Evaluation of a CGG Repeat Primed PCR system designed for detection of Fragile X expanded alleles in clinical prenatal samples.

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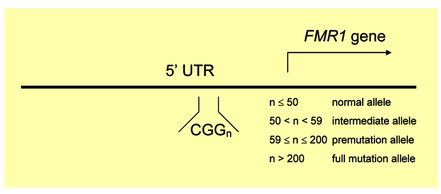
INTRODUCTION

Fragile X syndrome is associated with the expansion of (CGG)n trinucleotide repeats in the 5'-untranslated region (UTR) of the FMR1 (Fragile X Mental Retardation 1) gene.
Small expansions premutations (PM) do not cause FXS, but carriers have an additional risk of developing Fragile X tremor/ataxia syndrome (FXTAS) in later life. Female PM carriers have a further risk of primary ovarian insufficiency (POI). Molecular diagnosis is based on an accurate and efficient sizing of the triplet repeat elements in this region. Routine molecular analysis includes PCR analysis of the allele sizes and Southern Blot (SB). The normal form of the CGG repeat is polymorphic and contains 6 to 50 trinucleotide repeats, with 29-30 repeats being the most common alleles. In general the triplet repeat is interspersed with 0-3 AGG triplets associated with risk of expansion.

Current conventional PCR amplification techniques are only successful for normal and small PM alleles, and are not informative for homozygous female repeat alleles. Additional SB analysis for categorization and sizing is very laborious, time consuming and involves large amounts of high quality DNA, which are not always available.

We evaluated a commercialized PCR based assay (Asuragen) using a large set of archived prenatal samples that were previously analyzed for FXS with conventional PCR and SB analysis in our Center.
We also tested the sensitivity of the triplet primed PCR assay by interrogating mock mosaic samples of leukocyte and prenatal gDNA.

Figure 1 :
***FMR1* gene and expansion categories**



MATERIALS AND METHODS

DNA was extracted by in house standard methods for all prenatal samples (chorionic villi & amniotic cells). Two mock mosaic samples were prepared by admixing the gDNA from (i) a normal and a PM allele; and (ii) a PM and a FM allele. The % mass of respectively the PM and FM was varied (20, 10, 5, 2.5 %).

The AmplideX FMR1 PCR kit from Asuragen has been designed to detect the full range of FMR1 CGG expansions (including PM and FM) by PCR and capillary electrophoresis (CE). The assay has capabilities for two types of tests : a 'gene-specific' PCR (Filipovic-Sadic 2010), and a triplet-primed PCR (Chen 2010). The triplet repeat PCR generates full length PCR products and CGG repeat primed PCR products analyzed in a single CE trace. The full length PCR products allow accurate sizing of any allele under ~250 repeats. Larger alleles appear as a characteristic peak at 1045-1050 bp. The CGG repeat primed PCR products yield a characteristic profile of individual peaks that can be used to resolve zygosity, identify AGG and flag the presence of any expanded allele. The triplet repeat PCR was used for all experiments.
All amplicons were evaluated on an ABI 3130XL Genetic Analyser. Asuragen supplies a data analysis macro (using files exported from GeneMapper v. 4.1) that semi-automates the process of calculating repeat sizes.

The triplet PCR assay was performed according to the manufacturer's instructions. The results of the samples were compared with previous results from SB analysis and our in house conventional PCR protocol.

Figure 2 :
Triplet repeat PCR data of a male chorionic villi sample with a PM of 86 repeats.

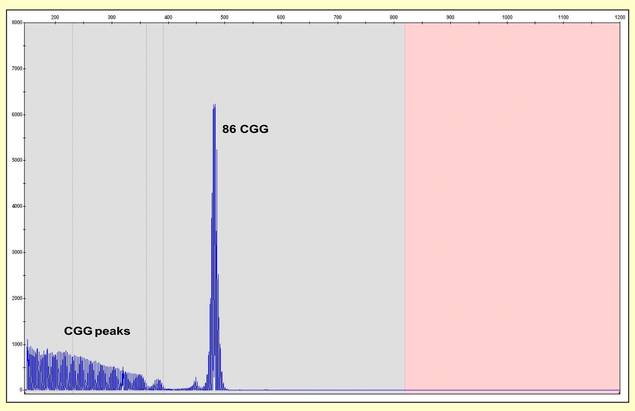


Figure 3 :
Triplet repeat PCR data of a male chorionic villi sample with a PM of around 160 repeats

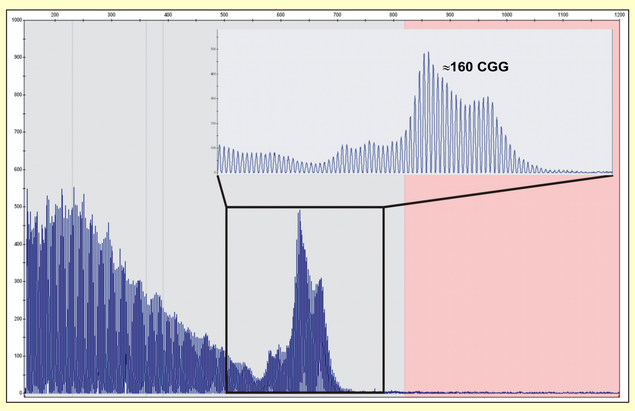


Figure 4 :
Triplet repeat PCR data of a mock mosaic sample of DNA from amniotic cells of a normal allele and a PM (2.5%)

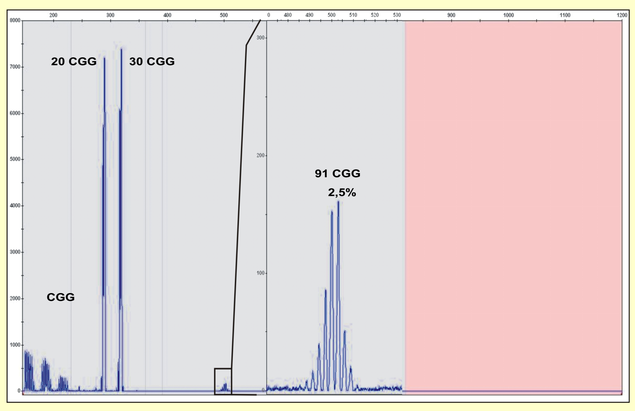
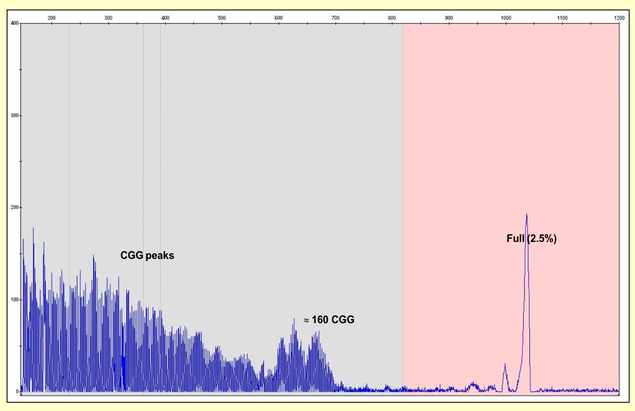


Figure 5 :
Triplet repeat PCR data of a mock mosaic sample of DNA from chorionic villi of a PM and a FM (2.5%)



RESULTS

A large set of archived prenatal samples, both males and females, with a variety of CGG alleles repeat numbers in the FMR1 gene were analyzed with the triplet-primed assay from Asuragen. All samples had previously been processed by both conventional PCR and SB.
In this set of clinical samples, 45 were procured from chorionic villi, while 11 samples resulted from cultured amniotic cells. The gender of all fetuses was known, 27 samples were female and 29 samples were male.
Across this full set of 56 prenatal samples 11, 10 and 14 samples had been characterised as respectively normal, intermediate and PM templates. In addition, the SB analysis had identified 20 FM and 1 mosaic PM-FM sample. In this pool of samples the intermediate alleles sizes ranged from 51 to 58 repeats, the PM alleles sizes from 59 to 160 repeats and the FM from > 200 up to 1350 repeat elements.
All samples were successfully amplified. 20/21 samples were categorised as FM alleles (with 1 sample as a PM-FM mosaic), consistent with the SB results. However, triplet primed PCR analysis also identified one DNA as a normal range amplicon that was previously scored as a FM sample by SB. Most probably, this discrepant result has to be regarded as incomplete digestion, with partially digested DNA generating additional fragments residing at the FM location, mimicking faintly an expansion.

The fragment size in base pairs was converted to the number of CGG repeats. Repeat sizes of the normal, intermediate and (small) PM alleles were compared with previous data. All sizes were within the limits of +/- 1 CGG repeat.

All amplification patterns of true homozygous female samples are readily distinguished as they never presented the typical uninterrupted triplet ladder profile seen with heterozygous expanded samples.

The sensitivity of the assay was assessed with artificial mosaic samples. Mock mosaic PM and FM samples were prepared by admixing respectively various proportions of a normal with a PM or a PM with a FM allele sample at final concentration of 20, 10, 5 or 2.5% of respectively the PM or FM. Both experiments were completed with gDNA extracted from leukocytes, but only mock mosaic samples of a normal and a PM (91 repeats) template from amniotic cells, and a PM and FM template from chorionic villi were available for investigation.
The analytical titration demonstrated that up to 2.5% of PM allele (91 repeats) of gDNA was clearly detectable in a background of normal (20 + 30 repeats) template. However, the identification of a PM (160 repeats) allele in the presence of a FM (1160 repeats) proved to be more difficult. An abundance as little as 10% of full length products masked the presence of the PM amplicon. The presence of the PM allele was only detected when the amount of FM template was ≤ 5%.

DISCUSSION

In this study we analyzed a large set of 56 archived clinical prenatal samples with a variety of CGG alleles repeat lengths numbers, including 21 FM along with a set of control samples (DNA from leukocytes and EBV cell lines) to evaluate a novel triplet primed PCR method.

1. FMR1 genotypes, ranging from normal over intermediate and PM to FM alleles, were accurately and quickly determined in prenatal and control samples, both male and female The Asuragen test amplified and correctly sized expansions up to 160 repeats (largest available PM allele within the prenatal sample set), compared to a previous maximum of around 120 repeats with an in house conventional assay.
   The results for all FM samples were concordant with corresponding SB analyses, except for one. This sample, previously characterized with SB as a large expansion was now sized in the normal range. This discrepancy is most probably due to incomplete digestion with EcoR I endonuclease, a problem that has previously been reported in literature for the FMR1 locus.

2. Homozygous samples, usually representing more than a 1/4 of all female samples, currently must be processed by SB. However, the triplet primed PCR is capable of resolving unambiguously the zygosity of all female samples. Female samples with a single allele in the normal range and no extension of the triplet-primed peaks can now assumed to be homozygous for the normal allele thereby reducing or eliminating the need for SB.

3. The assay is also sensitive to size mosaicism. Indeed, a clincal mosaic sample with a PM and a FM was clearly identified. In artificial prenatal mosaic samples, it was possible to amplify and detect a PM allele present in a normal template background at a final concentration of only 2.5 %. However, the identification of a PM-FM mosaicism is more complex. Although, the presence of a large PM allele did not impact detection of the much longer FM allele, the latter largely masks a proper discovery of the PM allele. Only at a FM amount of 5% and 2.5% was the PM peak detectable.

4. The FMR1 triplet repeat is not a pure CGG repeat. Interspersed AGG elements are known to be present in many FMR1 alleles.
   This assay is also able to identify AGG interruptions. The AGG profile of PM alleles has been proposed to be associated with the risk of expansion.

In summary, this triplet primed PCR assay is able to accurately size and categorize the CGG elements of the FMR1 gene and support molecular diagnosis with a limited requirement for SB analysis.

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