CLINICAL ARTICLE

Preimplantation genetic diagnosis of X-linked adrenoleukodystrophy with gender determination using multiple displacement amplification

Belén Lledó, Ph.D.,[a] *Rafael Bernabeu, M.D.,*[a,b] *Jorge Ten, Ph.D.,*[a] *Francisco M. Galán, Ph.D.,*[a] *and Luigi Cioffi, Ph.D.*[c]

[a] Bernabeu Institute of Fertility and Gynecology, Alicante, Spain; [b] Chair of Reproductive Medicine, Miguel Hernandez University, Elche, Spain; and [c] Fertility Center of Reproductive Medicine, Salerno, Italy

Objective: To evaluate the use of multiple displacement amplification (MDA) for whole genome amplification in the preimplantation genetic diagnosis (PGD) of X-linked adrenoleukodystrophy.
Design: MDA was used to amplify the whole genome directly from a single blastomere. MDA products were used for polymerase chain reaction (PCR) analysis of two polymorphic markers flanking the ABCD1 gene and a new X/Y marker, X22, to sex embryos in an X-linked adrenoleukodystrophy PGD program.
Setting: Fertility and gynecology private center in Alicante, Spain.
Patient(s): A couple in which the wife is a carrier of the ABCD1 gene mutation (676A→C) that was previously identified in her family.
Intervention(s): MDA of single blastomere and PCR tests for PGD.
Main Outcome Measure(s): The ability to analyze single blastomeres for X-linked adrenoleukodystrophy using MDA.
Result(s): The development of an MDA-PGD protocol for X-linked adrenoleukodystrophy allowed for the diagnosis of five embryos. These were biopsied on day 3 of culture and analyzed. One embryo was an affected male and one embryo was a female carrier. Three healthy female embryos were transferred 48 hours after biopsy. Unfortunately, no pregnancy was achieved.
Conclusion(s): The MDA technique is useful for overcoming the problem of insufficient genomic DNA in PGD and allows the simultaneous amplification of different targets to perform a diagnosis of any known gene defect and a sexing test by standard methods and conditions. (Fertil Steril® 2007;■:■–■. ©2007 by American Society for Reproductive Medicine.)

Key Words: MDA, multiple displacement amplification, PGD, preimplantation genetic diagnosis, ALD, X-linked adrenoleukodystrophy

Couples with genetic disorders including single-gene defects, sex-linked conditions, or chromosome rearrangements face a reproductive risk. Preimplantation genetic diagnosis (PGD) is a diagnostic tool to avoid inheritance of genetic disease by transferring unaffected IVF embryos and represents an alternative to prenatal diagnosis (1).

PGD first consisted of the selection of female embryos for patients at risk of transmitting X-linked recessive diseases (2), and usually female embryos are transferred. Fifty percent of those female embryos are carriers, who later in life will have a 50% chance of transmitting their defective X-chromosome to the next generation. Fifty percent of the discarded male embryos are unaffected, which represents not only an ethical problem but diminishes the pool of embryos suitable for transfer.

Advances in molecular biology allow the development of specific diagnosis in a single cell for gene defects. Indeed, the fifth report of the European Society for Human Reproduction and Embryology (ESHRE) PGD Consortium (3) lists over 40 monogenic diseases for which PGD has been applied. Furthermore, for families with an identified X-linked recessive disease-causing mutation, nonspecific diagnosis by sex identification can be considered a substandard method (4), and it might be considered as a monogenic disease performing specific molecular diagnosis.

Despite the significant advantages provided by PGD, the setting up and testing of molecular diagnoses on a single cell is work intensive, difficult, expensive, and time-consuming. Labor-intensive development and validation of highly sensitive amplification strategies for single-cell

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Reprint requests: Belén Lledó, Ph.D., Instituto Bernabeu. Avda. de la Albufereta, 31, 03016, Alicante, Spain (FAX: 0034-96-515-13-28; E-mail: blledo@institutobernabeu.com).

diagnosis are required, usually using nested polymerase chain reaction (PCR), whole genome amplification (WGA), or fluorescent PCR methods. The main disadvantage of nested and fluorescent PCR is the difficulty in choosing primers for multiplex PCR (5). On the other hand, the main disadvantages of WGA are the generation of nonspecific amplification artifacts, incomplete coverage of loci, inefficiency of microsatellite amplification, and the generation of DNA less than 1 kb long (6). For those reasons, PGD requires a technique that would be able to amplify the single-cell DNA with a high fidelity that suits the diagnosis of any known single-gene disorder by the standard PCR technique.

Multiple displacement amplification (MDA) is an isothermal WGA technique based on the used of ϕ29 DNA polymerase and random primers. The ϕ29 polymerase combines high processivity with a strand displacement ability leading to the synthesis of DNA fragments >10 kb and favoring uniform representation of sequences (7). MDA is a technique that is used in the amplification of very low DNA quantities in clinical samples (6). Sequence representation in the amplified DNA assessed by multiple single-nucleotide polymorphism analysis is equivalent to genomic DNA, and amplification is superior to PCR-based methods (8).

Adrenoleukodystrophy (ALD) is an X-linked recessive disorder that is secondary to a mutation in the ABCD1 gene (in the terminal segment of the long arm of the X, i.e., Xq28) and results in peroxisomal beta oxidation defect and the accumulation of the saturated very long chain fatty acids in all tissues of the body. The manifestations of the disorder occur primarily in the adrenal cortex, the myelin of the central nervous system, and the Leydig cells of the testes. The choice method for X-linked ALD PGD is sexing. The advantage of relying on sexing only comes from its nonspecificity and suitability for all couples at risk for X-linked conditions. Indirect diagnosis without gender determination has been used (4), but to avoid misdiagnosis due to the fact that polymorphic markers segregate with sexual chromosomes and allele dropout (ADO), a gender determination could be included.

We describe for the first time haplotype and gender determination using MDA for PGD of X-linked ALD that enables the selection of both male and female unaffected embryos. This approach offers an alternative to sexing, which is frequently used for X-linked disorders and which results in the discarding of all male embryos, including the 50% that would have been normal, thus increasing the chance of pregnancy and avoiding the loss of healthy male embryos.

The aim of this work was to increase the reliability of PGD for X-linked ALD and to improve our ability to respond in a fast and safe way because of the ability to obtain enough quality DNA by MDA from a single cell for multiple PCR analyses.

MATERIALS AND METHODS

Lysis of Single Cells

Lymphocytes from a female heterozygous for the DXS1073, DXS9901, and X22 loci were separated from blood by centrifugation over Ficoll, washed, and resuspended in PBS. Single cells were collected and transferred to 0.2-mL PCR tubes containing 0.5 μL of alkaline lysis buffer. The samples were kept at −80°C at least 30 minutes. Cells were lysed by incubation at 65°C for 10 minutes (9). Lysis was then stopped by adding 0.5 μL of neutralization buffer (9).

MDA Protocol

Cells lysates were used directly for MDA. WGA by isothermal MDA was achieved using bacteriophage ϕ29 DNA polymerase, exonuclease-resistant phosphorothioate-modified random hexamer oligonucleotide primers, and reaction buffer according to the manufacturer's instructions (Amersham Biosciences, UK) in a 20-μL reaction at 30°C (16 hours). The reaction was terminated by incubation at 65°C for 10 minutes to inactivate the enzyme and the amplified DNA stored at −20°C.

PCR Analysis

To use linkage analysis in the PGD of X-linked ALD, two extragenic polymorphic markers (DXS1073 and DXS9901), which flank the ABCD1 gene, were amplified using 1 μL of MDA products. The primers used were described by Gigarel et al. (4), and the forward primers were labeled at 5' with 6-FAM. PCR for the markers was carried out using the TaKaRa LA Taq kit (Takara Bio, Shiga, Japan). A reaction mix in a total volume of 25 μL containing 100 pmol of each primer, 200 mM dNTPs, 1× buffer was provided by the manufacturer, and 1 U of DNA polymerase was provided by the TaKaRa LA Taq kit. PCR was performed as follows: 5 minutes at 95°C, 35 cycles of 45 seconds at 95°C, 45 seconds at 55°C, and 45 seconds at 72°C followed by 5 minutes' extension at 72°C. Two microliters of the PCR product was mixed with 2 μL of loading buffer denatured by boiling for 5 minutes and loaded on the ABI PRISM 3100 Sequencer. The results were processed using the GeneScan Analysis software.

Sexing of human DNA by PCR-based methodology can be accomplished by amplifying X-Y homologous genes. To assess the sex status of embryos, a new X/Y chromosome marker, X22 (10), was detected by fluorescent PCR. The forward primer (5'-TAATGAGAGTTGGAAAGAAA-3') was 5' labeled with 6-FAM, while the reverse primer (5'-CCCATTGTTGCTACTTGAGA-3') was unlabeled. PCR amplification was performed for 25 cycles at the following temperatures: 95°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1 minute. The amplification products were sized using an ABI 3100 DNA sequencer and Genescan software.

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Patient Description and Informativity Test

The 29-year-old wife carried a causative mutation 676 A→C in the ABCD1 gene that causes X-linked ALD. Her husband was 33 years old, and he did not present any type of clinical alteration of interest. The affected wife was heterozygous at the DXS1073 and DXS9901 loci. She showed an allele of 126 bp and an allele of 128 bp for DXS1073. Moreover, she carried two alleles of 141 bp and 136 bp for the DXSS9901 locus. The 126-bp and 141-bp alleles cosegregate with X-linked ALD in this family. The husband carried a 126-bp allele for the DXS1073 locus and a 136-bp allele for the DXS9901 locus. To identify the sex chromosomes, an X22 informativity test was performed. The wife was homozygous for the X/Y marker and carried two 223-bp alleles for this locus. The husband carried two alleles of 200 bp and 219 bp. We could identify the Y-chromosome from the husband by the 200-bp allele. This study had the approval of the Instituto Bernabeu Review Board.

Stimulation Protocol and Intrascytoplasmic Sperm Injection Procedure

In the previous cycle oral contraceptives were given. A long protocol was used including leuprolide acetate agonist (Gonapeptyl Depot; Ferring, Madrid, Spain) in the previously midluteal phase. After pituitary desensitization was obtained, a combined protocol, using human FSH (Gonal F; Serono, London, UK) and hMG (HMG-Lepori; Farma-Lepori, Barcelona, Spain), was given. Ovarian response was monitored by transvaginal ultrasound and plasmatic E_2 levels. Ovulation was induced with 250 μg of recombinant hCG (Ovitrelle; Serono, London, UK). Oocytes were aspirated 36 hours after hCG administration by a transvaginal ultrasound-guided needle aspiration under sedation. Surrounding oocyte cumulus and corona radiata cells were removed by a brief exposure to 80 IU/mL of hyaluronidase (Hyase; Vitrolife, Göteborg, Sweden) followed by gentle pipetting. Intracytoplasmic sperm injection (ICSI) was carried out 4 hours after oocyte retrieval on a heated stage (Tokai Hit Thermoplate, Model MATS-U505R30, Japan) at 37°C, which was mounted on an inverted microscope (Nikon Eclipse TE200, Japan) equipped with Hoffmann modulation optics and a Narishige (Narishige, Japan) micromanipulation system. Microinjection was performed according to Van Steirteghem et al. (11). Only metaphase II oocytes were injected and then incubated individually in 30-μL droplets of G1.3 medium (Vitrolife AB, Kungsbacka, Sweden) covered with sterile equilibrated mineral oil (Ovoil; Vitrolife, Göteborg, Sweden) at 37°C in an atmosphere of 6% CO_2. Fertilization was assessed 16–18 hours after ICSI. Further development was evaluated on the morning of day 2 and again at day 3, when embryos were evaluated before biopsy.

Blastomere Biopsy of Cleavage Embryos

Five 6- to 8-cell embryos were biopsied on the morning of day 3. A noncontact, 200-mW diode laser system (Saturn, Research Instruments, Cornwall, UK) coupled to an inverted microscope was used to deliver 2–4 laser pulses of 4.900 ms to the zona pellucida, creating a funnel-shaped hole. One clearly nucleated blastomere was then gently aspirated through the hole.

Lysis and PCR Analysis of Embryos

Single blastomeres were collected and transferred to 0.2-mL PCR tubes containing 0.5 μL of alkaline lysis buffer. Lysis of the single blastomere, MDA amplification, and PCR analysis were performed as described for single lymphocytes.

RESULTS

Single Lymphoblasts Test

MDA was successful in 10/10 single lymphocytes. The quality and quantity of the amplified DNA match with previous studies (12). A total of 30 PCR analyses for each polymorphic marker were performed. The amplification efficiency obtained during the preclinical test on single lymphoblasts was 100% (90/90) for all markers. The ADO rate was 6.7% (2/30) for DXS1073 marker, and no ADO was detected in the amplification of the X22 and DXS9901 alleles. They all fall within the limits set in the ESHRE PGD Consortium's guidelines (13): amplification efficiencies are >90% and ADO rates are <10%. None of the blanks showed contamination.

Clinical X-Linked ALD PGD Program

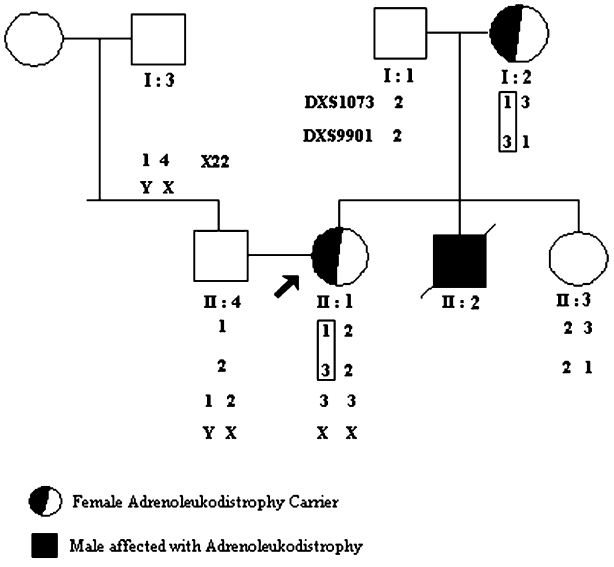
To be able to perform the PGD linkage analysis for X-linked ALD, the couple had to be informative, that is, the healthy X-chromosome of the female partner had to have been identified using at least one marker (DXS1073 and DXS9901) and the X22 had to identify the Y-chromosome from the male partner to sex the embryos. Segregation studies of the family were performed and showed that the markers DXS1073, DXS990, and X22 were informative (Fig. 1).

Twenty-two cumulus-oocyte complexes were retrieved, and 16 metaphase II oocytes were injected, of which 11 showed fertilization. On the morning of day 3, five of the 11 embryos had developed normally and could be biopsied.

The PCR reactions for the diagnosis of five biopsed embryos were performed in triplicate. The results obtained were consistent in the three PCR reactions. Table 1 shows the allele sizes amplified from the five embryos. The amplification efficiencies for DXS1073, DXS9901, and X22 were 12/15, 15/15, and 15/15, respectively. In addition, only ADO in DXS1073 was reported. Figure 2 shows the electropherograms obtained from the clinical PGD for X-linked ALD in a single blastomere. Three embryos were determined to carry the nonaffected female haplotype, one embryo was shown to be an affected male, and one was a female. The three healthy embryos were transferred. Unfortunately no pregnancy was achieved.

FIGURE 1

Pedigree of family. The markers DXS1073, DXS9901, and X22 were informative. The arrow indicates the proband of the family. The haplotype segregating with X-linked ALD is marked with a box.



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DISCUSSION

For recessive X-linked disease, sexing is the method of choice. Unfortunately, this approach has several disadvantages: First, we must bear in mind that from a theoretical point of view, half of the male embryos could be healthy and will not be placed, which means not only a decreased pregnancy rate but raises an ethical matter of concern. Second, the replacement of carrier female embryos cannot be avoided, thus the transmission to the offspring of the disease is not definitely stopped. This, together with the advances in molecular biology, has led to the fact that, if the mutation in a given family is known or can be analysed through linked markers, the couples who wish to undergo PGD will rather decide on a specific DNA diagnosis rather than a simple sexing (14). Accordingly, it becomes a monogenic disease with a gender determination requirement because we must discern between a resulting male with only one X-chromosome and an ADO female.

For monogenic disease diagnosis in embryos, single-cell PCR was the first technique developed for the analysis of DNA from single cells, and it is a demanding labor-intensive technique. Working up new diagnoses for PGD is very time-consuming because primers have to be designed to amplify the mutation and/or linked markers at the same multiplex reaction. PCR conditions have to be optimized for that and to reduce the risk of ADO and amplification failure.

Isothermal rolling circle amplification with random hexamer primers and ϕ29 polymerase was first described for circular DNA (15). Surprisingly, these reagents will also readily amplify linear, human genomic DNA in a cascading, strand displacement reaction that is termed MDA (16). This new tool has the potential to significantly expand the role of PGD in the diagnosis of single-gene disorders.

Until now, MDA has been used for the diagnosis of only four monogenic diseases (12, 17, 18), where the amplification rate of the different markers ranged from 85% to 95% with an ADO rate between 10% and 34%. According to ESHRE guidelines, optimization of the MDA protocol as we described in our previous work (12) produces better amplification and ADO rates that fall within the limits, and it becomes a protocol useful for clinical PGD. We describe the first MDA-PGD for X-linked ALD with gender determination. MDA produces enough DNA from a single cell to allow for multiple PCR analyses. Moreover, MDA as an initial step in PGD may be applied to multiple genetic analyses using standard procedures to diagnose a wide spectrum of single-gene defects. Moreover, using comparative genome hybridization, single-cell molecular karyotyping is now possible (19).

We have described an efficient and reliable method for the diagnosis of X-linked ALD with gender determination at the

TABLE 1

Allele sizes amplified from the five biopsed embryos.

Embryo no.	DXS1073	DXS9901	X22	Diagnosis
1	128–126	136–136	223–219	Normal female
2	128	136–136	223–219	Normal female
3	126–126	136–141	223–219	Carrier female
4	—	141	223–200	Affected male
5	128–126	136–136	223–219	Normal female
Amplification efficiency	12/15	15/15	15/15	
ADO	Yes	No	No	

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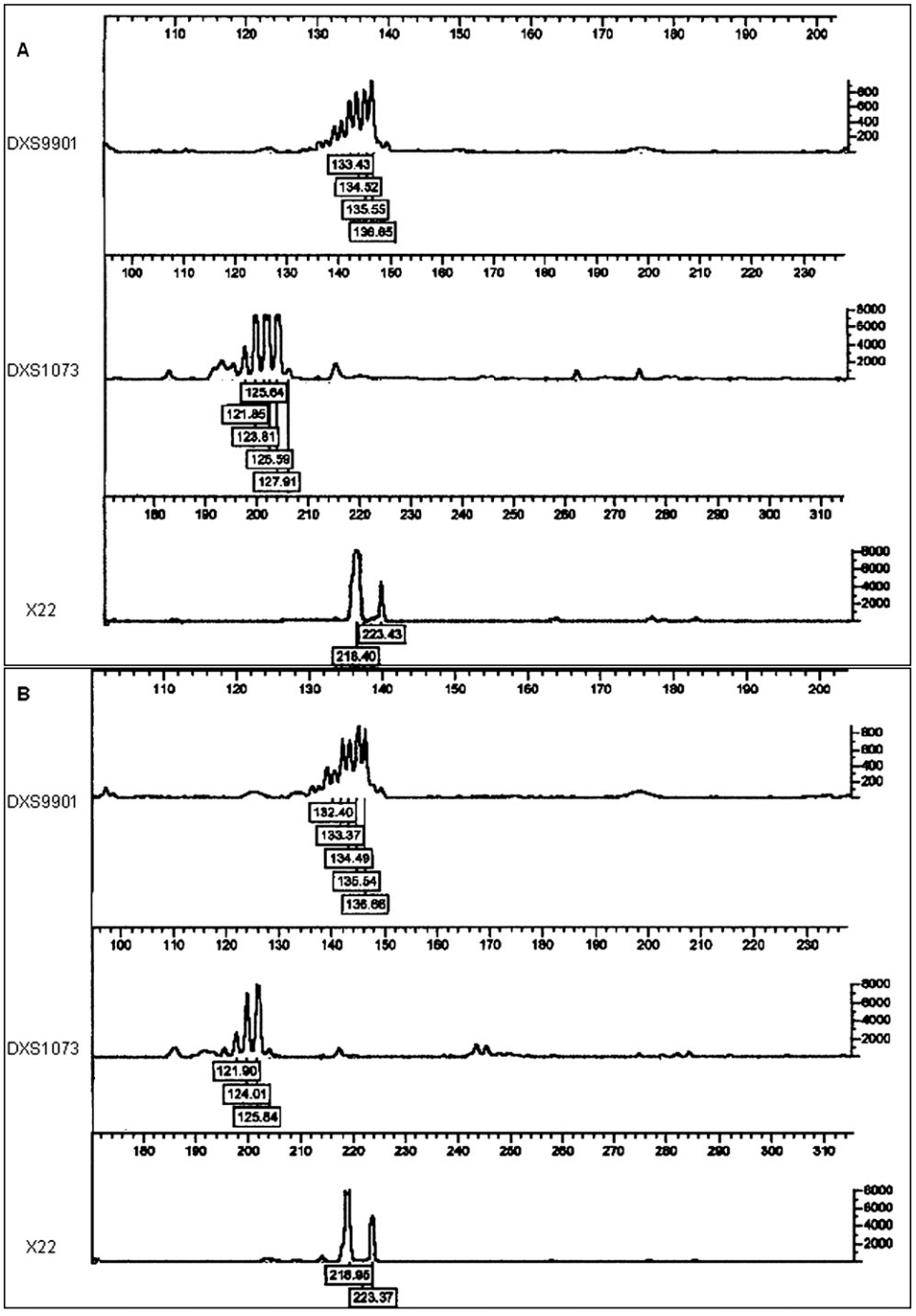
FIGURE 2

Electropherograms of the amplification from MDA products in the clinical PGD for X-linked ALD at a single blastomere. (A) Healthy female embryo; (B) carried female embryo; (C) affected male embryo.



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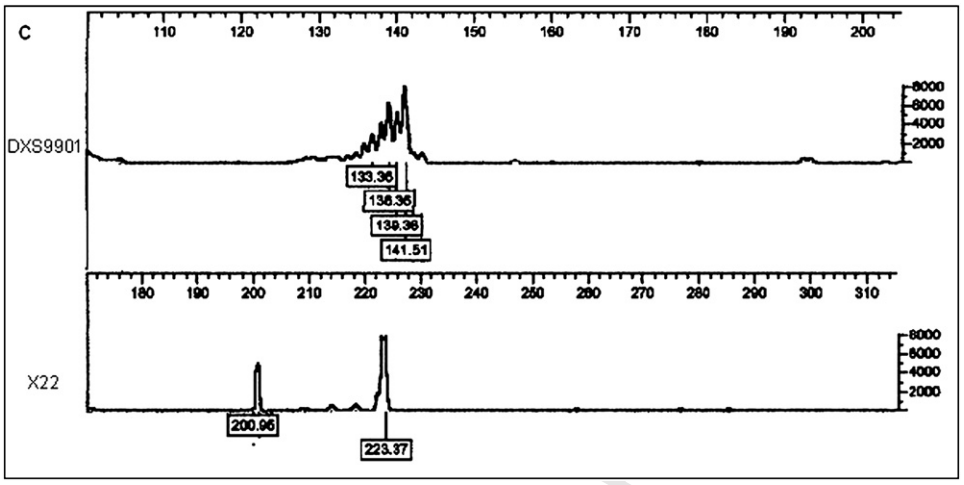
single-cell level using standard PCR procedures. The availability of enough DNA, thanks to the MDA reaction, allows for the creation of replicates from one blastomere. Furthermore, a different target amplification could be performed, which would result in a very accurate diagnosis. Moreover, a gender determination could be achieved that included only an X/Y-specific marker. MDA would become a universal first step in PGD.

The amplification efficiency and accuracy in blastomeres seems to be similar to that in lymphocytes. We detected

FIGURE 2 CONTINUED



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one amplification failure in one case performed in PGD for X-linked ALD. The use of two markers and a gender determination allowed us to diagnosis the embryo and explain that the possible cause would be a failure in the PCR, as amplification was detected from the same blastomere for more markers. The prevention of MDA was not the cause of the nonamplification.

According to ESHRE guidelines, the ADO rate should be determined in a preclinical test at the single-cell level and should be as low as possible (preferably <10%). Therefore, assessment of ADO is critical to evaluate any new technique for PGD and has important implications. Usually, for PCR, gender determination with a single-cell protocol includes the amelogenin gene as a sex marker. In humans, the amelogenin gene is present on both the X- and the Y-chromosomes. However, there are size differences in this gene between these chromosomes, which have also been used for sexing in forensic casework and prenatal diagnosis. In PGD amelogenin sexing dropout of the Y signal could lead to the transfer of a male embryo with an X-linked disorder or an XO karyotype that could lead not only to a baby with Turner's syndrome, but also, as sexing is done for X-linked disease, to a girl with the genetic disease (14).

We used a new X/Y marker, X22, to avoid the misdiagnosis caused by ADO. Since X22 is a pentanucleotide (AAATA) present on both the X-and Y-chromosomes in the pseudoautosomal region PAR2 and highly polymorphic (12 alleles), samples for heterozygous partners were expected to show two peaks, presumably different in length from each other. Moreover, X22 has a high diagnostic value for the detection of gonosome aneuplodies (20). The low rate of ADO reported using MDA, mainly in X22 amplification, and the use of this new highly polymorphic X22 marker might overcome the problem of testing the sex status of embryos. And it could be a better approach for sexing embryos because the absence of a Y-specific product of amelogenin could cause ADO to occur.

In conclusion, MDA could produce enough DNA from a single cell for multiple STR analyses, including an X/Y marker of gender and gonosome aneuploidy determination, which would avoid the multiplex optimization protocol for the detection of markers. MDA could be used clinically in PGD with standard protocols because very high amplification and low ADO rates are provided. The method we report for the diagnosis of X-linked ALD should be applicable to most patients carrying ALD as long as they are informative for the markers.

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Belé. Lledó, R. Bernabeu, J. Ten, F. M. Galán, and L. Cioffi

Alicante, Spain; Elche, Spain; and Salerno, Italy

We report the first clinical use of multiple displacement amplification from single cells for adrenoleukodystrophy preimplantation genetic diagnosis.

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