Arq Neuropsiquiatr 2005;63(3-A):564-570

LABORATORIAL DIAGNOSIS OF FRAGILE-X SYNDROME

Experience in a sample of individuals with pervasive developmental disorders

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ABSTRACT - Fragile X syndrome is a frequent genetic disease associated to developmental disorders, including learning disability, mental retardation, behavioral problems and pervasive developmental disorders (autism and related conditions). We studied a sample of 82 individuals (69 males and 13 females) presenting with pervasive developmental disorders using three techniques for the diagnosis of fragile X syndrome (FXS). Cytogenetic analysis detected the fragile site in four males, but only one showed a consistent positive rate. Molecular study based on the PCR technique was inconclusive for most females (92.3%), which where latter submitted to Southern blotting analysis, and for one male (1.4%), excluding the FRAXA mutation in the remaining male individuals (98.6%). Molecular tests using the Southern blotting technique confirmed only one positive case (1.2%) in a male subject. These results showed that Southern blotting analysis of the FRAXA mutation has the best sensitivity and specificity for the diagnosis of FXS but also validated the PCR technique as a confinable screening test.

KEY WORDS: PCR, molecular diagnosis, FRAXA, autism, mental retardation, pervasive developmental disorders.

Diagnóstico laboratorial da síndrome do cromossomo X frágil: experiência em uma amostra de indivíduos com distúrbios invasivos do desenvolvimento

RESUMO - A síndrome do cromossomo X frágil (SXF) é uma doença genética freqüente associada a distúrbios do desenvolvimento neurológico, incluindo dificuldades de aprendizagem, retardo mental, problemas comportamentais e distúrbios invasivos do desenvolvimento (autismo e correlatos). Estudamos uma amostra de 82 indivíduos (69 homens e 13 mulheres) apresentando distúrbios invasivos do desenvolvimento, utilizando três técnicas para o diagnóstico da SXF. A análise citogenética detectou a presença do sítio frágil em quatro homens, porém apenas um deles com percentagem consistente. O estudo molecular baseado na técnica da PCR foi inconclusivo para a maioria das mulheres (92,3%), as quais foram posteriormente submetidas a análise por Southern blotting, e para um homem (1,4%), excluindo a mutação FRAXA nos demais homens (98,6%). O teste molecular usando a técnica de Southern blotting confirmou apenas um caso positivo (1,2%) em um indivíduo do sexo masculino. Tais resultados mostraram que a técnica de Southemblotting para análise da mutação FRAXA apresenta a melhor sensibilidade e especificidade para o diagnóstico da SXF, mas também valida a técnica da PCR como um teste confiável para seu rastreamento.

PALAVRAS-CHAVE: PCR, diagnóstico molecular, FRAXA, autismo, retardomental, distúrbios invasivos do desenvolvimento.

Fragile X syndrome (FXS) was inittialy reported in 1943 by Martin and Bell[1] in a large family showing X-linked mental retardation. The first laboratorial method for its diagnosis was described by Lubs in 1969[2], who observed a fragile site [fra (X)] at Xq27-28 in samples cultured in folic acid deficient medium. After that, several different fragile sites were described in the same chromoso-

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Received 16 August 2004, received in final form 27 December 2004. Accepted 15 March 2005.

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mal region, denominated FRAXA, FRAXD, FRAXE, and FRAXF. FRAXA is the most common of these sites and it is associated with mutations in the *FMR1* gene, responsible for the FXS, while FRAXE (associated to the FMR2 gene) causes non-specific mental retardation and FRAXF seems to cause no abnormal phenotype[3,4]. The molecular basis of FXS was discovered in the early 1990´s[5-8] and consists of a dynamic mutation characterized by expansions of an unstable CGG repeat in the 5´ end of the *FMR1* gene. It was found that normal individuals have 6 to 54 repeats (normal allele) whereas affected individuals present more than 200 repeats (expanded allele or full mutation). In addition, phenotypically normal male and female carriers were found to have intermediate size alleles (pre-expanded alleles or pre-mutation).

FXS has a prevalence of one per 5000[9], representing one of the most common genetic disorders. Its clinical spectrum comprises some somatic changes, such as macrocephaly, large testes, marphanoid build habitus, long face, and protruding jaw. These features are only identifiable or more pronounced in males after puberty. Cognitive impairment is present, ranging from speech delay and learning disability to severe mental retardation. Besides, behavioral signs may be present, including hyperactivity, self-biting, poor eye contact, shyness, stereotyped movements, and other autistic features[10,11]. This wide range of symptoms may lead the clinician to consider the differential diagnosis of FXS in several situations, especially among individuals with different abnormalities of the neurological development, including the pervasive developmental disorders (PDDs), a group that comprises autism, atypical autism, and Asperger syndrome.

We undertook the present study aiming to compare cytogenetics, PCR and Southern blotting techniques for the laboratory diagnosis of FXS in a sample of individuals with PDDs diagnosed by the DSM-IV criteria. These techniques were applied since both, chromosomal abnormalities and the FXS, are important etiological factors in this group of neuropsychiatric conditions.

METHOD

We evaluated a total of 82 patients (69 males and 13 females) with PDDs from the Genetics and Neurology Clinics of our University Hospital. Parents or legal guardians were invited to join the study by signing a consent form approved by a Research Ethics Committee. After clinical evaluation, blood samples were collected for cytogenetic and molecular tests. All patients were also submitted to screening for inborn errors of metabolism, TORCH sorologies, and neuroimaging studies[12]. Three individuals with clinical diagnosis of Down syndrome were also tested for the FXS due to the possibility of co-occurence of both disorders, considering their high incidence in the general population.

Chromosomal analysis followed the routine cytogenetic temporary culture in folic acid deficient medium (M-199, with addition of 5-fluoro-2'-deoxyuridine 0.0025 mg/ml). The cultures were incubated for 96 hours at 37°C for the expression of the fra(X) site. The cell divisions were arrested in metaphases by adding colchicine $4x10^{-5}$ M for 30-40 minutes before harvesting the cultures. The samples were fixed in a solution of 3:1 methanol-acetic acid, spread in slides, and submitted to G-banding technique. Manual analysis in light microscope included at least 50 metaphases in male and 100 in female subjects[13].

For the molecular investigation we initially used the polymerase chain reaction (PCR) technique for specific amplification of the CGG repeat in the 5´end of the *FMR1* gene, according the protocol described by Fu et al.[6]. After amplification, the samples were submitted to electrophoresis in a 1.8% agarose gel at 24 V for 15 hours together with a 250 bp molecular weight marker. Samples were then transferred into Hybond N+ nylon membranes and hybridized with an α-^{32}P 3´-end labeled $(CGG)^6$ probe.

To confirm the PCR results in all subjects and to investigate the female patients, a second molecular test based on the Southern blotting technique was used. Samples were submitted to digestion with Pst I and Eco RI restriction enzymes. The resulting fragments were separated by electrophoresis in 0.8% agarose gel at 15 V for 16 hours together with a 1 kb molecular weight marker. After that, samples were transferred into Hybond N+ nylon membranes and hybridized with the pfxa3 probe (Oncor®) marked with an α-^{32}P isotope[14,15].

RESULTS

Table 1 shows the comparative findings obtained with the three techniques, as well as correlation with gender and the specific PDD diagnosis. Cytogenetic analysis (Fig 1) revealed the fra(X) in four patients with autism, three of them with low frequency (1%, 4%, and 2%) and only one patient with a significant positive result (17%). Karyotype was altered in other six individuals, including three patients with trisomy 21 and autism, one patient with a maternally inherited pericentric inversion of a chromosome 9 [inv(9)(p11q11)mat] and atypical autism, one patient with a maternally inherited Robertsonian translocation 15/21 [rob(15;21)(p10q10)mat] and atypical autism, and one patient

Table 1. Results according to the three techniques used for the laboratory diagnosis of the FXS and correlation with gender and the pervasive developmental disorder (PDD) diagnosis.

Patient (gender)	PDD diagnosis	Cytogenetics	PCR CGG alleles	Southern blotting FRAXA
T.L.B.S. (male)	autism	fra(X) 1%	normal	normal
E.C.S. (male)	autism	fra(X) 4%	normal	normal
C.R.T.S. (male)	autism	fra(X) 2%	normal	normal
E.C.M. (male)	autism	fra(X) 17%	no PCR product	expanded
E.C.C. (male)	atypical autism	rob(15;21)	normal	normal
M.C.X.A. (male)	atypical autism	inv(9)	normal	normal
D.G.M.D. (male)	autism	46,Xyqh+	normal	normal
J.A.B.G. (male)	autism	47,XY,+21	normal	normal
J.V.S.A. (male)	autism	47,XY,+21	normal	normal
T.V.S. (male)	autism	47,XY,+21	normal	normal
other 46 males	autism	normal	normal	normal
6 females	autism	normal	normal	normal
other 5 males	atypical autism	normal	normal	normal
4 females	atypical autism	normal	most inconclusive*	normal
8 males	Asperger syndrome	normal	normal	normal
3 females	Asperger syndrome	normal	normal	normal

* PCR results were definitive in only one female by showing the presence of two normal size bands.

Fig 1. Fragile site at Xq27.3 (arrow) as observed in the kary - otype of individual E.C.M.

showing an increased length in constitutive heterochromatin of the long arm of the Y chromosome (Yqh+) and autism. All the subjects with abnorm a l cytogenetic findings were males.

Molecular studies based on the PCR technique revealed the presence of normal size alleles in all male subjects except for one patient, thus excluding the presence of mutations in most individuals. The only individual with inconclusive PCR results after repeated experiments is the same who presented 17% of fra(X) cells in the chromosomal analysis (Fig 2). The remaining three male individuals with low percent of fra(X) cells in the cytogenetic analysis showed normal CGG alleles. In the females group, most patients showed only one band in the normal size range with the exception of one patient who showed two distinct bands in the normal size range. The observation of only one normal size band in females may indicate that the individual is homozygote for the normal allele. However, the presence of an expanded allele, which was not identified by this PCR technique, cannot be definitively excluded.

The only patient with a consistent positive fra (X) by cytogenetic evaluation was found to have the *FMR1* gene mutation by Southern blot analysis. We found that this patient was a mosaic for an expanded allele ranging from approximately 100 to 900 CGG repeats (Fig 3).

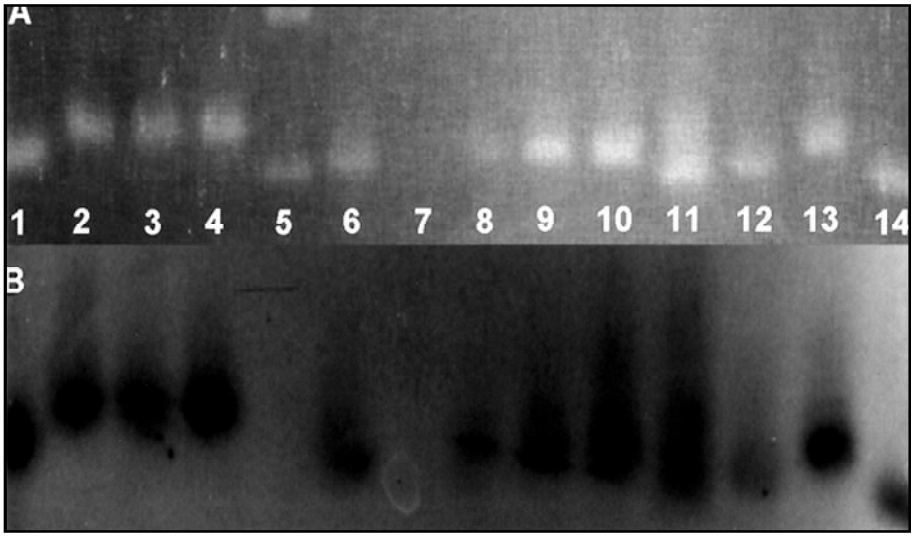

Fig 2. Molecular study based on the PCR technique showing pictures of the gel (A) and of the X-ray film obtained after autoradiography (B). Note the absence of PCR products in row 7, the presence of two distinct bands in row 11, and the presence of a single band representing normal alleles in the remaining rows. Row 5 corresponds to the molecular weight marker (250 bp).

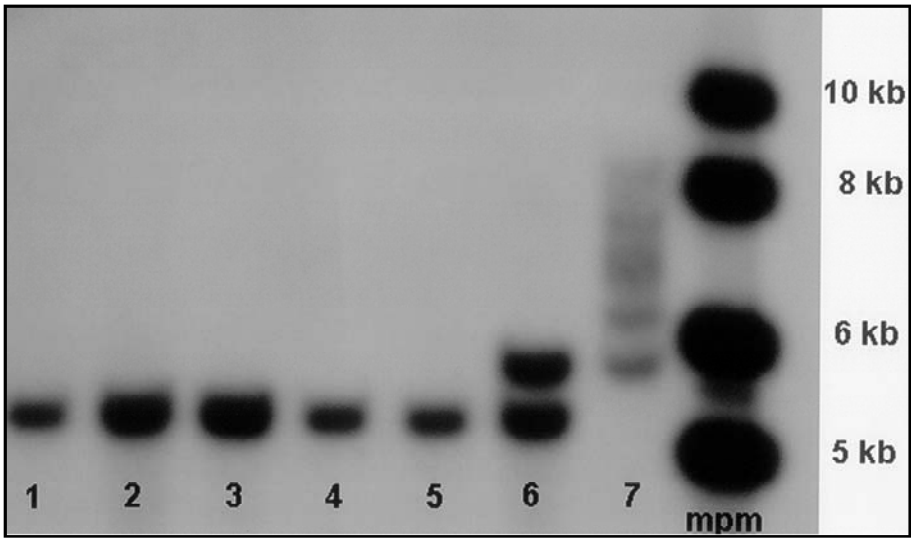

Fig 3. Molecular analysis following enzymatic digestion and Southern blotting. Normal alleles are shown is rows 1 to 5. A positive control female carrier is shown in row 6. Row 7 corresponds to patient E.C.M. presenting a mosaic pattern for the expanded CGG allele in the FMR1 gene; "mpm" refers to the molecular weight marker Generuler® 1 kb.

DISCUSSION

FXS may be present in about 0.8% of persons with autism[16], 0.5% of pre-scholars with speech delay[17], 0.56% of individuals with mental retardation[18], and 0.9% of subjects with non-specific mental retardation[19]. At first these frequencies may seem too low to justify laboratory testing for FXS in these patients. However, it is important to note that these conditions are very common in the general population and early intervention has a great impact in prognosis. In addition, the diagnosis of FXS is very important for adequate genetic counseling of the couple and, in extension, to other maternal relatives. Thus, laboratory detection of FXS is indicated in the etiological investigation of individuals presenting abnormalities in behavior, language, socialization or cognitive development, including the pervasive developmental disorders group.

Table 2. Advantages and disadvantages of each method for the laboratorial diagnosis of the FXS.

Method	Advantages	Disadvantages
Cytogenetics	Analysis of all chromosomes, allowing the identification of structural and numerical abnormalities besides the fragile site	Time consuming Risk of false negative results Does not differentiate FRAXA from FRAXE Does not detect carriers Does not identify point mutations
PCR	Quick result Uses small amount of DNA Dispenses the use of radioisotopes (thus reducing custs and becoming feasable in less equiped laboratories)	Confinable only for non-FXS males Not informative for female subjects and affected males Risk of false inconclusive results Risk of non detecting carriers Does not identify point mutations
Southern blot	Confiable for male and female subjects, including carriers	Time consuming and expensive Uses large amount of DNA Includes radioactive step Does not identify point mutations

Since the discovery of the molecular basis of FXS, cytogenetic tests for its diagnosis have been less used but were not totally abandoned. Southernblotting technique is the most used laboratorial method but several alternative approaches have been developed in the last decade. These include PCR with radioactive probe or silver stain, reverse transcription PCR (RT-PCR), methylation-sensitive PCR (MS-PCR), and immunohistochemical analyses. As for any laboratory technique a number of methodological issues are involved in the accurate diagnosis of FXS. The advantages and disadvantages of each method we used in the present work are summarized on Table 2.

Among the three techniques, cytogenetic analysis showed the highest rate of altered results in the present sample by the detection of three individuals presenting structural chromosomal abnormalities that could not be diagnosed by molecular study of the FRAXA mutation alone. One of them showed an increased length on the long arm of chromosome Y, which has been previously reported in association with autism[20]. Another patient had a pericentric inversion of chromosome 9, situation that recent literature data suggest may be involved in genetic susceptibility to psychiatric disorders such as schizophrenia or even autism[21]. The third one presented a roberstonian translocation 15/21 and, again, literature data suggest that abnormalities of chromosome 15 have a higher prevalence in autism than in the general population[22].

Considering the technical aspects of cytogenetic analysis for FSX, usually a longer exposure to colchicine causes more chromosomal condensation, which turns fra(X) easier to be detected by microscopic analysis. We used a shorter exposure (30 to 40 minutes instead of 60 minutes) which is less likely to interfere with the detection of other cytogenetic abnormalities. Although cytogenetic evaluation was sensitive and specific for the diagnosis of FXS, the fact that it is based in a 96-hours cell culture, which can fail, may require re-testing in several occasions. When the culture is successful, the study demands many hours of microscopic analysis, especially for the female specimens. In addition, it is well known that this method may not detect all carrier females, and would miss most male carriers, since only a small proportion of their cells will express the fra(X)[10]. The best advantage of this method is that it makes it possible to detect other chromosomal abnormalities, including the other fragile X sites with one single test.

PCR is a rapid and versatile method for amplifying a target DNA sequence. Because it is a fast and simple method, PCR is ideally suited for mutation screening since it can yield results in a single day experiment. However, the optimal size range for PCR amplification is between 0.1 to 5 kb, which may limit its application. The size of the band on the gel corresponding to a fragment with normal CGG repeat in the *FMR1* gene has approximately 250-300 bp and it will be easily amplified in the PCR experiments. However, the expanded CGG allele may be too large and fail to amplify. Therefore, a male with a normal allele will be readily identified by PCR as having only one single band

in the normal size range (250-300 bp). On the other hand, the absence of bands could indicate that the individual has an expanded allele or that a technical problem occurred to interfere in the optimal amplification of the normal allele. Therefore, the PCR technique can easily identify normal alleles, but usually is not efficient for the detection of the expanded CGG alleles.

In our study, only one patient who had positive cytogenetic study had an altered PCR result. The three individuals with low percentage of fra(X) cells in the cytogenetic study had single normal CGG alleles, thus excluding the FRAXA mutation which was also confirmed by the Southern blotting analysis.

In females, normal PCR fragments with similar sizes will result in a single band, making it impossible to differentiate normal homozygote female from a carrier of the FXS mutation. In our study the PCR technique was not informative for most females, since only a single individual was heterozygote for the normal CGG, and consequently a normal non-carrier FRAXA subject. Considering this, Weinhäusel and Haas[23] described an alternative PCR technique using fluorescence analysis called methylation-sensitive PCR (MS-PCR) that was useful for the detection of carrier females, although distinction between pre-mutated and fully mutated females was sometimes difficult in their experience. In addition, it is important to note that, in order to confirm that the band in the gel truly corresponded to the CGG fragment, transfer and hybridization with specific radioactive probes were performed after the gel electrophoresis. As there was no other bands in our experience, we concluded that transfer and hybridization was unnecessary and that the analysis can be accomplished by agarosis gel electrophoresis stained with ethidium bromide, without the necessity of use of radioisotopes.

Finally, Southern blotting gave informative results for all patients and confirm that only one individual had the FRAXA mutation. It also lead to information in all females tested. However, this technique requires large amounts of well preserved DNA (approximately 50 μg) and it demands a week of laborious laboratory work to complete all the technical steps required, including isotope manipulation.

These results are similar to those of Haddad et al.[24] who also studied a sample of Brazilian boys with mental retardation using the Southern blotting technique and the PCR technique of Fu et al.[6] modified by the use of silver staining instead of labeling with an isotopic probe. In their study, a boy with mosaicism including pre-mutated and fully mutated allele was also reported and showed discrepant results in sequenced PCR tests, which was not seen in our work.

A final problem, common to the all three techniques discussed, is that point mutations in the *FMR1* gene will not be detected by any of them. Fortunately, this is a rare situation in FXS[25], and can be diagnosed only by gene sequencing. This approach should be considered if the diagnosis of FXS is highly probable and all other diagnostic techniques failed.

The specific digestion followed by Southern blotting showed the best sensitivity and specificity for the laboratory diagnosis of FXS and also allowed the detection of possible female carriers. However, if only this technique was used it would have missed the six patients with chromosomal abnormalities, an important group of etiologic factors of mental retardation. Thus, we suggest that this method should only be the first choice when the clinical diagnosis of FXS is highly probable or to investigate relatives of confirmed FXS subjects (especially females) for genetic counseling and prenatal diagnosis. In individuals presenting developmental disorders in which the diagnosis of FXS is probable but not the main hypothesis, chromosomal analysis should always be performed. Considering that small structural abnormalities can be detected only in prometaphasic chromosomes, this type of preparation is recommended. On the other hand, this procedure will make it more difficult to detect fra(X).

In conclusion, we stress the importance of cytogenetic analysis as a necessary tool to investigate individuals with developmental disorders. It should be performed in male and female subjects, preferable with techniques that allow high chromosomal resolution. To complement the investigation of these individuals, molecular analysis based on the PCR technique may be used as a screening test for male subjects since it is enough to exclude the FRAXA mutation in most of them, who are expected to be not affected by the FXS. In female subjects and in males with a major indicative of FXS or when the PCR method was not informative, the Southern blotting technique should be the method of choice

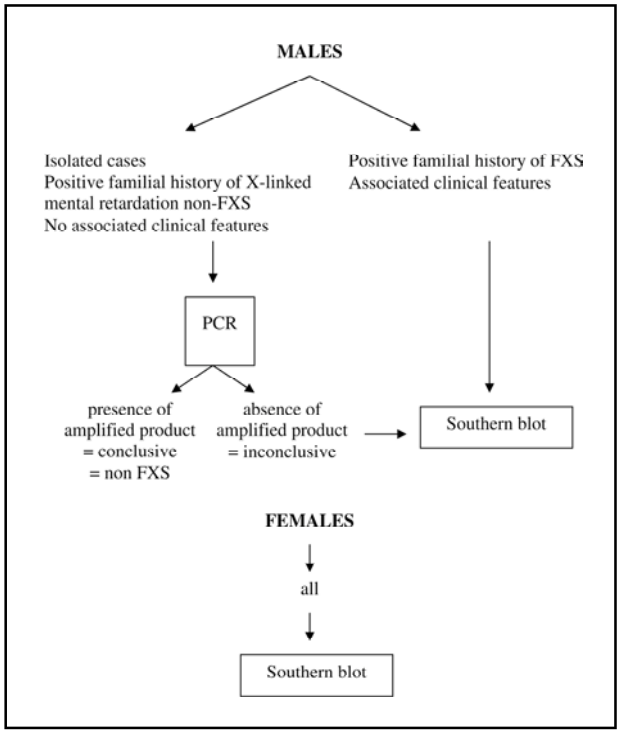

Fig. 4. Proposed algorithm for the laboratory diagnosis in Brazil of fragile X syndrome in male and female individuals with developmental disorders. These molecular tests are recommended to follow cytogenetic analysis, screening for inborn errors of metabolism, TORCH sorologies, and neuroimaging as part of the complementary evaluation of individuals with disorders of neurological development.

(Fig 4). The diagnostic strategy we propose, although not in accordance with other international guidelines for the laboratorial diagnosis of the FXS, represents a reasonable approach in Brazil, a country with limited access of families to clinical genetics services, heterogeneous distribution and conditions of laboratories, and limited governmental resources for medical genetics services.

Acknowledgements – The authors wish to thank all the patients and their relatives who participate of this research, as well as the colleagues from the Laboratório de Genética Molecular (Departamento de Genética Médica, FCM/Unicamp), especially Daniela Facchin, Tereza Cristina Lima e Silva, and Maria Eugenia Ribeiro de Camargo for valuable technical support in the PCR experiments. Thanks also to the staff from the Cytogenetics and Molecular Genetics (Women's and Children's Hospital, Adelaide, Australia), specially Drs. John C. Mulley and Agi K. Gereon for the helpful discussions on the Southern blotting analysis.

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