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ARTICLE

Improving preimplantation genetic diagnosis for Fragile X syndrome: two new powerful single-round multiplex indirect and direct tests

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Fragile X syndrome (FraX) is caused by the expansion of an unstable CGG repeat located in the Fragile X mental retardation 1 gene (FMR1) gene. Preimplantation genetic diagnosis (PGD) can be proposed to couples at risk of transmitting the disease, that is, when the female carries a premutation or a full mutation. We describe two new single-cell, single-round multiplex PCR for indirect and direct diagnosis of FraX on biopsied embryos. These tests include five unpublished, highly heterozygous simple sequence repeats, and the co-amplification of non-expanded CGG repeats for the direct test. Heterozygosity of the new markers ranged from 69 to 81%. The mean rate of non-informative marker included in the tests was low (26% and 23% for the new indirect and direct tests, respectively). This strategy allows offering a PGD for FraX to 96% of couples requesting it in our centre. A conclusive genotype was obtained in all cells with a rate of cells presenting an allele dropout ranging from 17% for the indirect test to 26% for the direct test. The new indirect test was applied for eight PGD cycles: 32 embryos were analysed, 9 were transferred and 3 healthy babies were born. By multiplexing these highly informative markers, robustness of the diagnosis is improved and the loss of potentially healthy embryos (because they are non-diagnosed or misdiagnosed) is limited. This may increase the chances of success of couples requesting a PGD for FraX, in particular, when premature ovarian insufficiency in premutated women leads to a reduced number of embryos available for analysis.
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INTRODUCTION

Fragile X syndrome (FraX, MIM#300624) is the most common cause of inherited mental retardation, affecting ~1 in 2500 individuals. FraX is caused by expansion of an unstable CGG repeat located in the 5′ untranslated region (NM_002024.5:c.-129_-127(6_>200)) of the Fragile X mental retardation 1 gene (FMR1) on the Xq27.3 chromosome band.[1–3] In normal individuals, the number of CGG repeats does not exceed 55. Alleles that contain between 55 and 200 CGG repeats are called premutations and can expand to a full mutation (>200 repeats) in the offspring when maternally transmitted. In individuals carrying a full mutation, methylation of the region leads to loss of transcription of the gene. Males with the full FraX mutation classically show varying degrees of cognitive and behavioural difficulties, moderate facial dysmorphism and macro-orchidism. Affected females usually present milder mental retardation, the severity of which depends on the ratio of X inactivation.

The risk of women carrying a premutation to transmit an expanded, fully mutated allele to their offspring is correlated to the number of repeats in the premutation, being close to 100% for premutations containing above 90 repeats. The frequency of female carriers is estimated to be ~1 in 260. Females carrying a premutation do not present any sign of the classical Fragile X symptoms, but 20% of them have a primary ovarian insufficiency (POI), leading to premature ovarian failure and infertility before the age of 40.[4]

Couples in which the woman carries a premutation or a full mutation can request a preimplantation genetic diagnosis (PGD) to avoid having an affected child. There are numerous technical difficulties associated with FraX PGD: (i) single-cell CGG-amplification is achieved for normal alleles only, limiting the use of a direct test to informative couples, that is, with CGG normal alleles of different sizes; (ii) GC-rich DNA content of the repeats perturbs the amplification of other loci (that is, microsatellites sequences) by single-cell multiplex PCR; (iii) currently used simple sequence repeats (SSRs) show limited or insufficient information for many couples requesting PGD. Moreover, women carrying a premutation who have POI often have poor oocyte retrieval, and therefore only few embryos can be analysed during PGD. Thus, a powerful PGD test is of fundamental importance to minimise the rate of un-diagnosed embryos.

The first PGDs for FraX were performed in 1995 and were solely based on the detection of non-expanded maternal and paternal alleles.[5] This method can only be offered to informative couples – this represents ~63% of the couples who request a PGD. Moreover, allele drop out (ADO) or amplification failure can lead to the failure to diagnose a large number of embryos. The first indirect method for FraX PGD was published in 2001. Using this test, Apessos *et al*[6] claimed to offer a PGD to 90% of the couples requesting for a PGD in their centre.

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CGG repeats are not amplified in the indirect test, and limited information from markers analysis, associated to recombination events and/or ADO can lead to misdiagnosis, even though the risk decreases with the number of markers used and the information they provide. A gold-standard test would include both CGG repeats amplification and linkage analysis in a single-round PCR test. To date, only one team has published such a test (using a nested-PCR) without preliminary whole-genome amplification.[7]

An approach combining multiple displacement amplification and fluorescent PCR for FraX PGD has been described. This includes the detection of non-expanded CGG alleles, linked SSRs and AMELY gene detection for gender identification. A low rate of amplification and a high rate of ADO led to 67 to 86% of successfully diagnosed embryos during the PGD cycles.[8,9]

PGD for FraX has been available in our centre since 1999. At that time, diagnosis relied only on normal CGG allele amplification and a Y chromosome marker (SRY). Then, we developed an indirect multiplex PCR for the detection of the following, widely used microsatellites markers: DXS998, DXS548, FRAXAC1 and DXS1215 combined with a Y chromosome marker. These tests were used alone or in combination (one biopsied cell per test) depending on whether CGG repeats were informative or not. This method is not fully adapted for all couples yet, as a large number of them are not informative for CGG normal repeats (36%) and show limited information from the linked markers cited above.

Here we present a highly informative, sensitive and specific new indirect test using newly identified microsatellite sequences for FraX PGD that increases the rate of diagnosed embryos and reduces the number of couples for which a PGD could not be offered. Moreover, four of these new markers could be simultaneously amplified with the normal CGG repeats in a single-round PCR amplification, giving an efficient direct test.

MATERIALS AND METHODS

The development of direct and indirect tests for FraX PGD included the following steps: (i) new FMR1-linked SSR markers selection and primer design; (ii) test for heterozygosity using 92 unrelated female DNA samples; (iii) determination of informative markers within referred couples for FraX PGD; (iv) single-cell multiplexing (including a gender-determination marker: AMELY, and the normal CGG-repeats detection for the direct test) and validation; and (v) application of the new indirect test for FraX PGD cycles.

Patients and clinical PGD

Since both direct and indirect tests have been available in our centre, 47 couples asking for a PGD for FraX were referred. Samples for each couple and relatives (ascendants, siblings, prenatal diagnosis or children) were collected. Genomic DNA from blood was extracted using QiaAmp DNA blood mini Kit from Qiagen (Frederick, MD, USA).

PGD cycles were performed for 18 couples. Patient ovarian stimulation, oocyte pick up, ICSI and biopsy procedures were carried out as previously described.[10] Two blastomeres were analysed per embryo, except for one cycle (one blastomere).

New markers design, heterozygosity testing and genotyping for couples asking for PGD

UCSC Genome Browser (http://genome.ucsc.edu/, hg19 Genome assembly) was used to identify large unpublished dinucleotide repeats nearby the FMR1 locus (Figure 1). Five dinculeotide repeat sequences, three located proximally to FMR1 and two distally (maximal distance 624 kb) were chosen for heterozygosity testing. Primers (Supplementary Table 1) were designed using Oligo7.0 software (Molecular Biology Insights, Inc. Cascade, CO, USA) and specificity was checked with PrimerBlast (NCBI). The maximal rate of heterozygosity was calculated by analysing 92 unrelated female DNA samples from our referrals (Figure 1).

The validity of the new markers was tested for couples asking for FraX PGD in our centre. Data were compared with those obtained with the markers included in our previous test. The validity of the markers was categorised as follows: fully informative (healthy and at risk maternal and paternal alleles are different), semi-informative (healthy and affected maternal alleles are different but one of them is the same than the paternal one), and not informative (healthy and at risk maternal alleles are the same, independently of the paternal one). Information regarding normal CGG repeats was also studied. In this case, couples were either informative (ie, healthy maternal and paternal alleles were different) or not informative (ie, healthy maternal and paternal alleles were the same). Premutated or mutated allele sizes were not determined because they are not visible at the single-cell level.

Single-cell testing

Six Epstein–Barr Virus transformed lymphoblast cell lines were used for PCR set up and validation: three control male cell lines (CFcN, SMNt and JOS063), two control female cell lines carrying two non-expanded CGG alleles of different sizes (AEC017 and HD080) and one female cell line carrying a FraX premutation (GM06905: CGG repeats 23 and 70),[11] (NIGMS Human Genetic Cell Repository). Single cells (lymphoblasts or biopsied blastomeres) were handled as previously described [10] and lysed in 2.5 μl lysis buffer (LB: 200 mM KOH, 50 mM DTT) for 10 min at 65 °C.

Diagnosis strategy for PGD was adapted depending on couples normal CGG repeats alleles and microsatellites markers. When CGG repeats were informative, the direct test was performed on one biopsied cell (the second cell was used for indirect test when applicable) or on two biopsied cells. When CGG repeats were not informative, one or two cells were used for indirect test. As the new indirect test has been developed, it was always preferred to the previously used one.

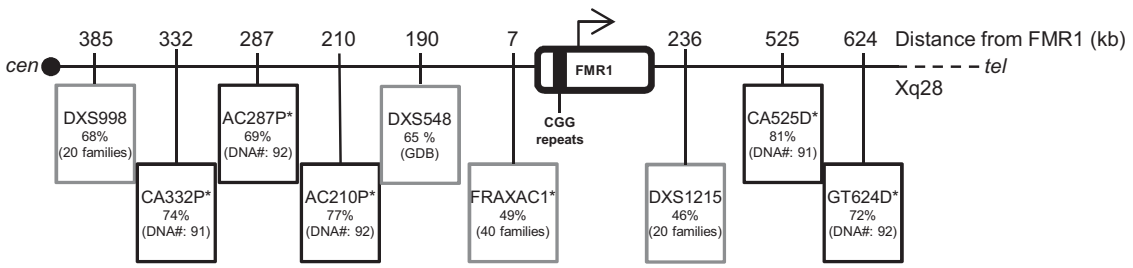

Figure 1 FMR1 genetic map. New described markers are in dark grey boxes. *: Markers included in the new indirect protocol. Percentage of maximal heterozygosity is given below each marker name according to CEPH families or GDB or after testing on 92 independent female DNA samples. Number of families or DNA tested is shown in parentheses. Distances (kilobases, kb) are not in scale. *cen*: centromere. *tel*: telomere.

PCR reaction

For indirect test, five new markers (FMR1-CA332P, FMR1-AC287P, FMR1-AC210P, FMR1-CA525D and FMR1-GT624D, see Supplementary Table 1 for sequences), were combined with FRAXAC1[12] and AMELY gene detection for sex determination[13] in a multiplex, single-round PCR test. A 47.5 μl reaction mix with primers (see Supplementary Table 1 for sequences and quantity per reaction) and a final concentration of 1 × QIAGEN Multiplex PCR Master Mix (Qiagen), 0.5 × Q-Solution (Qiagen) and 0.8 mM Tricine was added in the tube containing the denatured single-cell in LB. Conditions for amplification were as follows: initial denaturation (15 min at 95 °C); denaturation (30 s at 94 °C, except for the 10 first cycles: 96 °C), annealing (90 s at 52 °C) and elongation (90 s at 72 °C) for 45 cycles; final elongation (10 min at 72 °C).

For direct test, four new markers (FMR1-CA332P, FMR1-AC287P, FMR1-CA525D and FMR1-GT624D) were combined with normal CGG repeats detection[14] and AMELY sequence in a multiplex, single-round PCR test. A 51 μl reaction mix with primers (Supplementary Table 1) and a final concentration of 1 × QIAGEN Multiplex PCR Master Mix, 9% DMSO, 0.5 × dNTP500 (125 μM 7deaza-dGTP, 375 μM dGTP, 500 μM of each dATP, dTTP and dCTP), 0.7 × Q-Solution and 0.8 mM tricine was added in the tube containing the cell. Conditions for amplification were the same as the indirect test except that a total of 47 cycles was performed with an annealing temperature of 55 °C.

PCR products were run on an ABI3130xl or an ABI3500 automated sequencer with GeneScan 500 LIZ Size Standard (Thermo Fisher Scientific, Waltham, MA, USA).

RESULTS

Identification of highly informative markers linked to FMR1

Genomic map of the FMR1 gene (NM_002024.5) was studied and five potential new markers (FMR1-CA332P, FMR1-AC287P, FMR1-AC210P, FMR1-CA525D and FMR1-GT624D) consisting of large dinucleotide repeats flanking the gene (Figure 1) were tested for the rate of heterozygosity on independent female DNA samples. All five markers showed a higher maximal heterozygosity (from 69 to 81%) than that reported by the CEPH (foundation Jean Dausset) or by the Genome DataBase for the markers used in our previous test (from 46 to 68%).

The study revealed that in our cohort, 64% of couples asking for FraX PGD were informative for the CGG locus (Figure 2a). For the previous test, the rates of fully informative couples for the markers used ranged from 13 to 19%. For the five new markers, the rates ranged from 43 to 51%. Considering fully- and semi-informative couples, the total informativity for a single marker was increased (range 49–60% for the previously used markers *vs* 60–81% for the new markers).

When considering all the markers included in each test, global informativity was also improved. The mean rate of non-informative markers per couple within the test decreased from 46% for the previous indirect test (four markers) to 26% for the new indirect (six markers) and 23% for the new direct (four markers) tests, respectively ($P<0.05$ for both tests, Student test).

Information from the markers included in our previous indirect test was not sufficient for 28 couples requesting a FraX PGD (for details see Figure 2b). The reasons were a lack of informative marker (no possible indirect diagnosis) or of informative marker only on one side of the gene (not possible to highlight recombination events), or the presence of only semi-informative markers with identical paternal and unaffected maternal haplotypes (not possible to distinguish unaffected female embryos *vs* monosomy). Among them, 20 were informative for normal CGG repeats and were then eligible for a PGD by testing CGG alone (with SRY as a Y chromosome marker). In this case, two cells were analysed per embryo to minimize misdiagnosis risks due to ADO or contamination. Thus, considering our previous strategy, eight couples could not be offered a PGD in our centre. The new indirect test was not suitable for only four couples (no informative marker

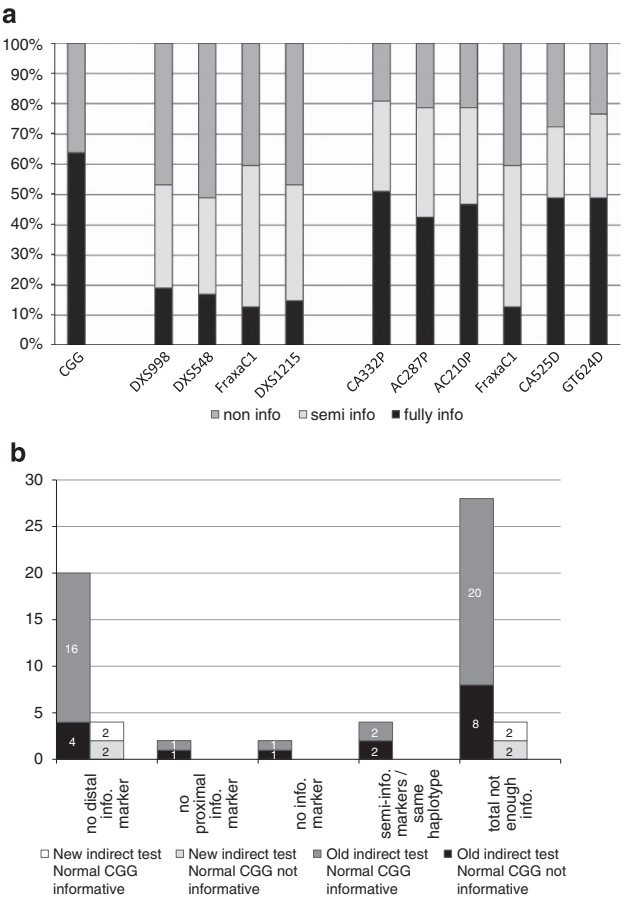

Figure 2 Couples informativity for FMR1-linked markers included in the PGD tests. (**a**) Informativity of CGG repeats and FMR1-linked microsatellites markers for 47 couples referred for FraX PGD. Markers are grouped by test (previous test in the middle, new indirect test on the right). For categorisation of markers (fully-, semi- or non-informative), see Material and Methods section. (**b**) Number of couples for which the previous or new indirect tests are considered as not sufficiently informative and the cause (no informative marker on one side of the gene, no informative marker at all, semi-informative markers only with identical paternal and safe maternal alleles preventing the discrimination between diploid and haploid cell at this locus). For each test, discrimination between couples informative and not informative for CGG repeats is made, as another possibility exist for informative couples by using the direct test.

located distally to FMR1), two of which were also neither informative for CGG normal repeats nor for DXS1215. Of these two couples, one resigned and a new test is being currently developed for the other.

Setting-up of a multiplex, single-round PCR

New indirect and direct tests were validated on single cells before application for PGD. Details are shown in Table 1 (values for the previous indirect and direct tests are shown as a reference). A PCR signal (for at least one marker) was obtained in 94% and 90% for the new indirect and direct tests, respectively. A successful amplification was obtained in >80% of cells for each marker and CGG repeats were successfully amplified in 82% of the cells in the direct test. A complete genotype (all markers with complete and correct genotype) was obtained in 63% and in 64% of the cells tested for the indirect and direct tests, respectively, and a conclusive genotype (missing/incorrect calls did not prevent correct genotype identification) was found in 100% for both tests. As a comparison, the previous direct test, which

Table 1 Single-cell validation

Name of the test	Nb of cells tested	PCR signal (%)	Correct signal DXS998 (%)	Correct signal CA332P (%)	Correct signal AC287P (%)	Correct signal DXS548 (%)	Correct signal AC210P (%)	Correct signal FRAXAC1 (%)	Correct signal CGG (%)	Correct signal DXS1215 (%)	Correct signal CA525D (%)	Correct signal GT624D (%)	Correct signal AMELY or SRY (%)	Complete genotype (%)	Conclusive genotype[a] (%)	Allele drop-out[b] (% female cells)
'New' indirect	80	75 (94%)	–	69 (92%)	72 (96%)	–	72 (96%)	62 (83%)	–	–	72 (96%)	70 (93%)	66 (88%)	47 (63%)	75 (100%)	17%
'New' direct	68	61 (90%)	–	56 (92%)	60 (98%)	–	–	–	50 (82%)	–	52 (85%)	60 (98%)	59 (97%)	39 (64%)	61 (100%)	26%
'Previous' direct	78	74 (95%)	–	–	–	–	–	–	62 (84%)	–	–	–	72 (97%)	60 (81%)	60 (81%)	29%
'Previous' indirect	59	58 (98%)	56 (97%)	–	–	57 (98%)	–	53 (91%)	–	50 (86%)	–	–	55 (95%)	43 (74%)	55 (98%)	25%

[a]Correct signals for at least four loci for the new tests, and at least three for the old indirect test. Corresponds to complete gentoypes for the 'old' direct duplex test.
[b]Female cells with ADO for at least one locus.

was a duplex showed only 81% conclusive genotype because the status of the cell could not be successfully determined as soon as the normal CGG repeats or SRY were not correctly amplified. The previous indirect test had a higher conclusive genotypes rate (98%) but, as specified earlier, included only one distal marker, which was not informative for number of couples. ADO rate (percentage of cells with at least one missing signal) was measured on heterozygous (female) cells only and ranged from 17% for the indirect test to 26% for the direct test. However, only FraxaC1 marker in the new indirect test shows a high (19%) rate of ADO when measuring the ADO rate per locus in female cells (all others present an ADO rate per locus $<10\%$, data not shown). Supplementary Figure 1 shows examples of amplification profiles for both tests.

PGD cycles

In total, between 2007 and 2014, 39 FraX PGDs have been performed for 18 couples, using CGG/SRY test only (17 cycles), previous indirect test only (7 cycles), both tests on one cell for each test (7 cycles), new indirect test (5 cycles), and both CGG/SRY and new indirect test on one cell per test (3 cycles). The new direct test has not yet been used for PGD application. Details of PGD results and outcomes are shown in Table 2. Indirect tests globally bring more confidence to diagnosis as they reduce the rate of non-diagnosed or misdiagnosed embryos compared with the CGG/SRY previous direct test alone ($P<0.05$, χ^2-test with Yate's correction). Indeed, the previous direct test alone shows more susceptibility to non-diagnosis or misdiagnosis as a weaker amplification signal or a simple ADO or contamination impaired diagnosis for a total of eight embryos (9.9%). Among the 29 embryos analysed with the previous indirect test alone, the status of six could not be discriminated between unaffected female and monosomy.

PGD cycles using the new indirect test alone or in combination with CGG/SRY gave rise to three pregnancies, and three healthy babies were born. In total, 14 babies (with the expected sex) were born after a PGD for FraX in our centre, using one of the tests described here. No prenatal diagnosis was performed, but one result was confirmed postnatally.

Focus on some particular cases

Even though it is hardly measurable, globally increased information retrieved from the markers included in the new indirect test and their number, compared with those included in the previous one, makes this new test more robust for FraX PGD. In some cases, the new indirect test clearly improved the diagnosis feasibility or quality.

For example, couple number 1 was referred to us as a PGD could not be offered in the other French centres, owing to a lack of informative CGG repeats and classical markers. Our new test including one fully- and one semi-informative marker on each side of the gene for this couple (Figure 3a), could be used for a PGD. A cycle was performed where eight embryos were analysed, one was transferred (and two frozen), a pregnancy ensued and a healthy boy was born.

Paternal and healthy maternal alleles for couple number 2 (who were not informative for CGG repeats) were identical for markers of the previous test, allowing the diagnosis of affected embryos, but preventing the distinction between an unaffected female and monosomic or haploid cell (Figure 3b). The previous test was initially adapted by adding an informative X-linked marker distant from the FMR1 locus (DXS8377) to distinguish diploid cells. This problem was solved by the presence of two proximal and one distal fully informative marker in the new test, which was successfully used for the third PGD cycle for this couple who gave birth to a healthy girl.

Table 2 PGD cycles

	CGG/SRY	*Previous*[a] *indirect test*	*CGG+previous indirect test*[b]	New[c] *indirect test*	*CGG+new indirect test*[b]	*Total*
Number of couples	9	4	5	5	3	18
Number of cycles	17	7[d]	7	5	3	39
Number of embryos (E) analysed	83	29	45	25	7	189
Amplification failure	2	1	4	0	0	7
Not diagnosed (% of analysed)	6[e] (7.2%)	0	1[f] (2.2%)	1[g] (4.0%)	0	8
Impossible to distinguish E status safe *vs* haploid	0	6	0	0	0	6
Cycles with reanalysis	8	1	6	3	1	19
Number of E reanalysed	36	1	19	9	2	67
Nb E confirmed (reanalysis or postnatally)	11[h]	1	15	8	2	37
Misdiagnosis (% of reanalysed)	2[i] (5.6%)	0	0	0	0	2
Amplification failure during reanalysis	22	0	2	0	0	24
Amplification failure or not diagnosed during PGD, diagnosed after reanalysis	1	0	2	1	0	4
Total not diagnosed or misdiagnosed (% of analysed with amplification)	9.9%	0.0%	2.4%	4.0%	0.0%	5.5%
Cycles with embryo transfer	17	5	6	4	3	35
Number of E transferred	25	6	8	5	4	48
Cycles with E frozen (after PGD)	5	2	1	2	0	10
Number of frozen embryos	10	4	1	4	0	19
Pregnancies						
hCG+	9	4	3	3	2	21
Fetal heart beats+(nb)	6 (6)	3 (3)	3 (4)	2 (2)	1 (1)	15 (16)
Miscarriage or termination of pregnancy (other cause)	1	1	0	0	0	2
Deliveries	5	2	3	2	1	13
Number of babies born	5	2	4	2	1	14
Sex confirmed	5	2	4	2	1	14

[a]DXS998, DXS548, FRAXAC1, DXS1215 and SRY (chromosome Y).
[b]One biopsied cell per test.
[c]FMR1-CA332P, FMR1-AC287P, FMR1-AC210P, FMR1-CA525D, FMR1-GT624D, FRAXAC1 and amelogenin (chromosome Y).
[d]One cycle (3E) with X-linked marker added to distinguish safe *vs* haploid embryos.
[e]For five embryos: not conclusive due to weak signals. For one embryo: two signals on one cell, one signal on the other (affected female with contamination on one cell or healthy female with ADO on one cell; amplification failure during reanalysis).
[f]Incoherent results between the two tests (affected/healthy male), affected male during reanalysis.
[g]Not diagnosed because of contradictory signals due to amelogenin contamination (diagnosed as healthy female when reanalysed).
[h]One E confirmed postnatally.
[i]ADO or AOF on normal CGG during PGD; 2 E reanalysed as healthy female and male, respectively.

DISCUSSION

FraX is one of the most common PGD indication in our centre after Huntington's disease, cystic fibrosis and myotonic dystrophy. In female premutation carriers, POI is responsible for a decrease in the number of oocytes retrieved per cycle and thus, reduces chances of transfer and pregnancy. Moreover, single-cell direct tests do not allow premutated and fully mutated alleles to be distinguished. Even if this was possible, the risk of mosaicism would be too high to transfer an embryo potentially containing both fully mutated and premutated blastomeres.[15] Thus, only male and female embryos carrying non-expanded CGG alleles may be transferred, that is, statistically half of the tested embryos. Finally, only a few embryos are eligible for transfer, and the PGD test has to be robust enough to ensure correct diagnosis.

Multiplex PCR using SSRs amplification is classically used for PGD of single-gene disorders and remains a fast, single-step and cost-effective procedure when compared with whole-genome techniques such as karyomapping,[16] whose main advantages are the absence of a couple- or disease-specific set up and the co-detection of aneuploidies. Moreover, such a targeted approach matches the French law that limits genetic analysis to the locus of interest and prohibits aneuploidy screening, and is still applicable to the large majority of couples as shown by our study.

A robust PGD test should be able not only to distinguish between a safe and an affected embryo, but also to highlight all the unexpected events that may happen during meiosis, fertilization or PGD experimental procedure, and thus to detect recombination, monosomy or trisomy (and therefore diagnose an abnormal embryo), ADO and contamination. An accurate interpretation depends on how informative the combination of markers can be for the diagnosis. In our new tests, globally increased information brought by the markers not only allows a better identification of unaffected/affected embryos but also normal/abnormal embryos (at least at the Xq27 locus). This was not only obvious in the particular case of couple number 2 but also in couples where almost fully identical paternal and maternal alleles prevent the discrimination between unaffected female and monosomic embryo/cell in case of ADO. In these cases, the new indirect test may increase the chances of transferring an euploid embryo.

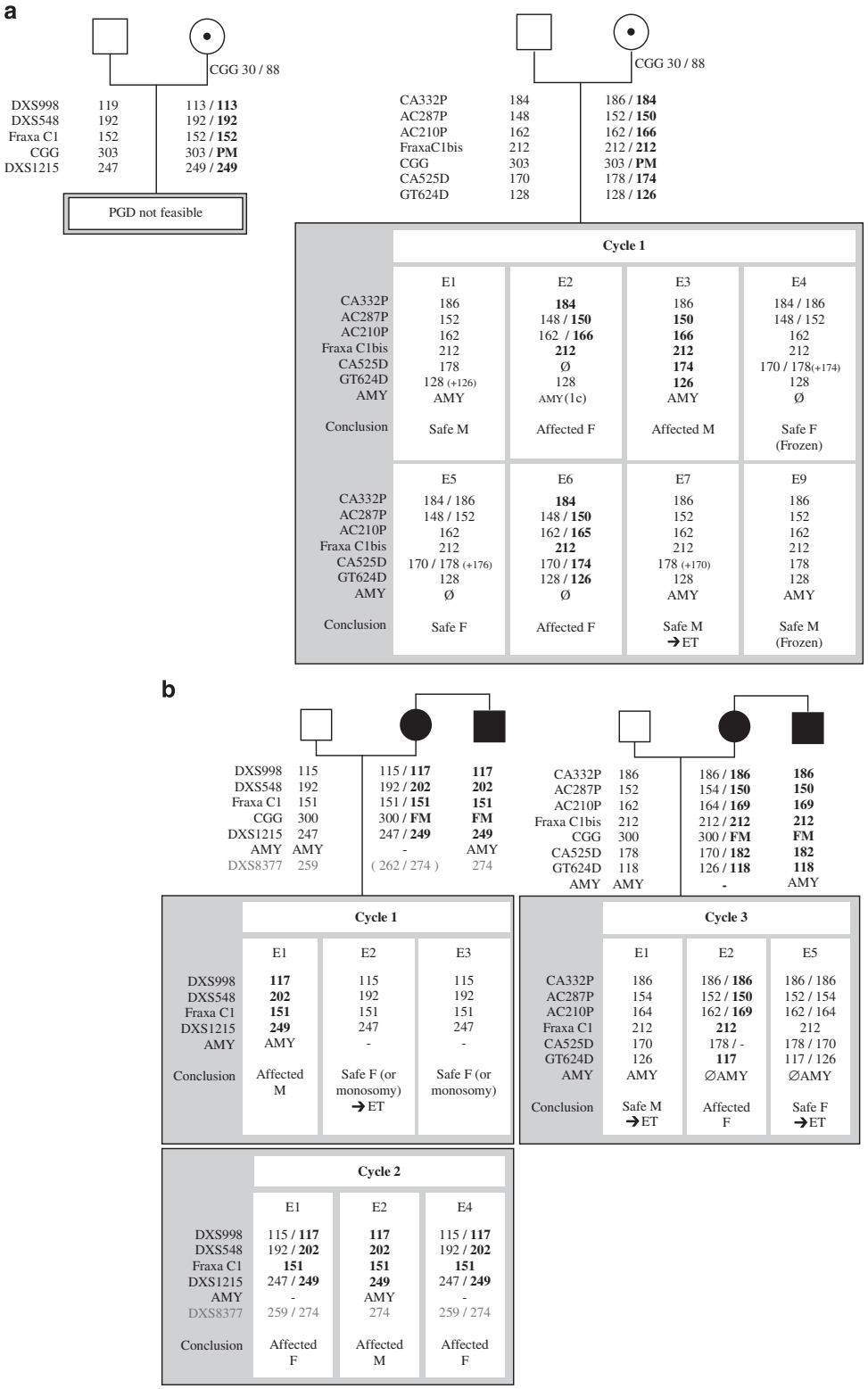

Figure 3 Particular cases. (**a**) In couple no. 1, woman carried a premutation (PM) and couple was not informative neither for CGG normal alleles nor for markers of the previous test. Markers of the new test were informative and affected maternal haplotype (in bold) was determined using two affected prenatal diagnosis DNA samples (not shown). Result of the first PGD cycle is shown. Embryo (E) 7 was transferred (ET, embryo transfer) and a pregnancy followed – a healthy boy was born. Numbers in parentheses show artefactual picks most probably due to minor contaminations. (**b**) Pedigree of couple no. 2 and three PGD cycles results. The woman and her brother carry a FraX full mutation (FM). Affected haplotype is shown in bold. With the markers of the previous test (cycle 1), safe female could not be discriminated from monosomic embryos as proved by the status of embryos E2 and E3. E2 was transferred but no pregnancy followed. A MTMX-linked (not FMR- linked) marker was added for cycle 2 but all embryos were affected. The new indirect test was informative and allowed the detection of monosomy so it was used for the last cycle (cycle 3). Two healthy embryos were transferred and a healthy girl was born.

Using our previous strategy, the direct test (CGG+SRY) was performed (on two cells) for couples not informative for markers but informative for normal CGG repeats. This test did not meet the best practice guideline recommendations for amplification-based PGD of the ESHRE PGD Consortium as it did not include polymorphic marker(s), which ensure a more accurate diagnosis by detecting the presence of ADO and contamination.[17] In our experience, two healthy embryos were indeed diagnosed as affected during PGD. These benign misdiagnosis due to ADO only have consequences on the number of transferable embryos, contrarily to contaminations that could lead to adverse misdiagnosis, that is, the transfer of an affected embryo.[18] Still, it is important to limit the number of benign misdiagnosis or of non-conclusive diagnosis because they do not only decrease the chances of pregnancy, but they also lead to the destruction of potentially healthy embryos, which is ethically debatable. For these reasons, when possible, the CGG repeats analysis was combined with our previous indirect test (one cell per test), but this was still insufficient for a large number of couples requesting a PGD in our centre. Moreover, the indirect test included only one marker located distally from FMR1 (DXS1215). This marker was rarely fully informative, increasing the risk of misdiagnosis in case of ADO and/or recombination. Our new tests include two or three markers on each side of the CGG repeats and the detection of the normal repeats for informative couples, allowing an overall better diagnosis quality.

In our centre, our new tests allow us to offer a PGD cycle to 44 couples out of 46 (96%). The two remaining couples are informative neither for CGG normal repeats nor for the distal markers of the previous and the new indirect tests. New distal markers have been designed and a new test is currently being developed. For couple number 1 (Figure 3a), the new indirect test was the only chance to benefit from a PGD, at least in France.

Best practice guidelines recommend to obtain $>90\%$ amplification efficiency for each locus in single-cell validation experiments.[17] In our experiments, two loci for each test are below this threshold (because of ADO, amplification failure, errors or contaminations), but this is probably due to the high multiplexing level and the co-amplification of CGG repeats in the direct test. ADO rate per locus in female cells is above the threshold fixed by the ESHRE guidelines ($>10\%$) for FraxaC1 in the new indirect test. However, the multiplexing with two other SSRs on the same side of the FMR1 gene (or the analysis of two cells per biopsied embryo if these are not informative) should avoid misdiagnosis due to ADO at this locus. Moreover, in our validation series, the number of successfully amplified loci is still sufficient as a conclusive genotype was obtained in 100% of the cells for both tests.

As we developed the new direct and indirect tests, we tested the couples for all available markers to choose the most adapted test. The decision was always made in favour of the new test. Therefore, as this seems to apply to the majority of couples, we may change our practice and initially only test markers of the new tests.

The use of the new tests may increase the number of transferable embryos per cycle by minimising the rate of non-conclusive diagnosis. Moreover, as the waiting list for a PGD in France is extremely long (1–2 years), embryo and oocyte vitrification can be proposed in order to preserve fertility of FraX premutated women with altered ovarian reserve parameters requesting a PGD in our centre. Overall, in the future, the chances of couples requesting a FraX PGD having a healthy baby may globally increase.

CONCLUSION

We developed one indirect and one direct single-round, single-cell PCR protocols using five highly polymorphic, not previously described, FMR1-linked SSRs. The high rate of heterozygosity of these markers increases global test informativity when compared with the classical set of described FMR1-linked markers. Moreover, co-amplification of CGG normal repeats and informative microsatellites within a unique PCR improves diagnosis for informative couples. Consequences are that (i) PGD for FraX can be offered to almost any couple asking for it in our centre, (ii) robustness of the test is increased and (iii) risks of non-diagnosis (and thus, loss of potential transferable embryos) or misdiagnosis are reduced.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Gallagher A, Hallahan B: Fragile X-associated disorders: a clinical overview. *J Neurol* 2012; **259**: 401–413.
2 Oberlé I, Rousseau F, Heitz D *et al*: Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science* 1991; **252**: 1097–1102.
3 Verkerk AJ, Pieretti M, Sutcliffe JS *et al*: Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* 1991; **65**: 905–914.
4 Conway GS, Hettiarachchi S, Murray A, Jacobs PA: Fragile X premutations in familial premature ovarian failure. *Lancet* 1995; **346**: 309–310.
5 Sermon K, Seneca S, Vanderfaeillie A *et al*: Preimplantation diagnosis for fragile X syndrome based on the detection of the non-expanded paternal and maternal CGG. *Prenat Diagn* 1999; **19**: 1223–1230.
6 Apessos A, Abou-Sleiman PM, Harper JC, Delhanty JD: Preimplantation genetic diagnosis of the fragile X syndrome by use of linked polymorphic markers. *Prenat Diagn* 2001; **21**: 504–511.
7 Malcov M, Naiman T, Yosef DB *et al*: Preimplantation genetic diagnosis for fragile X syndrome using multiplex nested PCR. *Reprod Biomed Online* 2007; **14**: 515–521.
8 Burlet P, Frydman N, Gigarel N *et al*: Multiple displacement amplification improves PGD for fragile X syndrome. *Mol Hum Reprod* 2006; **12**: 647–652.
9 Lee H-S, Kim MJ, Lim CK, Cho JW, Song IO, Kang IS: Multiple displacement amplification for preimplantation genetic diagnosis of fragile X syndrome. *Genet Mol Res GMR* 2011; **10**: 2851–2859.
10 Moutou C, Gardes N, Viville S: Multiplex PCR combining deltaF508 mutation and intragenic microsatellites of the CFTR gene for pre-implantation genetic diagnosis (PGD) of cystic fibrosis. *Eur J Hum Genet* 2002; **10**: 231–238.
11 Howard-Peebles PN, Friedman JM: Unaffected carrier males in families with fragile X syndrome. *Am J Hum Genet* 1985; **37**: 956–964.
12 Richards RI, Holman K, Kozman H *et al*: Fragile X syndrome: genetic localisation by linkage mapping of two microsatellite repeats FRAXAC1 and FRAXAC2 which immediately flank the fragile site. *J Med Genet* 1991; **28**: 818–823.
13 Levinson G, Fields RA, Harton GL *et al*: Reliable gender screening for human preimplantation embryos, using multiple DNA target-sequences. *Hum Reprod* 1992; **7**: 1304–1313.
14 Fu YH, Kuhl DP, Pizzuti A *et al*: Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell* 1991; **67**: 1047–1058.
15 Moutou C, Vincent MC, Biancalana V, Mandel JL: Transition from premutation to full mutation in fragile X syndrome is likely to be prezygotic. *Hum Mol Genet* 1997; **6**: 971–979.
16 Handyside AH, Harton GL, Mariani B *et al*: Karyomapping: a universal method for genome wide analysis of genetic disease based on mapping crossovers between parental haplotypes. *J Med Genet* 2010; **47**: 651–658.
17 Harton GL, De Rycke M, Fiorentino F *et al*: ESHRE PGD consortium best practice guidelines for amplification-based PGD. *Hum Reprod* 2011; **26**: 33–40.
18 Dreesen J, Destouni A, Kourlaba G *et al*: Evaluation of PCR-based preimplantation genetic diagnosis applied to monogenic diseases: a collaborative ESHRE PGD consortium study. *Eur J Hum Genet* 2014; **22**: 1012–1018.

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