Journal of Clinical Laboratory Analysis 23 : 24–28 (2009)

Comparison of PCR Methods for Detecting Fetal RhD in Maternal Plasma

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Background: Aim of this study was to establish the method yielding the highest sensitivity routinely used to determine fetal RhD type and gender from maternal cell-free plasma DNA in different periods of gestation.
Methods: Plasma DNA concentrations were measured from 46 pregnant women in different gestational periods and tested for RhD using three different PCR methods on exon 7: Thermal Cycler, Taqman method on LightCycler, and melting curve analysis on LightCycler. In addition, fetal gender was determined by PCR. Cell-free plasma DNA was measured in 100 healthy volunteers as a reference group.
Results: The mean value of cell-free plasma DNA in the reference group was 10.9 pg/μL mean, (standard deviation (SD): 3.66) in 50 healthy women and 12.7 pg/μL (SD: 8.2) in 50 healthy men. In the first trimester of pregnancy cell-free plasma DNA was 14.9 pg/μL mean, (SD: 4.2), in the second trimester 15.4 pg/μL mean, (SD: 4.96), and the maximum was achieved in the third trimester of pregnancy 15.6 pg/μl mean, (SD: 6.49). TaqMan probes had the same accuracy, when compared with Thermal Cycler technology (46 samples, 6 failures). Using real-time PCR with melting curve analysis 12 of 17 samples were correctly tested. Gender determination was correctly in 41 of 46 samples.
Conclusion: RhD determinations with Taq-Man and Thermal Cycler technology are useful methods for fetal RhD prediction. To increase the accuracy of RhD determination it is necessary to test on other exons in addition. J. Clin. Lab. Anal. 23 : 24–28, 2009.

Key words: fetal RhD; plasma DNA; PCR; cell-free DNA

INTRODUCTION

RhD-negative women with RhD-positive partners have a risk of alloimmunization from their newborn babies. Determination of RhD is highly important, because it is well known that RhD antibodies are one cause of hemolytic disease of the fetus/newborn (HDFN). In addition, male RhD-positive fetuses have a three fold higher mortality than female RhD-positive fetuses from HDFN caused by Anti-RhD antibodies (1).

For fetal RhD determination invasive procedures, such as amniocentesis or chorion villus sampling, have been reported (2,3).

The discovery of cell-free fetal DNA in maternal peripheral blood and the determination of fetal RhD by molecular biologically methods have opened up new possibilities for noninvasive prenatal diagnosis (4). Nowadays, new noninvasive prenatal diagnostic methods, such as PCR, have been developed for the determination of fetal RhD status using cell-free plasma DNA isolated from maternal peripheral blood (5).

Though PCR methods are known to have the highest sensitivities, in fetal RhD determination using maternal plasma DNA, these methods have different detection limits.

In our study we evaluated the method with the highest sensitivity for routine use in determining fetal RhD type from maternal cell-free plasma DNA in each period of gestation. In addition, we tested fetal gender and determined the concentration of cell-free plasma DNA in pg/μL. Furthermore, we investigated a possible

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Received 26 March 2008; Accepted 9 August 2008
DOI 10.1002/jcla.20282
Published online in Wiley InterScience (www.interscience.wiley.com).

correlation between plasma DNA concentrations and correctly determined fetal RhD type in normal pregnancy.

To determine a reference range, we evaluated a group of 100 healthy volunteers (50 nonpregnant, female, 50 male) aged between 21 and 64 years. Cell-free plasma DNA was measured by using fluorescent signal detection. For fetal RhD typing in 46 pregnant females, we used three different PCR methods: Conventional Block Cycler (GenXpress, Techgene, Duxford, UK), real-time PCR (LightCycler, Roche, Mannheim, Germany) with melting curve analysis, and real-time PCR (LightCycler) with TaqMan technology. For all different methods, the same primers on exon 7 were used.

MATERIAL AND METHODS

Probands

We included 46 healthy pregnant women (primipara) in our ethically approved study. The samples were collected from the Department of Gynecology with informed consent granted by the patient. We measured the concentrations of cell-free plasma DNA, gender, and RhD in all samples in duplicates.

The pregnant women consisted of 3 groups according to trimester of gestation: 9 samples were obtained from females in the first trimester, 26 in the second, and 11 in the third trimester of pregnancy.

For RhD determination real-time PCR (LightCycler system) using TaqMan probes were compared with a Thermal Cycler method in all samples. Additionally, in 13 samples we used the methods established on LightCycler with melting curve analyis.

To evaluate the reference ranges of cell-free plasma DNA, EDTA blood samples were taken from 50 healthy, nonpregnant women (aged from 21 to 64) and 50 healthy men (aged from 23 to 61).

Measurements of Cell-Free Plasma DNA

The DNA Isolation kit (Roche, Germany) was used for the isolation of cell-free plasma DNA from 800 μL of plasma. The isolated DNA was finally diluted in 50 μL of elution buffer and 5 μL was stained with Vistra Green.

The amount of cell-free plasma DNA was measured with the LightCycler system using the fluorescent signal detection at 530 nm. We have used the following protocol for measuring the calibration curve using commercial DNA standard (Human Placenta DNA, Sigma D-4642; Sigma, Vienna, Austria). The DNA standard was dissolved in TE Buffer pH 8.0 (TRIS-EDTA buffer, 1 mmol/L Tris HCL, 1 mmol/L EDTA, pH 8.0; Sigma) to yield the following DNA concentrations: 2.5, 10, 25, 50 ng/μL. Five microliters of the standard or sample was pipetted into precooled capillaries.

Vistra Green nucleic acid gel stain (stock: 10,000 × concentration in dimethyl sulfoxide (Amersham Bioscience, Freilburg, Germany) was diluted with TE buffer 1:10,000 and 5 μL was added to each sample or standard.

Subsequently, the samples were centrifuged at 800 × *g* for 30 sec. The emitted fluorescent signals were measured for samples and standards at 530 nm using LightCycler. The signals obtained from the standards were used for the calculations of the calibration curve. The amounts of the cell-free plasma DNA were expressed in pg/μL. Repeated measurements of 50 pg/μL DNA calibrators yielded an analytical imprecision (CV) of 3.08%.

RhD Determination

Thermal cycler

For the determination of the RhD on chromosome 1, we used following primers for exon 7: forward: 5′-CCC CAC AGC TCC ATC ATG-3′, reverse: 5′-CCA-CAT GCC ATT GCC GGC T-3′. The PCR conditions on a Thermal Cycler (GenXpress, Techgene) were as follows: denaturation: 94°C, 5 min; 50 cycles of 94°C for 15 sec; annealing: 56°C, 15 sec; extension: 72°C, 15 sec. The Mastermix contained 39.7 μL RNAse-free water (Sigma: W 4502), 1 μL forward primer (100 pml/μL), 1 μL reverse primer (100 pmol/μL), 5 μL 10 × buffer (15 mmol/μL), 1 μL dNTP (Deoxynucleotide Mix, 10 mM Solution, PCR Reagent D 7295), 0.3 μL Taq polymerase (GenXpress, Bio Therm DNA Polymerase, 5 units/μL) for each sample. We have added 2 μL of isolated DNA in Mastermix to obtain the total volume of 50 μL.

Real-time PCR with TaqMan probes

For the real-time PCR reaction using the LightCycler the same primers were used in addition with the TaqMan probe for exon 7 as follows: 5′-FAM-AGC TTG CTG GGT CTG CTT GGA GAG ATC-TAMRA-3. The PCR conditions on LightCycler were as follows: denaturation: 95°C, 7 min; 50 cycles of 95°C for 1 sec; annealing: 62°C, 5 sec; extension: 72°C, 10 sec. We developed a method using TaqMan probes according to the following protocol: 5.85 μL RNAse-free water, 1 μL forward primer (100 pmol/μL), 1 μL reverse primer (100 pmol/μL), 1 μL TaqMan (25 pmol/μL), 2 μL 10 × buffer (15 mmol/μL), 2 μL Mg buffer (25 mmol/μL), 0.4 μL dNTP, 1.5 μL BSA (Sigma, albumin from bovine serum, min. 96% electrophoresis, A 2153) and

0.25 μL Taq polymerase (GenXpress). Five microlitres of the isolated DNA was added in 15 μL Mastermix to get 20 μL final volume.

Real-time PCR with SYBR Green and melting curve analysis

For this PCR method, established on LightCycler, the same forward and reverse primer for exon 7 were used. The PCR conditions on LightCycler were as follows: denaturation: 95°C, 10 min; 35 cycles for 95°C 10 sec, annealing: 64°C, 1 sec; extension: 72°C, 10 sec; melting curve: melting peak at 89°C; The Mastermix contained 11.6 μL sterile water (Sigma), 2.4 μL $MgCl_2$ (25 mmol), 1 μL forward primer, 1 μL reverse primer, and 2 μL SYBR Mastermix (LightCycler, Fast Start DNA Master SYBR Green I). In 18 μL of the prepared Mastermix, 2 μL template was added.

After delivery umbilical cord blood was tested for RhD status using conventional serological methods: Dia Clon Anti-D (Dia Med AG; Cressier FR, Switzerland) and Dia Med-ID AB0/Rh Micro Typing System (Dia Med AG).

Gender Determination

SRY gene was detected using the LightCycler system. The primer sequences were: forward: 5′-TGG CGA TTA AGT CAA ATT CGC-3′; reverse: 5′CCC CCT AGT ACC CTG ACA ATG TAT T-3′. The protocol for SRY: 11.6 μL sterile water (Sigma), 2.4 μL $MgCl_2$ (25 mmol), 1 μL forward primer, 1 μL reverse primer, and 2 μL SYBR Mastermix (LightCycler, Fast Start DNA Master SYBR Green I). In 18 μL of the prepared Mastermix, 2 μL template was added.

β-globin was used as reference gene in all samples. The sequences of the primers for β-globin were as follows: forward: 5′-CAA CTT CAT CCA CGT TCA CC-3′ and reverse: 5′-GAA GAG CCA AGG ACA GGT AC-3′.

STATISTICS

Data were analyzed with STATISTICA for Windows, Ver. 6.0. Descriptive data are reported as mean, median, and SD. Statistical significance was determined by Wilcoxon matched-pairs test for nonparametric variables. Statistical significance was defined as $P<0.05$.

RESULTS

The calculated reference interval of cell-free plasma DNA in the group of 50 healthy, nonpregnant women was 10.9 pg/μL mean, (SD: 3.66, min: 4.8, max: 22.4;), and in the group of 50 healthy men 12.7 pg/μL mean, (SD: 8.2, min: 2.6, max: 79.0), respectively. We have demonstrated no significant increase of cell-free plasma DNA during the three trimesters of pregnancy. In the first trimester the amount of cell-free plasma DNA was 14.9 pg/μL mean, (SD: 4.2), in the second trimester 15.4 pg/μL mean (SD: 4.96), and the maximum was achieved in the third trimester of pregnancy 15.7 pg/μl mean, (SD: 6.49).

Plasma DNA of nonpregnant women when compared with plasma DNA in the first trimester of pregnancy was significantly increased: $P<0.05$. In addition plasma DNA of nonpregnant women was again significantly increased when compared with plasma DNA in the second trimester of pregnancy: $P<0.05$, whereas nonpregnant plasma DNA compared with DNA in the third trimester of pregnancy showed no significant difference (Fig. 1).

RhD determination using real-time PCR with TaqMan probes had the same accuracy, when compared with Thermal Cycler technology. Detailed results are summarized in Table 1. From 46 tested samples 40 were correctly determined using postnatal serological detection of RhD as the golden standard. In the first trimester of gestation, nine total samples were tested, of these two samples tested by the TaqMan system and three samples tested with conventional Thermal Cycler that incorrectly determined the RhD fetal type. In the second trimester of gestation, 26 total samples were tested, of these three samples tested with TaqMan method and two samples with Thermal Cycler that incorrectly determined the RhD fetal type. In the third trimester we tested 11 samples and had one incorrect Rh type in both methods. Seventeen samples of different gestational periods were additionally tested using real-time PCR with melting

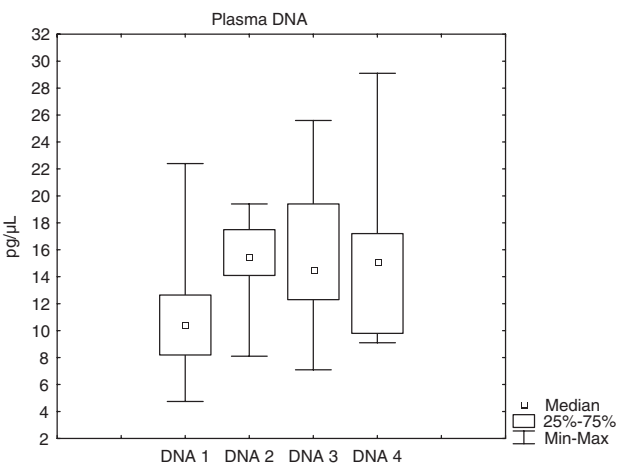

Fig. 1. Cell-free DNA concentrations show no significant differences during periods of gestations. **DNA 1** shows cell-free plasma DNA in 50 healthy nonpregnant women, **DNA 2** shows cell-free plasma DNA in the first trimester, **DNA 3** corresponds to levels in the second trimester, and **DNA 4** corresponds to levels in the third trimester of pregnancy.

TABLE 1. Results of RhD and Gender Determination in the (a) First Trimester of Pregnancy, (b) Second Trimester of pregnancy, (c) Third Trimester of Pregnancy, and (d) Results of all Three Trimesters of Pregnancy

Sample	DNA	Taq	LC	Thermo	SRY	Newborn Rh	Newborn Gender
(a) First trimester[a]							
1	8.4	pos		pos	m	pos	m
2	8.1	neg		neg	m	neg	m
3	17.5	pos		**neg**	**f**	pos	m
4	19.4	neg		neg	m	neg	m
5	19.3	pos		pos	f	pos	f
6	14.1	**pos**		**pos**	f	neg	f
7	15.5	pos		pos	m	pos	m
8	14.7	**pos**	**pos**	**pos**	**f**	neg	m
9	17.2	pos	pos	pos	f	pos	f
n/incorrect		**9/2**	**2/1**	**9/3**	**9/2**		
mean	14.9						
SD	4.2						
(b) Second trimester[b]							
1	10.5	pos		pos	m	pos	m
2	12.3	pos		pos	**f**	pos	m
3	8.6	pos		pos	m	pos	m
4	11.8	neg		neg	f	neg	f
5	12.7	**neg**		pos	m	pos	m
6	13.2	neg		neg	f	neg	f
7	7.5	pos		pos	m	pos	m
8	13.2	neg		neg	f	neg	f
9	14.2	pos		pos	f	pos	f
10	7.1	pos		pos	m	pos	m
11	14.4	neg		neg	m	neg	m
12	22.1	neg		**pos**	f	neg	f
13	21.8	pos		pos	m	pos	m
14	17.6	neg		**pos**	f	neg	f
15	20	pos		pos	m	pos	m
16	13.7	pos		pos	m	pos	m
17	19.4	neg	neg	neg	f	neg	f
18	25.6	neg	neg	neg	f	neg	f
19	14.8	pos	pos	pos	m	pos	m
20	17.1	neg	**pos**	neg	m	neg	m
21	17.1	pos	pos	pos	f	pos	f
22	23.5	**pos**	**pos**	neg	f	neg	f
23	10	pos		pos	f	pos	f
24	20.2	pos	pos	pos	m	pos	m
25	14.6	**neg**	**neg**	pos	**f**	pos	m
26	19	pos	pos	pos	m	pos	m
n/incorrect		**26/3**	**9/3**	**26/2**	**26/2**		
mean	15.4						
SD	4.9						
(c) Third trimester[c]							
1	9.2	pos	pos	pos	m	pos	m
2	15.15	neg	neg	neg	**f**	neg	**m**
3	11.8	neg	neg	neg	m	neg	m
4	9.1	neg	**pos**	neg	m	neg	m
5	17.2	**pos**		**pos**	m	neg	m
6	13.5	pos	pos	pos	f	pos	f
7	16	neg	neg	neg	m	neg	m
8	16.1	neg		neg	m	neg	m
9	29.1	pos		pos	m	pos	m
10	9.8	pos		pos	m	pos	m
11	25.6	pos		pos	f	pos	f
n/incorrect		**11/1**	**6/1**	**11/1**	**11/1**		
mean	15.6						
SD	6.5						
(d)[d]							
n/incorrect		46/6	17/5	46/6	46/5		
% incorrect		13%	29%	13%	11%		
mean	15.4						
SD	6.4						

[a]For RhD determination real-time PCR using TaqMan probes (**Taq**) were compared with a Thermal Cycler method (**Thermo**). In two samples PCR methods established on LightCycler with melting curve analysis (**LC**) were used. In the **Taq** group, the RhD type was incorrectly identified for two samples whereas three samples of the Thermal Cycler group were incorrectly identified. Using LightCycler with melting curve analysis one sample was correctly and one was incorrectly identified. **SRY** gene (detected using LightCycler system) was incorrectly determined in two samples. The mean of cell-free **DNA** in the first trimester was 14.9 pg/μL (SD 4.2).

[b]In the **Taq** group, the RhD type was incorrectly identified for three samples and two samples were incorrectly typed by the Thermal Cycler (**Thermo**). Using LightCycler with melting curve analysis (**LC**), three out of nine samples were incorrectly RhD typed using this method. **SRY** gene (detected using LightCycler system) was incorrectly determined in two samples. The mean of cell-free **DNA** in the second trimester was 15.4 pg/μL (SD 4.9).

[c]In the **Taq** group as well as the Thermal Cycler (**Thermo**) group, the RhD determination was incorrect for one sample. Using the LightCycler with melting curve analysis (**LC**), the RhD determination was incorrect for one out of six samples. **SRY** gene (detected using LightCycler system) was incorrectly determined in one sample. The mean of cell-free **DNA** in the third trimester was 15.6 pg/μL (SD 6.5).

[d]Results of all three trimesters of pregnancy are summarized. The mean of cell-free **DNA** in all trimesters was 15.4 pg/μL (SD 6.4).

curve analysis. In this group five samples incorrectly determined the RhD type.

Gender determination was done using conventional Thermal Cycler method.

From all 46 samples 41 were correctly determined. Gender misidentifications occurred in each trimester with two errors in first, two in the second, and one in the third trimester.

DISCUSSION

It has been shown that during pregnancy the concentrations of cell-free plasma DNA increase and they reach the highest values in the third trimester of pregnancy (6). We observed the same tendency in our data, although no statistical significance was found. This observation could be owing to a small sample number of patients in the first, second, and third trimester.

Though PCR methods are known to have high sensitivities and specificities in determining fetal RhD type using maternal plasma DNA, different analytical validities are reported for these methods (7,8). Finning et al. tested for RhD on three different exons in replicate (four times for each exon) (5). This group predicted the fetus to be D-positive when at least two positive signals are obtained from each RhD exon plus a total of three more positive signals from any exon. This means that at least 9 of 12 PCRs performed (75%) must be positive to render a positive result.

In a meta-analysis of 37 publications the overall diagnostic accuracy was 94.8% (9).

Recently, other authors showed 100% accuracy when tested on two different exons (8–12) from the 16th–36th week of gestation. In a study of Bischoff et al. RhD PCR results were compared between conventional and real-time methods. In this work the sensitivity of real-time PCR was 62 vs. 50% for conventional PCR (7). In our study RhD determination using real-time PCR with TaqMan probes had the same accuracy, when compared with Thermal Cycler technology. The accuracy in our study was 87–89%.

CONCLUSION

In our study we wanted to evaluate the method with the highest sensitivity appropriate for routine use to determine fetal Rhesus from maternal plasma. RhD determinations with both technologies are useful methods for fetal RhD prediction. To increase the accuracy of RhD determination it is necessary to test at least two exons (5,7,13) using a kind of duplex strategy for two specific RhD products. The detection of fetal RhD from maternal plasma DNA during pregnancy is certainly a promising noninvasive method, which should be implemented in clinical practice.

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