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Determination of Rhd Zygosity in India: To Prevent Hemolytic Disease of Newborn

Abstract

Objective: To access the prevalence of RhD heterozygosity rates in phenotypically RhD positive Indians using two different genotyping techniques.

Material and Methods: Husbands of women with RhD negative blood group, attending outpatient department were recruited for study. RhD zygosity was done in blood samples of Rh positive husbands by two different methods. DNA was isolated by using conventional phenol-chloroform method. For RhD zygosity determination two methods polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and polymerase chain reaction-sequence-specific primer (PCR-SSP) techniques were used for each sample.

Results: Of the 105 women with RhD negative blood group, husband's blood group was found to be Rh D negative in 5 (4.8%). Of the 100 blood samples that were found RhD positive, zygosity analysis showed that 26 were D heterozygous by both methods. Hence RhD heterozygosity rate in India is 26%.

Conclusion: Women with husbands having homozygous RhD positive (DD) are likely to have all fetus RhD positive (Dd) and hence not require molecular technique like fetal blood group determination from maternal plasma. While 26% of women whose husband are RhD positive by serology and heterozygous for RhD deletion by molecular methods require fetal blood group determination from maternal plasma. Hence, knowing the RhD zygosity of the husband, fetal blood group determination from maternal plasma is required to be done in one fourth of women having RhD isoimmunisation.

Keywords: RhD; Isoimmunisation; RhD negative; Heterozygosity

Research Article

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Abbreviations: PCR-RFLP: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism; PCR-SSP: Polymerase Chain Reaction-Sequence-Specific Primer

Introduction

Incidence of RhD negative varies between from less than 1% in the Asian population up to 35% in the Basque population. Women with RhD negative blood group have the risk of being isoimmunised during pregnancy, during delivery or very rarely during their in-utero life. If an isoimmunised pregnant women carries a Rh positive fetus it may cause complications like hemolytic disease of newborn, hydrops fetalis and even intrauterine death of fetus. In spite of availability of Anti D for prevention of RH isoimmunisation, it is still prevalent in 0.8 to 1.5 percent of pregnancy [1]. Prevalence rate of Rh isoimmunisation in India is not known. While anti D prophylaxis is used yet still it is not having a universal coverage leading to frequent cases of rh isoimmunization. The routine policy in the country for a Rh negative nonisoimmunized women with RhD positive husband is to give 300 mcg anti D polyclonal intramuscular injection at 28 weeks and then within 72 hours after delivery after doing baby's blood group.

Management of RhD negative pregnant women warrants doing molecular technique like RhD zygosity of the husband. Determination of fetal RhD blood group can be done either from maternal plasma which is a non-invasive method or from fetal tissues being invasive in nature.

The rhesus D antigen is the most important blood group antigen determined by a protein anti D which remains the leading cause of hemolytic disease of the newborn. The antigen of the Rh blood group are carried by proteins coded by two genes, RHD and RHCE, that are located at chromosomal position 1p34.1 – 1p36. Both genes, encompasses 10 exons and their structure are highly homologous. RhD gene is flanked by two homologous DNA segments of about 9000bp length, the upstream and downstream rhesus boxes. In white persons the D negative phenotype is usually due to a deletion of the RhD gene that had occurred by an unequal crossing over between these rhesus boxes, leaving only a single hybrid rhesus box. The detection of this hybrid rhesus box has been applied to demonstrate the presence of the RhD deletion. Hence it became feasible to distinguish RhD homozygous (+/+) from RhD heterozygous (+/-) individuals. Such discrimination is of clinical interest, because all children of an RhD homozygous father will be D positive and at risk of hemolytic disease of the newborn if the mother has produced an anti-D. The risk of an affected child is 50% when the father is heterozygous for RhD.

We have performed RhD zygosity in RhD positive husband of Rh negative women by two molecular methods (polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and polymerase chain reaction-sequence-specific primer (PCR-

SSP)) and calculated the heterozygosity rates in India.

The aim of the manuscript is to determine the RHD heterozygosity of partners of RHD-negative pregnant women in India to reduce the number of fetal RHD typings in maternal plasma. The frequencies of RHD positivity varies greatly between different populations and data about the RHD allele frequencies and heterozygosity in a certain population is useful and interesting.

Material and Methods

Women with RhD negative blood group, attending outpatient department was done in all cases. RhD zygosity was done in those blood samples of husband where blood group was found to be RhD positive. DNA was isolated by using conventional phenol-chloroform method [2].

For detection of RhD deletion, the PCR-RFLP method was performed using the expand high fidelity PCR system [3]. Primer used was rez7 5' CCT GTC CCC ATG ATT CAG TTA CC 3' (nonspecific, 5' of rhesus box identity region) & rnb31 5' CCT TTT TTT GTT TGT TTT TGG CGG TGC 3' (specific for downstream rhesus box, 3' of downstream rhesus box identity region).Primer annealing was at 66°C, the PCR amplicon was digested with Pst I restriction enzyme. Amplicon were resolved on 1% agarose gel. The enzymatic digestion pattern indicates the presence of the hybrid rhesus box or downstream rhesus box. In D negative haplotype there are 3 Pst I site in the amplicon, resulting in 1888bp, 564bp, 397bp & 179bp fragments. D positive haplotype lacks one Pst I site, resulting in fragments of 1888bp, 744bp & 397bp while D heterozygotes shows both fragments of 744bp and 564bp as shown in Figure 1.

To see the concordance of PCR-RFLP results, a second molecular technique PCR-SSP method was performed as described by Paul Perco et al. [4]. This method includes an internal control to indicate appropriate amplification in all the samples. The desired gene portion is amplified using primer u1s 5' TGA GCC TAT AAA ATC CAA AGC AAG TTA G 3' specific for hybrid & upstream rhesus box and antisense primer rnb31 (same as for PCR-RFLP) specific for hybrid & downstream rhesus box. The amplification was done under following condition: denaturation at 95°C for 10 minutes, following 40 cycles of 92°C for 20 seconds, 64°C for 30 seconds and 68°C for 5 minutes and the reaction was completed finally at 72°C for 5 minutes and the PCR products were visualized on 1.5 % agarose gel as shown in Figure 2. Both the methods were done in duplicate.

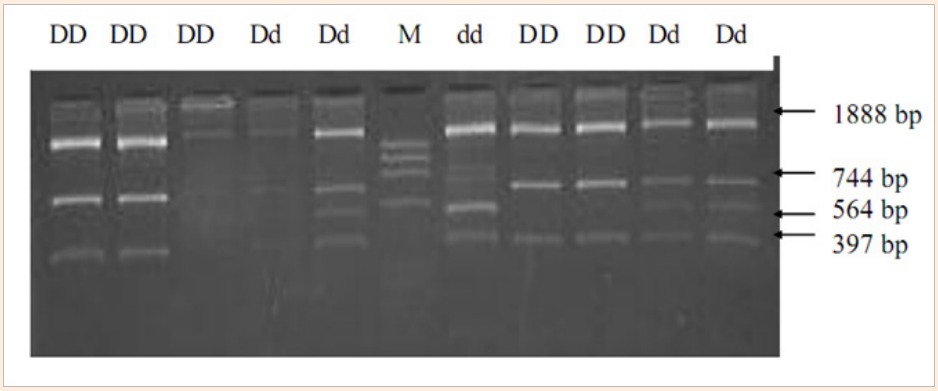

Figure 1: Gel Picture showing the PCR-RFLP pattern in different RhD zygosity.

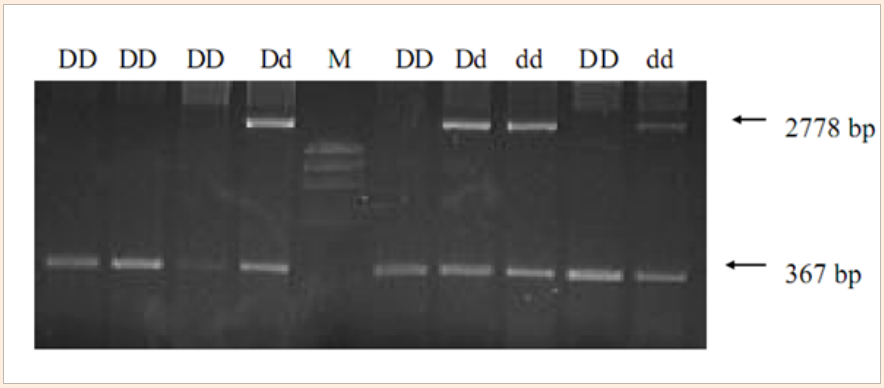

Figure 2: Gel picture showing the PCR-SSCP pattern in different RhD zygosity.

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Those women who were pregnant and husband were RhD heterozygous, with rh antibody titre more than the critical titre (i.e. > 1:16) were counselled about the need for fetal blood grouping and carrying 50% chance of having Rh negative fetus; thereby with normal fetal outcome.

Results

Of the 105 women with RhD negative blood group, husband's blood group was found to be RhD negative in 5 (4.8%). Of the 100 blood samples that were found RhD positive, zygosity analysis by both observer showed that 26 were D heterozygous by SSCP method (26%). When compared with the PCR-RFLP result by both observers it was found that the same samples were found to be heterozygous for RhD gene. There was no discrepancy observed between the two methods.

Discussion

Martine GHM et al. [5] reported that the RhD gene deletion due to unequal crossing over is the mechanism of RhD negative in Asian & Black persons from South Africa, Curacao & Ethopia. The D negative phenotype in the white persons is mainly caused by RhD gene deletion, although 14 different D negative RhD allele have been described with low frequencies (1: 1500) in Caucasian persons [6]. In other ethnic groups (especially in black persons), D negativity is frequently caused by the aberrant RhD genes; \ RhDψ (allele frequency 0.0714) and/or (C)cde (allele frequency 0.036) [7-9].

Several methods have been described for RhD zygosity determination, such as D antigen density, linkage disequilibrium and associating D negativity with polymorphisms in RHCE. These approaches are indirect and are not therefore very reliable.

Wagner & Flegel [10] have elucidated the organization of the Rh gene locus and proposed a model for the mechanism causing the RhD negative haplotype. They also suggested two different PCR methods for RhD zygosity determination by specific detection of the RhD negative genotype. One of the methods is based on PCR-RFLP technology. Both the hybrid rhesus boxes and downstream rhesus boxes are amplified with primers rez7(consensus) and rnb31(downstream specific). Owing to the Pst I restriction site present in the part predicting the identity region of only the upstream rhesus box (present in the amplified rhesus box), the RhD positive and RhD negative locus are distinguished. PCR-RFL prequires a reliable digestion of the PCR products, otherwise incomplete enzyme digestion may lead to false results .This disadvantage is circumvented by PCR-SSP methods which uses an internal control to indicate appropriate amplification in all the samples. However we found both the methods to be concordance in all 100 samples.

In developing countries like India, resources are limited. The antenatal investigations protocol includes maternal blood group and if negative, paternal blood group. If paternal group is Rh positive, the next step is to get an indirect coomb's test. Paternal zygosity testing is not included in the testing panel. Newer methods like non-invasive fetal typing in maternal plasma are neither carried out within the country nor is cost acceptable as of now. We must still appreciate that the later method is not affected by non-paternity, while paternal zygosity methods are.

The method described in the manuscript requires serological RhD typing of both mother and father and an additional fetal typing in a fourth of RhD negative woman instead of fetal typing for all RhD negative women. Prevalence of the RhD zygosity has not been much reported from India. We have compared both the methods for discrimination of D+/D+ from D+/D- partners of D negative mothers.

Conclusion

Fetal blood group determination from maternal plasma is required to be done in one fourth of women having RhD isoimmunisation. Women with husbands having homozygous RhD positive i.e. DD are likely to have all fetus RhD positive i.e. Dd and hence not require molecular technique like fetal blood group determination from maternal plasma which are not available in the country as of now. It is that 26% of women whose husband are RhD positive by serology and heterozygous for RhD deletion by molecular methods require fetal blood group determination from maternal plasma. Hence knowing the RhD zygosity of the husband, fetal blood group determination from maternal plasma is required to be done in one fourth of women having RhD isoimmunisation.

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