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Comparative Value of Real Time and Conventional PCR in Antenatal Gender Determination

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- DAZ gene
- X-linked genetic disorder

Abstract

Invasive procedures including chorionic villus sampling and amniocentesis in sex-linked diseases increase the risk of fetal loss. Therefore, Noninvasive fetal gender determination using cell-free fetal DNA (cffDNA) in maternal plasma may be promising. Fifty pregnant females with gestational age ranging from six to ten weeks were included. cffDNA were extracted from maternal plasma and amplified by real time and conventional PCR for identification of SRY, DYS14 and DAZ genes as specific genetic markers for male-bearing pregnancies. In general, sensitivity and specificity of real time PCR was better than conventional PCR. However, sensitivity of DYS14 gene and specificity of SRY gene by real time PCR was equal to those of conventional PCR. Sensitivity of DYS14 gene was the highest and sensitivity of SRY gene was the lowest. However, combination of the three Y-chromosomal sequences in the diagnosis increased the accuracy of the test, which is suitable for clinical application.

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Introduction

The determination of fetal gender during the first trimester of pregnancy for those with a family history of sex-linked disease has turned out to be fundamental today to guarantee their optimal management. In recessive X-linked disease, caused by a mutation on the X chromosome, just male fetuses are affected. In congenital adrenal hyperplasia, just female fetuses can develop external genitalia virilization. The complete antenatal diagnosis can only be done through invasive testing utilizing chorionic villus sampling (CVS). Nevertheless, CVS is infrequently difficult to perform and is accompanied by a risk of miscarriage evaluated to be around 0.5–1% [1]. Therefore, sonographic determination of fetal gender in the first trimester; as a non invasive antenatal diagnostic method, has been attempted by some utilizing trans-vaginal or trans-abdominal ultrasound. However, it is only reliable in the absence of risk of sexual ambiguity and only at the end of the first trimester, which is a relatively late time for diagnosis of fetal gender particularly in the presence of high risk of sex-linked genetic diseases [2].

In pregnant females, the cell-free fetal DNA (cffDNA) identification in their serum and plasma [3] offered an alternative source of fetal genetic material permitting possible recognition of the Y chromosome sequences in the mother's blood This allows early identification of the fetal gender in with minimum invasive intervention. The possible sources of fetal DNA entering the maternal plasma include direct transfer of DNA, hematopoietic cells and the apoptotic syncytiotrophoblast of the placenta, with placenta being the predominant source [4].

Actually, cffDNA can be detected in the maternal circulation at 5 weeks of gestation [5,6] and it represents 10%–20% of the total DNA that circulates in the maternal plasma[7,8].Moreover, maternal plasma cffDNA was undetectable 4 to 30 minutes following delivery as it is rapidly cleared from the maternal circulation; making it an ideal possible source suitable for antenatal investigations with no false impact on results [6,9].

Fetal gender determination utilizing maternal plasma cffDNA are mainly restricted to the paternally acquired sequences that are absent in the maternal genome, for example, the SRY, DYS14 and DAZ genomic sequences, which are situated on the Y chromosome. Consequently, a male-bearing pregnancy is the only way to detect these genomic sequences [10].

The current technique most commonly utilized in identification of particular cffDNA sequences is the polymerase chain reaction (PCR).Various PCR types have been investigated, with the quantitative real time PCR being the most prevalent[11],because it has the privilege of both high sensitivity and closed detection system, thus decreasing the contamination risk[10].

The aim of this study was to evaluate the sensitivity and the specificity of real time PCR in comparison to conventional PCR for gender determination in the first trimester and to demonstrate their impact on clinical practice. We did not assess the ethical outcomes of such a test, which have already been inspected somewhere else [12].

MATERIAL AND METHODS

Sample collection and preparation

Peripheral blood samples were collected from 50pregnant women with gestational age between 6th to 10th weeks; detected by trans-abdominal ultrasound, who were referred to Gynecology and Obstetrics Department, Benha University Hospitals, Egypt. Five men and five non-pregnant women were also included in this study as positive and negative controls respectively. Twin pregnancies were excluded from this study. Written informed consents were taken before blood sampling. The protocol of this study was approved by the Ethical Committee of Benha Faculty of medicine. Four ml whole peripheral blood from each case was collected in a sterile vacutainer tube containing EDTA. Blood samples were centrifuged at 4000 rpm for 10 min and the upper plasma layer was pipetted without disturbing the buffy coat and transferred into 1.5ml Eppendorf tube. Further micro centrifugation of the plasma was performed at full speed (15000 rpm) for 5 minutes. The upper plasma layer was obtained and stored at -80°C until further processing.

Detection of SRY, DYS and DAZ genes in the maternal cffDNA

- **DNA extraction**

cffDNA was extracted from 200µl of the plasma samples using GeneJet Whole Blood Genomic DNA purification Minikit (Thermo Scientific, Lithuania) according to the manufacturer's instructions. The DNA was eluted in 80µL of elution buffer. Purity and concentration of the purified DNA was assessed by Nanodrop 2000 (Thermofisher scientific, USA) and the extracted DNA was then stored at - 80°C until further processing.

- **Amplification of cffDNA**

PCR amplification was performed using primers with specific sequence for detection of SRY, DYS14[13], and DAZ genes [14]as specific genetic markers for male-bearing pregnancies and ß-globin gene as an endogenous positive control [15] to assess the presence of sufficient cell-free DNA after extraction. Table (1) shows the sequence of these primers with their amplicon size.

I. Quantitative real time PCR

The amplification reactions contained25ng of cffDNA,0.6µl of forward primer, 0.6µl of reverse primer, 10µl of QuantiTect® SYBR® Green PCR (Qiagen, Germany)and nuclease free water up to 20µl as a final volume. A sample from adult male was used in each reaction as a positive control. In addition, a sample from adult female was used in each reaction as a negative control. A non-template control, containing nuclease free water, was used in each run to detect the contamination. The PCR conditions were set in Rotor-Gene Q (Qiagen, Germany) according to the following program: initial denaturation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 30 s, annealing as shown in Table (2) for 30 s and extension at 72 °C for 30 s. Specificity of the amplification product was assessed by analyzing melting curves using Rotor-Gene Q system software.

Table (1): primer sequence of SRY, DYS14, DAZ and ß-globin genes with their amplicon size

Gene	Forward	Reverse	Amplicon size (bp)
SRY	TGGCGATTA AGTCAAATT CGC	CCCCCTAGTA CCCTGACAAT GTATT	137
DYS14	GGGCCAATG TTGTATCCT TCTC	GCCCATCGGT CACTTACACT TC	84
DAZ	TACCTCCAA AGCACCAGA GC	AATCTACCCA TTCCCGAACC	205
ß-globin	GTGCACCTG ACTCCTGAG GAGA	CCTTGATACC AACCTGCCCA G	101

Table (2): Annealing temperature of SRY, DYS14, DAZ and ß-globin genes

Gene	Annealing temperature
SRY	49 °C
DYS14	52°C
DAZ	51°C
ß-globin	55°C

II. Conventional PCR

Amplification was done using MyTaq^{TM}RedMix supplied by Bioline, UK. The PCR mix contained 10ul of Taq PCR master Mix 2x, 0.6µl of each primer, 25ng of cffDNA and nuclease free water to reach a final volume of 20ul. T100 thermal cycler, Bio-Rad, Singapore was used for amplification according to the following program: initial denaturation at 95ºC for 1 min, 45 cycles of denaturation at 95 ºC for 15 s, annealing as shown in Table (2) for 15 s and extension at 72 ºC for 30 s then hold at 4 ºC.

Agarose gel electrophoresis

Tenµl of each amplified DNA &CentiMark PCR marker (Vivantis, Malaysia) were separated on 2% agarose gel containing 0.3µg/ml of ethidium bromide. The bands were visualized using UV transilluminator (254nm) and photographed.

Anti-contamination measures

All procedures were performed by female operators only inside a biologically safety cabinet in order to avoid contact with exogenous male DNA**.**

Statistical analysis

It was done using SPSS 18. Age was presented as mean±SD. Qualitative data were presented as number and percentage. All the results of conventional and real time PCR were analyzed and compared with the birth outcome to calculate the sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV)of the applied method. In real time PCR, ROC curve was used to predict cutoff values of cycle threshold(Ct) of SRY, DYS, and DAZ genes' amplification curves with the optimum sensitivity and specificity. P value <0.05 was considered significant.

Results

In this study, fetal gender determination was performed for 50 antenatal cases with an age ranging from 19 to 38years with mean age ± SD of 26.9±5.6 years and gestational age ranging from6th to 10th weeks of pregnancy with a mean gestational age ± SD of 7.8±1.4 weeks. Real time PCR and conventional PCR were used for fetal gender determination. Birth outcome results were ascertained in all cases, including 27 male-bearing pregnancies and 23 female-bearing pregnancies. Gender prediction was calculated by comparing

the PCR results with birth outcome. The results of gel electrophoresis of the genes included in the study are presented in figure (1).

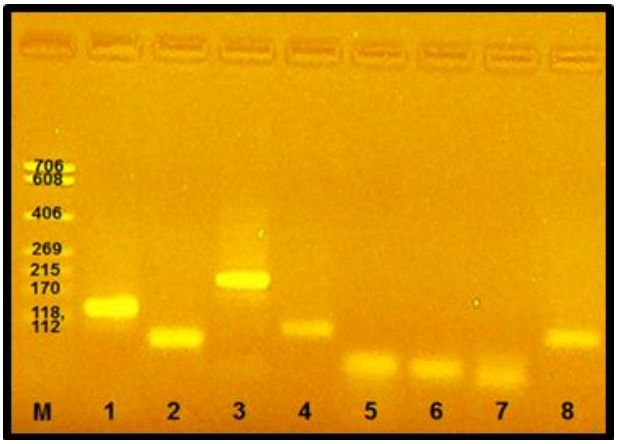

Figure (1): Detection of SRY, DYS14, DAZ and ß-globin sequences in maternal plasma by conventionl PCR. Lane M represents CentiMark PCR marker (Vivantis, Malaysia); lanes 1, 2, 3 and 4 represent SRY, DYS14, DAZ and ß-globinlocus respectively fromDNA of male-bearing pregnant woman; lanes 5, 6, 7and 8 represent SRY, DYS14, DAZ and ß-globinlocus respectively from DNA of female-bearing pregnant woman.

In the real time PCR, cut off values with the optimal sensitivity and specificity were determined for the Ct of the amplification curves of SRY, DYS, and DAZ genes. If the Ct was below or equal to the cut off value, the test was considered positive for male and if below it, the test was considered negative for male.

The number and percentage of true and false positive and negative results are presented in Table (3). Validity characteristics of using the individual genes alone or in combination in fetal gender determination are presented in Tables (4,5).Moreover, when the three genes were negative in both real time and conventional PCR, the birth outcomes were all female (100%).

Since real time PCR showed higher sensitivity and specificity than conventional PCR, a scoring model for interpreting the results obtained by combining the results of real time PCR of the three genes was constructed and presented in Table (6).

DISCUSSION

Invasive antenatal diagnostic methods such as CVS and amniocentesis should be carried out on a fetus having a risk of an X-linked recessive disorder. CVS is performed at the 10–11th weeks of gestation [16]. Thusly, maternal plasma of the included subjects of this study was acquired during the early gestational period between 6th to 10th weeks for fetal gender determination, so that it could be used as a "pre-test" prior to these invasive antenatal diagnostic methods. If a fetus has X-linked recessive inheritance risk and is shown to be female by our cffDNA testing method, the use of invasive antenatal diagnostic methods may be unnecessary [16].

Another use of fetal gender determination in non-invasive antenatal diagnosis is as a "screening test" to figure out whether dexamethasone administration should be proceeded to a pregnant woman with the risk of bearing a fetus with congenital adrenal hyperplasia [17].For the avoidance of virilization of the external genitalia of a female fetus, dexamethasone should be administrated when the pregnancy is perceived. If the pregnant woman at risk is appeared to be bearing a male fetus by the "screening test", then dexamethasone administration can be ceased [16].

In this study, ultrasonographic scan was performed within the first 7 weeks of gestation, to exclude twin pregnancies before blood sampling and to minimize false positive results that may occur due to existence of a vanishing male of a

Table (3): The number and percentage of true and false positive and negative results of both real time and conventional PCR.

Outcome	Result by PCR	Real time PCR			Conventional PCR		
		SRY	DYS	DAZ	SRY	DYS	DAZ
		Number/ total number of males or females (%)					
27 males	True positive	23/27 (85.2)	26/27 (96.3)	25/27 (92.6)	22/27 (81.5)	26/27 (96.3)	24/27 (88.9)
	False negative	4/27 (14.8)	1/27 (3.7)	2/27 (7.4)	5/27 (18.5)	1/27 (3.7)	3/27 (11.1)
23 females	True negative	22/23 (95.7)	23/23 (100)	23/23 (100)	22/23 (95.7)	19/23 (82.6)	22/23 (95.7)
	False positive	1/23 (4.3)	0/23 (0)	0/23 (0)	1/23 (4.3)	4/23 (17.4)	1/23 (4.3)

Table (4): Sensitivity, specificity, PPV and NPV ofSRY,DYS14 and DAZ genes in prediction of fetal male gender.

Real time PCR							
Gene	**cutoff value**	**Sens.%**	**Spec.%**	**PPV%**	**NPV%**	**AUC**	**P**
SRY	≤ 34.1	85.2%	95.7%	95.8%	84.6%	0.904	<0.05
DYS14	≤ 31.2	96.3%	100%	100%	95.5%	0.981	<0.05
DAZ	≤ 32.9	92.6%	100%	100%	91.7%	0.963	<0.05
Conventional PCR							
Gene	**cutoff value**	**Sens.%**	**Spec.%**	**PPV%**	**NPV%**	**AUC**	**P**
SRY	-------	81.5%	95.7%	95.7%	81.5%	0.886	<0.05
DYS14	--------	96.3%	82.6%	86.7%	95%	0.895	<0.05
DAZ	--------	88.9%	95.7%	96%	88%	0.043	<0.05

Table (5): Validity characteristics of using different combinations of the three genes as compared to single gene in prediction of fetal male gender

Real time PCR						
Positive gene(s) for male sex	**Sens.%**	**Spec. %**	**PPV%**	**NPV%**	**AUC**	**P**
SRY + DYS14	100%	95.65%	96.43%	100%	0.999	<0.05
DYS+DAZ	100%	100%	100%	100%	1.00	<0.05
SRY + DAZ	100%	95.65%	96.43%	100%	0.998	<0.05
SRY+ DYS14 +DAZ	100%	100%	100%	100%	1.00	<0.05
Conventional PCR						
Positive gene(s) for male sex	**Sens.%**	**Spec. %**	**PPV%**	**NPV%**	**AUC**	**P**
SRY + DYS14	96.3%	82.61%	86.67%	95%	0.956	<0.05
DYS+DAZ	88.89%	95.65%	96%	88%	0.968	<0.05
SRY + DAZ	96.3%	91.3%	92.86%	95.45%	0.972	<0.05
SRY + DYS14 + DAZ	100%	100%	100%	100%	1.00	<0.05

Table (6): The scoring model for interpretation of results of real time PCR obtained by combining the results of the three genes

	SRY	DYS14	DAZ	Final result	Explanation
Real time PCR result	Positive	Negative	Negative	Likely female (Technical failure so repeat the test)	Due to the lower sensitivity of SRY gene than the other 2 genes
	Negative	Positive	Negative	Likely male (Repeat the test)	Due to highest sensitivity of DYS14 compared to the other 2 genes. DYS has 100% specificity
	Negative	Negative	Positive	Likely male (Repeat the test)	Both DYS14 and DAZ are of high sensitivity, although DYS14 sensitivity is slightly higher. DAZ has 100% specificity.
	Positive	Positive	Negative	Likely male(repeat the test)	This combination has 100% sensitivity but specificity is not 100% (95.65%)
	Negative	Positive	Positive	Report male	This combination has 100% sensitivity and specificity
	Positive	Negative	Positive	Likely male (repeat the test)	This combination has 100% sensitivity but specificity is not 100% (95.65%)
	Positive	Positive	Positive	Report male	This combination has 100% sensitivity and specificity
	Negative	Negative	Negative	Report female	By exclusion of the presence of Y-chromosome sequences

male twin during the time of sampling and disappeared in the resulting weeks of pregnancy and just a baby girl was born. This is expected to bring about a false positive outcome in around 0.3%-0.7 % of cases [18].

Maternal plasma was preferred instead of serum in this study. Despite the fact that the absolute concentration of fetal DNA in maternal plasma and serum is the same, the relative amount of fetal DNA is less in serum than in plasma. This is because of more background maternal DNA resulting from cellular lysis that occurs during coagulation [7].However, Honda et al., [19] stated that maternal serum had higher sensitivity in detecting fetal DNA than that of maternal plasma, which might be attributable to the efficiency of the used DNA extraction method from either serum or plasma in this study.

Moreover, in this study, further micro centrifugation of the plasma was performed at full speed (15000 rpm) for 5 minutes to avoid contamination by residual circulating cells from previous pregnancies. It was reported that a second micro centrifugation step as well as the g-force and the time utilized are essential to acquire truly cell-free plasma [20].

The optimal Y chromosome DNA marker for early noninvasive fetal gender determination must be short and present in multiple copies only in the Y chromosome[21],the criteria which led us to choose DYS14 and DAZ genes, for this study. Although SRY gene is a single copy gene [22], it was also included in this study because it is specific to Y chromosome[22].

This study showed that the sensitivity and the specificity of SRY gene in fetal gender determination by real time PCR were 85.2% and 95.7% respectively. This finding is considered acceptable despite being the lowest sensitivity among the other two genes. This lowest sensitivity could be due to SRY gene is a single copy gene, unlike DYS14 and DAZ genes which have

multicopy sequences. So early in the first trimester of pregnancy when the fetal DNA copy numbers are low, SRY gene couldn't be detected giving false negative results. Therefore, a multicopy sequence is more sensitive, efficient, and accurate than the single-copy SRY in cffDNA assessment [22].

Nearly similar results were achieved by Jacob et al., 2015(84% and 92.8%) [23]. However, higher sensitivity results obtained by Rijnders et al., (97.2%)[24] and Davalieva et al., (89.2%)[25]. This higher sensitivity results could be attributed to longer time range of pregnancy that led to high probability of detecting cffDNA due to the gradual increase of its concentration with the more gestational age[26].

On the other hand, the sensitivity and the specificity of SRY gene in fetal gender determination by conventional PCR were 81.5% and 95.7% respectively. Zolotukbina et al., reported higher sensitivity results (94.5%)[27].They used nested PCR in which two rounds of PCR were performed using two pairs of primers for Y-chromosomes specific sequence, which explain higher sensitivity obtained by this study than our study.

False positive results of SRY gene were detected in only one sample by either real time PCR or conventional PCR and it was the same sample. This could be due to male DNA contamination that is magnified by PCR and conceals the real results. DNA contamination of the samples was the cause of the false positive results in previous studies [27, 28].

Regarding sensitivity and specificity of the multicopy DYS14 and DAZ genes in determination of fetal gender by real time PCR, the obtained sensitivity and specificity of DYS14 gene in this study were 96.3% and100% respectively. These findings were close to that obtained by Martinhago et al., 2006(92% and 100%)[29] and Aghanoori et al., 2012 (91.8% and 100%) [30].However, the obtained sensitivity and specificity of DYS14 gene by conventional PCR were 96.3% and 82.6% respectively. These findings were consistent with the sensitivity reported by Honda et al (95%)[16].

In addition, the obtained sensitivity and specificity for DAZ gene in this study were 92.6% and 100% respectively and the obtained sensitivity and specificity of DAZ gene by conventional PCR were 88.9% and 95.7% respectively. Vainer et al., [21] reported that one gene from the DAZ family is located in chromosome 3. Therefore, they used primer design to avoid the fragments of genes that are common for the autosomal copies and Y-specific copies. This explains the higher specificity (100%) using either conventional or real time PCR obtained by their study, which was more than our study that demonstrated lower specificity by conventional PCR.

Overall, the differences observed between the results of this study and previous studies could be explained through several factors. These factors include differences in the methods used for fetal DNA extraction, efficiency of the methods used[14],differences in the DNA sequence targeted for amplification, primers used[31], gestational age at which the sample is withdrawn, pregnancy time range, number of subjects included in the study and presence of possible contamination[14].

Our results observed that DYS14 PCR amplification have much better sensitivity than that

of DAZ, which may be due to the twofold higher number of copies of the DYS14 versus the five amplicons created by the DAZ primers [32]. Nonetheless, the DYS14 sequence has considerable homology to sequences other than the Y chromosome that could falsely characterize female fetuses as male[32].This fact explains false positive results of DYS14 gene observed in this study by conventional PCR. Whereas in real time PCR, a cutoff value was employed for the studied genes to differentiate between the true- and false-positive data and hence real time PCR showed higher specificity than that of conventional PCR.

On the other hand, detection of male fetal sex in maternal plasma by multicopy DAZ gene has a low risk of a false-negative results resulting from de novo DAZ deletions that occur in a frequency of 1/4,000 [33].

False-negative and false-positive results in gender determination utilizing cffDNA are a matter of concern. Therefore, we tried to shed light on possible causes of false-negative and false-positive results.

Regarding false-negative results, the main reason for false-negative results is failure to recognize the Y-chromosome sequences, which either occur due to cffDNA low concentration at an early gestational age[14]or might be associated with different cffDNA yield by various DNA extraction methods[22].Also, it has been demonstrated that cffDNA is fragmented into <300-bp segments[34, 35].However, the GeneJET™ Whole Blood Genomic DNA purification Mini kit used in this study is designed for rapid and efficient purification of high quality genomic DNA from whole blood and related body fluids. Furthermore, lower number of copies of the used gene has a role in obtaining false-negative results as occurs with SRY gene.

On the other hand, false positive results can result from technical issues, for example, contamination or clinical abnormalities, for example, a confined placental mosaicism/chimerism or the presence of a non-identical vanishing twin[10], which is excluded, in this study.

Different combinations of the three Y-chromosome sequences were used to maximize test accuracy. The sensitivity and specificity, PPV and NPP of real time PCR were higher than those of conventional PCR, when 2 genes of the three are positive. However, if the 3 genes results either by using real time or conventional PCR were the same, the PPV and NPV were 100%, indicating that if the test outcomes of the 3 genes are positive, the fetus is considered a male and there is 100% probability of being born a male and 100% probability to be a female if the test outcomes of the 3 genes are negative. Therefore, it is possible to apply the method presented in this study in fetal gender determination, prior to applying invasive antenatal diagnostic methods, due to its high probability of correct prediction.

A scoring model was done for reporting fetal gender, utilizing real time PCR; since it was more sensitive and specific than conventional PCR, in determination of fetal gender. This scoring model was designed by combining the results of the three genes. If DYS14 and DAZ are positive or the 3 genes are all positive or all negative, the results are conclusive; otherwise, the test should be repeated.

Another scoring model was previously designed by Fernandez-Martinez et al., 2012 [22].

In general, real time PCR is better than conventional PCR in determination of fetal gender. The operational advantages of real time PCR over conventional PCR are quantitative ability, speed, higher sensitivity and reproducibility[36]. Therefore, it may be used instead of conventional PCR in diagnostic routines.

To conclude, noninvasive fetal gender determination in maternal plasma can beadded to diagnostic routines to be used in clinical practice, decreasing the demand for the invasive diagnostic methods in women having a high risk of sex-linked genetic disease.

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