The Determination of Fetal RhD Status from Maternal Blood in Serbia

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Abstract The aim of this research was to demonstrate: The specificity, sensitivity, the positive and negative prediction value of the PCR method in the detection of the fetal RhD status from maternal blood in various weeks of gestation. Which was the earliest gestational week, when is possible to get a reliable result? How did the presence of RhD antibodies in sensitized mothers affect the results? This was a prospective clinical study within a multidisciplinary project approved by the Ministry of Science of the Republic of Serbia, 2011. The study was approved by the Ethics Committee of the Medical Faculty in Belgrade. Ninety six RhD negative patients had participated in the study, with written consent. The sensitivity of the determination of the RhD status of the fetus from the mother's blood after the 7th GW in our sample was 98.6%, with a specificity of 92%. The positive predictive value of the test was 97% and the negative predictive value was 95.6%. False positive results were detected in 2.6% of cases and 4.5% of cases were false negative. This is a reliable test which should be used in monitoring of pregnancies of RhD negative mothers.

Keywords Fetal RhD · Pregnancy · Maternal blood

Introduction

Rhesus D antigen negative mothers make up 10% of total patients. Monitoring of these pregnancies includes the determination of the fathers RhD status. Should he be a

homozygous, all fetuses will be RhD positive. If he is a heterozygous least 25% of fetuses may be RhD negative. For the first group of couples, it is necessary to determine the antibodies and apply RhD immunoglobulins, while the determination of the fetal blood type from maternal blood is advised to the second group. This test has a positive predictive value of a 100% and a negative predictive value of 97% [1, 2].

Through use of the cell free fetal deoxyribonucleic acid method it is possible to determine the RhD status of the fetus from maternal blood, which is a form of non-invasive fetal screening. The reliability of the method is 99–100% [3, 4]. Geifman-Holtzman meta analysis [5], has shown that the results are unreliable in 3% of cases, most often due to an insufficient amount of cf fDNA, or different variants of the D antigen on the membrane of the erythrocytes.

The indications for fetal RhD determination are:

1. Expecting mothers who are immunized so that, in case of RhD negative fetus, diagnostic procedures for monitoring and evaluating the degree of fetal anemia would not be applied.
2. Expecting mothers who are not immunized, with heterozygous partner, where prophylaxis should be applied, if the fetus is positive.

The Aim

The aim of this research was to determine:

1. The specificity, sensitivity, the positive and negative prediction value of the PCR method in the detection of the fetal RhD status from maternal blood in various weeks of gestation.

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2. Which was the earliest gestational week, when is possible to get a reliable result?
3. How did the presence of RhD antibodies in sensitized mothers effect the results?
4. The specificity and sensitivity of the Polymerase chain reaction method in detection of fetus gender through maternal blood in different weeks of gestation.

Methods and Materials

Ethics

This was a prospective clinical study within a multidisciplinary project approved by the Ministry of Science of the Republic of Serbia, 2011. The study was approved by the Ethics Committee of the Medical Faculty in Belgrade. Ninety six RhD negative patients had participated in the study, monitored on the Clinic of Gynecology and Obstetrics Clinical Center of Serbia, while the analyses of the fetal RhD status and sex were done according to the already stated protocol in the “Konzilijum” laboratory.

Patients

The expecting mothers were divided into two groups:

1. Group 1 consisted of 20 pregnant women with Rh alloimmunization.
2. Group 2 consisted of 76 pregnant women who were non immunized.

All pregnant women delivered their babies in Clinic for obstetrics and gynecology, where the blood type and sex of the child was determined after birth.

PCR RhD

Samples of EDTA blood of RhD negative women were received in the genetics laboratory within a maximum of 6 h of venipuncture. Plasma was separated by centrifugation at 3000 rpm/10 min and stored frozen at -20°C until the insulation. Free-floating DNA from the plasma was isolated from Macherey–Nagel commercial NucleoSpin Plasma kit according to the manufacturer’s instructions. In parallel with the isolation of plasma sample in duplicate, and was isolated by the same amount of RNase free water as a negative control monitored the entire procedure. Cff-DNA is eluted with 30 μl of the elution buffer.

In order to prevent false-positive and false-negative results and verify the presence of fetal DNA, in the sample is required the use of CFF-DNA control, as well as the presence of sex determinišuće region of the Y

chromosome (SRY) gene as a marker of male DNA. The lack of such controls can lead to false-negative results.

The isolated samples were tested for the presence of RhD gene (exons 7 and 10), so that the complete deletion of the RHD gene could be detected. Sex determinative, Y SRY gene related detect the presence of male-cff DNA.

Real-time analysis is performed on an Applied Biosystems 7500 instrument under the following conditions: 2 min at 50°C followed by 10 min at 95°C , then 45 cycles of: 15 s at 95°C and 60 s at 60°C . Probes and primers have been disclosed previously (PB Gahan, Nucleic Acids and Circulating Plasma and Serum, 147 https://doi.org/10.1007/978-90-481-9382-0_20, C Springer, 2011) and available courtesy TADEJA DovDrnovLek from Center for Transfusion Slovenia. PCR mixes contained 5 cff-DNA, primers, probes, and TaqMan Universal Master Mix in a final volume of 20 ml.

PCR Gender

For each sample in duplicate were analyzed: two exon RhD gene, SRY control the presence of the Y chromosome, and a beta-globin control the presence of the DNA-Cff. Negative control was put in isolation one copy for RHD exons, beta globin and SRY. As a positive control samples were used known RhD positive male DNA also released in a single copy for RHD exons, Beta globin and SRY are regarded positive amplification with each Ct value of less than 40tog cycles (Sample RhD and SRY gene). The fetus is RhD having amplification in 2/2 of the replicates for both exons analyzed RhD gene is regarded as RhD positive. A fetus that has Rh gene amplification or positive for the presence of SRY gene in 2/2 replicates and tested positive for the presence of Beta-globin is considered RhD negative. Each RhD negative, negative SRY, Beta-globin positive is issued as RhD negative, except in cases of pregnancy under 20 weeks. In these cases, a definitive result is issued after repeating the analysis after entering pregnancy in the 20th week of pregnancy. When such a pregnant women exposed to any risk during the procedure prior to the issuance of the final result, analysis of the fetal RhD factors should be treated as a non-informative.

Statistics

Statistical analysis was carried out by Chi square test. Specificity, sensitivity, the positive and negative predictive value of the diagnostic methods, both in nonimmunized and immunized pregnant women, according to the following formulas:

Sensitivity = number of true positive/number of true positive + number of false-negative

Specificity = number of true negative/number of true negative + number of false positive

The positive predictive value = number of true positive/number of true positive and false positive

The negative predictive value = number of true negative/number of true negative and false-negative.

Results

The average age of the expectant mothers in the first group was 31 years, and in the second group 31.5 years. The percentage of women over 30 was 67%, and over 40 was 16.7%. The statistical analyses of the Chi square test did not show any significant difference between the groups.

The fetuses in the study had shown to be RhD negative in 23 of the cases or 24%, while 73 were RhD positive which makes up 76%. Statistically there were no differences between sensitized expecting mothers and women who were not sensitized. (Chi square 0.339, $p < 0.5$).

Analysis of the neonatal blood groups of immunized mothers showed a match in 17 cases (85%), 2 samples were repeated and 1 discrepancy (5%). In the group of nonimmunized pregnant women coincidence was noted in 69 babies (91%), 5 samples were repeated (6.5%), a mismatch was found in 2 newborns or 2.6%. Statistically there is no difference between the groups (Chi square 1.237, $p < 0.5$).

Three results that have not match were in 2 cases of male positive and 1 case of negative female. One patient from the first and two patients from the second group.

The analyses of the weeks of gestation when the maternal blood was taken showed that 14 tests (13.6%) were done before the 12th week of gestation, 31 tests (30%) were done between the 12th and 20th week, 38 (36.9%) were performed between the 20th and 30th gestational week and 20 (19.4%) tests were done after the 33rd week of gestation. The results have shown that the tests can be performed even in early pregnancy. In the case of one pregnant woman in her 8th week of pregnancy the results showed the fetus was RhD positive female, and in another case the fetus was RhD negative male. Both of these cases show that there were enough fetal cells present in order to get the results. The statistical analyses of the Chi square test did not show any significant difference between the groups. (Chi square 7.7, $h < 0.5$).

The analysis of the repeated results revealed that in one case, the repeated positive result was of a male fetus, 1 male negative, while all others were negative female fetuses, which samples, according to all the recommendations, should be repeated, especially if it is made before the 20th week of gestation which is evident from the Table 1. Only one sample was taken after the 21st GW and was

repeated in the 33rd GW, all the other samples were analyzed between the 7th and 14th GW.

The sensitivity of the determination of the RhD status of the fetus from the mother's blood after the 7th GW in our sample was 98.6%, with a specificity of 92%. The positive predictive value of the test was 97% and the negative predictive value was 95.6%. Analysis of the sex of the fetus was appropriate for all newborn babies.

Discussion

Denis Lo has shown in his study the possibilities of fetal RhD genotyping through using cf DNA from maternal blood. The advantage of this method is that it is completely non-invasive, so the risk for loss of pregnancy does not exist. It also eliminates the possibility of sensitizing the mother after fetomaternal hemorrhage, which occurs after the invasive diagnostic procedures when a needle passes the placenta. His study showed that the test is reliable after 12 GW, due to insufficient amount of DNA and fetal cells in maternal blood in smaller gestations. It is early enough that the prophylaxis or treatment of these pregnant women with anemic fetuses can be planned. If RhD negative mother is carrying an RhD negative baby, no RhD prophylaxis or antibody testing are necessary [6]. Our results showed that analysis can be successfully performed after 8th week of gestation.

Bench Clausen analyzed 12,668 pregnant women and their newborns, and concluded that 61.8% were RhD positive babies, and 38.2% were RhD negative. The sensitivity of the test on his material was 99.9% and the percentage of false negative was 0.087%. 11 cases showed a false negative result, a false positive in 41. Repetition was performed in 274 analysis, so 37.1% of women avoided unnecessary RhD immunoglobulin treatment. Their recommendation is that this analysis should be done after 25 GW of the pregnancy [1]. RhD distribution in our study was 24% Rh negative and 76% Rh positive fetuses.

A.T. Wikman in Sweden analyzed over 4000 pregnancies mean age of 10 GW and found 58.3% to be RhD positive fetuses, 37.7% RhD negative fetuses and 4% of the results which were repeated. After repeating only 14 remained non-affirmative, probably due to poor expression of the RhD gene. The rate of false negative was 2.4 and 1.1% false positive. His recommendation is that the analysis should be done after the 8th GW. The results of the second phase of the study showed that the specificity of this test is 99% from the 8th to the 22nd GW, and 100% after the 22nd GW [7]. We showed 2 false positive (2.7%) and 1 (4.3%) false negative fetus in our group.

S. Lyn Chitty's study involved 2288 patients and got 4913 results. 436 fetuses underwent one analysis, 1132

Table 1 Comparison of the results

	False +	False –	Sensitivity	Specificity	NPV PPV
Banch Clausen	0.03%	0.0087%	99.9%	Not available	Not available
A.T. Wikman	1.1%	2.4%	Not available	99%	Not available
Lyn S. Chitty	0.7%	0.8%	99.3%	Not available	Not available
Ranjit Akolekar	Not available	3.5%	98.2%	100%	100% 96.5%
Gefmann-Holtzman	Not available	Not available	98.6%	95.4%	Not available
Allan T. Bombard	Not available	Not available	97.2%	96.9%	98.6% 94%
E. Breuer	Not available	Not available	Not available	100%	99.6%
Plesinac S.	2%	1%	98.6%	92%	97% 95.6%

fetuses two, 667 three and 53 four analyzes. The average gestational age of the pregnant women was 18.6 GW. For 8% of the samples the result was not obtained, 18 fetuses were false positive, 2890 were RhD positive and 19 false negative. The sensitivity of the test increased with gestational age [8].

Ranjit Akolekar performed a study which included 591 pregnant women, the mean gestational age of 12.4 GW (GW 11–14). In 86% of the analyzed samples the adequate amount of DNA was detected. In 42 pregnant women a variant RHD gene was detected as in African populations. From the adequate results 338 fetuses were positive and 170 negative. In 332 positive fetuses this finding is confirmed after birth, as well as 164 negative. Six fetuses were originally considered negative which was not confirmed after birth. The sensitivity of the test was 98.2% (95% CI 96.1–99.3) and the specificity was 100% (95% CI 97.2–100). The positive predictive value was 100%, and negative predictive value was 96.5%. 3.5% were false-negative results due to lack of concentration of cfDNA in the mother's blood. There is a possibility to try to determine the optimum gestation for taking a sample. Suggestions would be: 12 GW, 26 GW, or 12 GW, but in the case of negative results the testing should be repeated [9].

We also suggested that if the result was female negative before 12 WG the test should be repeated after 20th weeks of gestation.

Gefmann-Holtzman [5] showed in a metacentric study that the specificity of the test was 95.4%, sensitivity was 98.6%, PPV 99% and NPV 92.1%. Our study results showed the sensitivity of the determination of RhD status of the fetus from the mother's blood after 8 week of gestation was 98.6%, and the specificity was 92%.

Allan T. Bombard in the first cohort validation study had 236 samples with an average gestational age of 12.4 GW. RhD phenotype of the newborn was positive in 69.1% and negative in 30.9%. 0.9% of the results were inadequate. Analysis of the prenatal maternal blood taken, showed 6.8% inadequate findings, 4.7% RhD gene variants. RhD positive result was obtained with 140 samples, of which 2 were false positive and RhD negative findings in 67 fetuses, of which 4

were false negative. The reliability of the test was 97.1% (95% CI 93.5–98.8), test sensitivity was 97.2% (95% CI 93.0–98.9) and specificity was 96.9% (95% CI 89.5–99.1). The positive predictive value of the test was 98.6% (95% CI 94.9) [10]. The positive predictive value of the test was 97% and the negative predictive value was 95.6%.

E. Breuer, in his study tested the exons 7 and 10, and intron 4 which is located far enough from the first two exons. In a sample of 230 pregnant women he received a 100% reliable method of prenatal screening for fetal RhD, with a positive predictive value of 99.6% [11].

Conclusion

1. The sensitivity of the determination of RhD status of the fetus from the mother's blood after 8 week of gestation in our sample was 98.6%, and the specificity was 92%.
2. The positive predictive value of the test was 97% and the negative predictive value was 95.6%.
3. False positive results were detected in 2.6% of cases and 4.5% of cases were false negative.
4. The presence of RhD antibodies had not significantly changed test results.
5. A larger significance had the weeks of gestation and fetal gender. RhD negative female fetuses required repeated analyses after 20 GW.
6. Analysis of the sex of the fetus was appropriate for all newborn babies.
7. Only limitation of the method is female RhD negative baby in pregnancy less than 8 weeks of gestation.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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