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RESEARCH LETTER

Rapid protocol for pre-conception genetic diagnosis of single gene mutations by first polar body analysis: a possible solution for the Italian patients

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Pre-implantation genetic diagnosis (PGD) (Handyside *et al*., 1990) has become an established clinical approach for prevention of genetic disorders. Today it represents an important complement to traditional prenatal diagnosis, offering couples who have a known genetically transmittable disorder the option to diagnose the specific disease on embryos before a clinical pregnancy has been established.

PGD is usually performed by testing single blastomeres removed from cleavage-stage embryos (6–8 cell). An alternative approach is represented by first (1PB) and second (2PB) polar body (PB) testing (Verlinsky *et al*., 1990, 1997). Analysis of PBs might be considered an ethically preferable way to perform PGD for couples with moral objections to any micromanipulation and potential discarding of abnormal embryos (Kuliev *et al*., 2006). It may also be an acceptable alternative for countries in which genetic testing of the embryos is prohibited (Tomi *et al*., 2006), precluding the ethical debate concerning biopsy of human embryos.

To date, PGD has been performed for the above purposes only after fertilization of oocytes, by genetic analysis of 1PB, or sequential analysis of 1PB and 2PB, allowing only those that are predicted to be normal to proceed to syngamy.

In 2004, the Italian Parliament enacted a restrictive law regulating *in vitro* fertilization (IVF) techniques, imposing many limitations (Benagiano and Gianaroli, 2004). According to this law, PGD on embryos is forbidden for any purposes. Therefore, the only option for couples at high genetic risk for prevention of genetic diseases is 1PB testing, but before oocyte fertilization (so-called pre-conception genetic diagnosis, PCGD), provided that results of genetic testing are achievable within a reasonable time to prevent *in vitro* ageing of the oocytes. In fact, there is only a very narrow window of time available for PCGD, but if the 1PB biopsy is performed soon after oocyte collection (Magli *et al*., 2006) and follows a rapid diagnostic protocol, oocyte insemination could be carried out according to the results of the genetic analysis.

We overcame the time restriction problem by developing a rapid protocol for diagnosis of single gene mutations of maternal origin on 1PBs, which fits with the restrictions imposed by the new Italian regulation. The whole procedure can be completed within just 4 h, making it realistic to fertilize the oocytes predicted to be free of mutation within a time frame compatible with a late •ICSI (~6 h after oocytes collection).

The protocol was adapted for diagnosis of cystic fibrosis (CF) and ß-thalassemia (ßT) mutations, the two most common genetic diseases in the Italian population. However, a similar approach can be applied to any genetic disorder, autosomal recessive, X-linked or autosomal-dominant of maternal origin, provided that the disease-causing gene has been mapped.

The procedure involves a fluorescent multiplex polymerase chain •reaction PCR analysis of highly polymorphic short-tandem repeat (STR) markers, closely linked to the disease-causing genes, to identify the haplotype associated with the maternal mutation. A panel of six highly polymorphic STR markers (Table 1) flanking each gene were selected for haplotype analysis, to ensure sufficient informativity in all cases. The co-amplification of several markers also reduces the risk of amplification failure and increases the assay accuracy by allowing the detection of potential allele dropout (ADO) occurring in multiple markers, which would lead to the diagnosis of a recombinant heterozygous oocyte as hemizygous, thus reducing substantially the risk of misdiagnosis. In fact, in such a case, misdiagnosis is only possible in

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Table 1—Single-cell multiplex PCR from single lymphocytes and first polar bodies: amplification efficiencies and allele dropout (ADO) rates

	Lymphocytes					First polar bodies				
STR marker/gene region	No. cells analysed	Cells with total PCR failure	No. of cells with a PCR signal (%)[a]	No. of informative cells[c]	ADO (%)[b]	No. cells analysed	Cells with total PCR failure	No. of cells with a PCR signal (%)[a]	No. of informative cells[c]	No. of heterozygous cells[d]	ADO (%)[b]
D11S4146[a]	130	10	116 (96.7)	73	3 (4.1)	78	6	70 (97.2)	45	29	2 (6.9)
D11S988[a]	130	10	116 (96.7)	101	2 (2.0)	78	6	70 (97.2)	63	39	2 (5.1)
D11S4181[a]	130	10	118 (98.3)	104	4 (3.8)	78	6	72 (100.0)	63	38	2 (5.3)
D11S1760[b]	130	10	112 (93.3)	99	3 (3.0)	78	6	68 (94.4)	63	39	1 (2.6)
D11S1338[b]	130	10	116 (96.7)	88	5 (5.7)	78	6	70 (97.2)	54	33	2 (6.1)
D11S1331[b]	130	10	118 (98.3)	88	5 (5.7)	78	6	71 (98.6)	54	32	2 (6.3)
D7S2847	128	7	114 (94.2)	69	1 (1.4)	69	5	60 (93.8)	40	24	1 (4.2)
IVS17b-TA[c]	128	7	115 (95.0)	115	6 (5.2)	69	5	61 (95.3)	64	36	3 (8.3)
CFTR ex 10ΔF508	128	7	116 (95.9)	116	5 (4.3)	69	5	62 (96.9)	64	36	2 (5.6)
IVS1[c]	128	7	117 (96.7)	73	5 (6.8)	69	5	61 (95.3)	40	23	2 (8.7)
D7S677 b	128	7	113 (93.4)	59	3 (5.1)	69	5	60 (93.8)	32	17	1 (5.9)
D7S23[b]	128	7	112 (92.6)	98	4 (4.1)	69	5	59 (92.2)	56	31	2 (6.5)
D7S486	128	7	115 (95.0)	103	5 (4.9)	69	5	62 (96.9)	56	32	2 (6.3)

[a] The amplification rate for each marker is calculated on the totality of cells with a positive amplification signal for at least one locus.
[b] ADO rates for individual loci are calculated only from samples showing heterozygosity for those loci.
[c] Number of cells in which the female carrier was heterozygote for the specific marker.
[d] Number of 1PBs in which a recombination event has occurred.

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the very unlikely hypothesis that ADO of the wild-type allele occurs in all amplified markers.

The optimization of the multiplex PCR protocols was first performed on single lymphocytes, collected from 16 female carriers, determining the best condition to obtain reliable and reproducible results from single-cell amplification. Parameters such as amplification efficiency and ADO rate for each marker used in the multiplex PCR were also determined. A total of 258 single lymphocytes were individually tested with two different multiplex protocols, amounting to a total of 1676 loci analysed. A positive amplification signal was obtained in 120/130 single lymphocytes (overall amplification rate 96.7%) for βT protocol, and in 121/128 cells (overall amplification rate 94.7%) for CF protocol. Amplification rates were generally high for all loci tested, ranging from 92.0 to 98.3%. Amplification failed for all the markers/loci tested in 17 lymphocytes. The ADO rates varied among the different loci investigated, ranging from 2.0 to 6.8%, with an average ADO rate of 4.0% for βT protocol and 4.6% for CF protocol.

Biopsy of 1PB was performed on 147 oocytes that failed to become fertilized, obtained from 16 patients undergoing ART procedures combined with PGD for βT and CF. 1PBs were tested for both βT protocol (78 1PBs) and for CF protocol (69 1PBs). PCR was successful in 72 out of 78 1PB (92.3%) for βT protocol, and in 92.8% (64/69) of the cells for CF protocol. Amplification failed for all the markers/loci tested in 11 1PBs, totally. The amplification efficiency of the individual STR markers ranged from 92.2 to 100%, with an overall amplification rate of 97.5% for βT protocol and 94.6 for CF protocol. A complete genotype (i.e. a PCR signal for each locus tested) was obtained in 90.3% of the amplified 1PBs with βT protocol, and in 87.5% with CF protocol.

1PBs showed a high recombination rate for both CF (56.2%) and βT (61.1%) genes. ADO of at least one STR marker was detected on 9 recombinant heterozygous 1PBs with βT protocol and 10 with CF protocol, for a total of 24 ADO occurrences. The ADO rates of the individual markers varied from 2.6 to 8.7% (5.2% on average for βT protocol and 6.5% for CF protocol). A reliable haplotype was obtained in 136/136 (100%) of the 1PB with positive PCR results analysed with both protocols (Table 1).

Although the above results indicate the suitability for clinical application of the procedure presented here, some limitations must be considered.

PCGD by 1PB testing only provides information about the maternal genotype; it cannot be used in cases of paternally derived autosomal-dominant disorders. Moreover, pre-implantation •HLA matching (Verlinsky *et al*., 2001; Fiorentino *et al*., 2005) would be not possible.

Furthermore, the high rate of heterozygosity found in 1PBs (56.2 and 61.1%, for CF and ß-T genes, respectively) greatly reduces the number of oocytes available for selection because no assertion on the status of the corresponding oocytes could be made.

A poor ovarian response to hormonal hyperstimulation, is also known to have a major impact on the number of the retrievable oocytes and, consequently, on the number of oocytes available for analysis, reducing the chance of finding mutation-free oocytes to be fertilized.

Despite its limitation, PCGD by 1PB testing might be very helpful for Italian couples at genetic risk that, fearful of having babies with genetic illnesses and unwilling to accept a possible pregnancy termination, have been forced to seek a PGD treatment abroad to circumvent restrictions of Italian law, by resorting to so-called reproductive tourism. It may avoid the difficulty of being away from home for a long period in a foreign country, which makes the already psychological situation of at-risk couples all the more difficult. This procedure can also give hope to many couples who are unable to obtain that service abroad because of their limited economic means. The possibility to perform PCGD in Italy can give them the opportunity to have free access to IVF techniques, which is covered by public health insurance, re-enabling the constitutional right of equality of access to heath care.

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