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# The causes of misdiagnosis and adverse outcomes in PGD

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The European Society of Human Reproduction and Embryology PGD Consortium has collected data on PGD cycles and deliveries since 1997. From 15 158 cycles, 24 misdiagnoses and adverse outcomes have been reported; 12/2538 cycles after polymerase chain reaction and 12/12 620 cycles after fluorescence *in situ* hybridization. The causes of misdiagnosis include confusion of embryo and cell number, transfer of the wrong embryo, maternal or paternal contamination, allele dropout, use of incorrect and inappropriate probes or primers, probe or primer failure and chromosomal mosaicism. Unprotected sex has been mentioned as a cause of adverse outcome not related to technical and human errors. The majority of these causes can be prevented by using robust diagnostic methods within laboratories working to appropriate quality standards. However, diagnosis from a single cell remains a technically challenging procedure, and the risk of misdiagnosis cannot be eliminated.

**Key words:** PGD / preimplantation genetic screening / misdiagnosis / contamination / allele dropout

## Introduction

### What is misdiagnosis?

Many processes are involved in the successful execution of a PGD treatment cycle, the intention of which is the transfer of one or more embryos free from the genetic disorder, abnormality or gender for which the test is applied. The development of an affected pregnancy or birth of an affected child is not necessarily misdiagnosis *per se*. Examples include the decision to transfer an embryo with limited diagnostic information that results in an affected pregnancy or the evolution of a pregnancy affected with a disorder other than that for which a test was applied. In this respect, some misdiagnoses are, in fact, calculated risks with unwanted outcomes. Moreover, the discovery of an error following embryo transfer that does not lead to a pregnancy at all still constitutes a misdiagnosis but has no physical consequences for the patient involved.

Strictly speaking misdiagnosis should only apply when a technical procedure has failed, is inaccurate or has been incorrectly interpreted. Misdiagnoses may be sample specific, single cell specific, or technique specific. Many are independent of technique, sample type or sample quantity in that they could occur owing to general system failures, e.g. human errors. Such system failures should be considered avoidable and should be the first to be eliminated. Examples of system failures include mislabelling and misidentification of labelled samples.

## Severity of misdiagnosis

Misdiagnosis can be considered to be either adverse or benign. Adverse misdiagnoses are those that result in a severe adverse event for the patient (including the birth of an affected child or termination of an affected pregnancy in cases where the embryo transferred

was thought to be unaffected). In the case of benign misdiagnoses, examples include the birth of an unaffected child carrying a mutation when the transferred embryo was thought to be free of the tested mutation. Alternatively, misdiagnosis may be identified after confirmatory testing on untransferred embryos—a procedure that should be performed at least periodically as a quality assurance measure (Thornhill *et al*., 2005; PGDIS, 2008; Thornhill and Repping, 2008). Benign misdiagnoses can be considered 'near misses'. In either case, the opportunity to perform root cause analysis, identify possible process changes and a plan for both corrective and preventative action should be embraced. In the case of adverse misdiagnosis, lessons can be very painful to patients and staff, the consequences significant and the negative publicity severe. Indeed, one could argue that the relatively low reported rate of misdiagnoses may be a combination of failure to achieve pregnancy and live birth following a misdiagnosis and the absence of evidence of misdiagnosis (carrier versus normal baby born who goes untested). For this reason, it is difficult to provide an accurate estimate of the global misdiagnosis rate and in-house estimates are likely to be more reliable. Nevertheless, misdiagnosis rates have been estimated [usually quoted as $<1\%$ for polymerase chain reaction (PCR) cases and $<5\%$ for fluorescence *in situ* hybridization (FISH) cases] and both published and in-house estimates should be available as patient information to allow informed consent for PGD (Thornhill *et al*., 2005).

## General causes of misdiagnosis

There are a number of possible causes of misdiagnosis (Table I). Some causes are specific to the technology or test methodology involved (i.e. PCR or FISH based tests), but many apply equally to both PCR and FISH. An example is cumulus cell contamination that can affect both FISH (by inadvertently providing an additional 'female' nucleus) and PCR (by inadvertently providing both maternal alleles) diagnoses. In either case, it is vital that all cumulus cells are removed prior to biopsy. Technique specific errors and limitations are dealt with in subsequent sections.

Examples of errors that can occur irrespective of the technology include sample mislabelling and misidentification (which is true of any diagnostic test) and unprotected intercourse during a treatment cycle which can lead to the production of supernumerary *in vivo* developed embryos that are untested and thus whose genotype is unknown (Thornhill *et al*., 2005). Mislabelling can be controlled using robust

**Table I Summary of the causes of misdiagnosis in PGD**

| Factor | Error type | PCR | FISH | Examples of preventative action |
|---|---|---|---|---|
| Unprotected sex | Human | √ | √ | Written instructions for patients to avoid unprotected sex during treatment. Use of oral contraceptive pill |
| Mislabelled tube or slide | Human | √ | √ | Comprehensive, robust labelling system and SOP |
| Misidentified tube, slide or embryo | Human | √ | √ | Appropriate witnessing procedures |
| Misinterpreted report | Human | √ | √ | Appropriate training, report and counselling |
| Transfer of wrong embryo | Human | √ | √ | Appropriate training, report, witnessing and counselling |
| Use of incorrect probes or primers | Human | √ | √ | Appropriate witnessing procedures |
| Haploid cells | Intrinsic (embryo) | √ | √ | Removal of second cell. Use of informative-linked STR/SNP markers |
| Chromosomal mosaicism | Intrinsic (embryo) | √ | √ | Removal of second cell. Use of informative-linked STR/SNP. Test polar bodies. Develop test to determine origin of aneuploidy (PCR only) |
| Probe or primer failure | Extrinsic (technical) | √ | √ | Adequate pre-clinical validation. Use whole genome amplification to allow repeat sample testing (PCR only) |
| Maternal contamination | Extrinsic (technical) | √ | √ | Remove all cumulus cells prior to biopsy |
| Paternal contamination | Extrinsic (technical) | * | | Use ICSI to introduce only a single sperm into the oocyte (PCR) |
| Operator contamination | Extrinsic (technical) | √ | | Wear appropriate protective clothing in a controlled environment |
| Carry-over contamination | Extrinsic (technical) | √ | | See above. Use dedicated reagents and equipment in controlled environment |
| Allele dropout | Intrinsic (technical) | √ | | Remove more than 1 cell at biopsy. Include up to three informative-linked STR/SNP markers when performing analysis |
| Wrong segregation analysis | Human | √ | √ | Ensure validation is overseen by experienced/licensed molecular biologist (PCR) or cytogeneticist (FISH) |
| Uniparental disomy | Intrinsic (embryo) | √ | | Removal of more than 1 cell at biopsy. Include up to three informative-linked STR/SNP markers when performing PCR analysis |

FISH, fluorescence *in situ* hybridization; SNP, single nucleotide polymorphism; STR, short tandem repeat; SMA, spinal muscular atrophy; SOP, standard operating procedure.

labelling systems for both the therapeutic entities involved in PGD (i.e. oocytes and embryos) and the diagnostic samples (polar bodies, blastomeres, etc). Both barcoding and radio frequency identification (RFID) systems are currently being considered for routine application in IVF for this purpose. Irrespective of these advanced systems, more conventional systems can be used. In the case of microscope slides, simple printed sticker labelling systems are superior to that of a pencil/pen in terms of legibility, longevity and durability. The need in many regulatory environments to retain samples along with their associated documentation for many years makes such robust labelling a necessity. The amount and type of information recorded on labels can also prevent sample mix-up. For example, a unique identifier must always be present on the tube or slide and is particularly important for preimplantation diagnostics because a series of samples can be generated from the same patient containing the same patient name, date of birth and processing date but will differ only in the embryo and blastomere number. The critical step for individual embryo labelling arises post-biopsy. Coupled with appropriate labelling is the act of witnessing key steps to ensure reliable identification. Such measures are mandatory when handling gametes and embryos in some regulatory environments, e.g. the UK (HFEA, 2007). Once again, barcoding and RFID may facilitate witnessing without the need for additional staff.

As with any diagnostic test, inadequate assay validation can lead to misdiagnosis since the test performance may not be reliable, reproducible or accurate. This is particularly important for single-cell testing as the low template available is sensitive to only minor changes in lysis, sample integrity and equipment or reagent performance. Assuming appropriately trained staff, robust, appropriate and proven technology, properly calibrated equipment and quality-controlled reagents are used when developing a new clinical test, it is still important to develop and work to a validation plan irrespective of methodology used. In PCR cases, one should determine acceptable and achievable accuracy, amplification efficiency and contamination rates and work towards predetermined goals. In FISH cases, one should determine acceptable and achievable hybridization and accuracy rates and work towards predetermined goals.

Such quality control and quality assurance measures underpin effective clinical testing and are an essential part of an accredited diagnostic laboratory (Thornhill and Repping, 2008). Another key component to ensure high quality test performance is the use of external quality assessment schemes (EQAS). EQAS has been demonstrated to improve the quality of diagnostic testing (Kettelhut *et al*., 2003) and by analogy should reduce misdiagnosis rates if routinely incorporated into the PGD arena. Until recently, accreditation and EQAS was restricted to routine clinical diagnostic laboratories and notably absent from most PGD testing laboratories (Corveleyn *et al*., 2008). The slow conversion of PGD laboratories to accreditation probably lies in their origins within therapeutic IVF laboratories versus mainstream diagnostic laboratories. Another obstacle to accreditation is the perceived difficulty of applying some aspects of the accreditation process to single-cell diagnostics. One specific challenge to the incorporation of EQAS is the fact that only a single cell is available for analysis, whereas most diagnostic EQAS involve shared samples. However, EQAS are currently being developed for both PCR- and FISH-based single-cell assays (see Discussion). Many of the causes of misdiagnosis are avoidable by taking preventative action (Table I) and following the principles of quality management present in modern accredited diagnostic testing laboratories. A summary of the numbers of misdiagnoses reported to the European Society of Human Reproduction and Embryology (ESHRE) PGD Consortium are shown in Table II.

## Polymerase chain reaction

The possible causes of misdiagnosis for PGD based on PCR can generally be categorized as human factor, technical pitfalls (extrinsic) and innate quality of cell samples (intrinsic) (Table I). Prior to the clinical PGD test, all mutations and linked informative markers should be confirmed and all protocols need to be vigorously tested in house.

### *Inherent limitations of PCR*

Allele drop-out (ADO) and contamination, are the main inherent pitfalls of single-cell PCR and can potentially lead to an adverse misdiagnosis.

In ADO PCR products are detected from only one of the two target alleles. ADO may arise during the PCR reaction, such that the PCR primers anneal to one of the two target alleles with lower efficiency (or not at all). Alternatively, it may be observed when in fact the biopsied cell is haploid for the target locus. The latter cannot be avoided. However, the former can be minimized if the cell lysis and PCR reaction conditions are optimized, and PCR amplicon labelling and detection systems with high sensitivity are used. However, ADO is a chance phenomenon (even when optimally standardized PCR protocols are used) and whatever its cause, all PCR-based genotyping methods should include internal monitoring

**Table II Misdiagnosis reported to the European Society of Human Reproduction and Embryology PGD Consortium**

| Indication | Method of diagnosis | Number of misdiagnosis reported to consortium | Total number of cycles to PGD/PGS | % Misdiagnosis |
|---|---|---|---|---|
| Sexing for X-linked disease | FISH | 2 | 803 | 0.25 |
| Sexing for X-linked disease | PCR | 2 | 65 | 3.08 |
| Translocations | FISH | 3 | 2514 | 0.12 |
| PGS | FISH | 6 | 8822 | 0.07 |
| Social sexing | FISH | 1 | 481 | 0.21 |
| Monogenic disorders | PCR | 10 | 2473 | 0.40 |
| Total | FISH and PCR | 24 | 15 158 | 0.16 |

Reference: Harper *et al*. (2008); PGS, preimplantation genetic screening.

to detect it. This is achieved by incorporating, along with the disease-specific allele, at least two informative-linked markers, as close as possible to the disease-causing gene/mutation (Thornhill *et al*., 2005), preferably flanking markers (i.e. one located upstream and one downstream of the mutation) in order to detect recombination. Polymorphic microsatellite markers and single nucleotide polymorphisms (SNP) are both suitable, and their analysis should identify the occurrence of ADO, which will be seen as a discrepancy in genotype between the linked loci analysed in each cell. ADO can be further reduced or minimized by taking two cells at biopsy, although this may have an impact on the viability of the embryo, and/or using at least three linked markers when testing for a specific mutation.

Contamination is the other major cause of misdiagnosis when performing PCR-based PGD cycles. Contamination from either the cumulus cells or extraneous sperm cells can be precluded by applying correct procedures during the stages of embryo fertilization and biopsy (Thornhill *et al*., 2005). ICSI must be performed for all PCR diagnosis to avoid paternal contamination and cumulus cells must be removed prior to biopsy to avoid maternal contamination. Contamination from these sources is not caused by the PCR-step *per se*.

Other sources of contamination include operator contamination or amplicon carry-over contamination, and single-cell protocols are particularly vulnerable to both of these (Lewis *et al*., 2001). The first stages of the PGD procedure are most vulnerable to operator contamination, including the embryo biopsy, cell lysis and the first PCR. Carry-over contamination may arise if stringent conditions are not applied during any of the stages of PCR. Thus, contamination from both sources can be minimized by the application of stringent conditions including performing embryo biopsy, pre- and post-PCR procedures each in separate and preferably exclusive UV-treated areas, the use of exclusive equipment and finally the use of stringently prepared one-use reagent aliquots. Besides the inclusion of several negative controls and blanks at all stages, the occurrence of chance contamination should be monitored in each cell sample. The latter can be achieved by the inclusion of polymorphic microsatellite markers alongside the disease-specific assay, whereby the detection of spurious or supernumary microsatellite alleles inconsistent with those expected from the parents implicate the presence of contaminating DNA.

Certainly, since the initial application of PCR-based PGD cycles, there have been marked improvements in the quality of reagents and the available equipment, and together these have facilitated the development of more robust PCR-based PGD protocols. With current protocols such as multiplex PCR (i.e. the co-amplification of 3–4 or more linked markers together with the gene region containing the disease causing mutation) and preimplantation genetic haplotyping (Renwick *et al*., 2006), many misdiagnoses caused by ADO or contamination may have been avoided.

It is often difficult to trace the reason for misdiagnosis, mainly due to the fact that the same sample cannot be re-analysed following single-cell PCR. However, systematic re-analysis of untransferred embryos may help to highlight the more prevalent causes of misdiagnoses, so that we have a more complete evaluation of the accuracy and limitations of PCR-based PGD protocols. In addition, the emergence of whole genome amplification as a universal first-step prior to targeted mutation analysis (Renwick *et al*., 2006) should facilitate the re-testing of any ambiguous samples and provide opportunities for both confirmatory testing and EQAS with other laboratories.

Mosaic uniparental disomy is also a theoretical possible cause of misdiagnosis, misrepresenting a heterozygous embryo as homozygous when the mutation alone is analysed. Multiplex PCR often reveals apparently haploid patterns in blastomeres, which could also be due to uniparental disomy.

### *PCR misdiagnoses reported to the ESHRE PGD Consortium*

Among the 2538 PGD cycles performed until the end of 2005 using PCR-based protocols, a total of 12 adverse misdiagnoses were reported; 10 were for autosomal monogenic diseases and 2 were following PCR-based sexing for X-linked monogenic diseases. In 10 of the pregnancies, the misdiagnosis was detected following a prenatal diagnosis; six of these pregnancies had termination of pregnancy, whereas the other four pregnancies were delivered. The other two misdiagnoses were detected following the birth of an affected child (summarized in Table III).

It is difficult to find a posteriori causes for misdiagnoses in PGD for monogenic diseases following single-cell PCR. However, based on partial re-analysis results attempted for the spinal muscular atrophy misdiagnosis reported in data collection VII (Table III), the authors concluded that contamination was the cause of the misdiagnosis, leading them to adopt subsequently a 2-cell biopsy policy (Daniels *et al*., 2001). The misdiagnoses for myotonic dystrophy type 1 (reported in data collection I) was also likely to have been caused by contamination (Sermon *et al*., 1998).

According to the centre, the misdiagnosis for the β-thalassemia of data collection II was likely caused by misidentification of samples (tube switch), a pitfall inherent in any laboratory procedures that can be avoided through strict adherence to well designed standard operating procedures.

ADO was presumed to be the cause for the misdiagnoses in two cycles for sexing and the β-thalassemia misdiagnosis of data VIII.

The misdiagnoses in the sexing cases were probably due to non-amplification of the Y sequences, due to incorrect choice of primers, which would be avoided by the use of multiple loci on the Y chromosome.

For the two CMT1A (Charcot-Marie-Tooth 1A) cases, re-investigation of the family showed that the segregation analysis based on the one linked marker was incorrect and that affected embryos had systematically been selected and transferred (Inge Liebaers, personal communication).

For the other misdiagnoses referred to in Table III (familial amyloid polyneuropathy, fragile-X and two for cystic fibrosis), the centres could offer no explanation.

Most of the above misdiagnoses in which the causes were implicated could have been avoided if multiplex PCR using multiple disease-associated loci had been used as standard. As shown by the CMT1A example, even double blastomere biopsy is not a failsafe for misdiagnosis, whereas a multiplex PCR in the CMT1A region would have given a correct segregation analysis. The occurrence of misdiagnosis on the one hand and the birth of affected children after PGD on the other hand should be significantly reduced if there is a thorough pre-clinical set-up by a skilled team of molecular biologists, followed by a robust multiplex PCR on the biopsied blastomere. In addition, if prenatal diagnosis is carried out, it is preferable to use a different test than the one used for the PGD cycle.

**Table III Misdiagnosis after PCR**

| Indication | Method used | PND-post-natal | Outcome | PGD Consortium report number |
|---|---|---|---|---|
| **Monogenics** | | | | |
| Myotonic dystrophy type 1 | PCR | PND | TOP | I |
| B-thalassemia | PCR | PND | TOP | II |
| Familial amyloid polyneuropathy | PCR | PND | Born | IV |
| Cystic fibrosis | PCR | PND | Born | II |
| Cystic fibrosis (1 of twins) | PCR | Post | Born | IV |
| CMT1A | PCR | PND | Born | VII |
| SMA | PCR | Post | Born | VII |
| CMT1A (twins) | PCR | PND | TOP of both twins | VII |
| B-thalassemia | PCR | PND | TOP | VIII |
| Fragile X | PCR | PND | Born | VIII |
| **Sexing for X-linked disease** | | | | |
| 46 XY in retinitis pigmentosa | PCR | PND | Born | IV |
| 46 XY in Duchene muscular dystrophy twin | PCR | PND | TOP of one twin | III |

PND, prenatal diagnosis; TOP, termination of pregnancy; CMT1A, Charcot-Marie-Tooth 1A.

## Fluorescence *in situ* hybridization

Like any laboratory technique, FISH has a number of recognized limitations, which can lead to incorrect interpretation of results and a potentially adverse outcome. There are ways to minimize the effect of these limitations, but patients must be counselled that, although FISH is highly accurate, it is not foolproof and there is a recognizable risk of failure.

There are two predominant methods for preparation of blastomere nuclei on microscope slides. These are the Tween:HCl method first published by Coonen *et al*. (1994) and the more traditional fixation method that uses methanol and acetic acid (Tarkowski, 1966). Both methods require a degree of technical expertise and produce excellent results when performed well. However, when performed poorly both can result in signals that are difficult to interpret. No method is necessarily better than the other and individual laboratories should use the method with which they have the most expertise and that works most effectively under their own conditions.

### *Inherent limitations of FISH*

No FISH probes, whether produced in-house or obtained commercially, have 100% hybridization efficiency. Commercial probes should include a report of the efficiency on known normal cells and it usually ranges from 95% to 99%. This means, however, that one in every 20–100 cells that is actually euploid for the particular chromosome will give a spurious result. Hybridization failure can also occur if the DNA is inappropriately prepared, particularly if it is not fully denatured at the target hybridization site. Some probes also demonstrate cross-hybridization to sites on other chromosomes. This should be documented when purchasing commercial probes and should be accurately determined by analysis of known normal metaphase chromosomes for in-house developed probes (Thornhill *et al*., 2005). Ideally, these probes should not be used for interphase single-cell FISH, but if this is unavoidable then known cross-hybridization must be taken into account during signal scoring and interpretation.

Sometimes the target regions of the two copies of chromosomes being subjected to FISH can lie on top of each other during nuclei fixation or spreading. This is unavoidable and results in two signals of the same colour overlapping and being interpreted as one signal. Occasionally, the signal will appear unusually large, which can prompt reprobing of the nucleus with a probe located elsewhere (e.g. the telomere) on the chromosome in question (Colls *et al*., 2007). This can resolve the chromosomal copy number in the nucleus (Daphnis *et al*., 2005).

Signals from different chromosomes, and hence different fluorochromes, can also overlap. If a red and a green signal overlap, a yellow spot is produced which can be misinterpreted if a yellow fluorochrome is also used. It is important to analyse each plane of colour separately to identify individual fluorochromes. Some laboratories produce additional FISH colours by proportional labelling of probes. That is, a yellow signal is produced by mixing two probes with identical hybridization targets but one labelled with green fluorochrome and the other with red fluorochrome. In this circumstance, observing individual planes of colour will not resolve overlapping signals. It would be impossible to determine if a yellow signal was the proportionally labelled probes for that chromosome or the unfortunate overlapping of two different targets that were labelled red and green, respectively.

Another inherent limitation of the FISH technique is interpretation of closely adjacent signals that are labelled with the same fluorochrome. Chromosome target DNA can split and it is sometimes very difficult to differentiate between a split signal that represents one copy of that chromosome and two separate signals representing two chromosomes that are closely adjacent to each other. Most FISH scientists use the signal interpretation guidelines of Hopman *et al*. (1988), which state that separate signals need to be at least one signal width apart. Signals closer than this and of similar size are interpreted as one split signal. There will be occasions, however, where two very closely located signals will in fact represent two separate chromosomes. This scenario can also be resolved by reprobing the

nucleus with a probe located elsewhere on the chromosome in question.

Cumulus cell contamination in FISH could lead to a misdiagnosis as cumulus nuclei may appear very similar to blastomere nuclei. Great care must be taken to remove cumulus cells from oocytes or zygotes to minimize the chance they are inadvertently biopsied along with the blastomere (Thornhill *et al*., 2005). Additionally, the blastomere should be carefully observed, tracked and located during the fixing or spreading process to ensure that the correct nucleus is analysed.

### *Chromosomal mosaicism in early human embryos*

The existence of chromosomal mosaicism, in which different blastomeres have a different chromosomal complement, is well documented (Harper *et al*., 1995; Munne *et al*., 1997; Voullaire *et al*., 2000) and affects up to 50% of early human embryos. This means that the blastomere biopsied for PGD and FISH may not represent the rest of the embryo and could result in an adverse outcome. This is not a misdiagnosis, *per se*, as the FISH result on the biopsied cell could be correct, just different from the remainder of the embryo. Mosaicism exists in embryos and cannot be corrected, so it is an inherent limitation of the FISH technique when used in PGD. Some laboratories biopsy and analyse two cells from each embryo in an effort to detect mosaicism. Although this provides some value, there still may be undetected mosaicism in the cells remaining in the embryo, and there is likely to be a cost to the viability of the embryo by biopsying two cells (Cohen *et al*., 2007; Goossens *et al*., 2008). Most published data on chromosomal mosaicism comes from the analysis of cells of embryos already identified as aneuploid after single-cell biopsy and PGD. In these embryos, the frequency of mosaicism is high, but chromosomal pattern is predominantly one of different aneuploidies rather than a mixture of euploid and aneuploid cells so the clinical impact on the decision not to transfer the embryo is minimal. It is impossible to determine the frequency of mosaicism in transferred embryos that have been diagnosed as euploid after PGD. However, if it was particularly high one would expect far more misdiagnoses after PGD for aneuploidy.

### *Technical difficulties*

FISH needs to be performed according to exact protocols. These may differ slightly between laboratories and individual laboratories should optimize protocols to suit their own conditions. There are a number of technical difficulties that may arise while performing FISH and these can lead to misinterpretation of signals and adverse outcomes.

The stringency conditions of the post-hybridization wash are critical. If the conditions are too stringent the signals will be weak and some may not be visible. If the wash is not stringent enough, there will be non-specific hybridization on other chromosomes that could be incorrectly interpreted as signals.

Dirt or debris can overly the nucleus after fixation or spreading of single cells for PGD. Small spots of dirt can be mistaken for signals. Most often, however, dirt fluoresces in every colour so it can be identified by observation of individual colour planes. Any spot that is visible through each filter combination is very likely to be dirt. The other complication caused by debris is that part of the nucleus can be obscured and there may be real signals underneath that cannot be seen. Dirt cannot be completely avoided but it certainly can be minimized by using clean slides and pipettes and fresh solutions made up in clean test-tubes within a clean-air environment.

Usually, the fixed or spread nucleus is relatively flat and all of the FISH targets can be visualized in a single focal plane. Sometimes the nucleus is thicker (particularly when using the Tween/HCl method) and signals are in different depths within the nucleus. It is imperative that a range of focal planes are scanned to ensure that all signals are visualized and it is often necessary to capture more than one image plane to accurately document every FISH signal.

It is relatively common practice in PGD for aneuploidy or chromosomal translocations to maximize the number of chromosomes analysed by performing more than one round of FISH on each nucleus. This is a very effective strategy but great care should be taken to minimize the persistence of signals from the first round of FISH as this could result in incorrect interpretation of signals in the second round. Signal persistence can be reduced by exposure of the nucleus to very bright light and washing the slides in 4× standard saline citrate for 10 min at room temperature. When reading second or subsequent rounds of FISH, the position of fluorochromes on the nucleus should be compared with the location of those same fluorochromes from previous rounds to ascertain that they are new signals rather than those that have persisted from previous rounds of FISH.

It is well recognized that multiple denaturation and renaturation of DNA, as required for second and subsequent rounds of FISH, causes DNA degeneration and reduced hybridization efficiency (Harrison *et al*., 2000). Appropriate quality assurance should be performed for all FISH rounds and outcomes taken into consideration when assessing signals.

### *FISH to detect unbalanced chromosomal rearrangements*

All of the pitfalls and limitations described above equally apply to FISH for aneuploidy testing and chromosomal rearrangements. However, to detect chromosome imbalance in embryos from translocation carriers, it is critical to use a combination of FISH probes that can account for all possible segregation patterns for the particular translocation (Thornhill *et al*., 2005). Several authors have reported relatively simple strategies of using two probes that are located on either side of the breakpoint on one chromosome and one probe anywhere on the other chromosome involved in the translocation (Conn *et al*. 1995) or alternatively using one sub-telomeric probe for each of the chromosomes involved in the translocation and a control centromeric probe (Scriven *et al*., 1998). Either method should detect all possible unbalanced forms of the translocation. In some cases, it is possible to use four probes, i.e. two probes either side of each breakpoint, and this can provide some extra certainty. It is critical that the planned probe combination is tested prior to application in the clinical PGD case to ensure that all probes are working optimally and that signals are clear and interpretable.

### *FISH misdiagnoses reported to the ESHRE PGD Consortium*

From the 12 620 cycles where the diagnosis was performed by FISH, there have been 12 misdiagnoses. Most of the reported misdiagnoses listed in Table IV could be explained by any of the FISH limitations or technical difficulties described above, and from the information provided, it is not possible to determine the cause of the adverse

**Table IV Misdiagnosis after FISH**

| Indication | Method used | PND-post-natal | Outcome | PGD Consortium report number |
|---|---|---|---|---|
| **Sexing for X-linked disease** | | | | |
| 45, XO, haemophilia A | FISH | PND | TOP | IV |
| 46, XY, haemophilia A | FISH | Post-natal | Born | VIII |
| **Translocations** | | | | |
| T13 after 45,XY,der(13;14)(q10;q10) | FISH | Miscarried | Miscarried | VI |
| 47,XX,+der(22)t(11;22)(q23.3;q11.2)mat | FISH | PND | TOP | III |
| 46,XY,der(15)t(13;15) (q25.1;q26.3)pat | FISH | PND | TOP | VII |
| **PGS** | | | | |
| T16 after first PB biopsy only | FISH | Miscarried | Miscarried | VI |
| T16 after first PB biopsy only | FISH | Miscarried | Miscarried | V |
| trisomy 16 | FISH | Miscarried | Miscarried | VI |
| trisomy 16 | FISH | Miscarried | Miscarried | VI |
| trisomy 21 | FISH | Post | Born | III |
| 47,XXX | FISH | PND | Lost to follow-up | VII |
| **Social sexing** | | | | |
| Requested male but female fetus | FISH | PND | TOP | III |

outcome. Exceptions to this include the two cases of trisomy 16 after first polar body biopsy. The trisomy could have arisen from non-disjunction in meiosis II or from fertilization of the oocyte by a disomy 16 sperm. An unbalanced fetus developed after PGD for the translocation between chromosomes 11 and 22 (47,XX,+der(22)t(11;22)(q23.3;q11.2)mat) and the reason for this is most likely because of the use of only two FISH probes that could not detect this unbalanced form of the translocation (Delhanty, 2004; Kyu Lim *et al*., 2004; Mackie Ogilvie and Scriven, 2004; Sermon *et al*., 2005).

When using FISH for sexing, it is advisable to include the sex chromosome probes in the first round of FISH and to reduce the number of other probes in this round to ensure an accurate diagnosis of sex. Second and third rounds of FISH are not as accurate as the first round as the DNA degenerates (Harrison *et al*., 2000).

## Conclusion

The true level of misdiagnosis in PGD may remain a mystery for several reasons: (i) some centres may be reluctant to publish this information; (ii) since embryo transfer *per se* does not guarantee pregnancy and live birth, a proportion of misdiagnoses must currently go undetected and (iii) there are misdiagnoses with no adverse medical consequences (e.g. birth of a child carrying a recessive mutation versus free from the mutation). The Consortium offers a forum to which misdiagnoses can be reported anonymously and, to date, a low rate has been observed (24/15 158, 0.16%), although the rate for PCR-based cycles (0.5%) is relatively higher than that for FISH-based cycles (0.1%). However, new misdiagnoses are reported with each Consortium data collection and PGD laboratories must be aware of the possible causes and ways they can reduce the risk. From the explanations given in this paper, it is likely that some of these reported misdiagnoses could have been prevented.

It is important for PGD centres to determine their individual misdiagnosis rates. PGD tests are developed on single cells from a variety of sources but the only time affected embryos are obtained is when a couple come through a PGD cycle. It is therefore important that confirmation of the diagnosis is performed on untransferred embryos, although it is recognized that this may not be permitted in some jurisdictions.

PGD laboratories should conform to ISO 15 189 or equivalent local standards and work with national diagnostic laboratory accreditation schemes, if available. The ESHRE PGD Consortium recommends that, where possible, PGD laboratories should be accredited or working towards accreditation. To this end, the Consortium is currently preparing a 'beginners guide to PGD lab accreditation'. In accordance with laboratory accreditation, it is essential that the PGD laboratory is run to the highest standards as with other mainstream diagnostic laboratories, with standard operating procedures in place and suitably trained staff. Accreditation schemes also require EQA. EQA allows objective monitoring of a clinical laboratory's technical, analytical and interpretative performance. The ESHRE PGD Consortium has been working with the EU and the UK EQAS to try to ensure that PGD/preimplantation genetic screening results are accurate, reliable and comparable. The Consortium has recently launched the FISH and PCR EQA for PGD.

The FISH EQA is being run by the Cytogenetics European Quality Assessment scheme (CEQA) and aims to assess the ability to analyse FISH signals for PGD/PGS and to ensure that the correct probe combinations are used. The FISH EQA is an online scheme in two phases. In phase 1, centres register and submit images of one of their own FISH cases (PGD or PGS) with details of the parents' karyotypes and the protocol used, reports and interpretative comments. This information will be assessed by a panel of experts and feedback provided to individual centres. In phase 2, cases validated by the expert

panel will be posted on the secure CEQA website and centres will be asked to analyse one PGD and one PGS case and their responses scored.

The PCR EQA is being run in collaboration with the UK National External Quality Assessment Scheme (UKNEQAS) for Molecular Genetics. The aims are to be able to test the ability to carry out feasibility work-up for PGD, the technical ability to do single-cell PCR and the interpretation and reporting of results. Single cells from commercially available cell lines will be sent to participating Centres to test the ability of each participating laboratory to carry out feasibility/validation work-up for PGD, technical ability to do single-cell PCR and interpretation and reporting of results.

Making a diagnosis from a single cell remains a technically challenging process. A combination of improved quality standards, more powerful technologies and the ability to re-test single-cell samples should reduce the risk of a misdiagnosis to a minimum.

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