Polymerase Chain Reaction Analysis as a Rapid Screening Test for Diagnosis of Fragile X Syndrome

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ABSTRAK

Sindrom 'Fragile X' lazimnya disebabkan oleh ketidakstabilan kembangan jujukan ulangan trinukleotid pada gen FMR-1. Warisannya adalah rantaian X diturunkan secara 'reduce penetrance' dan frekuensinya terus meningkat melalui beberapa generasi. Fenotip yang dimanifestasikan oleh sindrom 'fragile X' adalah tidak spesifik. Penganalisaan secara sitogenetik memerlukan pengalaman untuk mengenalpasti tapak rapuhan pada metafasis manakala analisis DNA seperti Pemblotan 'Southern' adalah sangat mahal apabila ia digunakan sebagai rujukan rutin saringan. Kajian ini bertujuan untuk memperkenalkan teknik berasaskan tindakbalas rantaian polimerase ('Touch down PCR') sebagai kaedah saringan sindrom 'Fragile X'. Sejumlah enam kes telah dikajii. Antaranya, satu kes merupakan kes sindrom Fragile X (T1) yang telah dikenalpasti dengan kaedah sitogenetik konvensional, dua lagi merupakan ibu (T2) dan bapanya (T3). Dua kes yang lain (T4 dan T5) telah dipilih secara rawak daripada pesakit yang masing-masing menunjukkan ciri-ciri dismorfik dan kerencatan perkembangan. Satu kawalan normal (TC) juga dimasukkan. Analisis sitogenetik terhadap tapak rapuhan telah dijalankan. Dua sistem kultur digunakan, termasuk kultur 'synchronized' limfosit dan kultur kurangan folat-thimidin. Pencelupan metafasa dari kultur 'fragile X' dianalisis untuk kehadiran tapak rapuhan pada kromosom X. Kariotip daripada pencelupan saluran-G dibuat dengan alat penganalisa imejan untuk kesemua sampel yang dikaji bagi menyingkirkan kehadiran ketidaknormalan kromosom. DNA diekstrak daripada sampel-sampel ini dan digandakan dengan rantai polimerase 'Touch-Down'. Analisis sitogenetik menunjukkan satu tapak rapuhan sensitif-folat pada lelaki yang disyaki dan tapak rapuhan negatif pada lima sampel yang lain. Kariotip daripada pencelupan saluran-G menunjukkan tiada tambahan ketidaknormalan kromosom. Kesemua DNA yang diekstrak mengganda dengan baik. Lima daripada sampel kajian tapak rapuhan negatif menunjukkan kehadiran produk pada saluran yang dijangka iaitu 522bp, yang menunjukkan tiada kembangan gen FRM-1. Namun, tiada produk PCR dikesan pada lelaki yang dijangkiti; keputusan ujian PCR menunjukkan beliau mungkin mengalami kembangan penuh gen FMR-1. Kajian kami menunjukkan bahawa ujian PCR adalah satu kaedah yang cepat dan spesifik untuk menyaring alel normal dan mutasi penuh pada gen 'Fragile X'. Kami mencadangkan ia digunakan sebagai kaedah pelengkap kepada analisis sitogenetik untuk mengesan gen FRAXA (*Folate Sensitive Fragile Site at Xq28*).

Kata Kunci: Gen FMR-1, Sindrom Fragile X, Polymerase chain reaction (PCR), Touch down PCR

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ABSTRACT

Fragile X syndrome is a result of an unstable expansion of $(CGG)^n$ trinucleotide sequences in the FMR-1 (Fragile X Mental Retardation 1) gene site at Xq27. In a normal person, n ranges from 6 to 40 repeats with an average of 30 repeats, whereas in a mutated FMR1 gene the sequence is repeated several times over (stuttering gene). Full mutation occurs when n equals 200 repeats or more. Where n equals 50 to 200 repeats, it is a premutation. Fragile X occurs when the FMR-1 gene is unable to make normal amounts of usable Fragile X Mental Retardation Protein, or FMRP. The amount of FMRP in the body is one factor that determines the severity of the Fragile X syndrome. A person with nearly normal levels of FMRP usually has mild or no symptoms, while a person with very little or no normal FMRP has more severe symptoms. The mechanism for the role of the FMRP gene is still being researched upon. However, it has been observed that large numbers of repeats (more than 200) inactivates the gene through a process of methylation and when the gene is inactivated, the cell may make little or none of the needed FMRP. Inheritance is X-linked with reduced penetrance and the frequency of occurrence goes up through generations. The phenotypic manifestations of fragile-X syndrome vary and are largely dependent on the size of the mutation or premutation. The identification of the fragile site on G banded metaphases is a time consuming and delicate process requiring experience and skill, however, molecular diagnosis using DNA analysis and Southern blotting, even though expensive, is more specific in determining the presence or absence of the gene. This study was aimed to establish a rapid polymerase chain reaction (PCR) based - touch down PCR, as a screening method for fragile X syndrome. A total of six cases were analysed. Of these, one was a known case of Fragile X (T1) diagnosed by conventional cytogenetics, two were from the latter's family members namely, his mother (T2) and father (T3), and the other two (T4 and T5) were randomly selected from patients presenting with dysmorphic features and delayed development respectively. One normal control (TC) was included. Cytogenetic analyses for detection of the fragile site was carried out in all cases. Two culture systems were used, namely the synchronised lymphocyte culture and the folate - thymidine deficient culture. Stained metaphases from the fragile X cultures were screened for the presence of the fragile site on the X chromosome. G-banded karyotyping was done using an image analyser to exclude presence of chromosomal abnormalities. DNA was extracted from these samples and amplified by touch-down PCR. Cytogenetic analysis revealed a folate-sensitive fragile site in the affected male, but none in the other five samples. G-banded karyotyping exhibited no additional chromosomal abnormalities. All extracted DNA samples were successfully amplified. Five of the samples showed presence of the product at the expected band at 552bp, excluding the presence of an expansion of CGG segment of the FMR-1 gene. The absence of a band in an affected individual, suggested a fully mutated allele of FRAXA (Folate Sensitive Fragile Site at Xq28). We succeeded in establishing a slightly modified touch-down PCR analysis. Our study indicates that PCR testing offers a rapid and specific method for screening of normal allele and full mutation of the fragile X gene. We suggest this technique to be applied as a complementary tool for cytogenetic analysis to detect the FRAXA gene.

Key words: FMR-1 gene, Fragile X syndrome, polymerase chain reaction (PCR), touch down PCR

INTRODUCTION

Fragile X syndrome is caused by repetitive trinucleotide repeats $(CGG)^n$, in the first exon of the fragile X mental retardation-1 gene (FMR-1) of the X chromosome (Fu et al. 1991). When affected individuals have a large amplification, the DNA of the entire 5' region of the gene becomes abnormally methylated. This is associated with methyllation of an upstream gene promoter region (a CpG island) which results in transcripttional suppression of FMR-1, resulting in disease. Therefore, most affected males have no detectable FMR-1 mRNA, and it is believed to be the cause of the phenotype (Warren and Nelson 1994). At least three folate sensitive fragile sites, namely FRAXE, FRAXD, and FRAXF that map to distal Xq are involved, but only FRAXA is associated with the fragile X syndrome (Parrish et al. 1994; Sheila et al. 2000). FRAXA is located on the chromosome X at position Xq27.3 (Jin and Warren 2000), near the end of the long arm. It is the most common cause of mental retardation. It is inherited as an X-linked dominant disorder with reduced penetrance with an incidence of 15-20% of all X-linked mental retardation cases (Pergolizzi et al. 1992; Tarleton and Saul 1993; Warren and Nelson 1994).

The severity of the disease is correlated with the length of the CGG tract. Affected males and females exhibit a massive expansion resulting in greater than 200 copies or may increase, up to 1000 repeats. Such expansions are called 'full mutations'. Nonpenetrant male and female carriers exhibit premutation alleles between 60 and 200 copies (Sarah et al. 2003).

Male carriers transmit only unstable premutations while female premutation carriers can have carrier offsprings with premutation or affected children with full mutations. The phenotypic manifestations of fragile X syndrome are not specific. These may include variable degrees of mental retardation, prominence of the jaw and forehead, dysmorphic ears, high-arched palate, macrocephaly, macroorchidism and hyperactivity. Males with fragile X syndrome usually have mental retardation and often exhibit characteristic physical features and behavior. Affected females exhibit a similar but usually less severe phenotype, likely the result of random X-inactivation patterns (Warren and Nelson 1994).

The usual diagnostic analysis for fragile X syndrome is based on the detection of a fragile site by cytogenetic analysis, following specific cell culture conditions that modulate deoxynucleotide triphosphate levels. This however, has been difficult and sometimes inadequate for diagnosis (Rousseau et al. 1991), because other fragile sites such as FRAXE also exhibit the fragile site characteristics as FRAXA mutation (Warren and Nelson 1994). Molecular diagnosis of the CGG expansion mutation has been developed using Southern hybridization methods. However, the Southern hybridization tool is relatively expensive, time-consuming, laborious and requires large quantities of genomic DNA (Soon et al. 2001). Methods using PCR technique appears to be rapid and efficient for screening (Brown et al. 1993). It utilizes flanking of specific primers to amplify a fragment of DNA spanning the repeat region. In this study, the PCR analysis was verified using DNA obtained from a fragile X family, two cases with unrelated mental retardation, and a normal control. Thus, the aim of this study is to establish a rapid and simple PCR method as a screening tool for fragile X syndrome.

MATERIALS AND METHODS

Samples

A total of six samples were analysed. One of the samples was taken from a known fragile X positive male patient (T1), who was referred to the Haematology Unit. In 1998. Blood samples were also collected from his mother (T2) and father (T3) for cytogenetic analysis. The other two samples (T4 and T5) were randomly

selected, that is from two patients presenting with dysmorphic features and delay in development. The control (TC) sample was taken from a normal healthy female, who was cyto-genetically negative for the FRAXA gene at the fragile site. A detailed family history was obtained from all these cases.

Cytogenetic Analysis

Peripheral blood lymphocytes were cultured according to standard laboratory protocol for fragile X analysis. A combination of the two-culture system was used. These included lymphocyte culture (Fragile X culture) under folic acid and thymidine deficient condition (media 199 with 5% bovine serum, antibiotics penicillin and streptomycin, and PHA) and the other was a synchronised lymphocyte culture (1640 media with 5% fetal bovine serum, antibiotics penicillin and streptomycin, PHA and thymidine 0.1 M final concentration). All cultures were incubated at 37 °C for 72 hours. The culture was harvested after adding 0.1 ml colcemid (10µg/ml) for 7 minutes for the fragile X culture. 0.2ml (20µg/ml) of colcemid was added 1 hour before harvesting time for the synchronised culture. The cultures were treated with 0.075M potassium chloride (hypotonic solution) and fixed in 3:1 methanol acetic acid mixtures. Slides were aged and Leishman banding was carried out. At least 50 to 100 well spread, stained metaphases were scored to determine the presence of the fragile site on the X chromosome. Fragile sites appear as non-stainning regions, chromatid gaps, or less frequently, as breaks near the terminal end of the long arm of the X chromosome. Banded metaphases from the synchronised culture were also analysed to exclude the presence of chromosomal aberrations. Karyotyping was carried out using an image analyser (CytoVision, Applied Imaging).

DNA Extraction

DNA extraction was performed at the time of diagnosis. Samples were collected in ethylenediamine-tetraacetic acid (EDTA) tube and DNA was extracted from whole blood using the standard protocol of lysis; proteinase K digestion, followed by extraction with a combination of phenol and chloroform and precipitation with cold ethanol. The extracted DNA is aliquot and stored at -20°C.

PCR Amplification and Analysis

The archived DNA samples were subjected to a touch-down PCR analysis based on a slight modification of a previously described method (Tan et al. 2000). In this modified method, the enzyme AmpliTaq polymerase was replaced with the enzyme AmpliTaq (gold) polymerase. Amplification was carried out in a thermocycler in 30 µl reactions containing approximately 300ng of DNA template, PCR buffer, 10 pmol each of primers g (forward primer) and f (reverse primer), 1.5 U of AmpliTaq (gold) polymerase (Perkin Elmer), 10% dimethyl sulphoxide (DMSO) and 200µM each dNTP. dGTP was substituted with 7-deaza-2' dGTP in a ratio of dGTP (1.2) : 7-deaza-dGTP (1.0) to amplify the mutated alleles. For the negative control, DNA template was replaced by RNase free water.

The reaction mixture went through a 'touch-down procedure'; denaturation at 95°C for 8 minutes, followed initially by 5 cycles of denaturation at 95°C for 90 seconds, annealing at 70°C to 66°C (decreasing by 1°C per cycle) for 60 seconds and elongation at 72°C for 120 seconds. The reaction mixture was then subjected to 35 cycles of amplification at 95°C for 90 seconds, 65°C for 60 seconds, 72°C for 120 seconds, and a final extension of 10 minutes at 72°C. The PCR products were analysed on 1.0% agarose gel containing ethidium bromide using the gel documentation system. The amplicon sizes were determined by using a 100bp

standard DNA ladder. The presence of a band of the correct size approximately 522bp for the normal alleles excludes the presence of an expansion mutation at the FRAXA locus. An increased number of CGG repeats revealed no detectable band on the gel, indicating full mutation of the FRAXA gene in an affected individual.

RESULTS

Results of the cytogenetic analysis showed no chromosomal aberrations in five (T2, T3, T4, T5, TC) out of six samples studied (Table 1.0). One sample (T1) demonstrated presence of a fragile-site. The fragile site appears as a gap or break on the long arm of the X chromosome (Figure 1.0). All of the samples however, showed cytogenetically normal karyotypes using banded metaphases.

Table 1: Summary of results of cytogenetic analysis, fragile site detection and PCR analysis of the FRAXA locus

Sample	Sex	Results		
		Cytogenetic Analysis		PCR Analysis
		Karyotype	Presence of Fragile site	Presence of 522bp product
T1	Male	46,XY Normal male karyotype	Yes	Absence
T2	Female	46,XX Normal female karyotype	No	Present
T3	Male	46,XY Normal male karyotype	No	Present
T4	Male	46,XY Normal male karyotype	No	Present
T5	Male	46,XY Normal male karyotype	No	Present
C	Female	46,XX Normal female karyotype	No	Present

PCR analysis was carried out on all genomic DNA samples. The migrations of PCR products were analysed. As presented in figure 2.0, the presence of a 522bp band (for the normal allele) was observed in lanes 1, 3, 4, 5 and 6, thus excluding the presence of an expansion mutation at the FRAXA locus. The sample (T1) from the known case of fragile X syndrome (lane 2) showed absence of this PCR product, (figure 2.0) indicating that he may have the full mutation.

The family history of the known fragile-X positive patient (T1) revealed that both of his male siblings have characteristics of mental retardation and facial dysmorphic features of fragile X syndrome. The family of the other five cases showed no history of mental retardation among their siblings and parents.

DISCUSSION

Over the past few years, the diagnosis of fragile X syndrome was based on the presence of a folate-sensitive fragile site at Xq27.3 (FRAXA) induced in cell cultures under conditions of folate deprivation. In this study, cytogenetic analysis of the affected individual (T1) showed fragile sites at the distal end of the long arm of chromosome X (figure 1.0) when cultured under folate deficient conditions. However, cytogenetic analysis was unable to determine whether the breakage or gap resulted from expansion of the FRAXA gene or from other fragile sites genes. For instance, other fragile sites such as FRAXE (Xq28), that is located approximately 600 kilobases distal to FRAX, also exhibit fragile sites under folate deficient culture (Knight et al. 1993). This has led to difficulty in the interpretation of fragile sites using the conventional cytogenetic method. Cytogenetic analysis is less sensitive for detecting affected individuals. It also results in a high degree of variability between individuals and laboratories.

Our PCR analysis utilises flanking specific primers to amplify fragments of

Figure 1: Partial karyotype of G-banded (left) and stained (right) fragile X chromosome (arrow indicating the fragile sites)

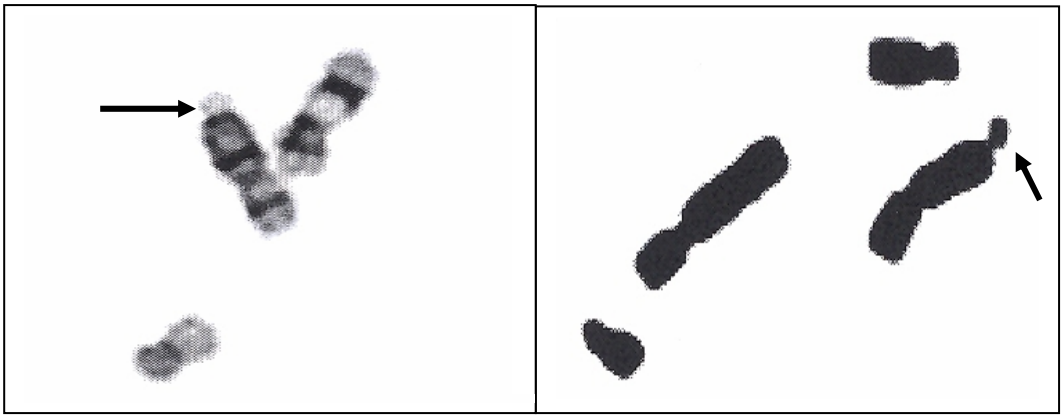
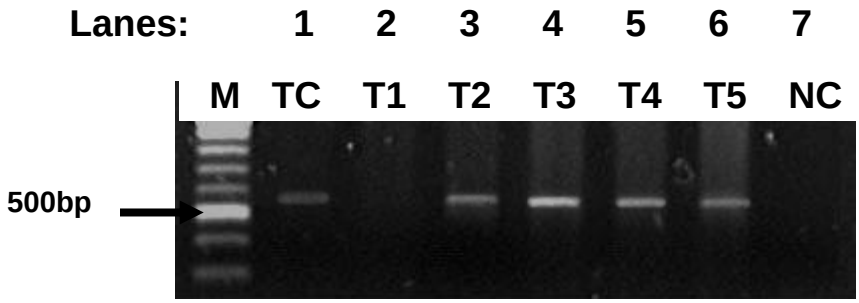


Figure 2: Gel electrophoresis showing Polymerase chain reaction (PCR) product of FRAXA locus. Lane 1 (light band) is normal female control (TC), lanes 2 to 6 are patients' samples (T1, T2, T3, T4, and T5 respectively). Lane 7 is a negative control (NC).M is the100bp DNA ladder



DNA spanning the repeat region. It amplifies the normal repeat size or those that are within the normal range, thus produces products of the expected size (figure 2.0). As expected, in the affected patient (T1), there is absence of the band, indicating full mutation of the FRAXA alleles, because the repeats are too large to be amplified (Soon et al. 2001; Wang et al. 1993). However, expansion involving other fragile site genes which demonstrated breakage under folate deficient conditions, will be amplified, and detectable bands will be observed on the electrophoresis gel. Thus, the PCR analysis is specific for detection of the FRAXA full mutation. It is also able to differentiate it from expansion due to other fragile site genes.

The modified PCR analysis as outlined

in this study is more sensitive and informative as a screening tool for detecting full mutation, compared to cytogenetic analysis. The method has its limitations in that it is unable to give an accurate size determination of full mutations and also to distinguish between normal and fragile X premutation carrier individuals. Affected patients showing absence of the expected band and females who show normal CGG allele should therefore be investigated further using Southern blotting. Although the Southern blot is the key diagnostic tool to detect the mutations of the fragile X syndrome, it is relatively laborious, time consuming, needs large quantities of DNA material, and is expensive. It is therefore not a suitable method for routine screening of fragile X syndrome.

Studies of family pedigrees are important because they help in excluding male transmission of fragile X and also to determine the carrier status. For instance, the family history of the affected male revealed that both of his male siblings also showed characteristics of fragile X syndrome, namely mental retardation and facial dysmorphic features. However, cytogenetic and PCR analyses were not carried out on the male siblings because they were residing abroad at the time of diagnosis. The paternal family history was not significant and revealed high intellectual capacities in all generations. The mother of the affected male was suspected to be an obligate fragile X carrier. Studies have shown that women who are pre-mutation carriers can have carrier offsprings with pre-mutations or affected children with full mutations (Warren and Nelson 1994; Soon et al. 2001; Pieretti et al. 1991). The chance that the allele will increase to full mutation depends on its size in the mother. All affected males and the majority of affected females inherit the mutation from their mother. The other two patients showed no family history of mental retardation.

PCR amplification of CGG repeats in the FRAXA fragment is difficult, and special conditions are required. The incorporation of 7-deaza-2' dGTP to stabilise secondary DNA structures allows successful amplification of the CG-rich region (Tan et al. 2000; Latimer and Lee 1991). However, PCR products with a high content of 7-deaza-2' dGTP, do not stain efficiently with ethidium bromide (Innis 1990; Latimer and Lee 1991). Presumably, the presence of 7-deaza-2' dGTP alters the electronic structure of bound ethidium so that it can no longer fluoresce (Latimer and Lee 1991).

To investigate whether these changes may be an appropriate alternative to the described procedure, we have used the ratio 1.0: 1.2 respectively of 7-deaza-2' dGTP to dGTP instead of 1: 1 ratio as previously described (Tan et al. 2000). We found that by changing the ratio of 7-deaza-2' dGTP to dGTP , our PCR reaction was able to successfully amplify the FRAXA allele within the normal range. Together with efficient staining by ethidium bromide direct visualization of the PCR product under ultraviolet light was obtained. Omitting 7-deaza-2' dGTP or lowering the ratio of 1.0 : 1.2 of 7-deaza-2' dGTP to dGTP is not recommended because it will increase secondary DNA structures in the PCR reaction. This may result in non-specific bands appearing during electrophoresis. The minor changes of using AmpliTaq Gold®DNA polymerase rather than AmpliTaq polymerase as a chemical hot start enzyme has resulted in the increased specificity and product yield without biological contamination of the PCR reaction. This modification has led to increased sensitivity of the reaction, diminishing the formation of non-specific product while allowing detection of low target copy number of the PCR product.

Interpretation of fragile X also poses some problems. The usual precautions for the prevention of contamination of the PCR product must be observed. Shadow or heteroduplex bands must be distinguished between true abnormal alleles when non-denaturing electrophoresis is used. Lane background and faint bands are other problems faced and these should be inter-

preted with caution as smears of expanded fragments can be very faint.

The PCR analysis carried out in this study is a basic and simple technique that is completely independent of methylation and is specific for the FRAXA locus. Our study indicated that PCR analysis using specific primers was more sensitive than cytogenetic analysis to screen for full mutation of the fragile site. Southern blot analysis should still be used for confirmation of the presence of an expanded allele. Typically, molecular diagnostic services should include a combination of both Southern blot-based and PCR-based analysis to cover instances in which one or both types of tests are needed. G-banded karyotyping should be routinely performed as constitutional chromosome abnormalities have been identified as frequently as FMR-1 mutations in mentally retarded individuals referred for fragile X testing. Laboratories often need to use more than one method because no single method can detect all types of mutations equally well or with equal weakness (Maddalena et al. 2000).

CONCLUSION

In conclusion, we have established a simple PCR analysis for screening fragile X syndrome. The PCR is an alternative method with advantages for mutational analysis. It is rapid, sensitive and uses minimal DNA. We recommend that laboratories use PCR as a screening tool for fragile X syndrome. Family history should also be evaluated to assist in the diagnostic screening and to determine the risk of having affected children. Southern blot should also be carried out to detect repeat trinucleotide and methylation status for samples that have failed to amplify (male) or show a normal allele (females). In spite of the advances in molecular testing, routine G-banded cytogenetic analysis should still be performed particularly for cases of unexplained learning difficulties and developmental delay to exclude other chromosomal abnormalities.

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