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Triplet primed repeat PCR – An approachable diagnosis method for detecting triplet repeat disorders -Friedreich Ataxia

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ABSTRACT

Diagnostic tests have been developed to detect triplet repeat expansion associated with many neurodegenerative triplet repeat disorders employing techniques including Southern blotting and PCR amplification of the gene expansion. Lack of precision and accuracy in determining the exact size of GAA repeats (triplet repeats in case of Friedreich ataxia) by conventional PCR amplification procedure led to improvisation of this method. This study was aimed to standardize a diagnostic procedure for detecting the GAA repeat expansion in suspected patients of Friedreich ataxia using improvised triplet Primed Repeat PCR technique. DNA samples required for the study was isolated from white blood cells of 50 healthy subjects and 5 suspected cases of Friedreich Ataxia, the isolated DNA were quantified and was used for performing the triplet primed PCR . The PCR products were screened through Capillary Electrophoresis to detect the exact number of GAA repeats present in the DNA samples. Among triplet repeat primed PCR performed in 50 control samples and 5 suspected FRDA patients, all healthy control subjects analyzed showed the electropherogram with peaks in normal limits and the suspected cases showed the peaks above normal repeat levels suggesting the pathogenic expansion. These findings suggests that the triplet repeat primed PCR assay can be used as a reliable and faster diagnosis technique to detect the pathogenic repeats expansions observed in triplet repeat disorders.

Keywords: Triplet repeat primed PCR, Friedreich Ataxia, Pathogenic expansions, Capillary electrophoresis, neurodegenerative triplet repeat disorders.

INTRODUCTION

Friedreich Ataxia (FRDA) is one of the most common hereditary ataxias with a prevalence of 1 in 50 000 in Caucasian population. It is less studied in Indian population. Recent study on FRDA families in Indian population by Singh *et.al.* (2010) describes existence of a common origin of FRDA in Indian and Caucasian populations [1]. The disease usually has a late onset with an average age ranging from 20-25 in most of the cases and in rare cases even at early age of 7. FRDA ultimately results in the degeneration of nerve tissue and neurons essential for directing muscle movement. Spinal cord becomes thinner and nerve cells lose some of their myelin sheaths. Campuzano *et.al.* (1996) reported that the most common molecular abnormality associated with FRDA was a homozygous expansion of GAA repeat in the first intron of the *frataxin* gene located on the long arm of chromosome 9 [2]. Frataxin is a mitochondrial protein thought to be playing an important role in iron metabolism [3, 4]. The normal repeat length is less than 39 where as Friedreich ataxia patients generally have repeats ranging from 100 to1700 [5, 6].

Even though the conventional PCR can amplify up to 100 triplet repeats approximately it's not completely reliable, the concept of triplet repeat primed PCR first came from the works of Warner *et.al.* in the year 1996 [7]. Triplet repeat primed PCR (TP PCR) provides a better amplification of the triplet repeats on the fluorescence trace leading to the rapid identification of large pathogenic repeats that cannot be amplified using flanking primers used in normal PCR. This approach will reduce the number of Southern blot analysis which is time consuming and labour intensive. The molecular diagnosis of the FRDA triplet expansion requires a different PCR protocol to amplify normal and mutated alleles along with the help of Southern blot analysis to determine the size of expansion accurately. This study was carried out by Ciotti *et.al.* in 2004 and actually is a modified version of the PCR technique developed by Warner *et.al.* in the year 1996 [8]. Diagnosis of FRDA are usually done by PCR amplification of the region containing the GAA repeat, followed by agarose gel electrophoresis of the PCR products to determine their size. However, in most of the cases where long expansions of GAA repeats occur, the PCR results were found to be inaccurate and require Southern blot for confirmation of the expansion size. Although, these artefacts may disappear on denaturing, gel purification and re-electrophoresis, results may still suffer from inconsistency. Even though TP PCR is able to amplify larger repeats, Southern blotting is still the gold standard technique to accurately determine the exact number of repeats. Only disadvantage of Southern blot is that it is labour intensive, time consuming and expensive also require larger amounts of DNA. Therefore when appropriately standardized, TP PCR can be used as the first and best method to assess GAA expansion in suspected cases.

MATERIALS AND METHODS

Selection of participants:

A total of 55 samples were analyzed to standardize the diagnostic procedure to detect the triplet repeat levels in FRDA. This included 50 healthy control subjects and 5 suspected cases of FRDA.

Technical information:

DNA samples required for the study was isolated from white blood cells by phenol chloroform method. Informed consent was obtained from human volunteers before drawing blood samples. DNA isolated from white blood cells of healthy subjects were quantified by NanoDrop1000™ Spectrophotometer. The DNA concentration ranged from 150-200 ng /µl, 55 samples were isolated and further analyzed for quality by running 1µl of it on a 1% agarose gel at 100 V for 10 minutes. Commercial primers obtained from Europhin mwg operon™ (sequences of P1, P3 and P4 are shown in Table 1) were dissolved in appropriate volumes of Milli Q water to obtain stock concentration of 1mM. Aliquots of stock primers are diluted to various concentrations and run on 2% agarose gel to assess concentration as well as purity. DNA with high concentration was taken for TP PCR along with three sets of primers. The TP PCR was performed as per the conditions showed in Table 2. The resulting PCR products were analyzed on 1 % agarose gel to rule out contamination and wrong results checking with the negative controls. Aliquots of the PCR product were analyzed on capillary electrophoresis by Applied Biosystems 3130 Genetic Analyzer™. Even though the same assay was carried out for all 55 samples as part of standardizing procedures, the best among the results clearly distinguishing the normal and pathogenic repeat expansions are included in the Figure 2a -b. All technical data used for standardizing are available on request.

Table-1: Primer sequences TP PCR -FRDA

TP-PCR PRIMER SEQUENCES
P1 : 5'-GCTGGGATTACAGGCGCGCGA-3'
P3 : 5'- TACGCATCCCAGTTTGAGACG-3'
P4 : 5' 6-FAM TACGCATCCCAGTTTGAGACGGAAGAAGAAGAAGAAGAAGAA-3'

Table -2: PCR condition TP PCR -FRDA

TP PCR CONDITION			
•	INITIAL DENATURATION	- 95°C- 5MINS	35 CYCLES
•	DENATURATION	-94°C-30 SEC	
•	ANNEALING	-60°C 30 SEC	
•	EXTENSION	-72°C 30 SEC	
•	FINAL EXTENSION	-72°C 10 MINS	
•	STORAGE	- 4 °C	

The TP PCR primers and PCR action:

The primer sequences and primer action employed for detecting GAA triplet repeat expansion in *frataxin* gene are shown in Figure 1 a-b. Here initially P1 primer gets attached to one side of the target region to be amplified where as at the other end the repeat containing P4 primer gets attached. These two primers together amplify the repeat sequence, as the P4 primer is 6 –FAM labeled the product formed is also fluorescently labeled. These PCR products produced by the two primers P1 and P4 again undergo amplification. The P1 act as primer for one end of the product

and P3 primer (with a sequence that does not have homology to any of the sequence present in human genome but having sequence same as that of the P4 primer tail sequence) acts on the other end and amplifies the product again. This second round of amplification maintains the specificity in amplification for these large pathogenic repeat sequences. As per calculation (represented in Figure-1a) including the primer sequences the expected product size of the triplet primed PCR is around 88bp for a person having 7 GAA repeats in the FXN gene.

RESULTS AND DISCUSSION

The triplet repeat primed PCR was performed for all 55 samples with the PCR condition and primer sequence mentioned in Table 2 and Table 1 respectively. Even though 2 among the suspected cases with Friedreich Ataxia failed to show up the pathogenic repeat expansion, three of them clearly displayed the repeats crossing the normal threshold values. Figure-2a represents the agarose gel electrophoresis picture and its corresponding electropherogram results for the healthy control sample run on lane 2 on the gel (Underlined with red line in Figure-2 a). The lane-1 of the same agarose gel is run with a 100 bp ladder. This typical electrophoresis picture and the corresponding electropherogram obtained after capillary electrophoresis represents clear distinguished peaks at both 88 bp and 148 bp. Product peak at 88 bp and 148 bp (shown with red arrows) correspond to 7 GAA and 27 repeats present in the DNA. The other peak smaller peaks could be accounted by unused primer P4. Figure-2b represents the agarose electrophoresis picture and its corresponding electropherogram results for a suspected patient sample of Friedreich Ataxia (Who was later confirmed for the disease using southern blot technique) run on lane 6 of the gel (Underlined with red line in Figure 2 b). The lane 1 in the same agarose gel is run with a 100 bp ladder. This typical electrophoresis picture and the corresponding electropherogram obtained after capillary electrophoresis represents clear distinguished peak at 487 bp along with other peaks as observed in control samples. Product peak at 487 (shown with red arrows) correspond to 140 GAA repeats present in the DNA. This undoubtedly falls under the category of pathogenic repeat expansion for the disease.

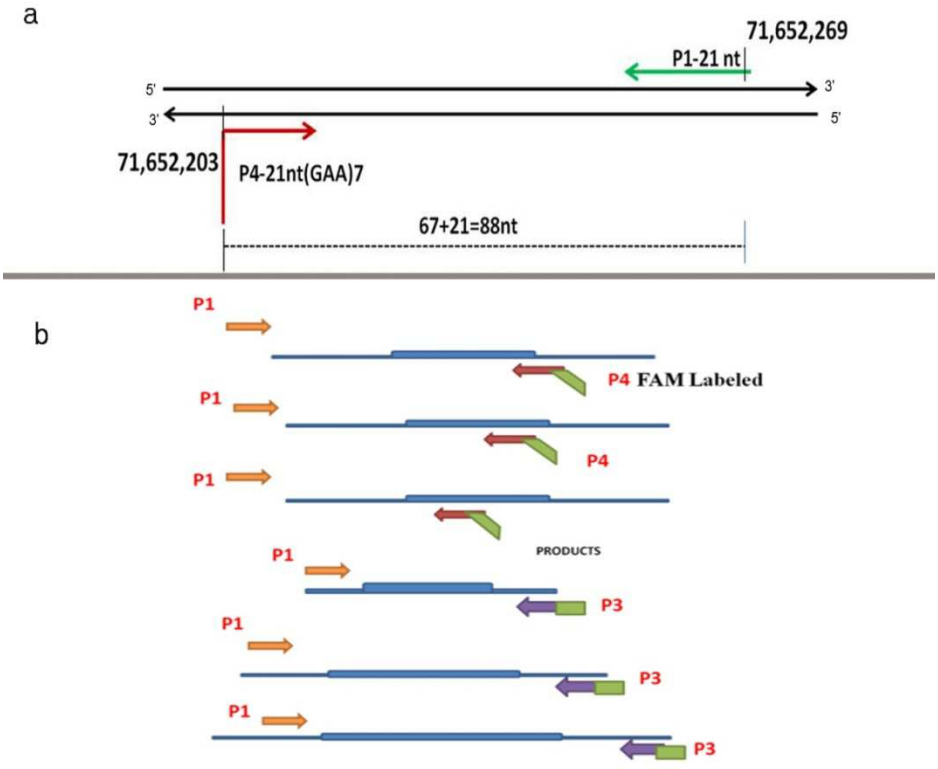

Figure 1 a: Expected PCR product size TP PCR-FRDA; Figure 1b: Primer action of FRDA TP PCR

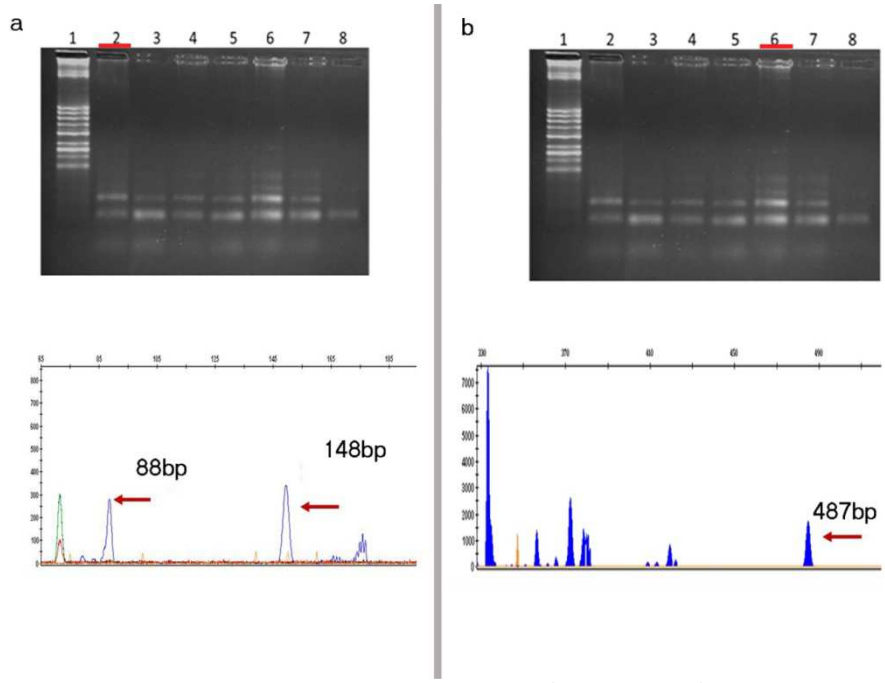

Figure 2 Figure 1a: Agarose gel picture and corresponding electropherogram of TP PCR assay from healthy control sample; Figure 2b: Agarose gel picture and corresponding electropherogram of TP PCR assay from FRDA patient sample

CONCLUSION

We have attempted to standardize Triplet repeat primed PCR based molecular method to quantify GAA repeats in DNA samples as described by Ciotti *et.al* (2004). About 55 samples of DNA were isolated from control individuals and suspected cases of FRDA and the TP PCR assay was carried out followed by the capillary electrophoresis of the products. The electropherogram were analyzed. The peaks in electropherogram of control samples falls under the normal repeat levels which is expected to be present in control individuals and crosses the normal threshold values in FRDA patient samples. However the quantity of the product formed in the TP PCR reaction appears to be much less as compared to electropherograms seen in the work of Ciotti *et.al*. We have attempted modifying some of the TP PCR reaction condition to improve product signal. Therefore when appropriately standardized, TP PCR can be used as a reliable technique to assess GAA expansion in suspected cases of FRDA. This methods need to be further modified to improve the product and signal yield where by the precision and accuracy of the technique can be increased.

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