Prenatal detection of trisomy 21 and 18 from amniotic fluid by quantitative fluorescent polymerase chain reaction

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Abstract
Prenatal diagnosis of fetal trisomies is usually performed by cytogenetic analysis on amniotic fluid. This requires lengthy laboratory procedures and high costs, and is unsuitable for large scale screening of pregnant women. An alternative method, which is both rapid and inexpensive and suitable for diagnosing trisomies even from single fetal cells, is the fluorescent polymerase chain reaction using polymorphic small tandem repeats (STRs). In this paper we present the preliminary results of a larger study comparing parallel prenatal diagnoses of trisomies 21 and 18 using cytogenetics with quantitative fluorescent polymerase chain reaction using STR markers. The results obtained by the two techniques were concordant in all cases. This is the first study reporting significant numbers of prenatal diagnoses using the quantitative fluorescent polymerase chain reaction. We believe that further studies on greater numbers of samples will determine the absolute reliability of this technique. These results also provide a model for diagnosis of trisomy from single fetal cells isolated from maternal blood.


Keywords: Down syndrome; prenatal diagnosis; fluorescent polymerase chain reaction

Chromosomal abnormalities are the most frequent genetic disorders observed in both live births and miscarriages. Trisomies, the largest group, accounts for 53% of all chromosome abnormalities in early fetal deaths, directly result in 17% of all fetal deaths, and are therefore a major cause of spontaneous abortion.†

The most common single trisomies are 21, 21, 16, 13, 2, and 18, of which only 21 and 18 are generally seen in live births. Even though more than two-thirds of trisomy 21 pregnancies result in fetal death, trisomy 21 (Down syndrome) has the highest birth prevalence (approximately 1 affected newborn in 700-800 births§). Trisomy 21 results in moderate to severe mental retardation, high risk of congenital heart disease, and other multiple malformations and is the major reason that older mothers seek prenatal diagnosis. The next most common trisomy observed in prenatal diagnosis is trisomy 18 (Edwards syndrome). Even though ~90% of trisomy 18 pregnancies result in fetal death, this syndrome has an incidence of 1 affected newborn in 1000 live births and results in severe mental retardation, multiple malformations, and is generally fatal before 1 year of age.

As the frequency of chromosomal abnormalities increases with maternal age,† trisomy screening by prenatal diagnosis (by amniocentesis or chorionic villus sampling) has become accepted by women over 35 years of age.‡ However, despite the extensive care of pregnant women over 35 years of age, the birth prevalence of Down syndrome remains high, as two-thirds of Down syndrome pregnancies occur in women under 35 years of age. Therefore to reduce significantly the birth prevalence of trisomies, a wide ranging screening programme of pregnant women has been suggested.

Current methods, such as cytogenetic analysis, for detecting chromosomal abnormalities often require lengthy laboratory procedures and incur high costs as well as significant delay in providing a diagnosis. These tests are therefore not suitable for large scale screening of all pregnant women. An alternative method, which is both rapid, inexpensive, and suitable for diagnosing trisomies, is the fluorescent polymerase chain reaction using polymorphic small tandem repeats (STRs). The quantitative nature of this technique allows the amount of PCR product to be determined and thus allows peak ratios to be calculated. Although the first description of this method was reported by Mansfield in 1993,§ there have been only a few reports§ applying this technique clinically to trisomy detection in prenatal diagnosis.

We report our experiences using a fluorescent polymerase chain reaction based investigation of D21S11 and D18S51 tetranucleotide STR markers to determine reliably trisomy 21 and 18 status from amniotic fluid samples.

Materials and methods
DNA PREPARATION
Cells from 1 ml amniotic fluid were collected by centrifugation at 15 000 rpm for 10 minutes. The supernatant was discarded and 100 μl of resin from ReadyAmp Genomic
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<table>
<thead>
<tr>
<th>Marker</th>
<th>Homozygous</th>
<th>Heterozygous</th>
<th>Total tested</th>
<th>Heterozygosity</th>
<th>Relative peak areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>D21S11</td>
<td>23</td>
<td>185</td>
<td>208</td>
<td>0.89</td>
<td>1.07, 0.052, 1.00-1.22</td>
</tr>
<tr>
<td>D18S51</td>
<td>14</td>
<td>195</td>
<td>209</td>
<td>0.93</td>
<td>1.09, 0.064, 1.00-1.26</td>
</tr>
</tbody>
</table>

Purification System (Promega, USA) was added to the pelleted cells. The subsequent extraction procedure was performed according to the manufacturer's protocol. Six microlitres from this DNA solution were used in each PCR reaction.

FLUORESCENT POLYMERASE CHAIN REACTION
PCR amplification was carried out in two separate assays, one with primers for marker D21S11 and the second with primers for marker D18S51 (Genomic Data Base). Primer sequences are listed in table 1. The D21S11 and D18S51 forward primers were labelled at the 5' end with 5-carboxy-fluorescein (FAM) and TAMRA labels. PCR products were performed in a total volume of 50 µl containing 6 µl of DNA solution prepared by the Promega kit, Taq polymerase buffer (Perkin Elmer), 200 µM of dNTPs, 10 pmol of each primer, 1.5 mmol/l MgCl₂, and 1.0 U AmpliTaq (Perkin Elmer). After denaturation at 95°C for five minutes, 30 cycles at 95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 45 seconds were performed in a Perkin Elmer 2400 thermal cycler. Final extension was for 10 minutes. The entire procedure took two hours.

RESULTS

In a 10 week period, 212 pregnant women underwent amniocentesis to rule out fetal chromosomal abnormalities either because of maternal age or previously detected ultrasound abnormalities. Informed consent was obtained from all patients. Ten millilitres of amniotic fluid were removed; 9 ml were used for cytogenetic analysis and 1 ml was stored for molecular genetic analysis. Samples from each patient underwent karyotyping and trisomy 21 and 18 diagnosis using D21S11 and D18S51 STR markers, respectively.

Of the 212 samples, seven showed an abnormal karyotype according to the cytogenetic results. Trisomy 21 was detected in four samples and trisomy 18 in a further three samples. These seven pregnancies were terminated and fetal liver tissues stored for further investigations. Molecular genetic analyses were performed on the stored amniotic fluid samples by amplifying STR markers on chromosomes 21 and 18. The number of homozygous and heterozygous samples for the examined markers and relative peak areas of heterozygous samples are shown in table 2. In all cases PCR results were consistent with cytogenetic results. Two trisomy 21 (fig 1) and one trisomy 18 (fig 2) samples showed a characteristic trisomic pattern with three STR peaks of similar intensity corresponding to three different alleles, while the other two trisomy 21 (fig 1) and two trisomy 18 (fig 2) samples showed a characteristic diallelic pattern with a fluorescent intensity ratio of 2:1.

Although ratio ranges of normal heterozygous and diallelic trisomic samples did not overlap, diallelic trisomic samples should be investigated further using other markers on each chromosome to determine the quantitative amplification of different alleles and thus the trisomy.
preliminary experiments show that fluorescent PCR amplification of STRs can reliably be used for the detection of trisomies of both chromosomes 21 and 18. Allelic drop out, as described by Findlay et al, was not observed during the PCRs (table 2, relative peak areas-range). However, to minimise the chance of misdiagnosis, the PCRs were repeated. The introduction of additional markers is also recommended and this is currently under investigation in our laboratory. STR markers on other chromosomes could also be used in a similar manner allowing detection of other chromosomal abnormalities by molecular genetic methods rather than cytogenetic analysis.

We believe that using two highly polymorphic STR markers on each chromosome would allow diagnostic accuracy to be increased in most of the cases investigated. In non-informative cases further polymorphic marker(s) could be amplified. We are currently using a multiplex PCR assay with several STR markers for chromosomes 21, 18, and 13 which will allow this technique to become even more valuable in the prenatal detection of trisomies. Quantitative fluorescent PCR was used in parallel with cytogenetic analysis for prenatal detection of trisomies as a "back up" in case of culture or cytogenetic analysis failure.

This technique allows detection of trisomies within four hours in urgent cases, allowing amniocentesis and possible termination to be undertaken on the same day. This is especially important when the pregnancy is relatively advanced as delay may lead to gestation exceeding the legal limit for termination. Amniocentesis takes about 10 minutes and DNA preparation from amniotic fluid takes only 50 minutes using the DNA purification system (Promega, USA) instead of a long phenol/chloroform extraction procedure. The length of PCR is two hours and the detection of one sample by the ABI 310 DNA sequencer takes 30 minutes. A time limit can be very important, for example in cases of preimplantation diagnosis.

As fluorescent PCR can also be performed from single cells, this technique appears to be suitable for prenatal diagnosis of trisomies from isolated fetal cells from maternal blood or in preimplantation diagnosis from blastomeres obtained by embryo biopsy. Further investigations, which are in progress, will determine the reliability of this technique in these fields.

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4 Ferguson-Smith MA, Yates JRW. Maternal age specific rates for chromosome aberrations and factors influencing them: report of a collaborative European study on 52965 amniosentences. Prenat Diagn 1984;4:3-44.
5 Mansfield ES. Diagnosis of Down syndrome and other aneuploidies using quantitative polymerase chain reaction


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