Original Article

An Effective Method for Detecting Y-chromosome Specific Sequences of Circulating Fetal DNA in Maternal Plasma During the First-trimester

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A B S T R A C T

Background and Aims: New advances in the use of cell-free fetal DNA (cffDNA) in maternal plasma of pregnant women has provided the possibility of applying cffDNA in prenatal diagnosis as a non-invasive method. One of the applications of prenatal diagnosis is fetal gender determination. Early prenatal determination of fetal sex is required for pregnant women at risk of X-linked and some endocrine diseases. The present study was carried out to perform an efficient polymerase chain reaction (PCR) method in order to improve sensitivity, specificity and accuracy of non-invasive fetal gender detection using fetal DNA in maternal plasma during 8th -12th weeks of pregnancy.

Materials and Methods: Thirty-five pregnant women with 8 to 12 weeks of pregnancy were selected for prenatal fetal sex determination. Maternal peripheral blood was collected and cffDNA was extracted from 3-ml of maternal plasma. Two multi copy Y-chromosome-specific region (DYS and DAZ) and a single copy gene (SRY) were amplified by real-time quantitative PCR. Amplification was labeled as positive, negative, or inconclusive according to a stringent algorithm.

Results: Using this method, the sensitivity and specificity of the real-time PCR assay was 100% and 93.8% for prenatal fetal sex detection, respectively.

Conclusions: It is concluded that fetal sex can be determined with a high level of accuracy by our algorithm, after 8 weeks of gestation with cffDNA analysis.

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Introduction

The presence of cell-free fetal DNA (cffDNA) in maternal plasma and serum was first recognized in 1997 [1], and its clinical applications continue to expand thereafter. Non-invasive prenatal diagnosis requires maternal serum analysis detecting the cell-free fetal DNA which avoids the risk of miscarriage associated with invasive procedures such as aminocentesis or chorionic villous sampling [2]. The origin of the cffDNA has remained uncertain although different hypotheses are supposed to derive from dead cells due to necrosis or apoptosis [3], or an active cells release [4] from the placenta or, more specifically, trophoblast as the most likely source [5]. The concentration of cffDNA is low in the first trimester but it rises in the second and third trimester of pregnancy [1]. The cffDNA has been shown to exist in plasma and serum of pregnant women as early as 6 weeks of gestation with concentrations rising during pregnancy and peaking prior to parturition and clears within several hours after birth [6]. The analysis of cffDNA in maternal serum and plasma is currently the method of choice for the noninvasive determination of fetal genetic traits [7].

Early prenatal determination of fetal sex is required for identifying pregnant women at risk of fetal aneuploidy and X-linked diseases. The identification of a male fetus indicates hemizygosity for the X chromosome and could indicate potential diseases if the mother is a carrier [8].

Several reports have been published by targeting Y-chromosome-specific genes in maternal plasma for fetal sex determination. Both the single-copy *SRY* gene sequence [9] and multi-copy regions of the Y chromosome such as *DYS14* marker sequence of the *TSPY* gene [10] and *DAZ* family [11] have been analyzed with polymerase chain reaction (PCR). Real-time quantitative PCR (RT-PCR), providing data in a real-time manner, is amenable to automation and less prone to contamination in comparison with conventional PCR methods [12]. Although results are encouraging, the diagnostic accuracy varies widely depending on the protocols and methods used with sensitivity and specificity ranging from 31% to 100% [13]. The present study was carried out to perform new PCR method so as to improve sensitivity specificity and accuracy of non-invasive detection of cffDNA in maternal plasma during 8th-12th weeks of pregnancy. The hypothesis was that early fetal gender determination using these tests are highly accurate when performed after 6 weeks using stringent reporting criteria that includes a third Y-chromosome-specific sequence for confirmation.

Materials and Methods

Study specimens

The study was performed during one year. Thirty five pregnant women who had visited the Taamin Ejtemayi Hospital, Shahrekord, were included in the non-invasive prenatal diagnosis (NIPD) of fetal sex. Fetal gender was unknown at the time of blood sampling and PCR analysis. It was confirmed later by

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ultrasonography at second trimester and finally at birth. All specimens were coded to facilitate blind testing. All subjects were needed to sign a written informed consent. The study was approved by Shahrekord University Ethics Committee, Shahrekord, Iran. Twin pregnancies and spontaneous miscarriages were not included in the study. Processing was preferably carried out by female personnel to avoid contamination with male genetic material.

Maternal blood samples were obtained during the first trimester of pregnancy (8 and 12 weeks of gestation). Ten mL of peripheral blood were collected into Ethylenediamine-tetraacetic acid (EDTA) containing tubes and centrifuged at 3500g for 10 minutes to separate plasma. The supernatant was then transferred to fresh tubes with care taken to ensure that the blood pellet remains intact. The plasma was then centrifuged at high speed centrifugation (10 minutes at 10000 g and 4˚C) to remove all residual intact cells. Supernatants were stored at -80˚C until DNA extraction.

DNA extraction from plasma

cffDNA was extracted from 3 ml of maternal plasma using the DNP® kit (Sinaclon, Karaj, Iran) according to the manufacture's protocol with some modifications. Reagents were increased proportionately to accommodate the 3 ml sample size. DNA was eluted in 100 μL of elution buffer (provided in the kit) and kept at -80˚C until PCR analysis.

Real-time PCR

The RT-PCR assays were performed using the Rotor-Gene Q 6000 RT- PCR machine (Rotor-Gene Q 6000 System, Qiagene, USA). The Y-chromosome-specific region- a multi-copy marker (DYS14)- was systematically analyzed in all cases to increase the sensitivity and specificity of the assay. An additional multi-copy sequence (DAZ) and a single copy gene (SRY) were implemented as a second-line test. The sequence and the accession number of used primers are presented in table 1. PCR reaction was set up in a final volume of 25 μl using 12.5 μl of SYBR premix EX Tag II (TaKaRa, China), with 400 nM of each primer, and 5 μl of extracted DNA. Amplification was performed for 45 cycles of denaturation (95˚C, 45 s) followed by annealing (63˚C, 30 s) and extension (72˚C, 30 s). The melting curve analysis was performed after the RT-PCR procedure. The fluorescence signals were recorded continuously during temperature ramp (70 to 95˚C). The number of amplification cycles required to reach a fixed threshold signal intensity is referred to as the cycle threshold (Ct). Identical thermal profiles were used for *DYS14*, *SRY*, *DAZ* and the *GAPDH*. In addition, a positive control was used in each reaction (DNA from a male) as well as a negative control (DNA of a non-pregnant female) and a non-template control, used to determine whether there was contamination of any reagent during the reaction procedure.

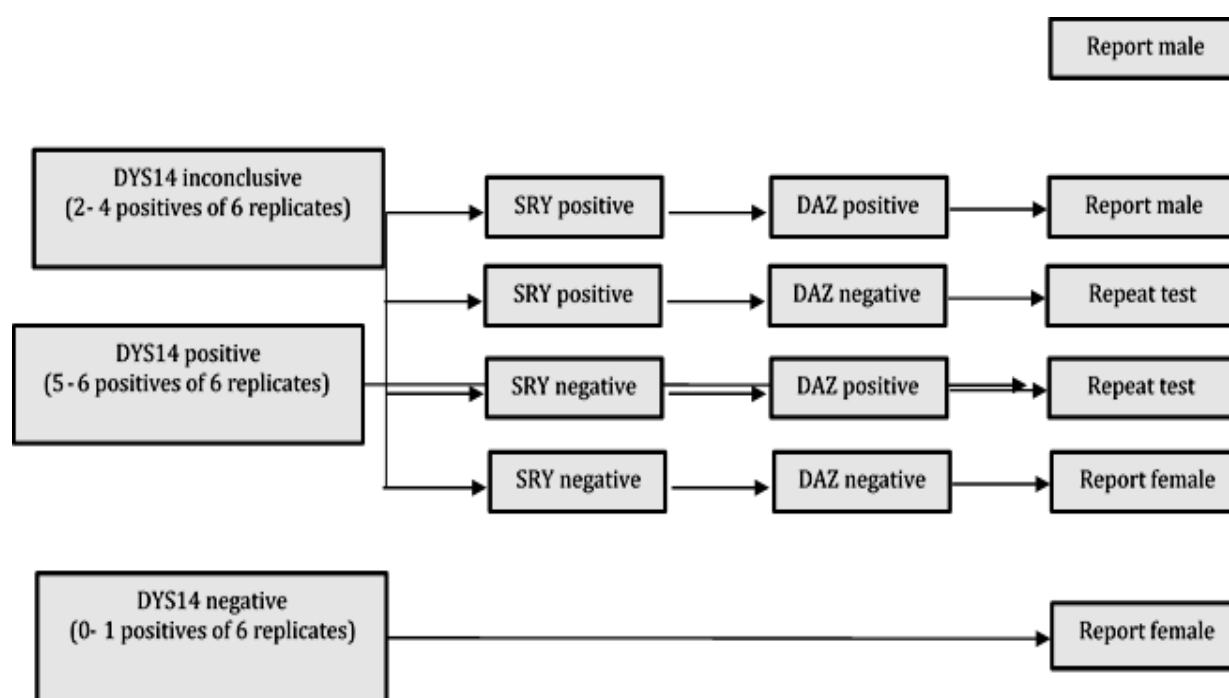**Table 1.** Sequence and the accession number of used primers

Gene name	sequence	Gene bank accession number
SRY	F: GGTACTCTGCAGCGAAGTG R: GTTGATGGGCGGTAAGTGG	MK011516.1
DYS	F: CGCTTTCCCTTCCATCGTG R: TCGTCAGTGATCAGGGCTG	NG-027958.1
DAZ	F: TACCTCCAAAGCACCAGAGC R: AGGCAGACACATAAGGAGAGC	NG_008286.1

Amplification was labeled as positive, negative, or inconclusive according to number of positive samples from six replicates of *DYS14*. Duplicate *SRY* and *DAZ* PCR was used as confirmation when inconclusive results were obtained. A sample was considered positive only when it had a melting point the same as the positive control. Samples were reported as male or female as shown in Figure 1. For positive results (male fetus), only one replicate of the *DYS14* gene was allowed to fail among six replicates. For negative results (female fetus), only one positive result for the *DYS14* gene was allowed among six replicates. For inconclusive results (test should be repeated), 2-4 positive result for *DYS14* gene was obtained and both duplicates of *SRY* and *DAZ* PCR failed to be positive or negative.

Statistical analysis

After collection of all pregnancy outcomes, descriptive statistics were performed using Prism 5 software (GraphPad Software, San Diego, CA). The Fisher exact test (two-sided) was used to determine the test sensitivity and specificity with 95% confidence intervals. The positive predictive value and negative predictive value were also evaluated.

Fig. 1. Algorithm used for fetal sex determination using RT- PCR.

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Results

The PCR efficiencies for the *GAPDH*, *DYS*, *DAZ* and *SRY* assays were 98.5%, 96.8%, 97.5 and 97.1%, respectively. Of 35 pregnant women, 18 delivered male newborns but 17 delivered female ones. Among the 18 women bearing male fetuses and 17 women bearing female fetuses, 16 (89%) and 11 (65%) had conclusive results upon first-line testing, respectively. After continuing with *DAZ* and *SRY*, the results got conclusive for the other male and five female fetuses remained. Test had to be repeated in 2 (5%) cases (1 male fetus and 1 female fetus) because of inconclusive results with the combined use of *DYS14*, *SRY* and *DAZ*. Finally, Y-positive signals were detected in all plasma samples of male pregnancies and only 1 of the 17 women bearing female fetus had a positive Y signal (5% false positive). Overall, the sensitivity of the RT-PCR was 100% as Y-specific PCR amplicons were detected in all of the male fetus samples. The specificity was 93.8% since Y-specific signals were detected in 17 women pregnant with a female fetus (Table 2). The sensitivity of the system was considerably increased by performing 6 replicates *DYS14* of each sample in RT-PCR analysis using cffDNA. As far as our experience is concerned, PCR tests done without a sufficient number of replication may miss a proportion of positive specimens, particularly at early pregnancies with a small amount of cffDNA present in maternal plasma.

Table 2. Statistical parameters of the study.

Samples analyzed	35
Male fetuses	18
Female fetuses	17
False Positive	1
False negative	0
Sensitivity (%)	100 (95% CI 84.9–100%)
Specificity (%)	93.8 (95% CI 76.8–93.8%)
Positive predicted value	94.7 (95% CI 80.5–94.7%)
Negative predicted value	100 (95% CI 81.9 –100%)

CI: Confidence interval

Discussion

The findings of this study demonstrate that noninvasive prediction of fetal sex from examination of cffDNA in maternal blood can be achieved with a high accuracy, using Y-chromosome single-copy and multi-copy sequences following a stringent algorithm. False-negative and false-positive results in sex determination using cffDNA are still a matter of concern. False-negative results due to failure to detect the Y-chromosome sequences may be

associated with significant differences in yielding cffDNA between different DNA extraction methods. It is important to note that cffDNA is fragmented into <300-bp segments and the extraction method should bear the ability for extraction of small fragments of DNA [14-15].

To date, several special kits for isolation of cffDNA from maternal plasma have been introduced [16]. All these kits are highly expensive so that the cost effectiveness of the process of cffDNA extraction needs to be considered. Here we used a new method for cffDNA extraction and showed that it was completely efficient. False-positive results are mainly the consequence of contamination during sample preparation or PCR procedure. RT-PCR reduces the risk of contamination because it has a closed system. Another reason for false-positive results is multi-copy sequences. It has been observed that a multi-copy sequence, such as *DYS14*, is more sensitive, accurate, and efficient than the single-copy *SRY* in the assessment of cffDNA, which is particularly important early in the first trimester of pregnancy when the copy numbers of fetal DNA are low [17]. When *DYS14* is used alone for prenatal fetal sex determination, a high frequency of amplification signals are obtained in female fetuses (35% vs. 63.9%) [18]. The drawback of the *DYS14* sequence, however, is that it has considerable homology to sequences other than the Y chromosome. This probably is the reason of false positive results by amplification of this gene [10]. Therefore, despite the high sensitivity of the *DYS14* sequence that provides the possibility of

detection for very low concentrations of cffDNA, various levels of amplification in female samples is an important problem with this sequence. To resolve this problem and distinguish between the true- and false-positive data, this study incorporated a second multicopy sequence of the Y chromosome, the *DAZ* sequence. *DAZ* PCR amplifications have much better sensitivity than *SRY* but lower sensitivity than *DYS14*. However, there are two reasons that may preclude the application of *DAZ* PCR assay as a first-line test for fetal sex determination: first, the presence of false-negative results in male fetal sex detection because of de novo *DAZ* deletions, which arise with a frequency of 1/4000 [19] and second, the twofold higher number of copies of the *DYS14* compared to *DAZ* sequence [10] and as a result the higher sensitivity for *DYS14* in detection of male pregnancies.

Another important point in preventing the false positive results is centrifugation method. It is demonstrated that fetal CD34$^+$ or CD34$^+$CD38$^+$ cells from the prior pregnancy persists in maternal blood up to 27 years after the last pregnancy [20]. Therefore, not only a second microcentrifugation step but also the g-force and time used are essential to obtain truly cell-free plasma. It has been shown that some false positives are due to the presence of a vanishing (male) twin, although this is only expected to cause a false-positive result in around 0.3%-0.7% of cases [21]. In prenatal fetal sex determination based on cffDNA, female fetuses are not detected directly but only inferred by a negative result for Y-chromosome-specific sequences. This can cause false-negative results

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because of undetectable levels of cffDNA. Some studies have used a combination of the *DYS14* and *SRY* sequences to improve sensitivity and specificity of the assay [22-23] or testing for only *DYS14* has been suggested in some other studies because of the higher sensitivity for this sequence [10-17]. Analysis based on only *DYS14* requires to set a cut off for threshold cycle (Ct) in qPCR analysis that may be inconsistant in the laboratories.

In this study, a case analyzed simultaneously with *DYS14*, DAZ and *SRY* and a robust algorithm for reporting fetal sex was designed to overcome the false-positive and false-negative results. The inclusion of two multicopy Y-chromosome-specific assays increased the sensitivity and matching the results with *SRY* increased the specificity as a whole.

Conclusion

Noninvasive fetal sex determination in maternal plasma can be translated into clinical practice for gender specific inherited disorders. We conclude that fetal sex can be determined with a high level of accuracy by analyzing cffDNA after 8 weeks of gestation using different Y specific loci and it is hoped that applying this method for non-invasive prenatal diagnosis can also be extended for single gene disorders. The need for invasive diagnostic testing in women with a high risk of sex-linked genetic disease can be significantly reduced through this test.

Conflict of Interest
The authors declare no conflict of interest

Acknowledgments

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