

Use of methylation sensitive polymerase chain reaction for detection of fragile X full mutation & carrier state in males

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Background & objectives: Fragile X syndrome is the most common cause of inherited mental retardation. It is characterized by the progressive expansion of polymorphic (CGG) trinucleotide repeats located in the promoter region of the *FMRI* gene located at Xq27.3. The typical dysmorphic features that help in diagnosis are very often subtle or absent especially in pre-pubertal children. Confirmation is by molecular diagnosis based on repeat size and methylation analysis of the *FMRI* gene. The present study was done to evaluate the utility of a methylation sensitive polymerase chain reaction (ms-PCR) method in the molecular diagnosis of fragile X syndrome in a select group of mentally retarded male children.

Method: We used a methylation sensitive PCR technique, which initially modified DNA by bisulphite treatment. Two sets of PCR primers one each for methylated and unmethylated DNA sequences, were used. In full mutations, PCR specific for the methylated sequences was designed to amplify the CpG dinucleotide region upstream to the CGG repeats in clinically affected males. In healthy males and carriers, the second set of primers would amplify the unmethylated DNA sequences. The amplified PCR product size would help to differentiate between normal and premutation repeat size.

Results: In all, 25 blood samples collected from mentally retarded male children and five from normal controls were tested. Analysis of cases revealed one full blown mutation and one carrier state. These were further confirmed by southern blotting.

Interpretation & conclusion: Unlike currently used methods, methylation sensitive PCR is a quick and accurate technique which could be used for the rapid screening of fragile X syndrome in mental retardation.

Key words Fragile X syndrome - full mutation - methylation sensitive PCR

Fragile X syndrome is the most common inherited cause of mental retardation accounting for approximately 1.5 per 10,000 of all cases of mental retardation about 1/4000 for males and 1/8000 for females¹. Clinical features show marked heterogeneity. Characteristic features such as long face with prominent mandible, large ears and macro orchidism are seldom noticed in pre-pubertal children². Mental retardation varies from mild to profound retardation with affected males being more severely affected. A CGG trinucleotide tandem repeat at the 5' untranslated region of the *FMR1* gene located at Xq27 has been found to be associated with this syndrome³. In normal unaffected individuals, the number of repeats ranges from 6 to 52 copies. In clinically unaffected (carriers) individuals, alleles in the so called premutation range may have between 52 and 200 copies of the repeat. In individuals with full mutations the repeat number ranges from 200 and above. In affected individuals with a full mutation, the expanded repeats in the 5' untranslated region of the *FMR1* gene and the 5' region surrounding the promoter of the *FMR1* become *de novo* methylated rendering it transcriptionally inactive⁴.

In view of great variability in presentation, clinical diagnosis is extremely difficult⁵. Numerous diagnostic methods have been developed for fragile X syndrome, including cytogenetic, Southern blot, polymerase chain reaction (PCR), methylation specific PCR (ms-PCR), reverse transcription PCR (RT-PCR), and immunohistochemical analyses⁶⁻¹⁰. The most commonly used diagnostic test for detection of fragile X syndrome is Southern blot analysis of *EcoRI* and *EagI* digested genomic DNA with the StB12.3 probe¹¹. The major disadvantage of the Southern blot method is its difficulty in distinguishing between large normal and small premutation alleles. Besides it is time consuming, expensive and inconvenient for rapid screening of large number of individuals. Conventional PCR has failed to identify DNA fragment containing large CGG repeats because of the very high C+G content. Some PCR based strategies which require subsequent Southern blotting have been reported to be reliable, but again are labour intensive. Methylation sensitive PCR is found to be a quick

and relatively inexpensive technique useful in the diagnosis of fragile X syndrome. Based on the method described earlier¹⁰, this test relied on the methylation sensitive conversion of cytosine residues "C" to uracil "U" by bisulphite treatment on single strand DNA. Primers were designed to amplify the antisense DNA strand only in affected patients (full mutations). As a result of mismatch between primers and bisulphite treated DNA, no PCR fragments would be obtained in normal individuals and carriers. The bisulphite treatment also reduces the C+G component of the region. The present study was carried out to ascertain the utility of methylation sensitive PCR for detection of full and premutation states of *FMR1* gene in a select group of mentally retarded male children in Mangalore, south India.

Material & Methods

Sample: The study sample consisted of 25 male children with mental retardation (IQ ranging from 35-70), aged 4-11 yr. A total of 38 children were selected during a three month study period (June 2003-August 2003) at the Department of Neurology, KS Hegde Medical Academy, Mangalore. Following detailed examination, out of the 38 children examined, those with Down's syndrome phenotype (a) and cerebral palsy (4) were excluded. The physical abnormalities noted included microcephaly in five (defined as head circumference below the mean for age and sex), low set ears in six, large prominent ears in three, prominent forehead in two and hyperextensible joints in three. Seizures were present in nine cases studied. There were two children with mild ataxia of stance and gait. In addition, five normal control samples [available from children being investigated for disorders other than mental retardation - anaemia (3) and fever (2)] were also included in the study.

DNA extraction and PCR amplification: DNA extraction was done using whole blood DNA extraction kit (Bangalore Genei, Bangalore). This was followed by bisulphite treatment and PCR amplification as per the method described earlier¹⁰. Accordingly, DNA was diluted in 50 µl of distilled water and 5.5 µl of 2M sodium hydroxide was added.

To create single stranded DNA, it was incubated at 37°C for 10 min and 30 µl of 10 mM hydroquinone was added to each tube. Hydroquinone was freshly prepared by adding 55 mg of hydroquinone to 50 ml of water. To this 520 µl of freshly prepared sodium bisulphite was added. Sodium bisulphite was prepared by adding 1.88 g of sodium bisulphite to 5 ml of water and pH was adjusted to 5.0 with sodium hydroxide. The reagents were properly mixed with DNA and a layer of mineral oil was added. An incubation period of 16 h was followed at 50° C. The single strand DNA was purified using Wizard DNA cleanup system (Promega, USA) and then desulphoned by adding sodium hydroxide to a final concentration of 0.3 M and incubated at 37° C for 15 min. The solution was neutralized by addition of ammonium acetate (pH 7.0). The DNA was precipitated with 4 volumes of ethanol, separated by centrifugation, dried and resuspended in 50 µl of double distilled water.

For the PCR amplification, the following primers (Bangalore Genie, Bangalore) were used for amplification of CpG island located upstream of the repeats- FR611R: CGT CGT CGC GTT GTC GTA C and FR690F: AAC CAC GAA CCG ACG ACG. These primers complimented the modified antisense strand and were specific for the amplification of methylated C present in affected individuals and normal *FMR1* gene on the inactive X chromosome. PCR amplification was performed in 50 µl volumes containing 0.2 mM dNTPs, 0.5 µM of each primer, 200 ng of bisulphite treated DNA and 1U of *Taq* polymerase (Bangalore Genei, Bangalore). The thermocycling programme consisted of 5 min denaturation at 94°C, followed by 32 cycles at 93°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds and a final extension of 10 min at 72°C in a PTC 100 Thermocycler (MJ Research Inc., USA). For amplification of fragments containing repeats, the following primers were used. FR526R: GGG AGT TTG TTT TTG AGA GGT GGG and FR754F: CAA CCT CAA TCA AAC ACT CAA CTC CA. The PCR products were separated by electrophoresis on 2 per cent agarose gel containing ethidium bromide and photographed using Gel Doc system (Hero Lab, Germany).

Positive Control was provided by Dr K. Majumdar, centre for cellular and Molecular Biology, Hyderabad, India. Negative samples were from healthy normal males. Negative control used in this study was PCR without addition of DNA.

Results

With the first set of primers (FR611R and FR690F), designed for the CpG island upstream of the trinucleotide repeats, an 80 bp fragment was amplified in affected males, where as in healthy and carrier males, no PCR amplification could be detected (Fig. 1). This is because of the mismatch between bisulphite treated DNA and primers. The second set of primers (FR526R and FR754F) amplified the fragment containing non methylated trinucleotide repeats. All normal samples showed bands at 280-300 bp. A 400 bp fragment was identified in a normal carrier males (Fig. 2). In normal and healthy transmitting male (carrier), following bisulphite treatment, the modified repeats in the antisense strand were amenable to PCR amplification. This is because the 3'-UUG-5' repeats contained approximately 33 per cent G as opposed to the high percentage (67%G) in the sense strand and enabled a detectable amplification following the use of primers FR526R and FR754F. However, in the affected males the high

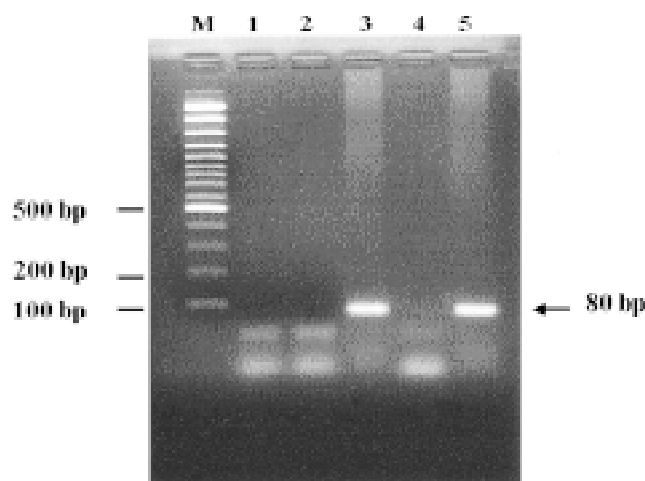


Fig. 1. Result of PCR using primers FR611R and FR690F for amplifying the methylated C residues. Lane M, GeneRuler100 bp DNA ladder plus (Fermentas, USA); lane 1, negative control (PCR without DNA); lanes 2 and 4, negative samples (healthy males); lane 3, full mutation; lane 5, positive control.

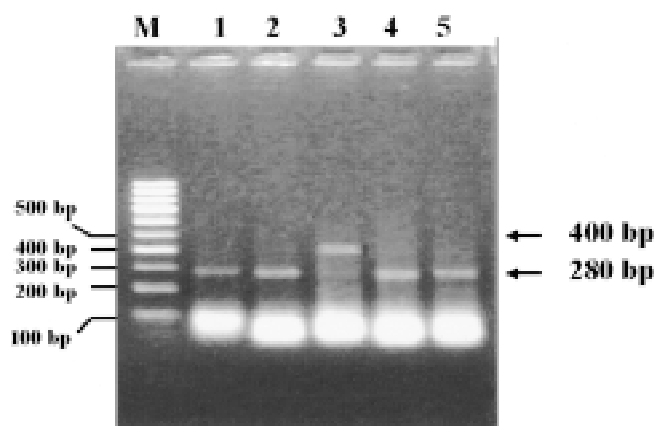


Fig. 2. Result of PCR with primers FR526R and FR754F which amplifies the bisulphite treated unmethylated DNA sequences. Lane M, 100 bp ladder (Bangalore Genei, Bangalore); Lanes 1, 2, 4, 5 normal; Lane 3, carrier.

C+ G content and the expanded size of repeats, prevented efficient PCR amplification resulting in no visible fragment by ethidium bromide staining. By this method, using both sets of primers, one affected male and one carrier was detected. These were further confirmed by Southern blot analysis.

Discussion

Fragile X syndrome is inherited as an X-linked semi dominant inheritance^{12,13}. Gender of carrier parent, gender of the offspring and the number of CGG repeats are important factors that influence disease expression. The complex pattern of inheritance poses an extraordinary challenge for accurate diagnosis and genetic counselling of affected families. Though a variety of clinical phenotypic characteristics has been described, none are singly or in combination helpful in definitive diagnosis¹⁴.

Though cytogenetic methods and PCR followed by Southern blotting have been used in Indian studies on Fragile X syndrome¹⁵⁻¹⁹, methylation sensitive PCR has not been reported. In our study with 25 samples only one case with full mutation and another with premutation (carrier state) were detected. The carrier state and healthy normal samples were clearly distinguishable by the size of the amplified PCR product, as also the fragile

X positive sample. The basic principle of this method is that it relies on the ability of bisulphite to deaminate C residues in a single strand DNA. A characteristic of the bisulphite treated DNA is that after modification, the sense and antisense strand are no longer complementary. Thus, the modified strands can be amplified separately by designing primer pairs specific for each of them. The C residues of all CpG dinucleotides flanking the CGG repeats as well as those of the CGG repeats are methylated in affected males and in the inactive X chromosome in females. The same C residues are however, unmethylated in healthy males, normal transmitting males and in the active X chromosome in females. The disadvantage of this methylation PCR is that it cannot reliably diagnose affected females with fragile X syndrome due to the fact that the inactive X chromosome is already methylated. During screening, all positive female samples will have to be subjected to Southern blot analysis for confirmation of diagnosis. Recently modifications have been incorporated into methylation sensitive PCR strategies which reliably differentiate normal from carrier and full mutations, in both females and males^{8,20}. All our DNA samples in the present study were from male children with mental retardation.

There is no specific treatment for fragile X syndrome. It is recommended that in the Indian context where financial constraints prevent large scale prenatal screening, DNA tests for fragile X syndrome should be done in all mentally retarded children without an obvious cause, along with genetic counselling of the involved families. Methylation sensitive PCR strategy is one of the most comprehensive methods available at present for the accurate diagnosis of fragile X carrier and disease state⁸. Compared with the commonly used Southern blot analysis, it is simpler, less time consuming and eliminates possible false positives due to partial digestion by methylation sensitive enzymes used in Southern blot analysis. The method does not require radioactive chemicals, biotin or digoxigenin. In addition, the small amount of DNA required for this test is advantageous for large scale screening of mental retardation.

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