Fetal Sex Determination using Non-Invasive Method of Cell-free Fetal DNA in Maternal Plasma of Pregnant Women During 6th–10th Weeks of Gestation

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Abstract
In previous years, identification of fetal cells in maternal blood circulation has caused a new revolution in non-invasive method of prenatal diagnosis. Low number of fetal cells in maternal blood and long-term survival after pregnancy limited the use of fetal cells in diagnostic and clinical applications. With the discovery of cell-free fetal DNA (cffDNA) in plasma of pregnant women, access to genetic material of the fetus had become possible to determine early gender of a fetus in pregnancies at the risk of X-linked genetic conditions instead of applying invasive methods. Therefore in this study, the probability of detecting sequences on the Y chromosome in pregnant women has been evaluated to identify the gender of fetuses. Peripheral blood samples were obtained from 80 pregnant women at 6th to 10th weeks of gestation and then the fetal DNA was extracted from the plasma. Nested PCR was applied to detect the sequences of single copy SRY gene and multi copy DYS14 & DAZ genes on the Y chromosome of the male fetuses. At the end, all the obtained results were compared with the actual gender of the newborns. In 40 out of 42 born baby boys, the relevant gene sequences were identified and 95.2% sensitivity was obtained. Non-invasive early determination of fetal gender using cffDNA could be employed as a pre-test in the shortest possible time and with a high reliability to avoid applying invasive methods in cases where a fetus is at the risk of genetic diseases.

Keywords: Fetus, Genetic material, Prenatal diagnosis, Sex determination

Introduction
Traditionally, early fetal gender determination has been performed using invasive techniques, such as chorionic villus sampling or amniocentesis. These procedures, however, still carry a risk of miscarriage around 1-2% and cannot be performed until 11 weeks of gestation (1). Also, reliable determination of fetal sex by means of ultrasonography cannot be done in the first trimester because of uncompleted development of the external genitalia (2).

Therefore, more efforts have been spent in developing prenatal diagnostic procedures that do not constitute a risk for the fetus, based on the analysis of fetal genetic material obtained from the fetal cells circulating in
maternal blood \(^ {3,4}\). Substantial advances have been made in the enrichment and isolation of fetal cells for analysis, but most techniques are time-consuming or require expensive equipment. In addition, these cells are very rare in maternal plasma (1 fetal cell per 10\(^6\) maternal cells) and they are unlikely to persist after delivery, including subsequent pregnancies \(^ {5,6}\). Further studies on tumour derived DNAs in the plasma of cancer patients open up the possibility that fetal DNA which originated from apoptotic trophoblasts of the placenta, may also be found in maternal plasma \(^ {7,8}\). Finally with the discovery of cell-free fetal DNA (cffDNA) fragments in the plasma of pregnant women carrying male fetus in 1997, reliable and accurate diagnosis became reality \(^9\).

More studies revealed that the concentration of fetal DNA in maternal plasma was found to be much higher than that present in the cellular fraction (25.4 GEq/ml in early stage of pregnancy and 292.2 GEq/ml in late stage of pregnancy) \(^ {10}\) and the post-partum clearance of cffDNA from the maternal circulation was rapid with a mean half-life of 16.3 min \(^ {11}\). Also, fetal DNA molecules are generally shorter than maternal DNA molecules (between 193 bp to 313 bp). Therefore, it can be distinguished from the maternal DNA by size separation \(^ {12}\). These findings have provided the opportunity to perform reliable genetic testing on cffDNA extracted from the maternal plasma at an early stage in pregnancy without interference from previous pregnancies for non-invasive prenatal diagnosis of paternally inherited disorders as well as fetal gender determination.

Using cffDNA in maternal plasma for fetal gender determination is mainly limited to those sequences which are absent from the maternal genome such as the \textit{SRY}, \textit{DYS14} and \textit{DAZ} that are located on the Y chromosome. Therefore, the only way to identify these sequences is through male-bearing pregnancies \(^ {13}\).

In this study, early determination of fetal gender using cffDNA can be considered as a non-invasive pre-test to determine whether invasive prenatal diagnosis should be performed on a fetus having a risk of X-linked disorders or not. Thus, invasive procedures can be avoided when the fetus is known to be female at an early gestational age, while prenatal diagnosis might be performed only for male fetuses. To achieve this goal, the following study using cffDNA in maternal plasma was performed on pregnant women during their 6\(^{th}\) -10\(^{th}\) weeks of pregnancy to obtain the required sensitivity, specificity and accuracy for a non-invasive prenatal test.

**Materials and Methods**

**Sample collection**

Peripheral blood samples were obtained from 80 pregnant women at their 6\(^{th}\) to 10\(^{th}\) weeks of gestation who were referred to Avicenna Infertility Clinic in Tehran, Iran during 2009-2010. Also in this study, five non-pregnant women and five men were considered as a negative and positive control. Before blood sampling, signed consent forms were obtained from all participants and the protocol of the study was approved by the ethics committee of Avicenna research institute. For each case, 5 ml peripheral blood was collected in a tube containing 200 µl of 0.5 M EDTA and immediately stored at 4 °C. Within 24 hr after collection, blood samples were centrifuged at 3000 g for 10 min and the upper plasma layer was carefully removed without disturbing the buffy coat, transferred into a new Eppendorf tube for storage at -20 °C until further processing.

**DNA Extraction**

Genomic DNA was extracted from 200 µl of the plasma samples using the QIAamp DNA Blood Mini kit (Qiagen, USA) as recommended by the manufacturer according to the manufacturer’s “Blood and Body Fluid” protocol. The extracted DNA was eluted in 50 µl of the elution buffer.

**Primers**

Due to the low concentration of cffDNA in maternal plasma, multicopy \textit{DAZ} and \textit{DYS14}
genes were used for detection of sequences on the Y chromosome in male-bearing pregnancies. Also, sequence of single copy \textit{SRY} gene was used as an internal control of gender determination. In order to assess the presence of sufficient cell-free DNA in an extraction, analysis of the \textit{ACTB} sequences was performed. All the pairs of primers were designed by using the Primer3 and Gene Runner software (Table 1).

**Nested PCR**

The fetal sex was identified by nested PCR technique to amplify the \textit{DYS14} and \textit{DAZ} sequences, whereas the semi-nested PCR was used for amplification of \textit{SRY} sequences. In the first round of PCR, the amplification reactions were set in a total volume of 25 μl containing: 2 μL of 10 ng genomic DNA, 2.5 μL of 10X PCR buffer (Roch, Germany), 1 μl of 10 mM dNTPs, 10 pmol of each primers, 1 unit of Taq DNA polymerase (Roch, Germany), 2.5 μl of Mgcl\textsubscript{2} (for \textit{SRY} and \textit{DAZ}) and a 3 μl of Mgcl\textsubscript{2} for \textit{DYS14}. Amplification was performed using a programmable thermal cycler gradient PCR system (Eppendorf, Germany) with an initial denaturation of 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec (for \textit{SRY}, \textit{DYS14}) and 64°C (for \textit{DAZ}), extension at 72°C for 30 sec and the final extension at 72°C for 10 min.

One μl of the PCR products were used as template for the second round of PCR. The reaction system and thermo-cycling condition were the same as those of the first round of PCR but they were carried out in 35 cycles. At the end of each round of PCR, amplification products were visualized by staining with ethidium bromide after electrophoresis on 2% agarose gel. To recognize contamination, a non template control, containing DNA free water was also added in each reaction.

**Anti-contamination measures**

As an anti-contamination measure, an aerosol resistant pipette tips were used for all liquids and separate areas were considered for all the steps of the analysis. In order to eliminate contact with exogenous male DNA, only female operators were selected in all procedures using a laminar flow hood.

**Statistical analysis**

At the end, all the obtained results were compared with the actual gender of the newborns to calculate the sensitivity, specificity and accuracy of the applied method. Kappa coefficient of agreement was calculated to evaluate the precision and correctness of the method and for evaluation of the clinical application, parameters such as Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were also analysed. Statistical analysis was performed using the SPSS statistical package (V.11.5).

**Results**

Statistical analysis of the data showed that from the total of 80 samples obtained from pregnant women with age range of 18 to 46 years (mean age=30.36) and pregnancy week range from 6 to 10 weeks (mean week=7.74), abortion was observed in 5 cases. Although the gender determination tests were conducted on these 5 samples, they were excluded from statistical analysis due to lack of knowledge.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5' → 3')</th>
<th>Primer name</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{SRY}</td>
<td>Forward: TACAGGCCATGCACAGAGAG</td>
<td>\textit{SRY}</td>
<td>Forward: AGTATCAGCCTGCAGAGAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCTTGAGTGTGTGGCTTTCG</td>
<td></td>
<td>Reverse: TCTTGAGTGTGTGGCTTTCG</td>
</tr>
<tr>
<td>\textit{DYS14}</td>
<td>Forward: AGCCCTGATCATCGACAGAGAG</td>
<td>\textit{DYS14}</td>
<td>Forward: AGGAAGACTGGGGCTAGAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGCAAGAGATGAAACAGTAGTGC</td>
<td></td>
<td>Reverse: ACCCTGACAGCAAAGGTGGA</td>
</tr>
<tr>
<td>\textit{DAZ}</td>
<td>Forward: TACCTCCAAGACACCAGACC</td>
<td>\textit{DAZ}</td>
<td>Forward: TACCTCCAAGACACCAGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: AATCTACCCATCTCCCGAAC</td>
<td></td>
<td>Reverse: TGAGGAGGCAATCTGGAAATC</td>
</tr>
<tr>
<td>\textit{ACTB}</td>
<td>Forward: GATGTTGGCCTGGGTCAGAAGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: CATTGTAGAAGGTTGTTGGGCAGAT</td>
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<td></td>
</tr>
</tbody>
</table>
of the actual fetal gender to compare with test results. Also, among the 80 samples, 16 cases with multiple gestations (9 identical twins, 6 non-identical twins and 1 triplet) were observed which were included in our statistical analysis.

**Nested PCR results**

In all the obtained samples, *ACTB* sequences were amplified (PCR product length=149 bp) indicating the presence of sufficient DNA in the extracted samples (Figure 1A). Regarding the *SRY*, *DYS14* and *DAZ* sequences, no positive bands of PCR were observed in samples during the first round of PCR. However, on the electrophoresis results of the second round of PCR (Figures 1B, C, and D), positive bands (PCR product length=143 bp, 122 bp and 156 bp, respectively) were observed for each *SRY*, *DYS14* and *DAZ* sequences in 40 samples similar to positive control DNA sample which was recorded as positive, and as a result the male gender. On the other hand, in the absence of positive bands of PCR for 2 or 3 of the mentioned sequences in fetal samples similar to negative control DNA sample. These were considered as negative and would represent the female gender. It should be mentioned that in all the 40 samples classified as male fetuses all the 3 marker targets were positive.

Comparison of the obtained results with the actual birth outcome indicates that from 50 baby girls born, 49 fetal genders were correctly diagnosed and only in one case, positive result was obtained. Also from 42 baby boys born, 40 genders were correctly diagnosed by using the *SRY*, *DYS14* and *DAZ* sequences (Table 2).

The sensitivity and specificity of the method used in fetal gender determination (with 95% confidence intervals) were respectively 95.2% (95% CI= 0.842 to 0.987) and 98% (95% CI= 0.895 to 0.996). Kappa coefficient of agreement in relevant test is 0.934 and the p-values=0.001 were considered stat-

<table>
<thead>
<tr>
<th>Gestational age</th>
<th>Result at birth</th>
<th>Result by Nested PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Weeks</td>
<td>Female 4</td>
<td>Male 1</td>
</tr>
<tr>
<td></td>
<td>Male 0</td>
<td>7</td>
</tr>
<tr>
<td>Total Gender at Birth</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>7 Weeks</td>
<td>Female 14</td>
<td>Male 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Male 0</td>
<td>7</td>
</tr>
<tr>
<td>Total Gender at Birth</td>
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<td>9</td>
</tr>
<tr>
<td>8 Weeks</td>
<td>Female 20</td>
<td>Male 0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Total Gender at Birth</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>9 Weeks</td>
<td>Female 8</td>
<td>Male 0</td>
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<tr>
<td></td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Total Gender at Birth</td>
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<td>4</td>
</tr>
<tr>
<td>10 Weeks</td>
<td>Female 3</td>
<td>Male 0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total Gender at Birth</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Total sensitivity of the test</td>
<td>95.2% (95% CI= 0.842 to 0.987)</td>
<td></td>
</tr>
<tr>
<td>Total specificity of the test</td>
<td>98.0% (95% CI= 0.895 to 0.996)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> False positive result  
<sup>b</sup> False negative result

Table 2. Comparison of test results by nested PCR with the actual birth outcome in 80 samples during 6<sup>th</sup> to 10<sup>th</sup> weeks of gestation

Figure 1. The agarose gel electrophoresis result of *ACTB* (A), *SRY* (B), *DYS14* (C) and *DAZ* (D) amplification by nested PCR analysis in fetal DNA samples extracted from the maternal plasma. Lanes 1 & 2: Fetal DNA, Lane 3: Male DNA as a positive control, Lane 4: non pregnant women DNA as a negative control and Lane 5: PCR reaction negative Control (Water). A) 149 bp Positive bands in lane 1 and 2 indicate the presence of sufficient DNA in the extracted samples. Observation of positive bands 143 bp B) 122 bp C) and 156 bp D) in lane 2 shows the amplification of the relevant genes and indicate that the gender of the fetus is male. The absence of positive band in lane 1 indicates the female gender of the fetus.
pistically significant in each analysis using 
*SRY*, *DYS14* and *DAZ* sequences. The PPV and 
NPV of the method used respectively were 
97.6% (95% CI= 0.895-0.996) and 96.1% 
(95% CI=0.868-0.989).

**Discussion**

In recent years, the existence of circulating 
fetal cell-free DNA in maternal blood which 
was discovered by Lo et al in 1997, created an 
outstanding revolution in non-invasive pre-
natal diagnosis. Fetal nucleic acids can be ob-
tained from a simple blood draw of the 
mother that is risk-free and highly cost ef-
effective compared to conventional invasive 
methods. Despite the low concentration of 
cffDNA (3.4% to 6.2% of total maternal 
DNA) and with the advent of molecular tech-
niques such as Polymerase Chain Reaction 
(PCR), nucleic acid based testing has become 
a valuable source for non-invasive prenatal 
diagnosis since nucleic acids can be amplified 
(10). Therefore, in this study by using nested 
PCR technique, 95.2% sensitivity was achiev-
ed in identification of male fetuses using 
*SRY*, *DYS14* and *DAZ* sequences. This is very close 
to the sensitivity obtained in previous studies. 
And in most cases, even significantly higher 
sensitivity was obtained.

Previous investigations have shown sensi-
tivity of 94% (Smid et al, 1999), 96% (Al Ya-
tama et al, 2001), 94% (Zolotukhina et al, 
2005) and 88.2% (Hong et al, 2006) (14-17). 
However in these findings, longer time range 
of pregnancy were considered, which led to 
high probability of identifying cffDNA be-
cause of the gradual increase of its concentra-
tion with increasing gestational age (18). In 
comparison, the results obtained in this study 
in the 6th to 10th weeks of pregnancy led to 
significantly better results.

Overall, the differences observed between 
the results of the previous studies and this 
study can be explained through the use of dif-
ferent methods of fetal DNA extraction, low 
concentration of cffDNA at an early gesta-
tional age, number of population, time range 
of the pregnancy and existence of potential 
contamination.

In this study, false positive result was ob-
erved only in one case that gender of female 
fetus, which was at the age of six weeks, was 
diagnosed as a male. Citing the theory of van-
ishing twins within the first 7 weeks of gesta-
tion in 0.3-0.7% of pregnancies (19), it can be 
concluded that during the time of sampling in 
the 6th week of gestation, there was a male 
twin that disappeared in the subsequent weeks 
of pregnancy and only the baby girl was born.

On the other hand, false negative results 
were obtained in two cases of pregnancies 
with non-identical twins which can be the 
result of extraction failure of the male twin 
DNA compared to the female twin due to its 
low concentration of cffDNA. Therefore if we 
do not consider multiple gestations in our 
study, we have reached a significant 100% 
sensitivity.

In comparison between total numbers of 
fetuses that were correctly diagnosed and total 
numbers of infant born, 96.7% accuracy was 
achieved in fetal gender determination. Also 
according to the Kappa coefficient of agree-
ment, which is in “almost perfect” agreement 
range between 0.81 and 0.99 (20), it can be 
concluded that the results are remarkably con-
sistent with the actual gender of the babies.

To check whether the method used in this 
study is suitable for clinical application or 
not, the parameters of PPV and NPV were 
calculated. The PPV and NPV indicate that if 
the test results are positive and the fetus is 
diagnosed as a boy, there is 97.6% probability 
be a boy and 96.1% probability to be a girl 
if the test results are negative. Therefore, this 
method can be used as a clinical method in 
determining the fetal gender due to its high 
probability of correct prediction, prior to ap-
plying invasive methods.

**Conclusion**

We hope that non-invasive fetal gender 
determination using cffDNAs in maternal 
plasma would allow us to obtain an early
knowledge of the fetal sex and adding to timely clinical management. This could reduce the need for invasive procedures in pregnant women carrying an X-linked disorder up to 50%. Also, hopefully in the near future, this method can be applied as a diagnostic tool for diseases of pregnancy such as pre-eclampsia or preterm labor, or for fetal anomalies such as aneuploidies.

Acknowledgement

We would like to thank all of the individuals who kindly accepted to participate in this study. Also, we are deeply indebted to the personnel of Avicenna Infertility Clinic for their assistance in sample collection and to Ms. H. Edalatkhah for their helpful advices. Financially, this study was supported by Avicenna Research Institute.

References