Validation of preimplantation genetic diagnosis by PCR analysis: genotype comparison of the blastomere and corresponding embryo, implications for clinical practice

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The aim of this study was to validate the overall preimplantation genetic diagnosis (PGD)–PCR procedure and to determine the diagnostic value. Genotyped embryos not selected for embryo transfer (ET) and unsuitable for cryopreservation after PGD were used for confirmatory analysis. The PGD genotyped blastomeres and corresponding embryos were compared, and morphology was scored on Day 4 post fertilization. To establish the validity of the PGD–PCR procedure and the diagnostic value, misdiagnosis rate, false-negative rate and negative predictive value were calculated. Moreover, comparison on the validity was made for the biopsy of one or two blastomeres. For the total embryo group (n = 422), a misdiagnosis rate of 7.1% and a false-negative rate of 3.1% were found. The negative predictive value was 96.1%. Poor morphology Day 4 embryos (Class 1) were over-represented in the embryo group in which the blastomere genotype was not confirmed by the whole embryo genotype. The misdiagnosis rate of Class 1 embryos was 12.5% and the false-negative rate 17.1%. Exclusion of these embryos resulted in a misdiagnosis rate of 6.1%, a false-negative rate of 0.5% and a negative predictive value of 99.3%. The two blastomere biopsies revealed a significant higher diagnostic value, lowering the misdiagnosis rate, whereas the negative predictive value remained the same. In conclusion, the two blastomere biopsies revealed a significant higher diagnostic value, lowering the misdiagnosis rate.

Keywords: preimplantation genetic diagnosis; preimplantation diagnosis; sensitivity; negative predictive value; embryo morphology

Introduction

Preimplantation genetic diagnosis (PGD) is a widely accepted alternative for prenatal diagnosis. The combination of assisted reproductive and molecular genetic techniques offers new possibilities for couples at a risk of monogenic disorders or chromosomal aberrations. It saves couples the difficult decision of pregnancy termination after prenatal testing.

The PGD procedure comprises in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI), followed by genetic analysis of one or two blastomere(s) biopsied from 4- to 10-cell cleavage stage embryo on Day 3 post fertilization (PF) (Handyside et al., 1990; Hardy and Handyside, 1992). By means of a hyperstimulation protocol, multiple oocytes and embryos are obtained. Biopsied embryos are genotyped either by fluorescent in situ hybridization to detect chromosomal aberrations or to determine the gender, or by PCR amplification to detect monogenic disorders. After blastomere analysis, one or two normal or unaffected embryos are selected from a cohort of embryos for embryo transfer (ET) to the mother.

Although the PGD technique has evolved, and precautions have been taken to reduce the risk of misdiagnosis to a minimum (ESHRE PGD consortium, Thornhill et al., 2005), several misdiagnoses have been published since the introduction of PGD (Hardy and Handyside, 1992; Harper and Handyside, 1994; Grifo et al., 1994; Sermon et al., 1998a; Sermon and Liebaers, 1999; ESHRE PGD consortium, Sermon et al., 2007). However, worldwide over 2000 children are born without indications that the PGD–PCR procedure is not valid or safe (ESHRE PGD consortium, Harper et al., 2008). In many PGD centres, it is good laboratory practice to test and validate the 1-cell PCR protocols on lymphocytes, buccal cells or blastomeres from surplus embryos before introduction into clinical practice. Nevertheless, in view of quality control, it is important to test the validity and diagnostic value of the actual PGD–PCR treatment. Especially, the negative predictive value, expressing the chance of the embryo transferred to be unaffected, provides important information.
For confirmation of the diagnostic procedure, the most ideal would be a diagnostic analysis of all the pregnancies and children born after preimplantation diagnosis. However, this is often not possible for practical, technical and ethical reasons. Therefore, a more comprehensive approach for confirmatory analysis would be the use of whole PGD genotyped embryos not suitable for transfer or cryopreservation (Ray et al., 1998; Sermon et al., 1998a; Emiliani et al., 2004). Using the latter approach, more complete data are obtained in the same period of time.

The aims of the present study were to validate and determine the diagnostic value of the overall PGD–PCR analysis and to compare the validity of the PGD–PCR analysis based on one and two biopsied blastomeres.

Materials and Methods

Patients and IVF/ICSI/PGD procedure

From 1995 to 2005, 80 couples at high risk of having offspring with a serious monogenic disorder underwent an IVF PGD–PCR procedure. Controlled ovarian stimulations, 188 in total, were performed as described earlier (Lund et al., 1996), and oocytes were retrieved under ultrasound guidance in 144 oocyte pick-ups (Table I). After 5 h of maturation, metaphase II oocytes were fertilized with ICSI (Van Steirteghem et al., 1993) followed by embryo culturing (Dumoulin et al., 2000). Embryo morphology grade was used as one of the parameters to assess the embryo quality. In the morning of Day 3 PF, blastomeres were successfully biopsied from 886 cleavage stage embryos. From cleavage stage embryos developed to the 4- to 7-cell stage one blastomere was biopsied. Two blastomeres were biopsied from embryos developed to the 8-cell stage and beyond. Biopsies were performed in CaCl2- and MgCl2-free medium with the help of a micromanipulator (Narisihige, ONO-121, Paes Nederland BV, Zoeterwoude, The Netherlands) mounted on an inverted microscope (Olympus, IX-70, Paes Nederland BV, Zoeterwoude, The Netherlands). Biopsied blastomeres were washed three times in washing buffer, containing phosphate-buffered saline solution with 1% polyvinylpyrrolidone molecular weight 360 000 (Sigma–Aldrich Chemie BV, Zwijndrecht, The Netherlands) and 0.1 mg/ml phenol red (Sigma, Zwinjdrecht, The Netherlands) and transferred to a 0.2 ml PCR tube. Blank samples were taken from the last washing droplet. PGD samples were stored at −20°C until PCR was performed.

PGD–PCR protocols for 13 different disorders and mutations were developed taking into account the specific characteristics in detecting these specific mutations on 1-cell. Autosomal recessive (AR) disorders caused by a homozygous mutation [Fanconi anemia, cystic fibrosis, spinal muscular atrophy (Dreesen et al., 1998), tyrosine hydroxylase deficiency and Leigh syndrome] were analysed using a simplex PCR protocol specific for that mutation only. AR disorders in which the index patients were compound heterozygous were analysed using a duplex (AR polycystic kidney disease) or triplex [Cystic fibrosis (Dreesen et al., 2000)] marker analysis PCR protocol. For myotonic dystrophy type 1 and Fragile X syndrome, caused by a triplet repeat expansion, a simplex PCR protocol was used detecting the repeat alleles within the normal range (Sermon et al., 1998b, 1999). In Huntington disease and spinocerebellar ataxia type 3 too the expansion could be analysed (Sermon et al., 1998a; Drüseuf et al., 2004). For other autosomal dominant (AD) disorders, a duplex PCR protocol for the mutation combined with a closely linked marker (Familial adenomatous polyposis and Tuberosis sclerosis type 1), or a duplex marker protocol (Marfan syndrome), was developed. In each cycle, one or two unaffected embryos were selected for ET in the morning of Day 4 PF. The selection was based on the well-known embryo score criteria (Dumoulin et al., 2000).

After obtaining informed consent of the couples involved, whole embryos not selected for ET and unsuitable for cryopreservation were collected. As not all couples gave informed consent, we were not able to dispose of all surplus embryos for confirmatory analysis. The zonae pellucidae were removed from the collected embryos by incubation in 1 U/µl pronase (Sigma). Embryos were collected on Day 4, washed three times in washing buffer and transferred to a 0.2 ml PCR tube. Collected embryos were stored at −20°C until PCR was performed. The protocol was approved by the local Ethics Committee.

PCR procedure

Prior to PCR, the alkaline lyses buffer was decontaminated from exogen DNA by UV-C irradiation for 1 h using an UV-C lamp type TUV 30W/G30TS life (Philips). The PCR mix used was decontaminated by restriction enzyme incubation or by UV-C irradiation. The DNA polymerase was not included in both decontamination steps, while the primers were not included in the PCR master mix using UV-C irradiation but were included using restriction enzyme decontamination. Blank samples were included in every PCR series to monitor DNA contamination. Cells were lysed by adding an alkaline lyses buffer [30 nM diihotriehitol (Pharmacia Biotech)/200 mM NaOH or KOH] followed by 10 min of incubation at 65°C. After cell lysis, PCR was performed using the ExpandTM Long Template or the Expand™ High Fidelity PCR System (Roche Diagnostics) or using Ampli-Taq Gold or Ampli-Taq Gold DNA polymerase (1.5 U/µl; Perkin-Elmer Netherland, Nieuwerkerk a/d IJssel, The Netherlands). Lysis by NaOH was applied when using the Expand™ Long Template or the Expand™ High Fidelity PCR System; lysis by KOH was used when employing with Ampli-Taq Gold or Ampli-Taq DNA polymerase. Thermal cycling was done on the GeneAmp® PCR System 9700 (Perkin–Elmer Applied Biosystems). The PCRs were all performed in a total volume of 25 µl and contained 1 × PCR buffer and 1.4–2.5 U of DNA polymerase, 0.2 mM dNTP from each of the four deoxynucleotide triphosphates (dGTP/ dATP/dCTP/dTTP) (Pharmacia). For neutralization of the alkaline lysis buffer, tricine (20 mM, pH 4.95; Sigma) was used in the PCR mix in combination with NaOH (Merek), and neutralization buffer (90 mM Tris–HCl, pH 8.3, 30 mM KCl and 20 mM HCl; Merck) was used in combination with the KOH (Merek) alkaline lyses buffer. Primer sets used in the PCR master mix varied from 5 to 20 pmol primer per sample. Of the primer sets, one primer was fluorescently labelled. PCR was started with an initial 2–10 min denaturation step at 95°C followed by 45–55 cycles of denaturation, annealing and elongation at the temperatures and times as described by the PCR protocol used. PCR products were analysed by gel electrophoresis on an ABI PRISM 377 Genetic Analyzer or by capillary electrophoresis on an ABI PRISM 3100 Genetic Analyzer followed by GeneScan analysis.

Study design

Four hundred and twenty-two surplus embryos were available for confirmatory analysis with the same PCR protocol used to genotype and diagnose the blastomeres. The embryo genotypes were designated as the gold standard. This is based on the fact that the genotype obtained from a total embryo is the result of more copies of template DNA compared with the blastomere analysis in which only one copy of each allele is present.

The genotypes from the biopsied blastomere(s) and the corresponding embryo were scored independently, compared and classified in a genotype outcome group. Blastomeres and embryos with concordant genotypes were defined as the blastomere genotype (alleles) exactly matched the embryo genotype. Discordant blastomeres and embryo genotypes were classified in the

<p>| Table I. PGD cycles performed from 1995 to 2005 for single-gene disorders using PCR. |</p>
<table>
<thead>
<tr>
<th>No. patients</th>
<th>No. cycles</th>
<th>No. OR</th>
<th>No. embryos diagnosed</th>
<th>No. cycles ET</th>
<th>No. FHB positives</th>
<th>No. babies born</th>
<th>% FHB/OR</th>
<th>% FHB/ET</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>188</td>
<td>144</td>
<td>793</td>
<td>130</td>
<td>31</td>
<td>35</td>
<td>21.5</td>
<td>23.8</td>
</tr>
</tbody>
</table>

OR, oocyte retrieval; ET, embryo transfer; FHB, fetal heart beat.
following three genotype outcome categories defined by the underlying phenomenon explaining the discordance: (i) ‘Allelic drop-out (ADO) explained’ defined as one parental allele was missing in the blastomere(s) genotype but was present in the embryo genotype due to ADO; (ii) ‘Contaminated’ defined as the blastomere genotype was not confirmed by the embryo genotype due to contamination of the blastomere; (iii) ‘Not confirmed’ defined as the genotype (alleles) of the blastomere did not exactly match the embryo genotype, and this could not be explained by ADO and/or contamination.

Moreover, the distribution of the embryo morphology grade of Day 4 embryos over the different genotype outcome groups was studied. Embryos were divided into morphology Classes 1–4 (with Class 4 being the highest and Class 1 the lowest morphology score) according to the amount of fragmentation, morphology of the blastomeres and, in the case of Day 4 embryos, the proportion of blastomeres undergoing the compaction process (Bolton et al., 1989; Tao et al., 2002).

For validation of the PGD–PCR procedure, the genotype outcome of the PGD-typed blastomeres and the confirmatory analyzed embryos were converted into a diagnostic outcome. In the conversion of the genotype outcome to a diagnostic outcome, the specific underlying characteristics and issues for different PGD–PCR protocols are taken into account. Once the diagnostic outcome groups are generated, they enable to summarize the overall PGD–PCR results because they are independent of the specific underlying characteristics and issues of the different PGD–PCR protocols. The blastomeres (T) and the confirmatory analyzed embryos (D) were designated as unaffected, T− and D−, or affected/aberrant, T+ and D+. A chromosomal aberrant blastomere or embryo with an unknown disease status was designated as T± or D±, respectively. A comparison was made between the diagnostic outcome of the blastomeres and the corresponding embryos. The validity of the PGD–PCR procedure was expressed by sensitivity (Se), specificity (Sp), accuracy (Ac) and likelihood ratio (LR). The Se was defined as the proportion of affected/aberrant embryos diagnosed correctly by PGD, whereas the Sp was defined as the proportion of unaffected embryos diagnosed correctly by PGD. The Ac was defined as the proportion of all embryos, affected/aberrant as well as unaffected, diagnosed correctly by PGD. Furthermore, the misdiagnosis rate and the false-negative and -positive rates were calculated. The diagnostic value was expressed by positive and negative predictive values. The positive predictive value was defined as the proportion of PGD analysis that predicted embryos correctly as affected/aberrant, and the negative predictive value was defined as the proportion of PGD analysis that predicted embryos correctly as unaffected. To validate the PGD–PCR procedure and to determine the diagnostic value a diagnostic-test analysis was performed: (i) on the total Day 4 embryo group, including all morphologies; (ii) on Day 4 embryos with Class 1 morphology alone (poor morphology embryos); (iii) on Day 4 embryos with Class 1 excluded. Validity of the PGD–PCR analysis based on one versus two blastomeres biopsied was compared.

The contingency χ²-test or the Fisher’s exact test was used for the comparison of categorical data. The analyses were conducted with the SAS version 8.2 computer package (SAS Institute Inc., Cary, NC, USA). All reported P-values are two-sided and were considered statistically significant when \( P \leq 0.05 \).

Results

Genotype and diagnostic outcome

An overview of the PGD–PCR analyses is given in Table I. Biopsied blastomeres were analysed in 144 cycles. In total, 793 embryos were genotyped for one of the 13 different disorders as described in the Materials and Methods section. Three hundred and forty were diagnosed as affected/aberrant and 453 as unaffected. In total, 241 unaffected embryos were transferred to 130 cycles, resulting in 31 pregnancies with a positive fetal heart beat. Thirty-five healthy children were born from 28 deliveries. The clinical pregnancy rate after PGD–PCR is 21.5% and 23.8% per oocyte retrieval and ET, respectively.

The genotypes of 422 blastomeres and their corresponding embryos obtained from 95 cycles were compared and assigned to one of the defined genotype outcome categories. In addition, the diagnostic outcome was determined (Table III).

Of the total of 422 genotyped blastomeres, 367 (87%) were concordant and confirmed by the confirmatory embryo analysis: 167 embryos with an unaffected diagnostic result (diagnostic outcome T−D−) and 200 embryos with an affected/aberrant diagnostic result (diagnostic outcome T+D+). Fifty-five initial PGD blastomere analyses gave discordant genotype results after confirmatory analysis of their corresponding embryos. In the ‘ADO explained’ genotype outcome group, ADO was observed in 32 of 422 (7.6%) blastomeres obtained from 26 cycles. Despite the occurrence of ADO, the diagnostic outcome of some blastomeres was confirmed by the embryo diagnostic outcome. This was the case in five embryos with an unaffected diagnostic result (diagnostic outcome T−D−) and eight embryos with an affected/aberrant diagnostic result (diagnostic outcome T+D+). On the other hand, ADO in the blastomere led to 19 embryos with false-positive diagnostic results (diagnostic outcome T+D−). However, no false-negative (T−D+) diagnostic outcome was observed (Table III).

| Table II. Diagnostic outcome PGD blastomere analysis (T) versus confirmatory whole embryo analysis (D). |
|--------------------------------------------------------|-------------------------------------------------|-------------------|
| D+ | Affected/aberrant embryos at confirmatory analysis | D− | Unaffected embryos at confirmatory analysis | Total |
| T+ | A (true positive) | b (false positive) | a + b |
| T− | Unaffected embryos at PGD | c (false negative) | d (true negative) | c + d |
| Total | a + c | b + d | n |

T, diagnostic outcome embryos based on biopsied blastomeres analysis during PGD.
D, Diagnostic outcome embryos based on confirmatory analysis whole embryos.
Sensitivity (Se): proportion of unaffected embryos at PGD.
Specificity (Sp): proportion of unaffected embryos at confirmatory analysis.
Accuracy (Ac): proportion of unaffected embryos at confirmatory analysis.
False positive (FP): proportion of affected embryos at PGD.
False negative (FN): proportion of unaffected embryos at PGD.
Misdiagnosis rate: proportion of affected embryos at confirmatory analysis.
Positive predictive value: proportion of unaffected embryos at confirmatory analysis.
Likelihood ratio (LR): LR+=Se/(1−Sp), LR−=(1−Se)/Sp.
In the ‘Contaminated’ outcome group contamination of the blastomeres gives a positive test result per definition, encompassing disease positive (three of four) as well as disease negative (one of four) embryos and hence, resulting in a false-positive (T+D−) test result (Table III). The contaminated blastomeres were obtained from four cycles.

In the ‘Not confirmed’ outcome group, 19 blastomere genotypes (4.5%) of the 422 blastomere/embryo genotypes were not confirmed by the whole embryo analysis. Of these, seven showed a true false-negative test result (T−D+), being a potential hazard for misdiagnosis. In three unaffected embryos, the blastomere genotype gave a positive test result leading to false-positive diagnostic outcome (T+D−). Although the blastomere genotypes were not confirmed by the embryo genotypes within this group, confirmed unaffected as well as confirmed affected/aberrant diagnostic results were observed in two and seven blastomere/embryos, respectively (Table III). The blastomeres and embryos of this group were obtained from 15 PGD cycles.

Comparing the blastomere and embryos in the ‘Not confirmed’ outcome group, we noticed a high number of poor morphology embryos. After studying embryo morphology distribution, a skewed distribution was observed in the ‘Not confirmed’ genotype outcome group, for Day 4 PF embryos (Fig. 1). On Day 3 PF, the morphology distribution in the different genotype outcome groups did not differ (results not shown). Of the 19 embryos in the ‘Not confirmed’ genotype outcome group, eight (42.1%) showed poor morphology and were scored as Class 1 embryos, whereas only 56 (13.9%) of the 403 embryos from the other groups scored as Class 1. Thus in the Not confirmed group, significantly more embryos were scored as Class 1 as compared with the other groups (P < 0.05).

**Validation**

Data on the validity and the diagnostic value of the PGD–PCR 1-cell analysis are calculated and given in Table IV. For the total embryo group, an Ac of 92.9% and a misdiagnosis rate of 7.1% were found. However, the Se of 96.9% giving a false-negative rate of 3.1% was obtained. The Sp observed was 88.3%. The LR for a negative test was 0.04. The negative predictive value of the PGD–PCR 1-cell analysis was 96.1%. The embryo group with morphology Class 1 embryos excluded differed significantly from the Class 1 embryo group alone. Although there was no significant difference found for the Ac (93.9 versus 87.5%) and the misdiagnosis rate (6.1 versus 12.5%), the Se was significantly higher (99.5% versus 82.9%; P < 0.001), as was the negative predictive value (99.3 versus 81.8%; P < 0.001). Consequently, the number of false negatives was lower (0.5 versus 17.14%; P < 0.001) (Table IV).

The results of a diagnostic-test analysis for one or two blastomeres analysed are given in Table V. No significant differences were found for one or two blastomeres within each embryo group for the Ac, Se, Sp and negative predictive value. However, the misdiagnosis rate

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### Table III. Conversion of genotype outcome to diagnostic outcome for the different blastomere/embryo genotypes outcome groups.

<table>
<thead>
<tr>
<th>Genotype outcome groups</th>
<th>Blastomeres/embryos compared (N)</th>
<th>Diagnostic outcome groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T−D−</td>
</tr>
<tr>
<td>Concordant</td>
<td>367</td>
<td>167</td>
</tr>
<tr>
<td>Discordant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADO explained</td>
<td>32</td>
<td>5</td>
</tr>
<tr>
<td>Contaminated</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Not confirmed</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>422</td>
<td>174</td>
</tr>
</tbody>
</table>

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Figure 1: The distribution of embryo morphology classes in the different genotype outcome groups with Class 4 being the highest and Class 1 (poor morphology embryos) the lowest morphology score. Vertical bars indicate the different embryo morphology classes and the percentage within the genotype outcome groups.

*Significantly different compared with the other genotype outcome groups; P < 0.05.
one might hypothesize that in the hypothetical situation, all embryos as a result, the ‘Not confirmed’ group might be smaller. Therefore, the PGD–PCR procedure, fewer Class 1 embryos are used for ET and, for ET, the diagnostic outcome will always remain unknown. In the children in our series). However, for the majority of the embryos used larger amount of data over a shorter period of time as compared with the specific characteristics and issues of the different types of genotype outcome to a diagnostic outcome, thus taking into account the confirmatory analysis approach of surplus embryos generates a by the T-ADO does not necessarily lead to false positives as demonstrated because of the precautions included in the PGD–PCR protocols to detect ADO. Hence, ADO will not lead to the transfer of an affected embryo and the potential birth of an affected child. We also showed that ADO may cause false-positive test results in PGD of an AD disorder caused by the detection of the non-risk allele of the unaffected parent alone, or the mutant allele, only in the case of a heterozygous AR disorder. In these cases, ADO will lead to the loss of embryos causative of monosomy can occur also explain ADO (Coonen Lissens and Sermon, 1997). Mitotic non-disjunction and anaphase lagging by which monosomy can occur also explain ADO (Coonen Lissens and Sermon, 1997).

## Table IV. Validation of the PGD–PCR analysis.

<table>
<thead>
<tr>
<th></th>
<th>Total group</th>
<th>Class 1 group</th>
<th>Class 1 excluded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>96.9% (218/225)</td>
<td>82.9% (29/35)*</td>
<td>99.5% (189/190)*</td>
</tr>
<tr>
<td>False negative</td>
<td>3.1% (7/225)</td>
<td>17.1% (6/35)*</td>
<td>0.5% (1/190)*</td>
</tr>
<tr>
<td>Specificity</td>
<td>88.3% (174/197)</td>
<td>93.1% (27/29)</td>
<td>87.5% (147/168)</td>
</tr>
<tr>
<td>False positive</td>
<td>11.7% (23/197)</td>
<td>6.9% (2/29)</td>
<td>12.5% (21/168)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>92.9% (392/422)</td>
<td>87.5% (56/64)</td>
<td>93.9% (336/358)</td>
</tr>
<tr>
<td>Misdiagnosis</td>
<td>7.1% (30/422)</td>
<td>12.5% (8/64)</td>
<td>6.1% (22/358)</td>
</tr>
<tr>
<td>LR (positive test)</td>
<td>8.30</td>
<td>12.01</td>
<td>8.00</td>
</tr>
<tr>
<td>LR (negative test)</td>
<td>0.04</td>
<td>0.18</td>
<td>0.006</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>96.1% (174/181)</td>
<td>81.8% (27/33)*</td>
<td>99.3% (147/148)*</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>90.5% (218/241)</td>
<td>93.6% (29/31)</td>
<td>90.0% (189/210)</td>
</tr>
</tbody>
</table>

*Significant different when Class 1 group compared with Class 1 excluded group; P < 0.001.

## Table V. Validation of one- and two blastomere(s) PGD–PCR analysis.

<table>
<thead>
<tr>
<th>No. blastomeres</th>
<th>Total group</th>
<th>Class 1 group</th>
<th>Class 1 excluded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>97.4% (76/78)</td>
<td>96.6% (142/147)</td>
<td>100% (59/59)</td>
</tr>
<tr>
<td>False negative</td>
<td>2.56% (2/78)</td>
<td>3.4% (5/147)</td>
<td>0.0% (0/59)</td>
</tr>
<tr>
<td>Specificity</td>
<td>85.7% (84/98)</td>
<td>90.9% (90/99)</td>
<td>84.0% (63/75)</td>
</tr>
<tr>
<td>False positive</td>
<td>14.3% (14/98)</td>
<td>9.1% (9/99)</td>
<td>16.0% (12/75)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>90.9% (160/176)</td>
<td>94.3% (232/246)</td>
<td>90.3% (84/93)</td>
</tr>
<tr>
<td>Misdiagnosis</td>
<td>9.1% (16/176)</td>
<td>5.7% (14/246)</td>
<td>9.7% (9/93)</td>
</tr>
<tr>
<td>LR (positive test)</td>
<td>6.82</td>
<td>10.63</td>
<td>6.25</td>
</tr>
<tr>
<td>LR (negative test)</td>
<td>0.03</td>
<td>0.04</td>
<td>0.000</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>97.7% (84/86)</td>
<td>94.7% (90/95)</td>
<td>98.8% (84/85)</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>84.4% (76/90)*</td>
<td>94.0% (142/151)*</td>
<td>93.5% (130/139)*</td>
</tr>
</tbody>
</table>

*Significant different; P < 0.05.

Table IV and V shows that compared for the one- and two-blastomere analysis found in the Class 1 excluded embryo group and the total embryo group is in favour of the two-blastomere analysis and seems lower. Although not significantly different, the same trend was seen for the false-negative rate, whereas the false-negative rate remains the same. On the other hand, two-blastomere analysis within Class 1 excluded and the total embryo group showed a significant increase for the positive predictive value compared with the single-blastomere analysis.

## Discussion

In this study, the validity of the overall PGD–PCR procedure and its diagnostic value was judged by means of a diagnostic-test analysis. Surplus embryos collected after PGD–PCR were analysed using the same protocol as in the initial blastomere analysis. Converting the genotype outcome to a diagnostic outcome, thus taking into account the specific characteristics and issues of the different types of PGD–PCR protocols, we demonstrated that the overall PGD–PCR procedure is a valid diagnostic test with a good diagnostic value.

The confirmatory analysis approach of surplus embryos generates a larger amount of data over a shorter period of time as compared with the studies on follow-up of PGD children (422 embryos versus 35 born children in our series). However, for the majority of the embryos used for ET, the diagnostic outcome will always remain unknown. In the PGD–PCR procedure, fewer Class 1 embryos are used for ET and, as a result, the ‘Not confirmed’ group might be smaller. Therefore, one might hypothesize that in the hypothetical situation, all embryos could have been used for confirmatory analysis, false-negative results will be lower, and Se and negative predicted value will be higher.

Compared the blastomere genotype with the embryo genotype, the ‘ADO explained’ genotype outcome group revealed 7.6% ADO in the blastomere genotypes as confirmed by the embryo genotype. ADO may cause false-positive test results in PGD of an AD disorder caused by the detection of the non-risk allele of the unaffected parent alone, or the mutant allele, only in the case of a heterozygous AR disorder. In these cases, ADO will lead to the loss of embryos for transfer. However, no false-negative test results were seen because of the precautions included in the PGD–PCR protocols to detect ADO. Hence, ADO will not lead to the transfer of an affected embryo and the potential birth of an affected child. We also showed that ADO does not necessarily lead to false positives as demonstrated by the T–ADO and T+D+ diagnostic outcome results. ADO can be explained by a number of causes. It can be caused by a technical failure due to inefficient or no priming of the PCR primers, or it can result from chromosomal mosaicism in embryos, like the presence of haploid cells in an otherwise diploid embryo (Harper, 1996; Lissens and Sermon, 1997). Mitotic non-disjunction and anaphase lagging by which monosomy can occur also explain ADO (Coonen et al., 2004).

The ‘Not confirmed’ genotype outcome group showed that in four-and-a-half percent of the analysed embryo genotypes, the initial blastomere genotype was not confirmed. Confirmatory analysis of embryos with PGD–PCR protocols in which polymorphic triplet
repeat alleles or polymorphic markers were used revealed that the discrepency between blastomere and embryo is mainly due to the presence of an additional parental allele or only one parental allele in the embryo. These embryos are possibly mosaic and chromosomally aberrant, at least for the chromosome where the marker is located. Mosaicism is a well-documented phenomenon in preimplantation embryos (Coonen et al., 1994; Munne et al., 1994; Harper et al., 1995; Delhanty et al., 1997; Munne et al., 1998, Coonen et al., 2004). Chromosomal aberrant embryos are most likely the result of metaphase I non-disjunction, a cleavage error of the first meiotic division, followed by mitotic segregation errors (Coonen et al., 2004). The former phenomenon can explain the loss of parental allele or the presence of an additional parental allele.

Conversion of the ‘Not confirmed’ genotype outcome group to a diagnostic outcome showed confirmed positive (T+D+) and negative (T−D−) test results, and a number of false-positive and false-negative test results. It is unlikely that a confirmed negative diagnostic outcome in the ‘Not confirmed’ genotype outcome group will be found. However, the confirmed negative test results were obtained from blastomeres with a normal unaffected genotype, while the remaining cells of the corresponding embryos showed an unaffected genotype with ADO. A plausible explanation could be that these embryos were chromosomally aberrant. The false-positive test results have a negative effect on the total number of embryos suitable for transfer. Nevertheless, they will not result in misdiagnoses with an adverse outcome, like the transfer of an affected embryo. On the contrary, the false-negative test results may lead to the transfer of an affected embryo and the consequent birth of an affected child. The misdiagnosis rate of the total re-analysed embryo group is 7.1%. However, the false-negative rate of the total re-analysed embryo group, reflecting the proportion of affected/aberrant embryos wrongly diagnosed as unaffected and leading to true misdiagnosis, was 3.1%.

On Day 4 PF, significantly higher numbers of morphology Class 1 embryos were observed in the ‘Not confirmed’ group (Fig. 1). In fact, from the seven false-negative embryos, six appeared to be morphology Class 1 on Day 4. The diagnostic-test analysis in the morphology Class 1 embryo group displayed a misdiagnosis rate of 12.5% and a false-negative rate of 17.1%. The high percentage of misdiagnosis and false negatives can probably be explained by the aberrant chromosomal constitution of these Class 1 embryos as was also demonstrated by Magli et al. (2007), who showed that the presence of fragments was associated with an increased incidence of chromosomal abnormalities. The higher number of Class 1 embryos in the ‘Not confirmed’ group became visible on Day 4 and was not evident on Day 3, the day on which the blastomeres were biopsied. Therefore, it is not possible to exclude these embryos, with a high false-negative rate, in advance from biopsy. By excluding the morphology class 1 embryos from the total embryo group, the misdiagnosis and the false-negative rates decreased from 7.1 to 6.1% and from 3.1 to 0.5%, respectively. This was at the cost of 27 (15.5%) of 174 embryos typed as unaffected by PGD and which were excluded for ET because they were Class 1. In three of the 130 transfers (Table I), only Class 1 embryos were transferred. However, from these transfers, no pregnancies were obtained. So one may conclude that the validity of the PGD–PCR analysis improves by rejecting Class 1 embryos from ET as demonstrated by the increase of the Se and the decrease of the LR of a negative test. The same effect was seen for the diagnostic value given by the negative predictive value reflecting the proportion of test negative blastomeres that indeed predict a disease-negative embryo correctly (Table IV).

Comparing PGD validity after biopsy of 1- or 2-cells revealed that the misdiagnosis rates decreased in favour of the two-blastomere analysis accept for the Class 1 embryo group, explained by the high false-negative rate. The Se and negative predictive value, the most important parameters for PGD analysis, remain the same. On the other hand, we observed decreased false positives and a significant increase of the positive predictive value in favour of the two-blastomere analysis. These results are in contrast with the study of Emiliani et al. (2004), who found no significant differences in Se, Sp and the negative and positive predictive values comparing PGD analysis based on the biopsy of 1- or 2-cells. Although the decreased false positives and the increased positive predictive value for two-blastomere analysis within the different embryo morphology groups could be biased due to our biopsy policy, still the analysis of a second blastomere reduces the chance that an embryo is erroneously typed as affected or aberrant due to ADO. Consequently, the number of false positives decreases and the positive predictive value increases, lowering the misdiagnosis rate. So the analysis of a second blastomere increases the number of embryos suitable for transfer as also reported by Goossens et al. (2007). They also showed that PGD after a two-blastomere biopsy increases the number of transferable embryos, moreover, they concluded that the biopsy of 1-cell significantly lowers the efficiency of a PCR-based diagnosis. However, the biopsy of two blastomeres and the effect on embryo viability remain subjects of debate (Cohen et al., 2007). It is still not clear whether the biopsy of two cells and the consequent higher number of transferable embryos due to a lower false-positive rate counter balances the diminished embryo viability. In view of these findings and with evolving the PGD–PCR analysis techniques, the biopsy of one blastomere for PGD analysis might be considered provided the PCR protocol is equipped for the valid analysis on one blastomere.

In conclusion, this retrospective study confirmed that PGD–PCR is a diagnostic valid method to select unaffected embryos for ET. We demonstrated that the rejection of Class 1 embryos for ET improved both the validity and the diagnostic value of PGD–PCR. Although the decrease of the misdiagnosis rate was not significant, 7.1–6.1%, the decrease of the false-negative rate was 3.1–0.5%. This was also demonstrated by the Se that increased from 96.9 to 99.5% and the negative predictive value that increased from 96.1 to 99.3%. Rejection of Class 1 embryos from ET on Day 4 is now implemented in our clinic. The validity of one- versus two-blastomere analysis appears to be comparable. The biopsy and analysis of a second blastomere does not affect the Se. However, the positive predictive value is improved preventing rejection of embryos for transfer due to a false-positive PGD result, as demonstrated by the decreased misdiagnosis rate from 9 to 4.5% in the Class 1 excluded embryo group. So, the biopsy of a second blastomere reduces the number of false positives and hence improves the misdiagnosis rate.

**Funding**

This work has been supported by grants from the Profilerings Fonds of the Academisch Ziekenhuis Maastricht and the Netherlands Organisation for Scientific Research ZON/NOW.

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Submitted on July 3, 2008; resubmitted on September 11, 2008; accepted on September 15, 2008