Triplet Repeat Primed PCR (TP PCR) in Molecular Diagnostic Testing for Friedreich Ataxia

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Friedreich ataxia (FRDA), an autosomal recessive neurodegenerative disease, is associated with an unstable expansion of a GAA trinucleotide repeat in the first intron of the *frataxin* gene on chromosome 9q13. Unequivocal molecular characterization of the FRDA triplet expansion requires the use of different PCR protocols to amplify normal and mutated alleles combined with Southern blotting analysis to accurately size the expansion. Nevertheless, expansion detection by PCR may be somewhat problematic in heterozygous individuals. The purpose of this study was to evaluate triplet repeat primed PCR (TP PCR) as a screening method for FRDA diagnosis in the diagnostic laboratory. Fifty-four cases referred either to confirm the diagnosis of FRDA or to detect carrier status were re-evaluated by the TP PCR method. The TP PCR assay correctly identified the FRDA status in all 54 individuals tested including homozygous expansions (9 individuals), heterozygous expansions (20 individuals), and non-carriers (25 individuals). Results showed 100% concordance with those obtained by Southern blot analysis. TP PCR allowed us to identify the expanded alleles or to demonstrate their absence in DNA samples where conventional PCR procedures failed to give a reliable result. TP PCR represents an additional valuable tool for mutation detection in FRDA patients and carriers, but also can be used as screening test in a diagnostic laboratory. (*J Mol Diagn* 2004, 6:285–289)

Friedreich ataxia (FRDA), an autosomal recessive disorder,¹ is the most common hereditary ataxia, with an estimated prevalence of 1 in 50,000^{2,3,4} and a carrier frequency of about 1 in 90 in the Caucasian population.⁵ Usually, the disease onsets between 5 and 15 years of age and is characterized by progressive ataxia of the limbs, loss of deep tendon reflexes and of vibration sense in the lower limbs, cerebellar dysarthria, and pyramidal signs.⁶ FRDA is associated with an unstable expansion of a GAA trinucleotide repeat in the first intron of the *frataxin*

gene on chromosome 9q13.⁷ Normal alleles contain 5 to 60 repeats. The range of the repeats in FRDA patients varies from 66 to 1700 repeats and results in a decreased expression of this gene.^{5,7–11} In about 96% of the patients, both alleles are expanded while 4% of patients are compound heterozygotes for a GAA expansion in the disease-causing range and an inactivating point mutation.⁷ FRDA shows broad clinical variability^{12,13} and therefore differential diagnosis may be sometimes problematic.

Molecular analysis can be relevant to confirm clinical diagnosis and to detect carriers. Molecular tests are usually performed by PCR amplification of the region containing the GAA repeat, followed by agarose gel electrophoresis of the PCR products to determine their size.^{7,8,14} Campuzano et al⁷ previously described two different PCR protocols: one, usually named short PCR, is proper in detecting and sizing of repeats within the normal range. However, PCR amplification of DNA from heterozygous individual may fail to amplify the expanded allele. Primers generating larger amplicons were used in a long-range PCR protocol referred to as Long PCR⁷ to alleviate the selective amplification of the smaller allele. PCR artifacts resembling large GAA expansion have been observed in individuals with two normal but significantly different-sized alleles and in those with a single short expansion (<200 repeats).¹⁵ These are believed to be heteroduplex-like molecules as they disappear under denaturing conditions or following gel purification and re-electrophoresis. In some cases, heteroduplexes can create misinterpretation of data and false-positive results.¹⁵ The report of the External Quality Assessment (EQA) scheme of the European Molecular Genetics Quality Network (EMQN) highlights the dangers of relying on Long PCR method alone in detecting heterozygotes because of the 11.7% genotyping errors detected (FRDA EQA scheme 2002, personal communication). Southern blotting represents the method of choice to analyze the GAA repeat region¹⁶ and to correctly size large expanded alleles. This method, both technically demanding and labor intensive, is not cost effective enough in the handling of a few samples.

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The triplet repeat primed PCR (TP PCR) method was developed by Warner et al¹⁷ to screen for expanded alleles in myotonic dystrophy. The PCR assay uses a locus-specific primer flanking the repeat together with paired primers amplifying from multiple priming sites within the repeat. Specificity is dictated by the fluorescently labeled, locus-specific primer. TP PCR gives a characteristic ladder on the fluorescence trace enabling the rapid identification of large pathogenic repeats that cannot be amplified using flanking primers. Subsequently, this approach was used to detect *frataxin* GAA repeat expansion^{18,19} and to analyze FRDA patients with interrupted GAA expansion.²⁰

The purpose of this study was to evaluate TP PCR as a screening method for FRDA diagnosis in the diagnostic laboratory of a Medical Genetics Department. This method was used to test 54 FRDA samples previously genotyped by Southern blotting. TP PCR allowed high-throughput screening of *frataxin*-expanded alleles.

Materials and Methods

Subjects

A series of 54 individuals was selected from individuals consecutively referred to the Service of Medical Genetics-Genova for molecular diagnosis of FRDA or for carrier detection. Fifty-four subjects, who were unequivocally genotyped by Southern blotting analysis, were included in the study.

Nine subjects had a clinical diagnosis according to Harding criteria.⁶ In nine patients the diagnosis was suspected, while nine patients had diagnosis of Friedreich ataxia with retaining reflexes (FARR) or cerebellar ataxia of unknown origin. Twenty-seven subjects, belonging to FRDA families, were referred to ascertain their heterozygous carrier status.

Short and Long PCR

DNA used for PCR amplification was extracted from venous blood leukocytes using standard methods. Amplification of normal and expanded alleles was obtained by PCR procedures previously described.⁷

Triplet Repeat Primed PCR (TP PCR)

TP PCR was performed according to protocol kindly provided by Dr. M. Schmitt (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique/Institut National de la Santé et de la Recherche Médicale (INSERM)/Université Louis Pasteur, Strasbourg, France, personal communication). Primer sequences used for FRDA TP PCR test were: P1 5'-GCTGGGATTACAGGCGCGCA-3', P3 5'-TACGCATCCAGTTTGTAGACG-3', P4 5'-6-FAM TACGCATCCAGTTTGTAGACGGAAGAAGAAGAAGAAGAA-3'. TP PCR assay was performed in a reaction volume of 25 μ l containing 200 ng genomic DNA, 1.5 mmol/L $MgCl_2$, 10 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 0.8 μ mol/L primer P1,

0.8 μ mol/L primer P3, 0.08 μ mol/L primer P4, 200 μ mol/L dNTPs each, and 2 units *Taq* polymerase (Eppendorf AG; Hamburg, Germany). The reactions were subjected to 30 cycles consisting of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds followed by a 10-minute extension at 72°C. PCR products were incubated at 95°C for 2 minutes and cooled on ice before loading and resolved by electrophoresis on an automatic sequencer (ABI 310; Applied Biosystems, Foster City, CA). Five μ l of each PCR product were added to 18 μ l of formamide (Sigma, St. Louis, MO, USA) and 0.5 μ l of Genescan 400HD [Rox] Size standard (Applied Biosystems). Each sample underwent TP PCR three times.

Southern Blotting

Southern blot analysis was performed on *Eco*RI or *Bsi*HKAI-digested (New England BioLabs, Inc.) genomic DNA.^{7,16} On Southern blot, the *frataxin* probe detects the FRDA expansion mutation with both restriction enzymes that determine normal fragment sizes of 8.2 and 2.4 kb, respectively. *Eco*RI digestion was used in a first series of individuals. The remaining samples were analyzed using *Bsi*HKAI-digested DNA. Samples previously digested by *Eco*RI were used as controls.

Twenty μ g of genomic DNA were digested by *Bsi*HKAI, electrophoresed in 1.3% agarose gel, and hybridized with a α -³²P-radiolabeled 453-bp genomic fragment (from position 366 to 828 in the GenBank sequence U43748) containing exon 1 of the *frataxin* gene including part of the 5' untranslated and part of intron 1. Blots were washed in saline sodium citrate (SSC) and exposed for autoradiography. The restriction fragment length was determined by comparison with a ladder (MassRuler DNA Ladder, High Range, Fermentas GmbH, St. Leon-Rot, Germany).

Results

Fifty-four individuals genotyped for FRDA expansion by different methods (short PCR, Long PCR, Southern blotting) were investigated by TP PCR. Southern blotting was used as reference method. Each genotype obtained by TP PCR assay was compared to the results of Southern blot analysis on tested blind samples. The genotypes obtained by Southern blotting were as follows: 25 subjects had two alleles with GAA repeats in the normal size range, 20 were heterozygotes for the expansion, and 9 had two alleles in the range of the GAA expansion (Table 1). The number of GAA repeats of FRDA chromosomes were evaluated in individuals both heterozygous and homozygous for the expansion. The estimated size ranged from 130 to approximately 1200 repeats.

TP PCR gave a characteristic trace of GAA expansion (representative electropherograms are shown in Figure 1). The reaction was successful and gave informative results in 54 of 54 individuals. TP PCR results showed 100% concordance with those obtained by Southern blot analysis (Table 1). Analysis by TP PCR demonstrated

Table 1. Molecular Analysis of Subjects Included in the Study ($n = 54$)

No. of cases	Indication	S.B. EcoRI	S.B. BsiHKA	Short PCR	TP PCR	Genotype
1	FRDA	exp/exp	exp/exp	null	exp	exp/exp
5	FRDA		exp/exp	null	exp	exp/exp
2	FRDA		norm/norm	norm/?	no exp	norm/norm
1	FRDA		norm/norm	norm/norm	no exp	norm/norm
4	Suspected FRDA		norm/norm	norm/?	no exp	norm/norm
3	Suspected FRDA		norm/norm	norm/norm	no exp	norm/norm
2	Suspected FRDA		exp/exp	null	exp	exp/exp
1	Cerebellar ataxia		exp/exp	null	exp	exp/exp
5	Cerebellar ataxia		norm/norm	norm/norm	no exp	norm/norm
1	Cerebellar ataxia		norm/exp	norm/?	exp	norm/exp
2	FARR		norm/exp	norm/exp	exp	norm/exp
10	Carrier testing		norm/exp	norm/?	exp	norm/exp
1	Carrier testing	norm/exp		norm/?	exp	norm/exp
6	Carrier testing	norm/exp	norm/exp	norm/?	exp	norm/exp
5	Carrier testing		norm/norm	norm/?	no exp	norm/norm
2	Carrier testing	norm/norm	norm/norm	norm/?	no exp	norm/norm
1	Carrier testing	norm/norm	norm/norm	norm/norm	no exp	norm/norm
2	Carrier testing		norm/norm	norm/norm	no exp	norm/norm

traces consistent with the presence of the GAA expansion in 29 of 54 (53.7%) samples (Table 1).

The whole series was also analyzed by short PCR: 12 of 54 samples showed two bands in the normal size range, 31 of 54 showed a single band in the normal

range, 2 of 54 showed one fragment in the normal size and one in the lower expanded range, and in 9 of 54 the PCR amplification did not show any fragment (Table 1). Conclusive information was obtained in 14 of 54 individuals (25.9%).

Figure 1. TP PCR representative electropherograms, aligned by size (bp). The size standard peaks are indicated in the top panel by vertical segments. The TP PCR signal consists of a ladder with 3bp periodicity, corresponding to the GAA repeat. Both normal and expanded alleles give peaks. The ladder peaks diminishes gradually with increasing product size. The ladder that extends beyond the threshold for the expansion is consistent with the presence of at least one FRDA chromosome. The threshold approximately corresponds to 250 bp, based on the observed size of the TP PCR product from the most frequent normal allele. **A:** The profile is consistent with the absence of expansion. The ladder demonstrates the amplification of a low number of repeats, below the threshold for expansion. This pattern was obtained from a non-carrier individual. **B and C:** Both profiles are consistent with the presence of expansion. The ladders extend along the electrophoretic run, beyond the threshold for the expansion. TP PCR patterns were obtained from a heterozygous individual (**B**) and a homozygous patient (**C**). The respective genotypes were assayed by the means of both short PCR and TP PCR: along the presence of the expansion demonstrated in both subjects, the short PCR pattern allowed to detect the presence of the normal allele in the heterozygous individual (data not shown).

Individual genotypes were determined combining short PCR data with those obtained by means of TP PCR as follows: 6 of 9 (66.7%) patients with clinical diagnosis of FRDA were carriers of the expansion on both alleles, while three patients (33.3%) had two alleles with GAA repeats in the normal size range. Among nine patients with suspected FRDA, 2 (23.3%) had two expanded alleles and seven (76.7%) had two alleles in the normal range. The two patients with FARR were heterozygous for the GAA expansion. Among seven patients with cerebellar ataxia of unknown origin, five had two alleles in the normal range, one had two expanded alleles, and one resulted heterozygous (Table 1). Seventeen of 27 possible FRDA carriers resulted heterozygous for the GAA expansion and 10 were carriers of two normal alleles.

Within the sample series, Long PCR was applied in a subset of 23 subjects, as its use was discontinued after the introduction of TP PCR. Combined data from Long PCR and short PCR provided 10 unambiguous genotypes. TP PCR analysis in the remaining 13 samples allowed to detect expansion in five homozygous and four heterozygous carriers. These data were consistent with Southern blotting results (data not shown).

Discussion

TP PCR technique has been developed for the screening of expanded CAG repeat alleles in myotonic dystrophy.¹⁷ Different groups subsequently used this approach to detect *frataxin* GAA repeat expansion.^{18,19,20}

The purpose of the present study was to evaluate this technique as screening method for a laboratory of a Medical Genetic Service in which few samples are occasionally sent for FRDA diagnosis or carrier status assessment. In fact, in our experience, about 50% of tests are performed on individuals belonging to FRDA families to detect carriers. Therefore, the laboratory priority in a screening FRDA test is the reliable detection of the expansion rather than its accurate sizing.

TP PCR method has many advantages and fits especially well for rapid handling and testing of a few samples as required in laboratory routine since the method is PCR-based, rapid, and not labor intensive. The analysis can be made using a small amount of DNA, it is a closed-tube system that does not require post-PCR handling, and has a high sample throughput.

To assess TP PCR method and introduce it in the routine diagnostic protocol for FRDA we performed the assay on a total of 54 individuals previously genotyped by combined techniques. Results showed a 100% concordance with those obtained by Southern blotting analysis. The reaction was successful on DNA samples of different quality and extracted by using different procedures thus showing the robustness of the method. Moreover, the reaction was performed at least three times for each sample with identical results demonstrating a high reproducibility of this technique.

The comparison between Southern blot and TP PCR did not reveal false positive and negative results suggesting that TP PCR assay has high sensitivity and specificity

for the frataxin GAA expansion both in homozygous and heterozygous subjects. We reported that this technique is a powerful and not labor intensive approach, which would reduce the recourse to Southern blotting in the preliminary screening. Nevertheless, TP PCR alone is not specific to ascertain the genotype and does not provide the size of expanded GAA repeat alleles.¹⁷ Therefore, we propose a step-wise protocol for the molecular analysis in FRDA.

The first step is aimed at establishing the genotype pertaining to the GAA expansion, that is, ascertaining the presence of one or two expanded alleles. It involves both short PCR and TP PCR. Short PCR is robust enough to detect normal alleles, while TP PCR provides a superior and consistent level of information about presence or absence of the expansion. By the means of the two combined assays, the genotype can be easily assessed in a short time. The accuracy of TP PCR joined with its rapid performance makes this first step applicable also to prenatal diagnosis in families in which the molecular characterization was previously obtained.

The second step should be accomplished when an accurate estimation of the GAA repeat size is needed, according to referral reasons and reporting standards. The completion of the first step implies a reduction in the number of samples to be processed for repeat sizing by either Long PCR or Southern blotting, as only expanded alleles should be evaluated. Long PCR, although able to identify expanded alleles, does not appear a method of choice because of the high rate of genotyping errors. In fact, confirmation by another method is recommended (EMQN report of the EQA scheme, 2002). The results obtained in our series confirmed this observation (data not shown). Therefore, Southern analysis could be considered the standard method to obtain a definite characterization of the GAA expansion.

It is worth emphasizing that, along the identification and characterization of expanded alleles, a complete molecular diagnosis of FRDA must include screening for point mutation in heterozygous individuals. This corresponds to the third step of the protocol, which should be limited to selected cases.

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