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Non-invasive prenatal determination of fetal RhD genotyping from maternal plasma: a preliminary study in Pakistan

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ORIGINAL ARTICLE

Non-Invasive Prenatal Determination of Fetal RhD Genotyping from Maternal Plasma: A Preliminary Study in Pakistan

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ABSTRACT

Objective: To determine the accuracy of the non-invasive pre-natal real-time polymerase chain reaction based fetal RhD genotyping from maternal plasma.

Study Design: Cross-sectional study.

Place and Duration of Study: Juma Health Sciences Research Laboratory, The Aga Khan University Hospital, Karachi, from July to December 2008.

Methodology: Cell-free plasma DNA from 21 D-negative women with D-positive spouse between 20-39 weeks of gestation was tested for the presence of exon 5 region of RhD gene using real-time polymerase chain reaction. β-globin was employed as the house-keeping gene. Sensitivity and specificity of the real-time PCR-based non-invasive fetal RhD genotyping was obtained by calculating proportion of the D-positive fetuses that were D-positive at birth as well.

Results: Of the 21 D-negative women 13 and 8 neonates were determined to be D-positive and D-negative, respectively, by serologic studies on cord blood samples at birth. RhD status was correctly determined in 17 of 21 cases. There were three false-positive and one false-negative results. The sensitivity and specificity of the assay was 92.3% (95% CI: 62.1, 99.6) and 62.5% (95% CI: 25.9, 89.8), respectively. The positive and negative predictive value of the assay was 80% (95% CI: 51.4, 94.7) and 83.3% (36.5, 99.1), respectively.

Conclusion: These preliminary results demonstrate the feasibility of non-invasive pre-natal diagnosis of fetal RhD status of D-negative mothers in Pakistan.

Key words: *Non-invasive pre-natal diagnosis. RhD genotyping. Cell-free fetal DNA. Real-time polymerase chain reaction.*

INTRODUCTION

Rhesus (Rh) D blood group incompatibility between a pregnant woman and her fetus is a significant problem due to the possibility of maternal allo-immunization and consequent severe hemolytic-disease of the fetus and newborn (HDFN), resulting in hydrops fetalis and fetal death.[1]

For D-negative immunized pregnant women with heterozygous partner, it is important to know the RhD type of the fetus to assess the risk of HDFN. This involves intrauterine testing by which the fetal RhD status can be predicted with high degree of accuracy by polymerase chain reaction (PCR) amplification of fetal DNA extracted from amniocytes or chorionic villi.[2] Pre-natal determination of RhD status in these cases is clinically useful because no further testing or therapeutic procedures will be necessary if the fetus is D- negative.[3] If the fetus is D-positive, further studies will be required to demonstrate the level of fetal hemolysis by invasive testing.[4] In Pakistan, invasive procedures carry as high as 5% risk of inducing spontaneous miscarriage[5] and is not widely available, with few centres carrying out invasive testing across the country. Furthermore, this procedure causes a significant risk of enhancing maternal antibody levels in allo-immunized women.[6] On the other hand, non-sensitized D-negative mothers are offered anti-D prophylaxis routinely in the third trimester.[7] This exposes them to unnecessary administration of anti-D as 40% of them carry a D-negative fetus.[8]

Based on the methods devised by the SAFE (special non-invasive advances in fetal and neonatal evaluation) network of excellence,[9] fetal RhD status can be identified using fetal DNA extracted from maternal plasma samples from D-negative women with serologically positive spouse. The objective of this study was to determine the accuracy of the non-invasive pre-natal determination of real-time polymerase chain reaction based fetal RhD genotyping from maternal plasma.

METHODOLOGY

Peripheral blood samples (5-10 ml) were collected from 21 D-negative pregnant women visiting the Aga Khan Hospital for Women and Children, Kharadar, Karachi (Aga Khan Health Services, Pakistan) between 20-39 weeks of gestation, during routine pre-natal visit from July to December 2008. Ten ml blood sample was also collected from D-positive woman to establish the real-time PCR system and to use as positive control. Informed consent was obtained from the women before

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drawing the blood. Approval for the study was provided by the Aga Khan University Hospital Ethics Review Committee. All the samples were brought to the Aga Khan University, Juma Health Sciences Research Lab within 24 hours of collection for further processing. Fetal RhD status was unknown at the time of collection; it was later confirmed with serological D typing on cord blood taken routinely at birth.

The EDTA blood was transferred into 15 ml polypropylene centrifuge tubes (Alpha Laboratories, Hampshire, UK) and centrifuged at 2760 rpm for 15 minutes. The exact volume of the blood and plasma obtained was recorded. Care was taken to ensure that the buffy coat was not disturbed. Maternal leukocytes obtained from the buffy coat were utilized as D-negative control. Plasma aliquots were prepared in 1.5 ml Appendorf tubes and stored at -80^{o}C until further processing. On the day of analysis, the plasma was thawed and again centrifuged at 14000 rpm for 15 minutes. Aerosol resistant tips (Molecular BioProducts Inc, San Diego, CA) were used for plasma separation and extraction of DNA.

DNA was extracted from the Rh-negative maternal leukocytes by use of the QIA amp Blood Mini Kit (Qiagen, Hilden, Germany) according to the protocol recommended by the manufacturer. An elution volume of 100 µl was used for the final washing. The purity and the yield obtained were measured using the mass spectrophotometer, Beckman_DU 650 (Beckman Coulter, Fullerton, CA) where the A260/A280 ratio between 1.8 and 2.1 was considered acceptable.

DNA was extracted from the plasma by use of the QIA amp Blood Mini Kit (Qiagen, Hilden, Germany). The volume of the plasma used was between 1-2 ml. The reagent volumes were scaled upto accommodate the increased plasma volume in each extraction. Extractions were performed in 400 µl aliquots and used 40 µl of proteinase K (Qiagen, Hilden, Germany) for digestion. The samples were loaded on the spin-column by repeated centrifugation and the DNA was eluted in 60 µl of elution buffer. Before elution, the columns and elution buffer were pre-warmed to 55^{o}C for 5 minutes on a heating block. DNA extraction was performed in a class-II safety cabinet (The Baker Company, Exton, PA).

Real-time PCR procedure was established for the RhD gene system according to the Safe Network Protocol with modifications. Real-time quantitative PCR analysis was performed in the Chromo4 Real-time PCR Detector (BioRad, Hercules, CA), which is a combined thermal cycler/fluorescence detector. In addition to the two amplification primers, RhD exon 5 forward 5'CGC CCT CTT CTT GTG GAT G3' and reverse 5'GAA CAC GGC ATT CTT CCT TTC3', a dual-labeled fluorogenic hybridization probe 5'(FAM) TCT GGC CAA GTT TCA ACT CTG CTC TGC T (BHQ) 3')[1] was also included. One fluorescent dye (6 carboxyfluorescein FAM) served as a reporter while its emission was quenched by a second reporter dye (black hole quencher BHQ). The primers and probe were targeted towards the exon 5 region of the RhD gene1. β-globin forward 5'GTG CAC CTG ACT CCT GAG GAG A 3'; β-globin reverse 5'CCT TGA TAC CAA CCT GCC CAG 3'; and β-globin probe 5'(FAM) AAG GTG AAC GTG GAT GAA GTT GGT GG (BHQ) 3' was used as a house-keeping gene to detect the presence of total DNA. The high-performance liquid chromatography-purified primers and probes were obtained from BioSearch Technologies Inc, (Novato, CA). The primers and probes arrived in lipholysed form which were then dissolved in 1X Tris buffer pH 8.0 and used at a final concentration of 100 mM.

All the samples were run in duplicate in a reaction volume of 25 µl with 5 µl DNA. Reaction conditions were as follows: 1X Go Flexi colorless buffer (Promega, Madison, WI), 5.5 mM $MgCl_2$ (Promega, Madison, WI), 0.2 mM of each DNTPs (deoxyribo-nucleotide phosphates) (Promega, Madison, WI), 450 nM forward and 450 nM reverse primers, 125 nM of TaqMan probe (BioSearch Technologies Inc., Novato, CA) and 1 U of Taq Polymerase (Promega, Madison, WI). PCR was performed for 50 cycles with 5 minutes initial heating at 95^{o}C, 15 seconds denaturation at 95^{o}C and 1 minute annealing and extension at 55^{o}C. To ensure quality of the RhD PCR assay, control reactions containing D-positive and D-negative DNA were included with every experiment. Water-blanks were also included with all the reactions as negative-control to detect possible contamination. Strict anti-contamination precautions were implemented throughout the experiments.

Positive predictive value was obtained by calculating proportion of D-positive neonates that were identified to have D-positive status antenatally. Negative predictive value was obtained by taking proportion of D-negative neonates that were determined antenatally to have D-negative status. The 95% confidence intervals were calculated using Epi Info version 6.[10]

The RhD PCR system was established according to the Safe Network protocol with minor modifications. To detect the sensitivity of the assay, the minimum detectable DNA was identified by serially diluting DNA enriched from a D-positive blood sample. A total of four 1:10-dilutions in distilled autoclaved water were prepared and ran with each assay (Figure 1A). Standard curve formation revealed that as the total DNA decreased, the minimum cycles required to produce detectable amounts of the fluorescent reporter molecule increased (Figure 1B).

RESULTS

The total minimum detectable DNA was 18 Genomic Equivalence (GE). Of the 21 D-negative women, 13 were identified to have D-positive neonates and 8 to

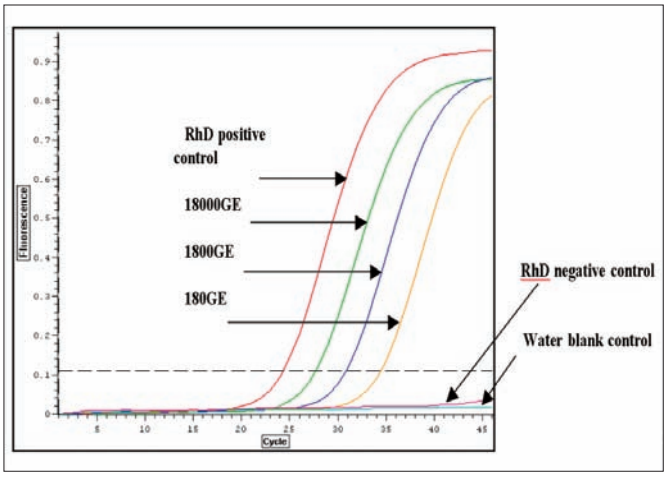

Figure 1A: Sensitivity of the RhD PCR system. The minimum DNA detected was 18GE.

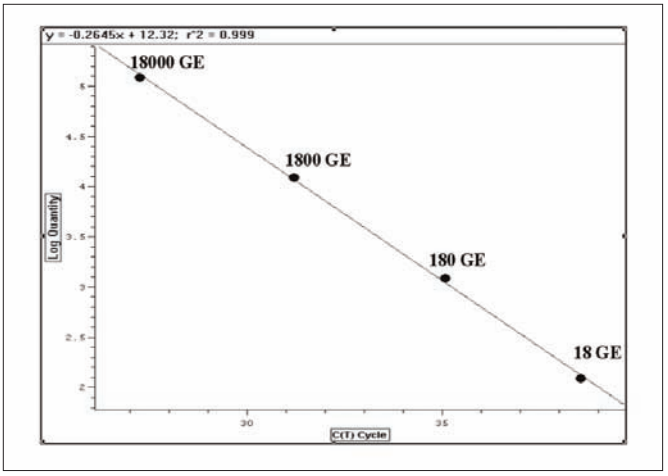

Figure 1B: Real time fluorogenic RhD PCR analysis of four-1:10 serially diluted samples. Standard curve depicts the relationship between DNA concentration and C_1 value. The C_1 value increases as the DNA concentration decreases.

have D-negative neonates at birth by serological testing on cord blood samples. Of the 13 D-positive neonates, 12 were antenatally correctly predicted to be positive for exon 5 of RhD gene sequence in maternal plasma with one false-negative result. Five out of 8 D-negative neonates were antenatally predicted to have absence of RhD gene sequence. There were three false-positive results (Table I). The sensitivity and specificity of the assay was 92.3% (95% CI: 62.1, 99.6) and 62.5% (95% CI: 25.9, 89.8), respectively. The positive and negative predictive value of the assay was 80% (95% CI: 51.4, 94.7) and 83.3% (95% CI: 36.5, 99.1), respectively. The level of accuracy in the 21 samples analyzed was found to be 80%. To validate the presence of DNA, β-globin

Table I: Results of RhD genotyping of fetuses of RhD negative women.

	Fetal RhD genotyping at birth (cord blood)		
Fetal RhD genotyping from maternal plasma	RhD-positive	RhD-negative	Total
RhD-positive	12	3	15
RhD-negative	1	5	6
Total	13	8	21

was employed as a positive control. β-globin gene was found in all the plasma samples. DNA extracted from D-positive maternal sample and D-negative maternal sample was included in all the assays. Figure 2 illustrates the results of the real-time RhD PCR system from DNA enriched from three maternal plasma samples, of which two were diagnosed to carry D-positive fetuses and one was diagnosed as carrying D-negative fetus.

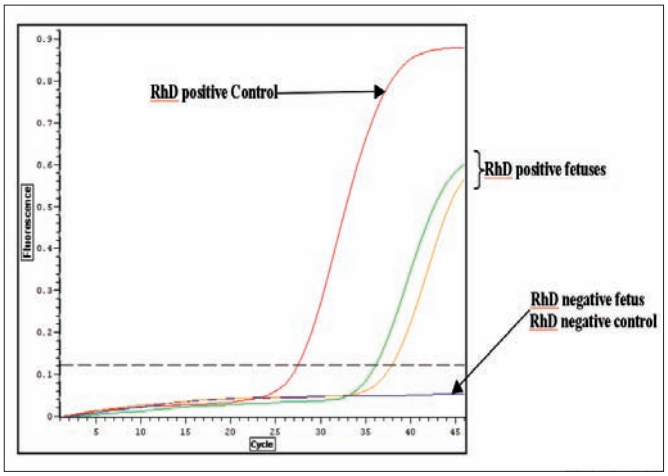

Figure 2: Presence of fetal RhD DNA in maternal samples using real-time PCR fluorogenic detection for the exon 5 region. The controls include D-positive and D-negative DNA from maternal white blood cells. The figure shows two D-positive fetuses and one D-negative fetus.

DISCUSSION

Our prospective preliminary study demonstrates 80% accuracy of non-invasive fetal RhD genotyping using DNA extracted from plasma of serologically verified D-negative women. SAFE Network is a European Union funded network that aims to optimize current protocols used for the non-invasive detection of the fetal RhD status so as to determine the efficacy of this technology in large multicentre clinical studies.[9]

Since the first report of successful isolation of cell-free fetal DNA from maternal circulation by Lo and his group,[11] several groups have utilized this novel technology for non-invasive identification of fetal RhD status from maternal circulation.[1,3,12,13] Currently, three centres across Europe namely in Bristol UK, Netherlands, and France are offering this state-of-the-art technology as a clinical service.[2,14,15] In Pakistan, this group is the first to carry out this preliminary investigation.

False-positive results could be due to genetic variations in our population since this assay only targeted the exon 5 region. The method employed was based on the Safe Network protocol, designed according to the genotypes found in the Caucasian population. Due to the genetic complexity of the RhD gene system, deletions in other regions which may give rise to a serological negative test could have resulted in RhD positive genotype.[13] This is commonly observed in people of African descent where the RhD pseudogene occurs with considerable

high frequency.[16] The Safe Network is currently devising a multiplex assay which will target all the commonly deleted regions.[17] However, the study is being carried out on the Caucasian populations which may comprise a different polymorphic pattern compared to our population. Even though strict anti-contamination techniques were employed, contamination could be possible during sample handling and transport resulting in false-positive outcomes. The false negative result noted in this study could be due to either absence of fetal DNA in the maternal plasma sample analyzed, low circulating fetal DNA concentrations, fetal DNA loss during sample processing or failure to amplify the DNA. Standard procedures use the Y-chromosome sequences as positive controls to prove the presence of fetal DNA in maternal circulation.[18] This cannot serve adequately as positive controls as they are only applicable to pregnancies carrying a male fetus.

Recently, it has been demonstrated that epigenetic modifications in fetal DNA differ from the maternal DNA in the placenta[19] and could be used as a positive control to confirm the presence of fetal DNA in the maternal plasma sample, hence, eliminating the possibility of false-negative results.[20]

Future work in Pakistan would require firstly, a large scale population study in order to determine the RhD gene sequence polymorphisms in various ethnic groups. This information would help us identify whether there is a complete deletion of the RhD gene or are there any variants existing according to ethnicity. This will help in correctly diagnosing the fetal RhD genotype by developing specific primer and probe combinations. Secondly, validation of the RhD PCR system would be required on a larger sample size in our population.

CONCLUSION

Maternal plasma based assay for non-invasive pre-natal diagnosis of fetal RhD genotype provided markedly accurate results obviating the need for invasive procedure and enabling targeting of pregnancies that need close monitoring. Further, fetal RhD typing in non-sensitized pregnant women in early pregnancy can allow reduced anti-D administration and less exposure to human blood products.

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