Data can sometimes be clustered simply by visual examination

basis for future studies into the causes and possible treatment of attempted suicide. (The second set of statistical techniques concerned with classification is known as discriminant or assignment methods. Here the classification scheme is known a priori and the problem is how to devise rules for allocating unclassified individuals to one or other of the known classes.)

Many methods have been suggested for constructing a set of clusters from numerical data, ranging from the visual examination of simple scatterplots of data (fig) to the fitting of complex statistical models. A comprehensive review is given in Everitt.² Among these methods, no single method is best in all situations and for all types of data. Different methods of cluster analysis can (and probably will) produce different solutions when applied to the same set of data. As a consequence, clustering techniques require great care in their practical application if misleading and artificial solutions are to be avoided.

The set of clusters produced by a clustering method can serve a variety of purposes. At one level they may simply represent a convenient method for organising a large dataset so that information may be retrieved more efficiently. In general, however, investigators are likely to be looking for their solutions to represent some more fundamental feature of the data, which is why the caveats offered above are necessary to prevent artefact becoming theory.

Peacock, Bland, and Anderson use cluster analysis in an attempt to identify women at risk of preterm birth. Seven binary variables are used to characterise each woman in the study who spontaneously delivered preterm. The method of cluster analysis used, association analysis, is monothetic—that is, division of the data into clusters is based on the possession or otherwise of a single specified characteristic. Division variables are chosen in a particular way, as described in the paper. The authors report a solution with three clusters, although the first two of these seem to be similar.

The use of cluster analysis in such a study is certainly a novel way of attacking the problem of identifying subsets of risk factors among highly correlated explanatory variables. It is unfortunate that the authors used a rather outdated clustering method, one based on divisions involving a single variable at each stage. It would be interesting to compare the results obtained with those given by a method such as latent class analysis,² which is also specifically designed for binary variables, but is underlined by a relatively sensible statistical model. Nevertheless, Peacock, Bland, and Anderson are to be congratulated for alerting other investigators to the advantages of using cluster analysis in this type of study.

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Optimal polymerase chain reaction amplification for preimplantation diagnosis in cystic fibrosis ($\Delta F508$)

Ke-Hui Cui, Eric A Haan, Ling-Jia Wang, Colin D Matthews

Abstract

Objective—To evaluate direct polymerase chain reaction amplification of mutation on single embryo cells for the routine preimplantation diagnosis of cystic fibrosis.

Design—Direct polymerase chain reaction amplification of mutation was performed to identify the cystic fibrosis $\Delta F508$ mutation in human blood DNA, single lymphocytes, embryos, and embryo cells obtained by biopsy. Preimplantation diagnosis was performed for a couple who were heterozygous carriers of the $\Delta F508$ mutation.

Setting—Laboratory for preimplantation diagnosis in a reproductive medicine unit.

Main outcome measure—Correct diagnosis of homozygous normal, heterozygous, and homozygous abnormal DNA of the cystic fibrosis $\Delta F508$ mutation.

Results—45 blood samples (18 homozygous normal, 17 heterozygous, and 10 homozygous abnormal) and 204 single lymphocytes from known sources showed 100% amplification and were diagnosed correctly. 17 human embryos and 52 normal nucleated embryo cells obtained by single cell embryo biopsy also showed 100% amplification. After a miscarriage of the initial pregnancy (diagnosed at preimplantation to be homozygous normal) in the heterozygous carrier couple, fetal tissue was confirmed to be homozygous normal.

Conclusion—Direct polymerase chain reaction amplification of mutation is a simple, fast, reliable test for the common cystic fibrosis mutation ($\Delta F508$) in blood DNA and single cells and should be applicable to routine programmes of general

screening, maternal blood examination, and preimplantation diagnosis.

Introduction

Cystic fibrosis is the most prevalent severe inherited disease in white people with an incidence of about one in 2500 births and a carrier frequency of about one in 25.¹ The disorder is caused by over 200 mutations in a gene called the cystic fibrosis transmembrane regulator, which is on chromosome 7q31.² The commonest mutation ($\Delta F508$) is the deletion of an amino acid (phenylalanine) at position 508 of the gene,³ which is responsible for 70% of cystic fibrosis cases.

Preimplantation diagnosis is a technique of prenatal diagnosis that avoids medical abortion. Precision of diagnosis—that is, as close to 100% accuracy as possible—is crucial⁴ and is particularly demanding technically, given that a single copy of the gene must be amplified successfully from a single embryo cell obtained by single cell biopsy. Precision of preimplantation diagnosis has been achieved in sex determination by polymerase chain reaction.^{5,6} Polymerase chain reaction is a method by which a targeted gene is greatly amplified by a polymerase enzyme with two short leading segments of the gene (primers) and other reagents by many cycles of suitable temperature changes in a thermal cycler. Precision of diagnosis is wholly dependent on the method used. Any inappropriate method is easily detected by basic experiments⁷⁻¹¹ and will be shown directly by misdiagnosis.¹² A technique that achieves near 100% precision of diagnosis would provide a scientific basis for routine clinical practice in preimplantation diagnosis.¹³⁻¹⁶

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This paper evaluates direct polymerase chain reaction amplification of mutation in single embryo cells to detect the common cystic fibrosis mutation $\Delta F508$.

Methods

BLOOD TESTING

Human blood DNA (which contains plenty of genes) was used to determine the ability of our own designed primers to detect the $\Delta F508$ mutation in cystic fibrosis. DNA from the whole blood of 18 homozygous normal, 17 heterozygous, and 10 homozygous abnormal subjects was extracted and prepared for amplification.¹⁷ All polymerase chain reaction mixtures were freshly combined and transferred to 0.5 ml tubes. Each tube contained 18 μ l sterile distilled water; 4 μ l 10 $\times$ reaction buffer (500 mmol potassium chloride and 100 mmol TRIS-hydrochloric acid per litre, pH 8.3); 1.5 μ l of each 10mM deoxynucleotide with either 1.6 μ l (0.2 μ mol/ml) of each normal gene primer (5'-GGCACCATTAAAGAAAATATCATCTTTG-3' and 5'-AGCTTCTTAAAGCATAGGTCATGTG-3'—designated as N tubes with 1 μ l phenol red 0.1 mg/ml water) or 1.6 μ l of each $\Delta F508$ mutation gene primer (5'-CTGGCACCATTAAAGAAAATATCATCTTG-3' and 5'-AGCTTCTTAAAGCATAGGTCATGTG-3'—designated as F tubes); 0.2 μ l ampli Taq DNA polymerase (5 U/ μ l); 6 μ l 25mM magnesium chloride; and 4 μ l (or 1.5 μ g) individual human blood DNA in a final volume of 40 μ l. DNA was denatured for six minutes at 94°C followed by 30 cycles of 94°C for one minute, 65°C for one minute, and 72°C for two minutes and a final extension at 72°C for 10 minutes. Amplified DNA results and marker (that is, DNA size control) were examined.

SINGLE LYMPHOCYTE TESTING

Single lymphocytes were used to test further the precision, sensitivity, and stability of amplification with the designed primers. Sixty eight homozygous normal single lymphocytes (from two homozygous normal males), 78 heterozygous single lymphocytes (from three heterozygous females), and 58 homozygous abnormal single lymphocytes (from two homozygous abnormal males) were diluted and individually transferred to 0.5 ml tubes containing 10 μ l buffer (10 mmol TRIS-hydrochloric acid, 50 mmol potassium chloride, and 2.5 mmol magnesium chloride per litre, pH 8.0). After DNA denaturing, freshly made mixture (30 μ l) containing 4 μ l 10 $\times$ reaction buffer, 6 μ l combined deoxynucleotides, 0.2 μ l Taq polymerase, 6 μ l magnesium chloride, and 1.6 μ l of each outer primer (5'-GCATAGCAGAGTACCTGAAACAGGA-3' and 5'-GACGTTTGTCTCACTAATGAGTGAAC-3') was transferred to the heat treated tubes containing the single lymphocytes.

FIG 1—Results of blood DNA amplification in 12 of 45 subjects tested for $\Delta F508$ mutation. NN1 to NN4 denote first to fourth homozygous normal subjects, NF1 to NF4 first to fourth heterozygous carriers, and FF1 to FF4 first to fourth homozygous abnormal subjects. M represents marker pUC 19 and B the blank. N represents normal gene and F mutant gene

FIG 2—Results of DNA amplification in 12 of 204 single lymphocytes tested for $\Delta F508$ mutation. NN denotes homozygous normal, NF heterozygous abnormal, and FF homozygous abnormal

The DNA was denatured for six minutes at 94°C followed by 30 cycles of amplification of the common (normal and mutant) sequences covering the mutation points (bases CTT) and temperature processed similar to that for blood DNA amplification. Another batch of N tubes (to detect the normal gene) and F tubes (to detect the mutant gene) were prepared with 40 μ l mixture containing the inner normal or mutation gene primers identical with those used in blood testing and placed in 1 μ l of first amplification product for 30 cycles of reamplification to identify separately the normal and mutant genes.

HUMAN EMBRYO BIOPSY AND EMBRYO CELL AND EMBRYO DIAGNOSIS

Four to eight cell human embryos from 31 couples were used. The embryos were secured by a holding pipette. A drilling pipette was approximated to the zona and acid Tyrode's solution (pH 2.3) gently expelled to dissolve the zona pellucida (the outer layer of the embryo). A single normal nucleated embryo cell was aspirated from each embryo with a biopsy pipette. All aspirated single embryo cells were transferred separately to 0.5 ml tubes for two stages of 30 cycles of amplification (as for single lymphocyte testing). Fifty two normal nucleated embryo cells obtained by biopsy from 52 embryos and a further 17 whole embryos were subjected to polymerase chain reaction amplification.

Preimplantation diagnosis of cystic fibrosis was performed for a couple who were heterozygous carriers of the $\Delta F508$ mutation. Briefly, using well established in vitro fertilisation techniques we recovered 18 oocytes and generated 16 embryos by fertilisation. Single cell embryo biopsy was performed on 14 embryos at the 6-10 cell stage. These 14 single normal nucleated embryo cells were subjected to DNA amplification.

ETHICS

Ethical approval was obtained for all aspects of the study.

Results

BLOOD TESTING

All N tube solutions (with normal gene primers) used to detect the normal cystic fibrosis gene fragments were red (owing to the phenol red) and easily differentiated from the colourless F tube solutions (with mutant gene primers), which were to detect the $\Delta F508$ mutation. Blood DNA from the 18 homozygous normal subjects showed normal gene fragments only (that is, positive amplification from N tubes, or N bands positive) (fig 1). All blood DNA samples from the 17 heterozygous subjects were positive for both normal and mutation gene fragments (N and F bands positive). All blood DNA samples from the 10 homozygous abnormal subjects were positive for mutation gene fragments (F bands positive) and negative for normal gene fragments (N bands negative). All the bands were bright and clear.

SINGLE LYMPHOCYTE TESTING

All 204 single lymphocytes tested showed 100% amplification and were correctly identified. The 68 single lymphocytes from homozygous normal sources all showed normal gene fragments (N bands) only (fig 2). All 78 heterozygous single lymphocytes were positive for both normal and mutation gene fragments (N and F bands positive). All 58 homozygous abnormal single lymphocytes were positive for mutation gene fragments (F bands positive) only. Weak common DNA bands (self control bands) derived from the initial set of primers were present.

FIG 3—Results of DNA amplification in seven of 17 single human embryos tested for F508 mutation. All were homozygous normal. (Notations as in figs 1 and 2)

FIG 4—Results of polymerase chain reaction amplification in eight of 52 normal nucleated embryo cells obtained by single cell biopsy from 52 human embryos tested for Δ F508. All were homozygous normal. (Notations as in figs 1 and 2)

DIAGNOSIS OF HUMAN EMBRYOS AND EMBRYO CELLS

Seventeen human embryos (fig 3) and 52 normal nucleated embryo cells (fig 4) obtained by single cell biopsy from a further 52 human embryos showed 100% polymerase chain reaction amplification. All were homozygous normal. The bands were bright and clear.

The results in the couple having clinical preimplantation diagnosis (August 1993) were two homozygous normal, seven heterozygous, and five homozygous abnormal embryos with 100% polymerase chain reaction amplification. Two homozygous normal embryos were transferred to the mother's uterus and five heterozygous embryos frozen. Pregnancy was established and confirmed by rising human chorionic gonadotrophin concentrations. At seven weeks, however, ultrasound examination indicated a failing pregnancy and uterine curettage was performed. Fetal tissue was confirmed to be homozygous normal.

Discussion

At present the most favourable material for use in preimplantation diagnosis is single embryo cells obtained by single cell biopsy.¹⁶ Gene analysis of single embryo cells should be simple, fast, sensitive, stable, and precise. A simple technique can help avoid

technical misdiagnosis and a rapid diagnosis can allow embryo transfer to be performed in the same in vitro fertilisation cycle. Sensitivity is especially important for gene amplification from the DNA of a single embryo cell, and polymerase chain reaction amplification is the most suitable technique. The stability of the technique allows the visualisation of bright, identical, and clear amplification products, thus avoiding the need for frequent changes of primers or reaction conditions. Precision of the diagnosis is the end point and the key to preimplantation diagnosis. Without precision, confirmatory procedures for prenatal diagnosis may be required. Furthermore, some couples object to aborting a fetus that they know to be genetically abnormal despite a wrong diagnosis of normality.

Low amplification rates in sex determination¹⁸ and incomplete amplification in the heterozygous condition—for example, resulting from "allele drop out," when one of the normal or mutant genes cannot be amplified—are closely related to a high fetal misdiagnosis rate. (Misdiagnosis is defined here as the actual fetal genetic condition not being consistent with the results of preimplantation diagnosis.) However, though the rate of amplification with the direct polymerase chain reaction is 100%, the possibility of misdiagnosis as a result of technical mistakes—for example, wrong labelling of tubes—or contamination, chromosomal abnormality, or gene mutation could still occur and should be explained to patients. The risk of misdiagnosis can be almost eliminated by strict procedures.

This study followed studies in a mice model (39 mice pups born with correct sex diagnosis) which included safety analyses⁷ and a study of human sex determination⁶, in which 100% amplification and correct diagnoses were achieved. Apart from experimental design,^{9,6} factors such as amplification conditions, quality of reagents (such as 10× buffer and magnesium chloride), quality control of the thermal cycler, single cell transfer, and even conscientiousness and skills of the operator are closely related to the amplification rate and precision of diagnosis. In human preimplantation diagnosis, selecting embryo cells with normal nuclei (rather than those degenerating or degenerated cells with nuclear morphological change) for single cell embryo biopsy is also crucial for 100% amplification.¹⁹

When DNA amplification is applied to blood or tissue samples many copies of the target sequence are present, facilitating a reliable and correct diagnosis. By contrast, sex determination in preimplantation diagnosis requires amplification of a single male cell which contains only one copy of the Y linked testis determining gene.⁶ Precise diagnosis from a fragment of the single copy target of this gene in a single cell is clearly more difficult. Furthermore, precise diagnosis of cystic fibrosis in preimplantation diagnosis requires amplification of a single copy target to detect a difference of only three bases and is more difficult than detecting a difference of more than 100 bases to sex an embryo. Thus the conditions of amplification for cystic fibrosis need to be more rigorous than those in sex determination. In these experiments conditions allowed 100% amplification and precision in the diagnosis of the cystic fibrosis (Δ F508) mutation with single embryo cells.

RISK OF MISDIAGNOSIS DUE TO CONTAMINATION

The risk of fetal misdiagnosis due to contamination in the diagnosis of X linked diseases is very small.⁶ Cystic fibrosis is an example of an autosomal recessive mutation disease in which misdiagnosis due to contamination can be analysed (fig 5). If only homozygous normal embryos are to be transferred without loss of the embryo cell (or DNA), then no fetal misdiagnosis will

* ** *** Degrees of risk after DNA contamination

FIG 5—Theoretical consequences of contamination in preimplantation diagnosis of cystic fibrosis (Δ F508)

occur by contamination. There are four reasons. Firstly, when a normal gene contaminates a homozygous abnormal embryo the DNA result is a heterozygous embryo. This embryo would not be transferred. Secondly, when a normal gene contaminates a homozygous normal or heterozygous embryo (about 1% in our study) there is no alteration of the DNA diagnosis. Thirdly, when a homozygous abnormal gene contaminates a homozygous abnormal or heterozygous embryo there is also no alteration of the DNA diagnosis. Fourthly, when a homozygous abnormal gene contaminates a homozygous normal embryo, then DNA misdiagnosis would occur but the embryo would be wasted and not transferred.

If only homozygous normal embryos are to be transferred with loss of the embryo cell (or DNA), or if heterozygous embryos are to be transferred, then in the 1% of cases in which contamination might occur there would be homozygous normal, heterozygous, and affected babies in a ratio of 1:2:1 when both parents are heterozygous carriers. It should be important to set up five or 10 blank control tubes to identify the amount of contamination in the same batch of samples for further calculation (to be detailed in a future report). If no contamination is found in the blanks of that batch and the cell contains a normal nucleus, then the diagnosis is also very precise, allowing heterozygous embryos to be confidently transferred.

OTHER METHODS OF DIAGNOSIS

Several different methods have been examined for the diagnosis of cystic fibrosis. Some primers were designed towards locus CS.7, which is located only close to rather than directly covering the mutation points of cystic fibrosis.²⁰ This method was later abandoned.

A method of direct electrophoresis of 95/98 bp (mutant gene/normal gene) bands by size difference and another method of heteroduplex formation (namely, normal-mutant gene combination) yielded an amplification rate of 84% in single human polar body and embryo cell experiments but with unclear and unstable bands.^{21, 22} Until recently the reason for heteroduplex rather than homoduplex formation (that is, normal-normal or mutant-mutant gene combination) was not thoroughly understood. The method was originally published (in 1975) for location of the position of the mutation and for sequencing²³ but only around half to three quarters of all mutations could be detected in the β globin gene.^{24, 25} The fidelity of the method was adversely influenced by the poor discrimination (or separation) rate (at the same separation temperature) of the normal and mutant homoduplexes of the cystic fibrosis gene, even with blood DNA.^{26, 27} In single cell experiments for the diagnosis of cystic fibrosis a correct diagnosis rate of only 80% (48/60)²⁸ rather than 100%²⁹ was reported.

Under these conditions the heteroduplex method has been used for preimplantation diagnosis in humans but the amplification rate was only 75% (12/16) with nucleated embryo cells.²⁹ It is clear that failure of amplification was due to failure of the technique and failure to differentiate normal and abnormal nuclei. Hence the likelihood of misdiagnosis was very high. Two misdiagnoses were reported with the same heteroduplex method for cystic fibrosis. The primers used²⁹ were also not suitable for single cell amplification (unpublished data). Thus a reported birth²⁹ does not mean that these three basic technical problems have been solved. Routine use of the two cell embryo biopsy^{18, 29} to patch these shortcomings of the diagnostic method is also inappropriate owing to safety problems. The use of "a series of pregnancies" (human fetal life) first rather than basic scientific experiments "to assess the method"²⁹ (or design) raises important ethical

Key messages

- A fundamental requirement of routine pre-implantation diagnosis is precision of diagnosis
- Misdiagnoses in sex linked diseases and cystic fibrosis are mainly due to limitations of methods
- A basic experimental design with 100% correct results when testing blood DNA, single lymphocytes, embryos, and single embryo cells combined with good clinical practice and success in an animal model were the key factors in good outcome of this study
- Applying direct polymerase chain reaction amplification of mutation in general screening, preimplantation, and prenatal diagnosis should lead to a substantial reduction in cystic fibrosis in the future

questions and has been the subject of severe and increasing criticism.^{4, 9, 13-15, 30}

In preimplantation diagnosis of cystic fibrosis polar body biopsy and double biopsy (polar body biopsy followed by embryo biopsy in the same embryo) have been reported.²² It was recognised that polar body biopsy "will not make it possible to establish genetic diagnosis of the embryo" because (a) paternal alleles cannot be analysed, (b) the test might not be efficient owing to loci crossing over, and (c) sex determination is not possible.²²

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Commentary: Preimplantation diagnosis raises a philosophical dilemma

J A Raeburn

Preimplantation genetic diagnosis followed by implanting healthy embryos offers high risk couples the option to decrease the risk of disease in their offspring without the dilemma of a prenatal diagnosis which may be followed by a termination of pregnancy. If the technology of preimplantation diagnosis were easy or inexpensive (which it is not) the approach would supplant prenatal diagnosis for several single gene disorders.

Ethics and basis of test

In principle the ethics of selecting healthy embryos to implant do not differ from those of early prenatal diagnosis with the selection of unaffected fetuses. However, because genetic tests before implantation can remove the need for abortion they are a preferred approach for couples who are offered the choice (L Jenkins and J A Raeburn, unpublished data, 1994). Cui *et al* describe and evaluate the technology required to carry out preimplantation diagnosis of cystic fibrosis in carrier couples (who have a one in four risk of having affected offspring).¹ The general reader does not require detailed knowledge of the relevant technology and scientific principles but ought to be aware of the possibilities and implications.

The basis of preimplantation testing is that single cells are obtained by biopsy from pre-embryos formed in vitro. The relevant gene sequence is then amplified by the technique of polymerase chain reaction, in which oligonucleotide sequences of about 24 bases long—which flank the relevant part of the gene—are used to amplify both the common cystic fibrosis mutation, $\Delta F508$, and the normal gene sequence. If the pre-embryo genotype is homozygous normal the two copies of the normal sequence in the cell (one copy from each parent) will be amplified. If the pre-embryo is of carrier genotype both a normal gene and a cystic fibrosis gene will be present and both will amplify, producing a mixture of normal and cystic fibrosis sequences. If the pre-embryo is abnormal (homozygous for the cystic fibrosis gene) only the cystic fibrosis sequences will amplify. Cystic fibrosis sequences are

clearly distinguishable from normal by having a three base pair deletion, which leads to a different electrophoretic mobility.

Carriers are not "abnormal"

The wonder of polymerase chain reaction techniques, and the likeliest cause of error, lies in the ability to amplify a single copy of the relevant sequence rapidly. Thus, though single cells contain only one copy of the gene conformation from each parent—or at the most two copies of each if DNA synthesis has occurred—polymerase chain reaction can in a few hours replicate with precision the target stretch of DNA by as much as a millionfold. The danger is that the pre-embryo cell could be contaminated with other DNA, thus invalidating the amplification procedure (and the whole test). To minimise this error Cui *et al* (backed by mathematical models) have analysed the effect of contamination in different circumstances. The risk of fetal misdiagnosis would be lowest if only identified normal homozygotes were implanted.

Therein lies the dilemma. Heterozygotes for cystic fibrosis mutations are not "abnormal," as would be implied by any decision not to implant them. Thus preimplantation testing carries the danger of discrimination. Selection before implantation is so straightforward conceptually that it could easily lead to "technological stigmatisation." Should we select only normal homozygotes, or should we counsel couples that in a small percentage of situations carrier embryos may not be distinguishable from abnormal?

BMJ readers will have strong views about this because all are heterozygotes for recessive mutations which in the homozygous state could be lethal. Well informed couples may or may not choose to accept some low degree of risk of having a cystic fibrosis pregnancy. That decision must not be based on technological aspects alone.

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