Amniocentesis for Fetal Rh Grouping by PCR : A Major breakthrough in Anti-D Prophylaxis and Management of Rh Negative Pregnancies

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OBJECTIVE – To study the feasibility, accuracy and usefulness of amniotic fluid (AF) PCR for fetal Rh grouping in Rh negative pregnant women. **METHODS** – Fetal Rh grouping by PCR was performed on AF obtained by ultrasound guided amniocentesis in 50 Rh negative (3 non-immunised and 47 immunized) pregnant women, between 16-30 weeks of gestation. Indication for amniocentesis in Rh non-immunized women was fetal karyotyping to rule out Down syndrome, while in Rh immunized women the indication was spectrophotometry for bilirubin levels to note severity of fetal red cell hemolysis. Amniotic fluid PCR for Rh grouping was done by the technique described by Bennett et al[1]. Fetal AF Rh group was correlated with neonatal serologic Rh group after delivery. **RESULT** – AF Rh grouping by PCR showed than one non-immunized and two immunized women (3/50 Rh-ve women) had a Rh-ve, fetus. There was 100% correlation of AF, PCR, Rh grouping PCR with fetal /neonatal serologic Rh group. **CONCLUSION** – AF Rh grouping by PCR is an accurate, simple, but major breakthrough in "customized" anti-D prophylaxis, and in intensive management of immunized pregnancies in Rh negative women.

Key words : amniocentesis, Rh group, PCR, pregnancy

Introduction

Management of an Rh negative pregnancy is an obstetric challenge. When the father is heterozygous Rh D positive, there is a 50% chance that the fetus will be Rh D negative and a 25% chance if zygosity is unknown. If the fetus is Rh D negative, there is no need for propohylactic anti-D and no risk of immunization. Lack of knowledge of the fetal Rh D status in cases of Rh isoimmunization, has led the obstetrician to give expensive intravenous immunoglobulin therapy, perform unnecessary invasive procedures like serial amniocentesis, intensive fetal monitoring, giving prophylactic corticosteriod therapy and even perform preterm delivery in an Rh D negative fetus[2]. In nonimmunised pregnancy, lack of knowledge of fetal Rh D status leads to unnecessary expensive antepartum anti-D prophylaxis after amniocentesis or chorionic villus sampling and even routinely at 28-30 weeks. Fetal cord blood sampling (cordocentesis) is the only method of diagnosis of fetal Rh group. Cordocentesis is a highly skilled procedure, difficult to perform before 24-28 weeks of gestation and associated with a 1% to 5% risk of fetal loss[3]. Hence it is not routinely performed for fetal Rh grouping in an Rh negative pregnant woman.

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Recently, cloning and characterization of the Rh gene locus has provided the molecular basis for the Rh positive and the Rh-negative phenotype[4-5]. Prenatal diagnosis of fetal Rh group has been reported by polymerase chain reaction (PCR) of aminoticfluid (AF)[5]. Since amniocentesis is a simpler and less risky procedure than cordocentesis, Rh grouping from AF is the preferred technique for prenatal determination of Rh group when indicated.

This study aimed to establish the technique of determination of fetal Rh group from AF by PCR in Indian population, to compare the accuracy of Rh D grouping by PCR with serological fetal / neonatal cord blood Rh grouping, and to assess its usefulness in the management of Rh negative pregnancies (non-immunized and immunized).

Material and Methods

The study was conducted on 50 Rh negative women attending the high risk ante natal clinic. Fetal Rh grouping by PCR was performed on AF in :

i) Rh non-immunized women undergoing amniocentesis for fetal karyotype to rule out Down's syndrome – 3 cases.

ii) Rh immunized women undergoing amniocentesis for spectrophotometry for bilirubin levels to note severity of fetal red cell hemolysis – 47 cases.

Fetal AF Rh group was correlated with fetal / neonatal cord blood Rh group by serology obtained either by fetal cordocentesis or collected from cord blood after delivery in Rh isoimmunised cases, or from fetal blood collected after delivery in non-immunised cases.

Inclusion Criteria

- Pregnancy with Rh isoimmunization requiring intravenous immunoglobulin (IVIG) therapy, serial amniocentesis or cordocentesis or intrauterine transfusion.
- Rh negative non-immunized women requiring amniocentesis for genetic indication.

Exclusion Criteria

- Blood stained AF

Procedure for amniocentesis

Under ultrasound guidance a G 23 disposable spinal needle was inserted into the amniotic cavity per abdomen, taking care to avoid the placenta (Fig.1). The stilette was withdrawn, a disposable syringe attached, and AF aspirated in amounts necessary for the required investigation (karyotype/spectrophotometry) besides an additional 10 ml for PCR.

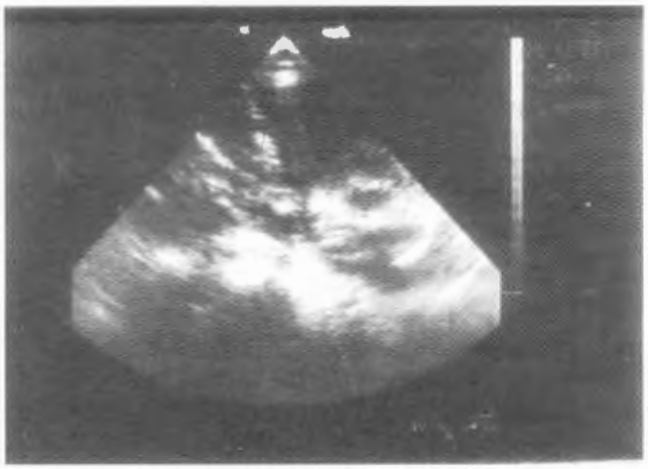

Fig 1 : Ultrasound guided Amniocentesis : showing needle in amniotic cavity

Technique of DNA preparation from amniotic fluid

Clear AF samples were taken and centrifuged at 10,000 rpm for 10 minutes.

The supernatent fluid was discarded and pellet was washed twice in 150 mM NaCl/25mM EDTA. It was centrifuged again at 10,000 rpm for 10 minutes, supernatent fluid was discarded and 220 microlitre NaCl-EDTA was added. Proteinase K. 6 µl (2.5mg/ml) and 20% SDS 22µl was added and was left overnight for digestion at 37°C. Three hundred microliters of saturated phenol was added to the Eppendorf tube and was mixed in the rotamixer at 10 rpm for 10 minutes. It was then centrifuged at 500 rpm for 10 minutes and the supernatent was transferred into another Eppendorf tube. The procedure was repeated after which 300 µl of chloroform : isoamyl alcohol : 24:1 was added and admixed in rotamixer at 10 rpm for 10 minutes and centrifuged at 5000 rpm for 10 minutes. The supernatent was transferred into another Eppendorf tube and this procedure repeated after which 12-15 microliter of 3M sodium acetate was added.

Twice the volume of chilled ethanol was added and mixed by inverting the tube. A fine thread like pellete was formed, which was centrifuged and washed twice with 70% ethanol. It was dried in a vacuum drier and 5-10 µl of distilled water was added and stored for PCR.

PCR was performed according to published literature[1]. Briefly, 2µl of template human DNA was used with 20 pmol each of 4 primers in a 20 µl reaction. Thirty cycles of denaturation for 1 minute 92°C annealing was done for 1 minute at 49°C, extension time was 1 minute at 72°C and final extension was for 9 minutes at 72°C. The products were run on a 3% gel containing 0.5 µl/ml of ethidium bromide and visualized under UV light.

Results

Amniotic fluid Rh group by PCR correlated with cord blood serologic Rh group in all cases (100% accuracy). Of the non-immunized Rh-ve group (3 cases), one fetus was Rh-ve by PCR and serology. Of the Rh immunized group (47 cases) two fetuses were Rh-ve by PCR and serology. In all 47 fetuses were Rh+ve by PCR and serology.

As accuracy of amniotic fluid PCR for Rh grouping during study was not yet confirmed, anti-D was given after amniocentesis in the one non-immunized woman with Rh negative fetus. After delivery, when cord blood serology confirmed a baby to be Rh negative, post-partum prophylaxis was avoided. For the same doubt of accuracy, the two Rh negative fetuses in isoimmunized group were followed up by twice weekly ultrasound and doppler only but no serial amniocentesis or cordocentesis were required. After delivery, cord blood serology confirmed Rh negative blood group.

The 45 Rh+ve isoimmunized fetuses were managed as routinely indicated by IVIG therapy, serial ultrasonography, amniocentesis, cordocentesis intrauterine transfusion, intensive fetal monitoring and planned delivery.

Discussion

After elucidation of the molecular basis of the Rh genotype[4,6], the determination of the Rh D blood type at the DNA level has become possible. Analysis of Rh group by DNA by PCR from 765 adult blood samples has been reported with 99.7% sensitivity by Yankowitz et al[7].

The Rh system is the most complex, and assays to define Rh genotye have been modified in response to increased understanding of the molecular biology of this blood group system[8]. Benett et al[1] were the first to perform Rh grouping by PCR in 15 AF samples and reported 100% accuracy. Similarly, several other authors have obtained good results in their work on prenatal diagnosis of Rh blood group by AF PCR when correlated with fetal blood serology. Sagot et al[9] reported 100% accurate results in 21 samples and so did Rossiter et al[10] in three samples, Spence et al[11] in 67 samples and Crombach et al[12] in 41 samples.. The fluorescent PCR based DNA test allowed easy, rapid and accurate determination of zygosity for Rh D gene, which is useful information for the clinical management of Rh negative preganancies[13]. Fetal Rh D group could be determined by PCR of AF as early as 14 weeks of gestation, thus allowing testing of fetal Rh D group at an earlier gestational age than by fetal blood sampling. Results of some other studies are given in Table I.

The occurance of D gene variants has led to errors in prenatal typing, especially in assigning a positive fetus as negative. Hence assessment of D gene in family studies with Rh genotype in blood would be very informative for at risk pregnancies[14].

Lighten et al[17] reported two cases of false negative diagnosis out of 135 patients with an accuracy of 98.6%. Repeated testing of these infants using the same PCR primer showed that they were Rh D positive. This indicated a methodological error. Van den Veyver et al[15] had 96.6% accuracy in diagnosing Rh group in 107 patients.

Table I : Accuracy of Amniotic Fluid PCR in Prenatal Diagnosis of Rh Blood Group

Author / Year	No. of cases	Period of gestation	Accuracy (Percent)
Benett et al[1], 1993	15	18-22 wks	100
Lighten et al[14], 1995	125	2nd trimester	98.6
Van den Veyver and Moise[15], 1996	107	22-40 wks	96.6
Dildy et al[16], 1996	147	99.7	
Present study, 2002	50	14-30 wks	100

AF PCR for prenatal diagnosis of Rh group is an accurate, simple and major breakthrough in "customized" anti-D propylaxis in Rh-ve, non-immunized pregnancies undergoing amniocentesis and in management planning of Rh immunized pregnancies. AF Rh grouping by PCR is recommended in Rh-ve non-immunized women at amniocentesis for any genetic indication (Down syndrome, biochemical disorder, etc) and for deciding on anti-D prophylaxis. It is also recommended in Rh immunized women at early pregnancy before starting IVIG therapy, in early pregnancy in severe Rh isoimmunization, at first amniocentesis for spectrophotometry and at first cordocentesis for hemoglobin and Rh grouping.

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