FOETAL RHESUS D AND SEX GENOTYPING FROM THE PLASMAS OF RH-NEGATIVE PREGNANT WOMEN IN DUHOK

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ABSTRACT

Background: This prospective study was done to evaluate the benefit of Real-time Polymerase Chain Reaction in the determination of RHD and SRY of the fetuses in the plasma of Rhesus D-negative enrolled ladies.

Methods: Thirty-nine pregnant Rh-negative females with (RhD+) partners were registered in this study. Blood samples were taken from those participants whose pregnancy age was from 20 -28 weeks for the purposes of fetal RHD genotyping and DNA extraction/purification. Real-time PCR was used to determine the RH and sex genotype using particular RHD, SRY, and GLO gene primers and probes.

Results: Out of the total 39 samples, 28/39(71.8 %) were positive for Rhesus D antigen and 11/39 (28.2 %) were negative. The results of prenatal fetal RHD genotyping based on exons 7, and 10 combined were concordant with postnatal RhD phenotyping in 94.9% of cases (37/39) with a sensitivity & specificity of 93.3 and 100% respectively, and precision was 100%. For the gene SYR, there was a concordance rate of 92.3% between the fetal sex genotype and the newborn sex with one false-positive (2.6%) and two false negatives (5.1%) results leading to a precision of 95.2%.

Conclusion: The study demonstrated great accuracy of the Real-Time PCR technique through the usage of cffDNA for the genetic study of the fetal RHD and SYR genes and implicates the effectiveness of this procedure for predicting the necessity of Anti-D immunoprophylaxis in pregnant women whose fetuses have a peril of hemolytic anemia of the newborn due to RhD incompatibility between partners.

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Keywords: Rhesus D, Sex prediction, Fetal DNA, Maternal's Plasma.

Among the highly complicated and polymorphous blood group systems found in human beings is the Rh blood group.(1)

Clinically, Only ABO is more significant than the Rhesus blood group system. The Rhesus system is made up of the specific genes of the RHD and RHCE that are of high homogeneity and are located on Exon 10 of chromosome 1. The phenotype for RhD negativity is brought on by whole absence of the RHD gene, a change that is frequent among people of Caucasians origin (15-17%).(2)

The D, C, E, c, and e antigens of the Rh group system are encoded by the RHD and RHCE and there are a minimum of 45 different antigens in this system.(3)

The fetus has a 50 % chance to become positive for RhD (RhD Ag), if the father is also positive but the mother is negative for Rhesus D antigen, alloimmunization may occur due to fetomaternal hemorrhage and the mother might produce alloantibodies against fetal RhD antigens during the pregnancy and after delivery resulting in severe hemolysis in the fetus and newborn baby (HDFN) in a following pregncncy.(4)

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Because the RhD status of the fetus cannot be defined till birth, it is common practice in many nations, including Iraq, to give prophylactic anti-D immunoglobulin antenatally to most of the pregnant RhD-negative women when there is Rhesus D incompatibility between both partners. Numerous women who are expecting and carry fetuses that are RhD-negative receive unneeded immunoglobulin D.[5]

After accurately determining the fetuses' RHD genotype, RhD-negative pregnant ladies can be purposefully offered the anti-D immunoglobulin.[6, 7]

This study's objective was to examine the concord rate of the genotyping study for RHD and SRY genes by RT- PCR with the newborn Rh phenotype and gender determination.

SUBJECTS AND METHODS

Study Design and participants: This prospective study was done at the Maternity Teaching Hospital, Duhok, Iraq. The study was conducted from December 2021 to December 2022 over a one-year period. At the obstetric clinic, 39 pregnant, healthy looking RhD-negative women who coupled RhD-positive men were recruited on purpose. The objectives of the study were explained to them, permission was taken from the enrollees of the study.

Ethical Considerations: Duhok Directorate General of Health's Research Ethics Committee approved this study with a registration No. 24102021-10-34 on 24/10/2021. Pregnant women who decided to participate, provided written informed consent. Respondents were given the option to participate voluntarily, were assured that the information collected would only be used for research and academic reasons, and were given the choice to withdraw from the study whenever they want.

Sample Collection and Preparation. Six mls of the blood were drawn from the participants and were put in two separate EDTA anticoagulated tubes and plasma prepared within the first 2 hours of sample collection. Blood samples were first centrifuged at 1200 g according to Hromadnikova et al. (8) protocol 1, plasma samples were then recentrifuged at 3000 g (protocol 2) for ten minutes, according to Lo et al. (9) protocol 2 and until further processing, the supernatants were collected and kept at -20 oC.

Inclusion criteria:

Pregnant women with Rh(D) negative and with RhD positive partners.

Gestation age between (20 – 28) weeks.

All of the pregnant women had never had a transplant and had not received blood transfusions during the previous three months before sample collection, however, pregnant women with gestational age < 20 weeks and those > 28 weeks were excluded from the study.

DNA Extraction and Purification: DNA extraction from 200 µl of plasma was done using AddPrep Genomic DNA Extraction Kit (addbioinc, Korea) along with the producer's instructions. After cfDNA was extracted, it was assessed using a Nanodrop 2000 spectrophotometer for concentration and purification at 260/280 for protein contamination, which was between 1.8 and 2.0, and 260/230 for organic contamination, which was between 2.0 and 2.2. The final volume of DNA eluted was 50 µl, and it was kept at -20°C until further processing. The PCR reaction's template consisted of three µl of DNA.

Real-time PCR Analysis:

The RhD genotype and fetal sex were determined by RT-PCR, by using particular probes and primers for the study's intended genes (Eurofins, Germany). In the present research, the exons (Seven and Ten) of the RHD gene were used to boost the assay's specificity, and the GLO and SRY genes were amplified to confirm that the sample contains DNA. Table 1 illustrates the primer and probe sequences. To ensure that DNA was present and of high quality in each sample, the GLO gene was used as a control (Lo et al. 1998b & Hromadnikova et al. 2003)[8,9]. Amplicons of GLO control genes were spotted in the studied samples.

Each amplification reaction was set up using the Universal Real-time PCR master mix-2 (SNP Biotechnology, Turkey) in a reaction volume of 25 µl. The smallest concentrations of the primer and probe that yield the greatest normalized reporter were determined by adjusting the primers and probes. RHD exon 10 and RHD exon 7 and GLO probes were utilized at doses of 100 and 200 nM, respectively. At final concentrations of 200 nM and 300 nM, PCR primers were used. In 8-well reaction optical tubes/strips (LightCycler 96, Roch), DNA amplifications were conducted. The initial denaturation stage of the PCR was planned to last 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Every sample was examined at least twice, and each experiment included a positive control (a male with the D phenotype) and a negative control (a female with the dd phenotype). A sample was considered positive when one or more individual replicate was positive at threshold cycle (Ct) 40.

Table1: RT-PCR Primers and Probes sequences (RHD, SRY, and Beta-Globin genes).

Gene	Primers (Forward & Reverse)	Probes
RHD exon 10	5-CTCCATCATGGGCTACAA-3 5-CCGGCTCCGACGGTATC-3	5-(FAM)AGCAGCACAATGTAGATGATCTCTCCA(TAMRA)-3
RHD exon 7	5-TGGCGATTAAGTCAAATTCGC-3 5-CCCCCTAGTACCCTGACAATGTATT-3	5(FAM)AGCAGTAGAGCAGTCAGGGAGGCAGA(TAMRA)-3
SRY	5-GTGCACCTGACTCCTGAGGAG-3 5-CCTTGATACCAACCTGCCCAG-3	5-(FAM)AAGGTGAACGTGGATGAAGTTGGTGG(TAMRA)-3
GLO	5-CCTCTCACTGTTGCCTGCATT-3 5-AGTGCCTGCGCGAACATT-3	5-(FAM)TACGTGAGAAACGCTCATGACAGCAAAGTCT (TAMRA)-3

Statistical Analysis: The results were expressed in median, frequency and percentage. The associations and concordances between genotype and postnatal phenotype data were determined using the X2 and Cohen's Kappa analysis. Additionally, diagnostic measures including specificity, sensitivity, and accuracy applied.

4. RESULTS

The study was carried out at Duhok Maternity Hospital, Duhok province/Iraq. Among the 60 Rh-negative pregnant women enrolled in this study, only 39 responded to recall for post-delivery neonatal RhD and gender phenotyping.

4.1 The social and demographic characters of the participants:

The mean age of the participant was 26.6 yrs, and the typical pregnancy age at blood collection sample collection was 24.7 weeks (range 20 – 28 weeks), pregnant women were categorized into three age groups. None of the ladies had a prior history of organ or bone marrow transplants and had not received blood transfusions during the previous three months. The social and demographic features of the pregnant enrollee are demonstrated in Table 2.

Table 2: Sociodemographic characteristics of the study of pregnant women:

Variable	Frequency	%
Age (Years)		
Group A-15-25	14	35.9
Group B- 25-35	20	51.3
Group C- >35	5	12.8
The educational level of pregnant women		
Basic	21	53.8
Intermediate (Intermediate or preparatory school)?	14	35.9
Advanced (Bachelor or Diploma)	4	10.3
Alcohol consumption		
Consumption	0	0
Non-consumption	39	100
Smoking habit		
Yes	3	7.7
No	36	92.3
Disease		
Diabetes mellitus	1	2.6
Normal	38	97.4
Consanguineous partners		
Yes	14	35.9
No	25	64.1

4.2 Prenatal genotyping of fetal Rhesus D and postnatal confirmation of its phenotype Thirty-nine pregnant RhD -- mothers with RhD + partners., RhD status diagnosis and gender analysis were done by prenatal non-invasive measures. From the 39 newborn babies taken in this study, 28/39(71.8 %) were RhD positive and 11/39 (28.2 %) were RhD negative. The results of real-time PCR for prenatal fetal RHD genotyping test showed 30/39(76.9%) were positive for RhD and 9/39 (23.1%) were negative. RhD phenotyping of the neonates showed 28/39(71.8 %) were RhD positive and 11/39 (28.2 %) were RhD negative. When compared to the serological results the concordance rate between genetic study and post-delivery rhesus D phenotype was 94.9%, with two false-positive (5.1% of the total) in patients numbered (8,23) and no false-negative results (0% of the total), the observed sensitivity and specificity were 100% & 81.8% respectively with a precision of 100% as shown in Table 4.

4.3 Maternal plasma fetal sex genotyping and postnatal phenotypic confirmation:

The gender determination of the 39 infants considered for this study showed 22/39(56.4 %) were males and 17/39(43.6%) were females. Fetal sex genotype by real-time PCR showed 21/39(53.8%) of fetuses were positive for the SRY gene and 18/39 (46.2%) were negative, giving a concordance rate of 92.3% between the fetal sex genotype and the newborn sex. One false-positive (2.6%) in patient number (16) and two false negatives (5.1%) in patients numbered (8,30) were recorded, leading to a sensitivity of 90.9% and a specificity of 94.1%, with a precision of 95.2%. Table 4 Furthermore, because fetal DNA was detected in all of the examined samples, the GLO gene used this finding as a positive marker for the presence of fetal DNA in maternal plasma.

Table 3: Fetal Sex, RHD genotype from maternal plasma, and their confirmation after delivery.

Fetal genotyping							
No.	**Age**	**Gestation weeks**	**Exon 7**	**Exon 10**	**SRY**	**Neonatal RhD phenotype**	**Gender of neonates**
8	35	28	-	+	-	-	Male
16	20	21	-	-	+	-	Female
23	31	21	-	+	+	-	Male
30	21	25	+	+	-	+	Male
31	25	20	-	-	-	-	Female

+: Positive, -: Negative

Table 4: Statistical analysis and diagnostic tests for fetal genotype & neonatal phenotype.

Genotyping	Neonatal RhD Phenotype						
Fetal RHD Genotype	RhD +	RhD --	Concordance	Cohen's Kappa	Precision	Accuracy	P-value
RhD Exon 7							
Positive	28(71.8 %)	0(0.0%)	100%	100%	100%	100%	<0.0001
Negative	0(0.0%)	11(28.2%)					
RhD Exon 10							
Positive	28(71.8 %)	2(5.1%)	94.9%	86.6%	93.3%	94.9%	<0.0001
Negative	0(0.0%)	9(23.1%)					
Fetal Gender	Neonatal gender						
SRY	Male	Female					
Positive	20(51.3%)	1(2.6%)	92.3%	83.9%	95.2%	92.3%	<0.0001
Negative	2(5.1%)	16(41.0%)					

DISCUSSION:

Based on the presence of Cell-Free fetal DNA materials in the maternal plasma, non-invasive prenatal diagnosis (NIPD) of the foetal RHD is performed.[5]

Genotype study of the fetal RHD may help in identifying fetuses having the peril of hemolysis before and during neonatal life.[3]

RhD typing needs to be done on more than a single region of RHD gene because the Rh system is complex and the possibility of getting erroneous findings.[10] Consequently, in this study, RHD-specific exons[7 & 10] were amplified as part of the genotyping assay for fetal RHD in maternal plasma.

Low cffDNA concentrations could lead to false-negative results, especially in the early weeks of gestation and can give false negative results, furthermore, with increasing gestational age, the quantity of cffDNA in plasma surges over the trimester of pregnancy.[11, 12], therefore in this study we selected pregnant women whose gestational ages were 20-28 week.

The incidence of false-positive rates for RHD and SRY genes was 2.6 and 5.1% respectively and was relatively lower when compared to the 5.9% reported by Oliveira et al.[1] and false-negative results for RHD was not reported in this study, similarly to prior studies by Moezzi et al., Aykut et al. and Hromadnikova et al.[8, 13, 14]. Regarding SYR genotyping, two false positive results were recorded with a lower sensitivity and specificity rate compared to those for Moezzi et al., Aykut et al. and Hromadnikova et al.[8, 13, 14], this is probably related to the age of gestation included in

their studies and this may be due to the fact that the quantity of circulating DNA of the fetus in maternal plasma increases sharply toward the end of pregnancy.[9]

The high sensitivity 100% and specificity 81.8% supports the administration of RhD immunoglobulin prophylaxis to all pregnant RhD-negative mothers carrying RHD-positive fetuses. Based on the study results, unnecessary anti-D prophylaxis would have been given to 28.20% of the RhD-negative pregnant women if the fetal RhD genotype is not determined, a similar finding was also reported by Otchere et al., Moezzi 2016, and Aykut et al.[13-15]. On the other hand, Bohmova et al., Haimila et al. and Hyland et al.[16-18] reported higher sensitivity and specificity of PCR method in determination of both fetal Rh D and sex, such variation may also be related to the sample size of the study and the gestational age of the enrollee.

Regarding the pre-natal testing for the SRY gene, 21/39(53.8%) of fetuses were expected to be males and the remaining 18/39 (46.2%) were females. After delivery, the predictable number of males and females was confirmed to be 22/39 (56.4%) and 17/39 (43.6%), respectively. (concordance = 92.3%, κ = 83.9%, diagnostic accuracy = 95.2%) (Table 3). There are variations in the research' sensitivity for genotyping fetal DNA from maternal plasma., our findings are similar to those reported by Zhou et al. and Aykut et al.[13, 19] but were contrasting and even higher than the 70.0% reported from Bischoff et al. study.[20]

This study also shown the feasibility and potential for clinical practicing of fetal Rhesus D genotyping from the maternal plasma, similar findings was reported by other researcher.[21]

CONCLUSION:

According to this study, noninvasive genetic study of the fetal SRY and RHD by PCR is very sensitive, accurate, and it may be beneficial for predicting RhD type of fetuses having risk of hemolytic disease of fetuses and newborns due to anti-D alloimmunization.

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الخلاصة

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الخلفية والأهداف: .....

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