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Molecular analysis of *FMR1* gene in a population in Southern Brazil: Comparison of four methods

Cinthia Ramos [a,c], Maristela Ocampos [b], Ingrid Tremel Barbato [b], Maria da Graça Bicalho [a,c], Renato Nisihara [a,d,*]

[a] Post Graduate Program in Gynecology and Obstetrics, Universidade Federal do Paraná, Curitiba, Brazil
[b] Neurogene Laboratory of Human Citogenetics and Molecular Genetics, Florianópolis, Brazil
[c] Department of Genetics, Federal University of Paraná, Curitiba, Brazil
[d] Department of Medicine, Positivo University, Curitiba, Paraná, Brazil

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ABSTRACT

Objectives: Fragile X syndrome (FXS) is caused by expansion of the number of cytosine-guanine-guanine (CGG) repeats in the regulatory region of the gene *fragile X mental retardation 1* (*FMR1*). The molecular diagnoses of FXS can be performed using two tests based on two different techniques, namely polymerase chain reaction (PCR) and Southern blotting (SB). However, both of these techniques have limitations. The purpose of this study was to evaluate the performance of the commercial FragilEase™ PCR kit for FXS diagnosis comparing to other laboratory methods.
Design: and methods: This study had a retrospective design. We analyzed the performance of the FragilEase™ PCR kit using 90 DNA samples from patients with clinical suspicion of FXS or a family history of the syndrome using capillary electrophoresis and compared with the results obtained for the same samples using PCR, SB, and AmplideX *FMR1* PCR.
Results: FragilEase™ PCR kit displayed high concordance with the results obtained using PCR, SB, and AmplideX *FMR1* PCR regarding the detection of normal, intermediate/gray zone, pre-mutation, and full mutation alleles, as well as female homozygosity and mosaicism. The replicate sizes found using the FragilEase™ PCR assay varied on average by two CGG repeats.
Conclusion: FragilEase™ PCR, as well as other commercially available kits, efficiently detect *FMR1* mutations and simplify the workflow in laboratories that performing FXS diagnoses.

1. Introduction

Fragile X syndrome (FXS) is considered the most common form of hereditary intellectual disability (ID) and the second most frequent cause of ID of genetic origin [1]. FXS is also associated with autism [2]. FXS is caused by expansion of the number of cytosine-guanine-guanine (CGG) repeats in the 5′-untranslated region of *fragile mental retardation 1* (*FMR1*), which is located at the Xq27.3 chromosome position. Large expansions in this region are associated with hypermethylation of the promoter region of the gene. Other less frequent causes such as deletions or point mutations in *FMR1* may also cause FXS [3]. FXS is classified according to the number of CGG repeats. Specifically, normal individuals have less than 45 CGG repeats, individuals in the intermediate or gray zone normally have 45–54 repeats, individuals with premutation have 55–200, and individuals with full mutation have more than 200

* Corresponding author.Department of Medicine, Positivo University, R. Prof. Pedro Viriato Parigot de Souza, 5300, Curitiba, 81280-330, Brazil.
E-mail addresses: renatonisihara@up.edu.br, renatonisihara@gmail.com (R. Nisihara).

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repeats [4]. Premutation alleles are unstable, and when transmitted by maternal meiosis, they increase the risk of full mutation in subsequent generations [5]. Premutation is associated with ovarian insufficiency [6]. Full mutation is associated with ID, autism, and dysmorphic features such as face, ear, and eyelid elongation [7,8]. It is currently recommended that tests for *FMR1* expansions are conducted in people with ID, autism, developmental delay, a family history of undiagnosed FXS or ID, and cerebellar ataxia, as well as women with infertility [4]. Many laboratories use polymerase chain reaction (PCR) to determine the number of CGG repeats. However, this technique has limitations, as it does not differentiate homozygous alleles in women and it hardly amplifies alleles containing more than 100–150 CGG repetitions [9]. When reflex testing is needed, Southern blotting (SB) is used. SB remains the gold standard for FXS diagnosis because it detects alleles containing more than 150 CGG repeats, mosaicism, and the *FMR1* methylation pattern. However, this time-consuming method requires a large amount of genomic DNA [10–12]. In recent years, commercial PCR-based kits that simplify the workflow in FXS diagnosis have been developed, and these tests can detect mosaicism, identify homozygous women, and accurately quantify the number of CGG repeats [9,10,13]. Such kits are intended to facilitate the diagnosis of FXS by replacing SB. The first test used in our laboratory was AmplideX *FMR1* PCR (Asuragen, Austin, TX, USA), and more recently, we gained access to the FragilEase™ PCR kit (PerkinElmer, Turku, Finland). Both kits are used to confirm the diagnosis of FXS.

Thus, in this study, we evaluated the performance of the FragilEase™ PCR kit for detecting *FMR1* mutations in comparison with PCR, SB, and AmplideX *FMR1* PCR.

2. Material and methods

This study had a retrospective design and was approved by the Research Ethics Committee of the Clinical Hospital, Federal University of Paraná. We analyzed the performance of the FragilEase™ PCR kit using 90 DNA samples from patients (26 men and 64 women) with clinical suspicion of FXS or a family history of the syndrome. Samples were sent to a laboratory in Florianópolis, Brazil specializing in the diagnosis of genetic diseases in the period of May 2013 to March 2018. All samples were previously analyzed for FXS using PCR, SB, and AmplideX *FMR1* PCR. The samples were divided into two groups by sex (Group 1, 12 mothers of children diagnosed with FXS, 15 women with infertility, and 37 women with clinical suspicion of FXS; and Group 2, 26 men with clinical indications for FXS research or family histories of the syndrome).

2.1. PCR and cycle sequencing

PCR was conducted using primers C (5′-gctcagctccgtttcggtttcacttccggt-3′) and F (5′-agccccgcacttccaccaccctcctcca-3′) [14]. The total reaction volume of 30 μl contained Expand Long Template PCR System products (Roche Diagnostics, Mannheim, Germany) including buffer 2 and 1 U of Taq enzyme, 500 μM dNTPs, 2.0 M betaine (Betaine B0300, Sigma-Aldrich, St. Louis, MO, USA), 0.33 μM of each primer, and 100 ng of genomic DNA extracted using the salting out method. The reactions used the following temperature conditions: denaturation at 98 °C for 10 min; 10 cycles of 97 °C for 35 s (denaturation), 64 °C for 35 s (annealing), and 68 °C for 4 min (extension); 25 cycles of 97 °C for 35 s, 64 °C for 35 s, and 68 °C for 4 min, including a 20-s increment for each cycle; and termination at 68 °C for 10 min [15].

The PCR product was subjected to electrophoresis using 2% agarose gel containing 0.05 mg of ethidium bromide. Electrophoresis was performed at 130 V for approximately 60 min in 1 × TAE buffer and visualized using ultraviolet translucent material (UVP, Upland, CA, USA).

2.2. Southern Blotting

The method described by Tassone et al. [16] was used. Specifically, 10 μg of DNA extracted using the salting out method were digested using *Eco*RI and *Nru*I. The DNA was separated on 0.8% agarose gel and transferred to a nylon membrane. After transfer, the membrane was hybridized with the *FMR1* genomic probe StB12.3. Hybridization and *FMR1* detection were performed using the manufacturer's protocol (Roche, Dig system). The membrane was exposed to X-ray film (super RX; Fuji Medical X-Ray Film, Bedford-Shire, UK) for 2 h.

2.3. Asuragen AmplideX FMR1 CGG-Primed PCR

To analyze AmplideX *FMR1* CGG-Primed PCR, 2 μl of DNA (20 ng/μl) were used. The reaction mix was generated according to the manufacturer's instructions (11.45 μl of GC-rich Amp Buffer, 0.5 μl of FMR1 F/R FAM-Primers, 0.5 μl of FMR1 CGG Primers, 0.5 μl of diluent, and 0.05 μl of GC-Rich Polymerase Mix). The PCR program consisted of an initial stage of denaturation at 95 °C for 5 min; 10 cycles of 97 °C for 35 s, 62 °C for 35 s, and 68 °C for 4 min; 20 cycles of 97 °C for 35 s, 62 °C for 35 s, and 68 °C for 4 min, including a 20-s increment for each cycle; and final extension at 72 °C for 10 min. PCR products were separated via capillary electrophoresis (CE) using an ABI3130 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA, USA). Data were analyzed using GeneMarker software V2.7.0 (SoftGenetics, LLC, State College, PA, USA).

2.4. FragilEase™ PCR

The reaction mix was prepared with 15 μl of PCR buffer solution, 2.6 μl of sample diluents, and 0.4 μl of Taq Polymerase. Two microliters of DNA (25 ng/μl) were used in each reaction. Samples were amplified with an initial denaturation step at 95 °C for 5 min

followed by 25 cycles of 98 °C for 35 s, 59 °C for 35 s, and 72 °C for 4 min and a final extension step of 72 °C for 10 min. FragilEase™ PCR products were separated via CE using an ABI3130 Genetic Analyzer sequencer with a 50-cm capillary tube and POP-7 polymer (Applied Biosystems). Samples were prepared using 2 μl of the PCR product with 2 μL of LIZ 1200 (Thermo Fisher Scientific) and 11 μl of Hi-Di Formamide (Thermo Fisher Scientific). These samples were heat-denatured at 95 °C for 2 min and cooled to 4 °C. The configurations of the ABI 3130 were as follows: 2.5 kv/20 s of injection time and 2800 s of running time.

The obtained results were analyzed using GeneMarker software V2.7.0. To calculate the number of CGG repeats for the samples, a linear regression curve was assembled from reference samples with known CGG repeat sizes. The indications of the genotypes followed the guidelines of the American College of Medical Genetics and Genomics [4].

2.5. Statistical analysis

Data were analyzed as frequency and contingency tables. The results are presented as frequencies and percentages. Numerical data were compared using Fisher's test and the chi-squared test. P values of less than 5% were considered significant.

3. Results

Repeat analysis using FragilEase™ PCR revealed the following frequencies:

- Among women (n = 64): 42 (65.6%) normal, 2 (3.2%) intermediate/gray zone, 15 (23.4%) premutation, and 5 (7.8%) full mutation alleles
- Among men (n = 26): 6 (23.1%) with normal results; 15 (57.7%) with full mutation, and 5 (19.2%) with mosaic premutation/full mutation

3.1. Concordance

Concordance was determined by comparing the repeat sizes of the samples previously analyzed using PCR, SB, and AmplideX *FMR1* PCR with the repeat numbers determined using FragilEase™ PCR.

Data for samples previously analyzed using PCR are presented in Table 1. FragilEase™ PCR revealed variations of −3 to +2 repeats for alleles featuring less than 56 repeats. For premutation alleles containing more than 56 repeats, there was greater divergence in the numbers of repeats.

Data for samples previously analyzed using AmplideX *FMR1* PCR are presented in Table 2. FragilEase™ PCR revealed variations of ±2 repeats in the normal, intermediate/gray zone, and premutation allele ranges. Only two samples had larger differences (−3 and −4 repeats, respectively). Data for the full mutation samples were concordant between the two techniques.

Data for samples first analyzed via SB and subsequently retested using FragilEase ™ PCR exhibited high agreement (Table 3).

4. Discussion

The present study demonstrated that commonly used techniques are effective for quantifying and detecting normal, intermediate/gray zone, premutation, full mutation, and mosaicism, being widely concordant regarding their results for diagnosing FXS. It is important to emphasize that the accurate diagnosis of FXS will improve patient counselling. FXS is a major cause of hereditary ID, and it is associated with autism and other disorders such as FXTAS and FXPOI. The disease remains underdiagnosed in Brazil [18].

Table 1
Comparison of samples previously analyzed by PCR and the FragilEase™ PCR.

Samples	Sex	PCR (A)		FragilEase™ PCR (B)		
		Genotype	Alleles	Genotype	Alleles	# (A)/(B)
NL01	F	NOR	30–40	NOR	32–37	+2,-3
NL12	F	NOR	28–40	NOR	28–40	0,0
NL81	F	NOR	28–40	NOR	28–37	0,-3
NL91	F	INT	28–46	INT	30–47	+2,+1
NL02	F	PRE	28–55	PRE	27–56	−1,+1
NL86	F	PRE	28–64	PRE	28-79*	0,+15
NL82	F	PRE	28–66	PRE	29-94*	+1,+28
NL84	F	PRE	20–66	PRE	18-84*	−2,-18
NL87	F	PRE	28–76	PRE	28-93*	0,+17
NL88	F	PRE	28–76	PRE	28-103*	0, +27
NL89	F	PRE	28–76	PRE	29-95*	+1,+19
NL83	F	PRE	28–78	PRE	28-101*	0,+23

F: female, M: male, N: normal, INT: intermediate/gray zone, PRE: premutation.
FM: full mutation, * no consensus was reached.

Table 2
Comparison of samples previously analyzed by PCR AmplideX *FMR1* and the FragilEase™ PCR.

Samples	Sex	Amplidex (A)		FragilEase™ PCR (B)		
		Genotype	Alleles	Genotype	Alleles	# A)/(B)
NL10	F	NOR	28	NOR	28	0
NL24	F	NOR	30	NOR	27	−3*
NL25	F	NOR	29	NOR	28	−1
NL26	F	NOR	28	NOR	28	0
NL28	F	NOR	29	NOR	28	−1
NL30	F	NOR	32	NOR	28	−4*
NL32	F	NOR	28	NOR	27	−1
NL33	F	NOR	29	NOR	29	0
NL34	F	NOR	29	NOR	27	−2
NL35	F	NOR	28	NOR	30	+2
NL43	F	NOR	18	NOR	20	+2
NL45	F	NOR	27	NOR	28	+1
NL47	F	NOR	28	NOR	29	+1
NL51	F	NOR	30	NOR	31	+1
NL53	F	NOR	29	NOR	30	+1
NL56	F	NOR	28	NOR	29	+1
NL60	F	NOR	29	NOR	28	−1
NL61	F	NOR	29	NOR	28	−1
NL65	F	NOR	29	NOR	28	−1
NL78	F	NOR	30	NOR	29	−1
NL29	F	NOR	29–30	NOR	27–28	−2,-2
NL39	F	NOR	28–35	NOR	27–34	−1,-1
NL41	F	NOR	22–36	NOR	21–36	−1,0
NL44	F	NOR	19–43	NOR	18–42	−1,-1
NL46	F	NOR	27–30	NOR	28–31	+1,+1
NL50	F	NOR	18–28	NOR	19–29	+1,+1
NL54	F	NOR	19–29	NOR	18–28	−1,-1
NL55	F	NOR	28–29	NOR	28–28	0,0
NL57	F	NOR	27–31	NOR	28–32	+1,+1
NL58	F	NOR	28–29	NOR	30–30	+2,+1
NL59	F	NOR	20–28	NOR	19–28	−1,0
NL66	F	NOR	28–37	NOR	27–37	−1,0
NL68	F	NOR	21–26	NOR	20–25	−1,-1
NL74	F	NOR	21–30	NOR	20–28	−1,-2
NL76	F	NOR	30–31	NOR	28–29	−2,-2
NL77	F	NOR	20–30	NOR	18–28	−2,-2
NL80	F	NOR	22–28	NOR	21–27	−1,-1
NL52	M	NOR	29	NOR	29	0
NL62	M	NOR	29	NOR	28	−1
NL67	M	NOR	31	NOR	30	−1
NL69	M	NOR	30	NOR	28	−2
NL72	M	NOR	30	NOR	29	−1
NL79	M	NOR	30	NOR	28	−2
NL63	F	INT	38–46	INT	37–45	−1,-1
NL36	F	PRE	27–81	PRE	27–83	0,+2
NL40	F	PRE	35–75	PRE	34–76	−1,+1
NL75	F	PRE	29–65	PRE	27–63	−2,-2
NL70	F	PRE	29–93	PRE	29–94	0,+1
NL73	F	PRE	29–79	PRE	28–78	−1,-1
NL42	F	FM	29–200	FM	29 - >200	0,0
NL90	F	FM	30 - >200	FM	28 - >200	−2,0
NL27	M	FM	>200	FM	>200	0
NL31	M	FM	>200	FM	>200	0
NL37	M	FM	>200	FM	>200	0
NL38	M	FM	>200	FM	>200	0
NL71	M	FM	>200	FM	>200	0
NL23	M	PRE/FM	138 - >200	PRE/FM	138 - >200	0,0
NL48	M	PRE/FM	95 - >200	PRE/FM	95 - >200	0,0
NL49	M	PRE/FM	102 - >200	PRE/FM	103 - >200	+1,0
NL64	M	PRE/FM	94 - >200	PRE/FM	94 - >200	0,0

F: female, M: male, N: normal, INT: intermediate/gray zone, PRE: premutation.
FM: full mutation, PRE/FM: mosaic premutation/full mutation, * no consensus was reached.

The results obtained using the FragilEase™ PCR were in agreement with those obtained using SB. Additionally, when we compared the two kits FragilEase™ and AmplideX *FMR1* PCR we observed few differences. The measure of the assay observed from 1 to 2 repeats of difference for the normal alleles, intermediate/gray zone and premutation. Two samples (NL24 e NL30) demonstrated larger disagreements in repeat size and these results could be attributed to analytical error. Nevertheless, these findings are comparable to the

Table 3
Comparison of samples previously analyzed by Southern Blotting and the FragilEase™ PCR.

Samples	Sex	SB	FragilEase™ PCR	
		Genotypes	Genotypes	Alleles
NL19	F	NOR	NOR	27–28
NL21	F	NOR	NOR	19–28
NL07	F	PRE	PRE	18–112
NL20	F	PRE	PRE	22–114
NL03	F	FM	FM	28 - >200
NL17	F	FM	FM	27 - >200
NL85	F	FM	FM	28 - >200
NL05	M	FM	FM	>200
NL06	M	FM	FM	>200
NL09	M	FM	FM	>200
NL11	M	FM	FM	>200
NL13	M	FM	FM	>200
NL14	M	FM	FM	>200
NL15	M	FM	FM	>200
NL16	M	FM	FM	>200
NL18	M	FM	FM	>200
NL92	M	FM	FM	>200
NL08	M	PRE/FM	PRE/FM	62 - >200

F: female, M: male, NOR: normal, PRE: premutation, FM: full mutation, SB: Southern blotting PRE/FM: mosaic premutation/full mutation.

performance of proficiency studies described by the College of American Pathologists [17]. For any borderline result between FXS allele classes, uncertainty of measurement should be stated on the report and taken into account when giving genetics counselling [5]. FragilEase™ PCR detected mosaicism for full mutation and premutation alleles. Among individuals with full mutation, it is estimated that 12–41% have mosaicism, i.e., one full mutation allele and one premutation allele [19]. Normally, these genotypes are difficult to detect using conventional PCR methods, and they require the use of SB [10].

At least 25% of female samples are homozygous, and they cannot be distinguished from heterozygous samples with an unamplified (and possibly expanded) allele using standard PCR methods [10], making it difficult to detect female homozygosity. FragilEase™ PCR safely detected the homozygous peak and revealed the absence of an expansion pattern in the female homozygous samples.

The use of commercial kits may reduce the need for SB in laboratories that diagnose FXS, as only samples for which information on the methylation status of *FMR1* is needed would require this technique. Because many laboratories restrict methylation evaluations to premutation and full mutation samples, it is estimated that only 2% of all samples would require SB [9]. Although SB is considered the gold standard for diagnosing FXS, the technique is manual and laborious with high costs. The use of more modern techniques would optimize the workflow in diagnostic laboratories.

In our country, the main limitation of the use of commercial diagnostic kits for FXS is the high cost compared with that of other PCR-based methodologies. For this reason, PCR remains a first-line test, and when reflex testing is needed, *FMR1* kits are used as second-line tests because they are more practical and less laborious than SB [20]. Thus, for commercial kits to become commercially viable as first-line tests, their costs must be reduced.

We conclude that FragilEase™ PCR, as well as other commercially available kits, efficiently detect *FMR1* mutations and simplify the workflow in laboratories that performing FXS diagnoses.

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Author contribution

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MO = Protocol/project development, Data collection or management, Manuscript writing/editing.
ITB = Protocol/project development, Data collection or management, Manuscript writing/editing.
MGB = Protocol/project development, Manuscript writing/editing.
RN = Protocol/project development, Data collection or management, Data analysis, Manuscript writing/editing.

Declaration of competing interest

None.

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