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A simple and reliable method of obtaining fetal DNA from maternal circulation; its accuracy and sensitivity

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ABSTRACT

The remarkable discovery of fetal DNA in maternal circulation has opened up enormous possibilities of fetal genetic investigations. Therefore, an attempt has been made to develop a simple and reliable method for obtaining fetal DNA from maternal blood and establish its accuracy and sensitivity, so that it can be useful for prenatal diagnosis in developing countries. Blood samples were collected from pregnant mothers (n=255) recruited after informed consent and recording of clinical parameters. DNA was extracted from plasma, analyzed by PCR, detected sensitivity, specificity and accuracy of method using diagnostic tool. Total DNA increased from 157.09 to 608.15 GE during first to third trimester. PCR using Y chromosome specific primers showed 87.88, 94.44 and 94.70 % correct diagnosis during 1st, 2nd and 3rd trimesters respectively. Percentage of sensitivity, specificity, positive predictive value and accuracy of the method showed a gradual increase (83-100%) with advancement in gestation age. The overall sensitivity, specificity and positive predictive value were 89, 98 and 97% respectively with 94% method accuracy.

Keywords: prenatal diagnosis, non-invasive, maternal plasma, cell-free fetal DNA, genomic equivalent.

INTRODUCTION

The invasive approach of obtaining fetal tissue is currently the standard method for prenatal DNA diagnosis. Many women are reluctant to undergo invasive testing, either due to significant risk of pregnancy loss or dilemma of terminating their pregnancy in case of disease diagnosis. According to a report in UK out of 700 000 pregnant women around 20 000 amniocentesis and 5

200 chorionic villus sampling (CVS) tests were conducted annually, with an estimated pregnancy loss of around 250 due to miscarriage [1]. Since an invasive test has a risk of up to 0.5% for pregnancy loss, research is aimed at developing a noninvasive, risk-free maternal blood test for fetal DNA analysis. The remarkable discovery of fetal DNA in maternal plasma/serum opened up enormous possibilities of fetal genetic investigation without the use of invasive procedures [2]. An attempt was made to develop a simple and reliable method of obtaining fetal DNA from maternal blood and establish its level of accuracy and sensitivity.

MATERIALS AND METHODS

Study design and Settings

An outpatient clinic in Department of Obstetrics and Gynecology, CSMMU, Lucknow. A blinded study.

Selection of participants

Pregnant women attending the Obstetrics and Gynaecology clinic at Queen Mary's Hospital, Chhatraprati Shahuji Maharaj Medical University (CSMMU) at Lucknow, India were recruited for the study after ethical approval from the Institutional Ethics Committee of CSMMU, Lucknow (No.348/R-Cell-09). Healthy pregnant women without pregnancy-associated complications were evaluated according to maternal and gestational age, blood group with Rh factor, number of previous pregnancies, abortions or miscarriages, and age of previous children (male or female). Blood samples from 255 pregnant women (19-42 years) were collected over a period of 19 months (May 2009-December 2010) after informed consent from all participants. The gestation ages when the blood samples were collected ranged from 2 weeks + 1 day to the time of delivery. The recruited women were categorized into first (<12 weeks), second (12-24 weeks) and third trimesters (>24 weeks) of gestation. Fetal gender was unknown at the time of blood sampling.

Collection and processing of blood samples

Three milliliters of maternal peripheral blood samples were taken in EDTA vials and kept at 4°C for 1 hour. After one hour, plasma was obtained by centrifugation of the whole blood samples at 3 000 rpm for 10 minutes and carefully transferred into fresh centrifuge tubes. The plasma samples were re-centrifuged at 8 000 rpm and the supernatants were collected once again and stored at -20°C until further use.

DNA Extraction and quantification of cell free DNA (cfDNA) from maternal plasma

DNA was extracted from 1.0 ml plasma by phenol-chloroform method with slight modifications. One ml of plasma was treated with 1x SDS/Proteinase K solution (0.5 mg/ml) (1:1), incubated overnight at 56 °C followed by phenol-chloroform (4:1) treatment and centrifuged at 7 000 rpm. The upper layer was transferred into fresh 15 ml centrifuge tubes and the same step was repeated again; DNA was precipitated by adding glycogen (0.1 µg/µl), ammonium acetate (7.5 M) and absolute alcohol. DNA pellet was obtained by centrifuging at 7 000 rpm; was washed with 70% alcohol at 10 000 rpm, dried and finally dissolved in TE buffer at 56 °C and stored at -20 °C until further use. Total circulating cfDNA (maternal + fetal) in plasma was quantitated by measuring A_{260} on Warburg formula (BioPhotometer, Eppendorf). The concentration was calculated as

genome equivalent (GE) by using the conversion factor of one diploid genome being equivalent to 6.6 pg of DNA per cell [3].

DNA amplification and analysis

PCR was performed in a 25 µl reaction mixture containing plasma DNA (43-96 ng), 10 pmol of each primer (IDT, USA), 200µM dNTPs, and 0.3 U of Taq DNA polymerase (Bangalore Genei, India) in a gradient master cycler (Eppendorf, USA). Initial experiments included primers for *β-globin* and *β-actin* as controls [4]. The *SRY* (R1, R2, R3, R4, R5) and *DYS392* primers were used to confirm the presence of fetal DNA. Primer sequences, annealing temperatures and amplicon sizes are shown in Table 1. The PCR products were resolved on 2% agarose gels and analyzed.

Table 1: List of primers, PCR conditions, and amplicon sizes

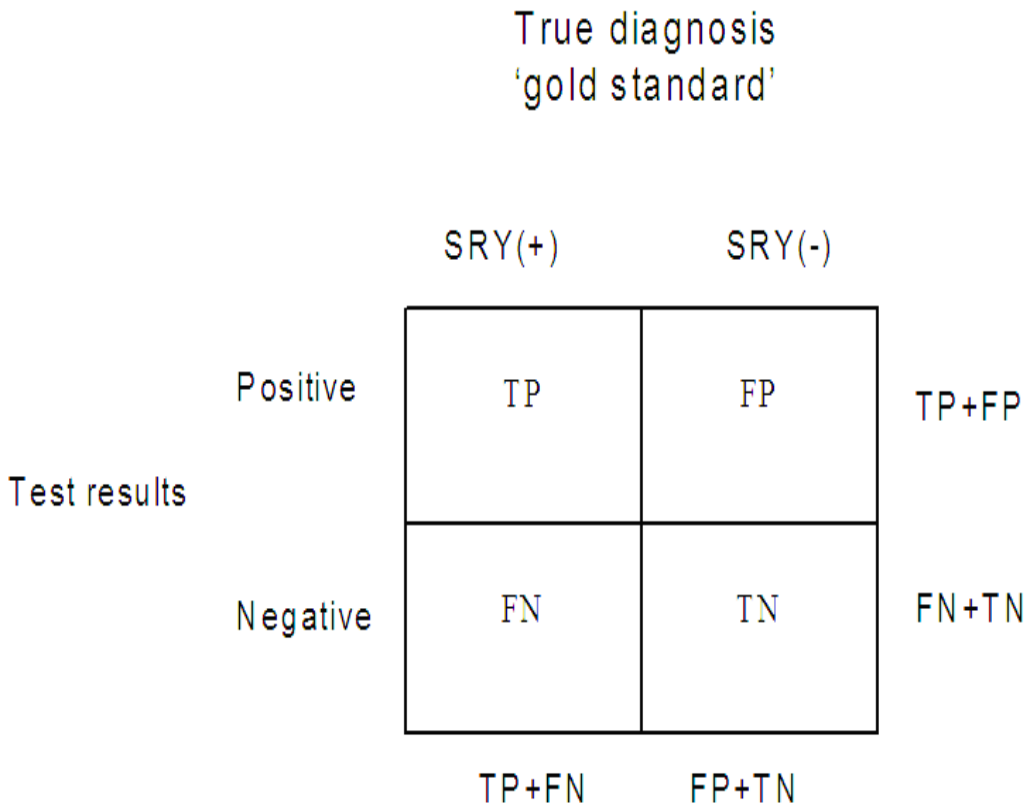
Loci	Primer sequences	Annealing temp. °C	Amplicon Size (bp)	References
β-actin	(F) 5'-GTGGGGCGCCCCAGGCACCA-3' (R) 5'-CTCCTTAATGTCACGCACGATTTC-3'	57	541	[5]
β-globin	(F) 5'-TCCTGAGGAGAAGTCTGCCG-3' (R) 5'-CCTTAAACCTGTCTTGTAAC-3'	60	109	[6]
DYS392	(F) 5'-TCATTAATCTAGCTTTTAAAAACAA-3' (R) 5'-AGACCCAGTTGATGCAATGT-3	60	253	[7]
SRY R1	(F) 5'-GGTCAAGCGACCCATGAAYGCNTT-3' (R) 5'-GGTCGATACTTATAGTTCGGGTAYTT-3'	55	231	[8]
SRY R2	(F) 5'-AAAGGCAACGTCCAGGATAGAG-3' (R) 5'-TGAGTTTCGCATTCTGGGATT-3'	58	107	[9]
SRY R3	(F) 5'-AAAGGCAACGTCCAGGATAGAG-3' (R) 5'-ACTTCGCTGCAGAGTACCGAA-3'	58	313	
SRY R4	(F) 5'-AAAGGCAACGTCCAGGATAGAG-3' (R) 5'-ATGTTACCCGATTGTCCTACAGC-3'	60	475	
SRY R5	(F) 5'-CCCGAATTCGACAATGCAATCATATGCTTCTGC3' (R) 5'-CTGTAGCGGTCCCGTTGCTGCGGTG-3'	65	612	[10]

Statistical analysis used

Statistical analysis was performed using PRISM software (version-7.0).

Data analysis for clinical applications

The results obtained from PCR and after delivery were compared against a reference ('gold') standard and results were tabulated in a 2 x 2 table also called the Criterion Standard Test (Figure 1). The sensitivity, specificity, predictive values and accuracy of the diagnostic test were estimated as per Figure 1. Accuracy of the method was calculated as total number of male and female bearing pregnancies correctly identified/ total number of pregnancies tested (Table 4) [11, 12].



True Positive (TP): Fetal DNA showing Y-chromosome specific sequences and male birth at delivery.
True Negative (TN): Fetal DNA not showing Y-chromosome specific sequences and female birth at delivery.
False Positive (FP): Fetal DNA not showing Y-chromosome specific sequences but male birth at delivery.
False negative (FN): Fetal DNA showing Y-chromosome specific sequences but female birth at delivery.

Specificity= TP/ (FN+TP)
Specificity= TN/ (FP+TN)
Positive predictive value= TP/ (FP+TP)
Negative predictive value= TN/ (FN+TN)

Figure 1. Estimation of sensitivity and specificity of diagnostic test [11]

RESULTS

Concentration of cfDNA in maternal plasma

Estimation of total plasma DNA *i.e.* A_{260} showed an overall increase in the DNA level in pregnant women (n=255) when compared to that in non-pregnant women (n=100). However, the normal plasma DNA level was almost similar to that in the 1st trimester (54.46 ± 30.66 pg/ml). Genomic equivalent (GE) of DNA (fetal and maternal) ranged from 157.09 to 608.15 in first to third trimester of pregnancy, with no significant increase (Table 2).

Table 2: Concentration of cell free DNA (cfDNA) in maternal plasma

Gestation age (GA) (Weeks)	Sample size (n=255	DNA conc. (Pg/ml)	Genomic equivalent (GE)	*P* value
< 12	33	54.46 ± 30.66	359.44 ± 202.35	**0.385**[a]
12-24	193	59.44 ± 30.29	392.30 ± 199.88	**0.493**[b]
> 24	274	61.41 ± 30.74	405.30 ± 202.85	**0.221**[c]

[a] *<12 vs 12-24 weeks of Gestation age*
[b] *12-24 vs >24 weeks of Gestation age*
[c] *>24 vs <12 weeks of Gestation age*

Detection of fetal DNA in maternal plasma

DNA extracted from maternal plasma of 255 pregnant women was subjected to PCR assay using *β-actin, β-globin, DYS392* and *SRY* specific primers. *β-actin* and *β-globin* primers were used to confirm the presence of DNA while the latter two primers were used to confirm the presence of fetal DNA. The PCR products of various primer sets are shown in Figure 2. Out of the 255 samples evaluated, 106 carried male fetuses while remaining 149 cases showed presence of female fetuses. The diagnostic results in all cases were also matched after delivery. Fetal sex was correctly diagnosed and matched with the PCR analysis of 29 out of 33 in the 1st trimester, 85 out of 90 in the 2nd trimester and 125 out of 132 in the 3rd trimester (Table 3).

Table 3: Presence of fetal DNA in maternal plasma using Y-chromosome specific primers and its confirmation after delivery

Gestation age (Weeks)	Samples (n)	PCR results		Correct diagnosis (%)
		SRY (+)	SRY (-)	
1st trimester (<12)	33	12	21	= 87.88 (29/33)
2nd trimester (12-24)	90	34	56	= 94.44 (85/90)
3rd trimester (>24)	132	60	72	= 94.70 (125/132)
Total cases (n)	**255**	**106**	**149**	

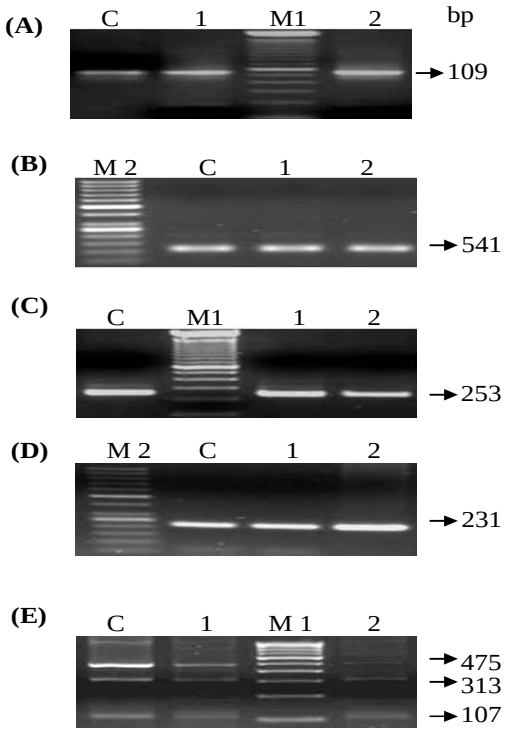


Figure 3. Agarose gel pictures showing PCR products using different primers. (A) *β-actin* (541 bp). (B) *β-globin* (109 bp). (C) *DYS392* (253 bp). (D) *SRY* R1 (231 bp). (E) Multiplex PCR using *SRY* R2 (107 bp), *SRY* R3 (313 bp) and *SRY* R4 (475 bp) primers. Lane C-control male DNA; M1-100 bp ladder; M2-50 bp ladder. Lanes 1, 2 cffDNA (*SRY* +ve).

Accuracy of method

Using TP, TN, FP and FN parameters the method sensitivity, specificity, positive predictive values and accuracy showed a gradual increasing trend from 1st to 3rd trimester. The overall sensitivity and specificity were 89 and 98 % respectively with 94% method accuracy (Table 4).

Table 4: Diagnostic tools (sensitivity, specificity, predictive values, accuracy) at different gestational ages

Diagnostic tools	Estimation	1st trimester (n=33)	2nd trimester (n=90)	3rd trimester (n=132)	Whole pregnancy (n=255)
True positive (TP)	Count in 2x2 table	10	30	54	94
False positive (FP)	Count in 2x2 table	02	01	-	03
False negative(FN)	Count in 2x2 table	02	04	06	12
True negative(TN)	Count in 2x2 table	19	55	72	146
Sensitivity (%) = True positive rate (TPR)	TP/(FN+TP)	83	88	90	89
Specificity (%) = True negative rate (TNR)	TN/(FP+TN)	90	98	100	98
1-Sensitivity (%) = False negative rate (FNR)	FN/(TP+FN)	17	12	10	11
1-Specificity (%) = False positive rate (FPR)	FP/(TN+FP)	10	02	-	02
Positive- predictive value (%)	TP/(FP+TP)	83	97	100	97
Negative- predictive value (%)	TN/(FN+TN)	90	93	92	92
Accuracy (%)	(TP+TN)/(TP+TN+FP+FN)	88	94	95	94

DISCUSSION

The aim of this study was to calculate the levels of cfDNA in maternal plasma at different gestational ages of pregnancy and to evaluate the presence of fetal DNA using simple PCR. Previously, circulating fetal cells in maternal blood presented an attractive starting material for NIPD, particularly for the diagnosis of fetal sex and chromosomal abnormalities. Various methods of fetal cell enrichment were developed with varying degrees of success [13, 14]. The unsatisfactory results with fetal cells due to their insufficiency in maternal circulation (around one cell per ml of maternal blood), low efficiency of enrichment and difficulties with chromosomal analysis because of abnormally dense nuclei [15, 16] led to the detection and isolation of cffDNA in maternal blood, amount of which was found to be significantly higher (by a factor of almost 1 000) [17]. However, the general problems associated with the detection of cffDNA in the maternal circulation are: low concentration of cfDNA in the circulation, out of which only ~3-6% is cffDNA; variation of total amount of cffDNA among individuals and fetus inheriting half its genome from mother. The major focus of recent research has been to overcome these difficulties and develop methods to enhance the amount of cffDNA and establish a cheap and safe procedure for routine clinical practice [18, 19]. Detection of cffDNA was initiated by using Y-chromosome specific sequences since this was fetal specific and ruled out any maternal contamination [1]. For example, the Y-chromosome specific *SRY* gene was used as a fetal marker [17], followed by diagnostic tests for X-linked disorders such as Duchenne Muscular dystrophy (DMD) and hemophilia, etc. In the present work, in addition to *SRY* markers, *DYS392* was also used.

A number of different types of PCR have been explored, of which the most popular is real-time quantitative PCR (Q-PCR) which accurately quantifies fetal DNA in maternal plasma and serum through identification of fetal gender by *SRY* and *RHD* genes [20]. Surprisingly, high mean concentrations of cffDNA (3–6% of total cfDNA) were detected using Q-PCR during early and late pregnancies, peaking at the time of delivery [17]. However, Q-PCR demands expensive equipment facility which makes its application difficult in a routine setup. Thus, to develop diagnostic facilities with limited financial resources, a simple, cost effective, reliable and replicable conventional PCR technique may be an alternative solution [18]. An attempt was made in this study to analyze the accuracy and sensitivity of conventional PCR with cfDNA obtained from mother's circulation.

In the present study, an overall increase in the DNA level was found in pregnant mothers when compared to non-pregnant women like reported earlier [21]. A significant correlation between early gestation age (10-12 weeks) and total fetal DNA concentration, as well as a slightly higher DNA concentration in the first and third trimesters than in mid-pregnancy were demonstrated [22, 23]. Our study also showed an increase in total cfDNA concentration from 157.09 to 608.15 GE of total DNA with advancement of gestation age (1st to 3rd trimester) (Table 2). While studies with conventional PCR could identify 95 and 100% male fetuses, sensitivity of Q-PCR was 100% [23, 24]. The identification of Y-chromosome specific sequences (*SRY* and *DYS392* gene) in the present study suggested gradual increase in sensitivity 83-89%, specificity 90- 100% and accuracy 88%-95% respectively from 1st to 3rd trimester (Table 4). Y-chromosome specific sequences could be detected as early as 2 weeks +1day of gestation age unlike earlier studies which could detect it at 5 weeks of gestation [25].

Regarding clearance of fetal DNA after delivery, there have been controversial reports. Some show that fetal DNA is cleared rapidly within a short time post-delivery and is not detectable during following pregnancies, thus enhancing the potential of NIPD [12, 26, 27]. However, Invernizzi et al. detected cffDNA of male offspring several decades after delivery in 22% of healthy women [28]. In the present study, out of 255 cases, 94 males and 146 females were correctly identified with 3 false-positives (FP) and 12 false negatives (FN). Out of 12 FN cases, 9 had record of previous male pregnancies, suggesting that fetal DNA is not completely cleared from maternal circulation even after delivery which seems to be affecting the diagnostic accuracy.

Quantity of fetal DNA in maternal plasma or serum has also been used as a marker for genetic disorders and complications of pregnancy [25]. A two-fold increase in cffDNA levels for fetuses with trisomy 21 and other pregnancy-related disorders such as preeclampsia (PE) [26], preterm labour [29] and intrauterine growth restriction (IUGR) [30] have been found when compared to normal cases. At a glance our findings suggest that the procedure of NIPD by obtaining fetal DNA especially at the time of first gestation and using simple PCR for analyses can be used as a low cost effective method for routine diagnostic purposes.

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REFERENCES

[1] Wright CF; Burton H. *Hum Reprod Update*, **2009**,15,139-151.
[2] Lo YMD; Corbetta N; Chamberlain PF; Rai V; Sargent IL; Redman CW; Wainscoat JS. *Lancet*, **1997**,350,485-487.
[3] Zimmermann B; El Sheikhah A; Nicolaides K; Holzgreve W; Hahn S. *Clin* Chem, **2005**,51,1598-1604.
[4] Nelson M; Eagle C; Langshaw M; Popp H; Kronenberg H. *Vox Sang*, **2001**,80,112-116.
[5] Kashiwagi H; Tomiyama Y; Kosugi S; Shiraga M; Lipsky RH; Kanayama Y; Kurata Y; Matsuzawa Y. *Blood* , **1994**,83,3545-3552.
[6] Zhong XY; Holzgreve W; Hahn S. *Br J obstet gynaecol*, **2000,**107,766-769.
[7] Nair SP; Peter S; Pillay VV; Remya UM; Krishnaprasad R; Rajammal B. *Ind J Hum Genet*, **2007**,13,69-72.
[8] Griffiths R; Tiwari B. *Mol Ecol*, **1993**,2,405-406.
[9] Chan KCA; Zhang J; Hui ABY; Wong N; Lau TK; Leung TN; Lo KW; Huang DWS; Lo YMD. *Clin Chem*, **2004**,50,88-92.
[10] Nagafuchi S; Seki S; Nakahori Y; Tamura T; Numabe H; Nakagome Y. *J Hum Genet*, **1992**,37,187-193.
[11] Attia J. *Aust Prescr*, **2003**,26,111-113.
[12] Galbiati S; Smid M; Gambini D; Ferrai A; Restangno G; Viora E; Camprogrande M; Bastonero S; Pagliano M; Calza S; Ferrai M; Cremonesi L. *Hum Genet*, **2005**,117,243-248.
[13] Jackson L. *Prenat Diagn*, **2003**,23,837-846.
[14] Sekizawa A; Purwosunu Y; Matsuoka R; Koide K; Okazaki S; Farina A; Saito H; Okai T. *J Obstet Res*, **2007**,33,747-764.
[15] Bianchi DW; Williams JM; Sullivan LM; Hanson FW; Klinger KW; Shuber AP. *Am J Hum Genet*, **1997**,61,822-829.
[16] Babochkina T; Mergenthaler S; De Napoli G; Hristoskova S; Sevgi T; Holzgreve W; Hahn S. *Haematologica*, **2005**,90,740-745.
[17] Lo YMD; Tein MSC; Lau TK; Haines CJ; Leung TN; Poon PMK; Wainscoat JS; Johnson PK; Chang AMZ; Hjelm NM. *Am J Hum Genet*, **1998,**62**,**768-775.
[18] Tufan NLS; Tufan AC; Kaleli B; Yildirim B; Semerci CN; Bagci H. *Turk J Med Sci*, **2005**,35,85-92.
[19] Frints S. 26th annual meeting of the European Society of Human Reproduction and Embryology **2010**;2010PHYSORG.com.
[20] Traeger-Synodinos J. *Mol Aspects Med*, **2006**,27,176-191.
[21] Lo YM. *Ann NY Ascd Sci*, **2000**,906,141-147.
[22] Farina A; Caramelli E; Concu M; Sekizawa A; Ruggeri R; Bovicelli L; Rizzo N; Carinci P. *Prenat Diag*, **2002**,22,148-152.
[23] Bischoff FZ; Lewis DE; Simpson JL. *Hum Reprod Update*, **2005,**11**,**59–67.
[24] Honda H; Miharu N; Ohashi Y; Samura O; Kinutani M; Hara T; Ohama Koso. *Hum Genet*, **2002**,110,75-79.

[25] Johnson KL; Dukes KA; Vidaver J; Shane L; Ramirez I; Weber WD; Bischoff FZ; Hahn S; Sharma A; Dang DX; Hire LM; Bianchi DW; Simpson JE; Holzgreve W; Elias S; Klinger KW. *Clin Chem*, **2004**,50,516-521.
[26] Lo YMD; Lau TK; Zhang J; Leung TN; Chang AMZ; Hjelm NM; Elmes RS; Bianchi DW. *Clin Chem*, **1999**,45,1747-1751.
[27] Smid M; Galbiati S; Vassallo A; Gambini D; Ferrari A; Gabriella R; Viora E; Pagliano M; Calza S; Ferrari M; Cremonesi L. *Clin Chem*, **2003,**49,1526-1528.
[28] Invernizzi P; Biondi ML; Battezzati PM; Perego F; Selmi C; Cecchini F; Podda M; Simoni G. *Hum Genet*, **2002**,110,587-591.
[29] Leung TN; Zhang J; Lau TK; Hjelm NM; Lo YM. *Lancet*, **1998,**352**,**1904-1905.
[30] Caramelli E ; Rizzo N; Councu M; Simonazzi G; Carinci P; Bondavalli C; Bovicelli L; Farina A. *Prenat Diagn*, **2003**,23,367-371.