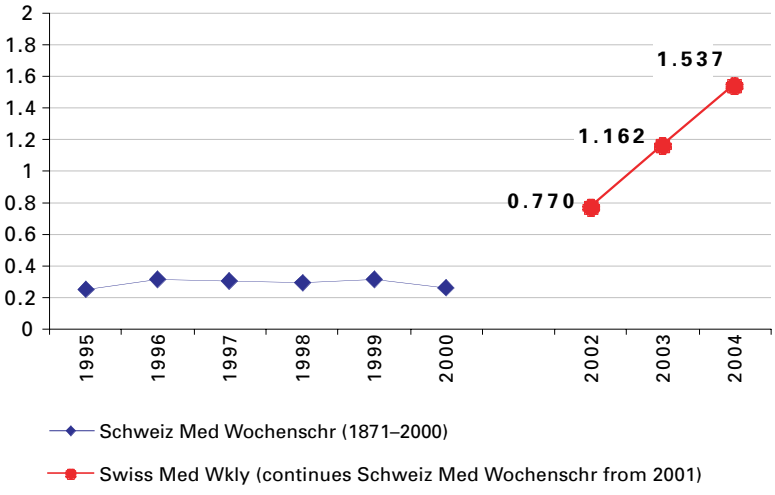

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