Prenatal Diagnosis of Sickle Cell Disease by the Technique of PCR

Praneeta J. Singh · A. C. Shrivastava ·
A. V. Shrikhande

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Abstract Sickle cell disease (SCD) is prevalent in Central India and causes major morbidity and mortality. There is a lack of prenatal diagnostic facility near population affected with SCD. This is the pilot study in our region with the aim to establish prenatal diagnostic facility for the couples carrying sickle cell gene in Central India, in order to help them take an informed decision regarding fetus affected with SCD and also to calculate sensitivity of polymerase chain reaction (PCR) technique in our set up with follow up high performance liquid chromatography (HPLC) of baby's blood sample. Fetal sampling was done by chorionic villous biopsy. Extracted DNA was subjected to amplification refractory mutation system (ARMS-PCR) to detect sickle cell mutation (GAG → GTG) in the sixth codon of β globin gene. Follow-up HPLC was done to detect baby's Hb pattern. Prenatal diagnosis of sickle cell anemia was offered in total 37 cases out of which one (2.7 %) fetal sample was inadequate. Total 26 (70.27 %) fetuses had AS Hb genotype, 3 (8.11 %) had AA Hb genotype and 3 (8.11 %) had SS Hb genotype while remaining 4 (10.81 %) were given AA/AS Hb genotype. All couples with SS fetuses opted for MTP. Follow up HPLC was performed in 24 cases, out of which 18 (75 %) were correlated and 6 (25 %) were mismatched. In present study sensitivity of ARMS-PCR was 75 %. ARMS-PCR is a simple technique to be established initially for providing rapid prenatal diagnosis to the couples with known sickle cell mutation. The sensitivity of ARMS-PCR can be increased by using suitable techniques to detect maternal cell DNA contamination.

Keywords Sickle cell disease · Prenatal diagnosis · ARMS-PCR · CVS

Introduction

Sickle cell disease (SCD) is an autosomal recessively transmitted hemoglobinopathy. It causes high infant mortality rate [1], poor quality of life along with decrease in overall life expectancy causing social and psychological trauma especially to the parents of affected children.

The cumulative gene frequency of haemoglobinopathies in India is 4.2 % [2]. Among various hemoglobinopathies, prevalence of Sickle cell gene is especially high in Central India [3, 4].

Detection of the single base pair mutations (GAG → GTG) in the sixth position of β globin gene, thereby encoding valine instead of glutamine, is the key for the prenatal diagnosis (PND) of sickle cell disease. In 1970s, prenatal diagnosis of sickle cell anemia was initiated by studying beta globin chain synthesis in fetal blood [5, 6]. Later on it was achieved by restriction endonuclease enzyme application on DNA cultured from amniotic cells [7, 8].

In early 1980s, with development of Polymerase chain reaction (PCR) by Mullis and his colleagues [9, 10], prenatal diagnosis of SCD using PCR was firstly done by restriction endonuclease enzyme digestion of PCR amplified product [11]. Then a modification of PCR technique, known as Amplification Refractory Mutation System (ARMS), was developed by Newton et al. [12] in 1989 and subsequently ARMS-PCR was used for the prenatal diagnosis of SCD [13–15], since it is a simple technique in comparison to other PCR based techniques and provide rapid results even for large number of samples [16].

P. J. Singh (✉) · A. C. Shrivastava · A. V. Shrikhande
Department of Pathology, Indira Gandhi Government Medical
College, Nagpur, India
e-mail: pranita.singh123@gmail.com

ANALYTE ID	%	TIME	AREA
P2	3.9	1.33	79820
P3	3.0	1.72	60324
Ao	52.3	2.47	1059929
A2	2.0	3.67	37445
S-WINDOW	38.6	4.45	783146
TOTAL AREA			2020664

F 0.0% A2 2.0%

Fig. 1 HPLC of husband showing AS pattern

By implementing safe and efficacious method of chorionic villous sampling (CVS) for prenatal diagnosis [17], it became possible to detect this point mutation in early pregnancy and provide couples an opportunity to take informed decision regarding fetuses affected with SCD.

In present study, prenatal diagnostic facility for couples carrying sickle cell gene was established with the aim to prevent birth of affected fetus and to calculate the sensitivity of ARMS-PCR by comparing results with baby's Hemoglobin pattern on follow up HPLC. We offered prenatal diagnosis of sickle cell anemia in 37 pregnancies by using CVS and ARMS-PCR.

Materials and Methods

Subjects

Present study was approved by the Institute's Ethics committee. Known cases of sickle cell anemia registered with the Institute and attending the sickle cell outpatient department were counseled for prospective PND. While couples with previously affected children with sickle cell disease and attending an antenatal clinic or the sickle cell outpatient department were counseled for retrospective PND. Maximum number of the couples i.e. 31 (83.78 %) belonged to Scheduled Caste and two (5.41 %) each to

ANALYTE ID	%	TIME	AREA
F	2.4	1.05	29781
P2	2.8	1.28	34375
Unknown 1	0.4	1.44	5429
P3	3.8	1.64	47248
Ao	53.7	2.33	664556
A2	3.3	3.52	41201
S-WINDOW	33.9	4.30	420220

TOTAL AREA 1242810

F 2.4% A2 3.3%

Fig. 2 HPLC of wife showing AS pattern

Scheduled Tribe, Teli and Kalhar community. Present study was carried out from 1 January 2006 to 31 October 2011, during which 37 cases were studied. One woman conceived twice during the study period and requested PND during both pregnancies.

Couples Hb Pattern

Peripheral blood samples of both the partners and their affected children were collected to determine their Hb pattern by performing Agar Gel Electrophoresis at alkaline pH [18] and High performance liquid chromatography (HPLC) [18] on VARIANT hemoglobin testing system supplied by Bio-Rad, using Beta Thalassemia Short Program. Couple Hb pattern on HPLC shown in Figs. 1 and 2. Couples with gestational age of more than 20 weeks were excluded from the study since MTP for fetus affected with SCD cannot be done after 20 weeks of gestational age due to Prenatal diagnosis act in India.

CVS and Fetal Genotype

After proper genetic counseling and explaining risks and complications associated with CVS procedure, informed and written consent in a prescribed proforma was taken from the couples who accepted PND. Couples not willing for CVS procedure were excluded from the study.

Fig. 3 Appearance of chorionic villi under dissecting microscope

Chorionic villous biopsy was the method of choice for obtaining fetal tissue as it can be safely performed during first trimester. It was done under the ultrasound guidance either through trans abdominal or trans cervical route depending on the position of the placenta and choice of operating obstetrician [19]. Adequate sample was obtained through both the routes [20]. The adequacy of sample was confirmed both by visual inspection and under dissecting microscope. Grossly, chorionic villi appear as free-floating, white fluffy structures while contaminating decidual tissue has amorphous appearance. As shown in Fig. 3, under a dissecting microscope, chorionic villi were identified as having distinctive branched appearance and were separated from maternal deciduas [21].

DNA was extracted from chorionic villi by Standard Phenol Chloroform extraction method [22]. Pure DNA sample appears as a viscous solution. After checking quality of the extracted DNA on Gel documentation system, ARMS-PCR was performed in thermal amplifier; Primus-96 Advanced Gradient Innovative PCR Technology, PeQ Lab.

ARMS-PCR

Amplification-refractory mutation system (ARMS) or allele-specific polymerase chain reaction (ASP) is a method for detecting any single base pair mutation or deletion. We used it to detect sickle cell allele by using two ARMS primers. One of the primers was designed to detect normal DNA sequence of β globin gene and is known as normal sickle cell specific primer. While other primer known as Mutant Sickle cell specific Primer, was designed to detect point mutation GAG $\rightarrow$ GTG at sixth position of

β globin gene which causes SCD and amplification with this primer occur only in presence of this sickle cell mutation.

For each DNA sample ARMS-PCR was put up in two tubes, one labeled as normal (N) and other as mutant (M). In each tube we added a common forward primer, ARMS mixture and Taq polymerase. Normal sickle specific primer was added in tube labeled as N and mutant sickle specific primer added in tube labeled as M. To check if the PCR system working properly and to avoid false negative result, a pair of internal control primers was added to each tube. Each of these primers were tested with known positive and negative control DNA samples under uniform and stringent PCR conditions [23, 24].

All the reagents were supplied by Chromus Biotech Pvt. Ltd.

Following primers sequences were used:

1. Normal sickle cell specific primer (CD6) primer sequence 5' $\rightarrow$ 3': N-CCC ACA GGG CAG TAA CGG CAG ACT TCT GCT
2. Mutant sickle cell specific Primer (A $\rightarrow$ T) HbS primer sequence 5' $\rightarrow$ 3': M-CCC ACA GGG CAG TAA CGG CAG ACT TCT GCA
3. Second Common primer (C1): 5'ACC TCA CCC TGT GGA GCC AC3'
4. Internal control (Inc1) : 5'CAA TGT ATC ATG CCT CTT TGC ACC 3'
5. Internal control (Inc2): 5'GAG TCA AGG CTG AGA GAT GCA GGA 3'

Fragment size of PCR product with normal and mutant sickle specific primer is 207 base pair and with internal control Inc1 and Inc2 is 860 base pair.

To interpret the result of ARMS-PCR, the amplified products with both normal and mutant sickle cell specific primers were first run side by side on Horizontal

Table 1 Interpretation of ARMS-PCR amplified product

S. no.	Amplification seen	Interpretation
1.	Amplification in N (normal sickle cell specific primer) No amplification in M (mutant sickle cell specific primer)	Mutation is absent
2.	Amplification in N (normal sickle cell specific primer) Amplification in M (mutant sickle cell specific primer)	Mutation is present in heterozygous condition
3.	No Amplification in N (normal sickle cell specific primer) Amplification in M (mutant sickle cell specific primer)	Mutation is present in homozygous condition

Fig. 4 Result of gel electrophoresis of ARMS-PCR amplified product, seen under UV light. *Lane 1* 100 bp DNA ladder. *Lanes 2 and 4* Fetal DNA samples with normal sickle cell specific primer. *Lanes 3 and 5* Fetal DNA samples with mutant sickle cell specific primer. *Lane 6* DNA of known AS patient with Normal Sickle cell specific primer. *Lane 7* DNA of known AS patient with mutant sickle cell specific primer. Interpretation: Fetal DNA of sample no.1 (*lanes*

2, 3) is showing PCR amplification with only mutant sickle specific primer. Fetal DNA of sample no. 2 (*lanes 4, 5*) shows no PCR amplification with either of the primers and internal control bands too are not seen. ARMS-PCR is to be repeated for this patient. Result: Fetal DNA of sample no. 1 is showing PCR amplification with only mutant sickle specific primer thus fetus is likely to be affected with sickle cell anemia; “SS” PATTERN

Submarine Gel Electrophoresis. 2 % agarose gel containing 0.5 µg/ml ethidium bromide was prepared and samples were run at 100 V for 45–60 min. After electrophoresis, bands were visualized under ultraviolet (UV) light in Gel documentation system (Gel Logic 200 imaging system, Kodak molecular image) which works on the principle of fluorescence. Ethidium bromide, an intercalating dye binds which specifically to nucleic acid, is excited by ultraviolet irradiation and emits fluorescence. Following ARMS reaction, presence or absence of a 207 base pair band with mutant sickle specific primer under UV light is diagnostic for the presence or absence of the sickle cell allele [23]. Interpretation of ARMS-PCR test result is shown in Table 1 and Fig. 4.

Interpretation of ARMS-PCR

A band of 860 base pair with internal control primers is seen in all the samples. As shown in Fig. 4, based on the mutation specific primer band of 207 base pair, the sample can be labeled as normal, heterozygous or homozygous for sickle cell mutation as shown in Table 1.

Follow Up HPLC to Know Baby's Hemoglobin Pattern

Baby's Hemoglobin pattern was determined by performing Hb electrophoresis and HPLC on either cord blood or peripheral blood sample collected from babies coming for follow up. In case of cord blood sample, repeat sampling

F	66.1	1.22	1328901
P3	0.5	1.70	9333
Ao	11.2	2.51	192016
A2	1.0	3.60	15122
S-WINDOW	9.4	4.42	161029
TOTAL AREA			1706401
66.1%	A2	1.0%

Fig. 5 Follow up HPLC of baby's cord blood sample showing FS pattern

was done at the age of 1 year in order to reevaluate levels of Hb A and Hb S in babies. Figure 5 shows baby's Hemoglobin pattern on HPLC of cord blood sample.

Table 2 Distribution of couples according to Hb pattern (n = 37)

Couples Hb pattern	No. of cases (n = 37)
AS–AS	35 (94.60 %)
AS–SS	02 (5.40 %)
Total	37 (100 %)

Fig. 6 Distribution of cases according to route of CVS for obtaining fetal tissue

Results

In present study, prenatal diagnoses of sickle cell anemia were offered to 37 couples carrying sickle cell mutation.

Couples Hemoglobin Pattern

Maximum number (94.60 %) of couples had AS pattern. Distribution of couples according to their Hb genotype is shown in Table 2.

Obstetric History

27 (72.97 %) couples had previously affected children with sickle cell disease while 10 (27.03 %) couples underwent prenatal diagnosis during first pregnancy.

CVS

30 (81.08 %) women came during first trimester and adequate fetal tissue was obtained in all 24 (80 %) cases by trans-abdominal CVS and 6 (20 %) cases by trans-cervical route. Out of 7 (19.92 %) second trimester CVS (13–17 weeks of gestational age), adequate fetal tissue was obtained in all 5 cases done through Trans-cervical route and out of two trans-abdominal CVS adequate fetal tissue was obtained only in one case while quality of DNA in other case was poor.

Distribution of cases according to the route of CVS is shown in Fig. 6.

Fetal Genotype by ARMS-PCR

Out of 37 cases, 26 (70.27 %) fetuses were carrier for sickle cell anemia as fetal DNA showed amplification with both mutant and normal sickle cell specific primer. Three

Table 3 Distribution of fetal Hb genotype reported by ARMS PCR

Fetal genotype	No. of cases (n = 37)
AA	03 (8.11 %)
AS	26 (70.27 %)
SS	03 (8.11 %)
AA/AS	04 (10.81 %)
No diagnosis possible due to improper DNA sample	01 (2.70 %)
Total	37 (100 %)

(8.11 %) fetuses were normal for sickle cell anemia as DNA showed amplification with only normal sickle specific primer. Three (8.11 %) fetuses had SS Hb genotype since DNA showed amplification with only mutant sickle specific primer and would likely suffer from sickle cell disease. Four (10.81 %) fetuses were reported as having AS/AA Hb genotype because DNA showed amplification with normal sickle specific primer along with a faint band of amplification with mutant sickle specific primer. These fetuses could be either carrier or normal for sickle cell anemia but would not suffer from sickle cell disease. In one (2.70 %) case no diagnosis was possible as no band of amplification was seen with either of the primers. Since a band of internal control was seen, any fault with PCR system was ruled out. Quality of extracted DNA seemed to be poor on gel documentation system and it was reported as improper DNA sample with a request of repeat sampling. Thus complete genotype for sickle cell anemia was given to 32 fetuses (86.49 %) at risk for SCD.

Distribution of fetal Hb genotype as reported by ARMS-PCR is shown in Table 3.

Follow Up of Pregnancies

In follow up, 28 (75.68 %) babies were reported to be delivered normally. Out of which 27 babies were born full term and one was born preterm. Three (8.11 %) fetuses were spontaneously aborted and all abortion took place after more than 6 weeks of CVS procedure. MTP was done in all three (8.11 %) cases with SS Hb fetus genotype, before 15 weeks of gestational age. In one (2.70 %) case MTP was done for fetal congenital abnormality. Rest of the two (5.41 %) pregnancies continued normally. Summary of follow up of pregnancies shown in Table 4.

Correlation of Fetus Genotype and Baby's Hemoglobin Pattern on Follow Up HPLC

Out of 26 fetuses with AS Hb genotype, 22 were delivered normally, one was spontaneously aborted, for one MTP

Table 4 Follow up of pregnancies

Follow up of pregnancies	No. of cases (n = 37)
Normal delivery	28 (75.68 %)
Spontaneous abortion	03 (8.11 %)
MTP for SS	03 (8.11 %)
MTP due fetal congenital abnormality	01 (2.70 %)
Pregnancy continued normally	02 (5.40 %)
Total	37 (100 %)

was done due to fetal congenital anomaly seen on ultrasonography and two pregnancies with AS fetuses continued normally. Out of 26 fetuses with AS Hb genotype, follow-up HPLC was done in 20 cases. Out of which 14 were correlated as having AS Hemoglobin pattern, two babies had AA Hemoglobin pattern and four had SS Hemoglobin pattern.

Out of three fetuses with AA Hb genotype, follow up HPLC was done in 2 babies and were correlated. While one was born preterm and died after 12 days. Follow up HPLC could not be done in this case. Out of four fetuses with AS/AA Hb genotype, two were delivered normally and both had AA Hemoglobin pattern on follow up HPLC. While remaining two were spontaneously aborted and no material was available for follow up HPLC. In all three cases with SS Hb fetus genotype, as MTP was done at their native places, no material was available for follow up.

Correlation of fetus Hb genotype reported by ARMS-PCR was done in 24 cases with Follow up HPLCs, out of which 18 (75 %) were correlated and 6 (25 %) were mismatched. Sensitivity of ARMS-PCR in present study was 75 %.

Correlation of fetus Hb genotype reported by ARMS-PCR with follow up HPLC of baby's peripheral blood sample shown in Table 5.

Discussion

Prenatal diagnosis for hemoglobinopathies has been available in various parts of the country with its main focus on β -thalassemias [25–27]. Though Sickle cell gene is quite prevalent in India with highest number of cases in Central India [4], Sickle Cell Anemia Prevention and Control Program has been neglected in country for many years. Earlier reports from India clearly indicate the need for developing such program for identifying couples at risk for sickle cell anemia by antenatal screening and developing centers for genetic counseling along with establishment of prenatal diagnostic facilities at places near the affected population [28, 29].

Until recently patients from Central India who needed prenatal diagnosis of SCD were referred to ICMR, Mumbai [27]; due to lack of established laboratory set up in this region. So we undertook this study in order to establish prenatal diagnostic facility of SCD for the couples from Central India with sickle cell gene.

During the study period, prenatal diagnosis for sickle cell disease was offered to 37 couples with sickle cell gene mutation. Despite high prevalence of sickle cell gene [3, 4], number of couples who underwent PND were less. This occurred mainly due to high number of women coming late for their first antenatal check up, mostly during last trimester, and had to be excluded from study. Colah et al. [30] reported similar finding that in India number of prenatal diagnoses done during first trimester were less

Table 5 Correlation of fetus Hb genotype reported by ARMS-PCR with follow up HPLC of baby's peripheral blood sample

Report of ARMS-PCR (fetus Hb genotype)	Total no. of cases reported by ARMS-PCR (n = 37)	Total no. of babies delivered (n = 28)	Total no. Follow up HPLC done (n = 25)	Baby hemoglobin pattern on follow up HPLC (n = 25)			Correlation of fetus genotype with follow up HPLC (n = 24)	
				AS	AA	SS	Confirmed	Mismatched
AS	26	22	20	14	02	04	14	06
AA	03	03	02	00	02	00	02	00
AA/AS	04	02	02	00	02	00	02	00
SS	03	00	00	NA	NA	NA	NA	NA
Improper DNA	01	01	01	01	–	–	Not applicable	Not applicable
Total	37	28	25	15	06	04	18	06

NA not available

because very few pregnant women came for antenatal check up during first trimester [30]. It was also found that the couples were reluctant to undergo prenatal diagnosis and were ready to accept a child born with sickle cell disease instead. This can be due to varied clinical presentation of sickle cell disease in India. Some cases show mild clinical picture due to high levels of Hb F [31]. Some couples refused to undergo CVS procedure due to unshakable belief of child being born with deformity owing to the CVS technique. In our study maximum couples belonged to low socio economic groups, were uneducated and lived remote areas. All these factors hindered with proper motivation and acceptance of PND. Similar problems were stated by Colah et al. [27] from India.

In our region sickle cell gene is also prevalent among Muslim community and they refused PND based on their religious belief. Anionwu et al. [32] stated that when prenatal diagnosis for sickle cell anemia is offered to a minority population, their cultural and religious view should also be considered since the variability of clinical picture for SCD complicates the issues of ethical and moral dilemmas.

At the start of the study period couples who already had children suffering from SCD accepted to undergo PND. While during later years of study period, couples requested for PND during their first pregnancy. This clearly shows increased awareness among target population since the start of study.

CVS

In present study, CVS was the method of choice for obtaining fetal tissue. Oladapo OT (2009) reported that CVS provides information earlier in pregnancy when it is emotionally relatively easier for parents to decide about MTP [33]. In Maximum (81.08 %) number of cases CVS was done in first trimester and in 19.92 % of cases CVS was done in second trimester (<20 weeks of gestational age); allowing safe Medical termination of pregnancy for affected fetus. These findings are comparable with previous studies done by Akinyanju et al. [34] and Arora et al. [15] who obtained fetal tissue in maximum number of cases by CVS in early pregnancy.

Spontaneous Abortion Following CVS

Olney et al. [35] stated that all spontaneous abortions occurring after CVS are not directly related to the CVS procedure. Arora et al. [15] from India reported that six fetuses were aborted spontaneously and all abortions took place more than 4 weeks after the procedure. In present study three spontaneous abortions occurred, each more than 6 weeks after the CVS procedure. Thus CVS

procedure could not be a direct cause of spontaneous abortion. Various other factors causing spontaneous fetal loss in a normal pregnancy could have played role in these cases.

Fetus Genotype

There are several methods to perform prenatal diagnosis of sickle cell anemia. We needed to establish PND facility for SCD in our set up using a suitable technique. Extracted DNA from Chorionic villi sample was subjected in parallel to ARMS-PCR and PCR followed by restriction enzyme digestion of PCR amplified product. While we were successful in obtaining results with ARMS-PCR, we fail to establish PND of SCD by restriction enzyme digestion of PCR amplified product with ddeI. Review of literature showed ARMS-PCR to be useful in cases with a known sickle cell mutation. As all our subjects were known sickle cell anemia patients, we gave reports based on ARMS-PCR alone.

Complete fetal Hb genotype for sickle cell anemia was offered in 32 cases (86.49 %). This is in concordance with previous study from India by Arora et al. [15] who gave precise prenatal diagnoses for various hemoglobinopathies by using ARMS method in maximum number of cases. In 4 (10.81 %) cases complete genotype diagnosis was not possible due to a faint band seen with mutant sickle specific primer and were reported as AS/AA genotype i.e. fetus may be normal or carrier for SCD. Though a complete genotype was not offered but we were able to rule out that fetus would not suffer from SCD. Similar findings were reported by Goosen et al. [36] and Tuzmen et al. [37]. The faint band seen with mutant sickle cell primer could be due to maternal contamination. Elles [38] reported that chances of ambiguous results in CVS are high because sometimes maternal cells are indistinguishable from fetal cells and may be included in cell analysis. Chorionic villi were meticulously separated from maternal deciduas under dissecting microscope but no other method was used to rule out maternal contamination. To avoid ambiguous results, more suitable technique should be used in parallel to detect any presence of maternal contamination.

Correlation of Fetus Hb Genotype with Follow Up HPLC of Baby's Peripheral Blood Sample and Sensitivity of PCR

On follow up HPLC, 18 (75 %) cases were correlated and 6 (25 %) were mismatched. As maternal contamination is major cause of diagnostic error, it is a prerequisite to separate villi from maternal deciduas under inverted or dissecting microscope [38, 39]. Very little amount of maternal DNA present in fetal sample can amplify with

either of ARMS-PCR primer and forms a band visible on gel electrophoresis, resulting in misdiagnosis. Batanian et al. [40] reported that VNTR-PCR (Variable number tandem repeat) done along with test procedure is useful for ruling in or out presence of any maternal DNA in the fetal test sample. Colah et al. [30] reported that in few cases diagnosis was incorrect due to maternal contamination in fetal sample. In 2001, Arora et al. [15] performed VNTR PCR for a hypervariable human DNA marker in selected cases to rule out possibility of maternal contamination. As we were in initial stages of establishing laboratory for PND of SCD, fetal DNA was obtained after separating chorionic villi from maternal decidual tissue under a dissecting microscope. No other standard procedure was used to rule out maternal contamination. In our study, 25 % mismatched cases show that ARMS-PCR alone is not 100 % sensitive and performing test like VNTR PCR, to detect the presence of maternal cell DNA contamination, in parallel with ARMS-PCR can increase the sensitivity of prenatal diagnosis by ARMS-PCR.

Overall ARMS-PCR is a simple technique to be established for PND of SCD as it gave good and rapid results in our set up. But it should be done in parallel with other standard procedures to avoid ambiguous results.

As all the three couples with affected fetus opted for MTP, our aim to prevent the birth of children affected with SCD and thereby reducing the incidence of sickle cell gene was achieved. Overall burden of sickle cell gene on society thus can be decreased by sensitizing the couples with sickle cell gene through proper genetic counseling and providing option of Medical Termination of Pregnancy for affected fetus. By creating awareness about the safety and efficacy of modern prenatal diagnostic techniques more couples can benefit in future.

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