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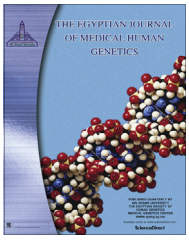

ORIGINAL ARTICLE

Fetal gender determination through Y-STR analysis of maternal plasma during the third trimester of pregnancy

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KEYWORDS

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Abstract *Background:* The passage of nucleated cells between mother and fetus is well recognized (Lo et al., 1989, 1996). As well as, cell-free fetal DNA in maternal plasma or serum is at present widely investigated as a source of fetal genetic material (Stanghellini et al., 2006) [18]. There has been much recent interest in the use of DNA derived from plasma or serum (Boland, 1996). This DNA can be utilized for molecular diagnosis as well as prenatal sex discernment.

Objective: To establish an easy, reliable, and completely safe method for fetal gender determination alternative to conventional exhausting current techniques applied in gynecologic hospitals and clinics, besides its further applications in forensic casework.

Methods: EDTA-Blood samples were taken from 30 pregnant women all in the third trimester of pregnancy, then plasma was separated from each sample, from which DNA was isolated using a QIAamp DNA Mini Kit, with special modifications done in the extracting procedure to concentrate and obtain minute quantities of fetal DNA, together with maternal DNA, from maternal plasma. In addition, bloodstain samples were taken from the husbands of women who were pregnant with male fetuses from which DNA was isolated using a QIAamp DNA Micro Kit for comparison. DNA quantification was done using a Real-time PCR utilizing Quantifiler Duo Kit. PCR was done using an AmpFlSTR Y-Filer Kit, then amplified products were typed using a 3130 Genetic Analyzer.

Results: Full and partial Y-STR profiles (6–17 STR loci) were obtained from all plasma samples taken from pregnant women with male fetuses, while negative Y-STR profiles (no single STR locus) were obtained from all plasma samples taken from pregnant women with female fetuses.

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Conclusion: It is recommended to use Y-STR profiling as an alternative technique for fetal gender determination during the third trimester of pregnancy, in addition to its significance in forensic casework.

1. Introduction

Prenatal sex discernment is the prenatal testing for discerning the sex of a fetus before birth. Prenatal sex discernment can be performed via different technical methods. Chorionic villus sampling (CVS) and amniocentesis are two rather invasive testing procedures. The difficulty of these tests and the risk of damage to the fetus, potentially result in miscarriage or congenital abnormalities. Obstetric ultrasonography, either transvaginally or transabdominally, can check for the sagittal sign as a marker of fetal sex. It gives a result in 90% of cases [1]. The accuracy of fetal sex discernment using obstetric ultrasonography depends on a number of factors including [2]:

1. Gestational age.
2. Precision of sonographic machine.
3. Expertise of the operator.
4. Fetal posture.

According to the 1982 World Health Organization and U.S. Department of Health and Human Services report, "Effects of Ultrasound on Biological Systems", stated that animal studies suggest that neurological, behavioral, developmental, immunological, hematological changes and reduced fetal weight can result from exposure to ultrasound. However, studies have since refuted, challenged or discarded findings that ultrasound may have a bearing on fetal birth weight or speech development [3].

Noninvasive prenatal diagnosis constitutes a long-sought goal in human genetics, where the passage of fetal nucleated cells into maternal blood is a well-recognized phenomenon [4–6], and it makes possible the use of these cells for noninvasive prenatal diagnosis [7,18]. Significant advances have been made in the enrichment and isolation of fetal cells for analysis [8,9]. However, most techniques are time consuming, labor intensive, or difficult to implement on a large scale. These limitations prompted for the investigation of alternative sources of fetal genetic material for molecular analysis [10].

Our study aims to launch an alternative technique to be applicable especially in the Middle East in all obstetric and gynecologic hospitals, to determine the gender of the fetus with a percent of accuracy reaching 100%, without subjecting pregnant women to the disadvantages of conventional current techniques. The work is carried out in accordance with The Code of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

2. Subjects and methods

Thirty peripheral blood samples were collected from 30 pregnant women aged from 20 to 35 years all in the third trimester of pregnancy (mean gestational age, 31.96; range, 27–39 weeks), where blood was collected in EDTA tubes. After the delivery of all women included in the study, it is indicated that 18 of them had delivered a girl and the other 12 had delivered a boy (one of them delivered a twin of 2 boys) (Table 1). According to the delivery results, 12 blood samples were collected from the husbands of women who were pregnant with male fetuses, in the form of bloodstain samples. All samples were taken from Saudi families after informed consents had been obtained from all women included in the study and their husbands and consent of the ethics committee of our university.

Six ml EDTA-Blood from each sample from a pregnant woman was centrifuged at 3000*g* for 20 min, and 2 ml plasma from each sample was carefully removed from collection tubes and transferred into plain polypropylene tubes. The plasma samples were then recentrifuged at 3000*g* for 10 min, and the supernatants were collected in fresh tubes. Samples were stored at −20 °C until further processing. Few blood drops were obtained from the thumb of each husband by using a sterile lancet, and deposited on a Whatman filter paper. Bloodstains were dried in air for half an hour, and put inside sterile plastic

Table 1 Week of pregnancy and gender of fetus for all women included in the study.

Sample identity	Week of pregnancy	Gender of fetus
ID 1	36	M
ID 2	31	M
ID 3	33	M
ID 4	30	M
ID 5	36	M
ID 6	38	M
ID 7	35	M
ID 8	32	M
ID 9	39	M
ID 10	34	M
ID 11	32	M
ID 12	33	M
ID 13	30	F
ID 14	31	F
ID 15	34	F
ID 16	34	F
ID 17	27	F
ID 18	28	F
ID 19	33	F
ID 20	27	F
ID 21	28	F
ID 22	33	F
ID 23	37	F
ID 24	29	F
ID 25	29	F
ID 26	28	F
ID 27	30	F
ID 28	31	F
ID 29	29	F
ID 30	32	F

containers. Samples were stored at 2–8 °C until further processing.

DNA (maternal and fetal) was extracted from separated maternal plasma samples using a QIAamp DNA Mini kit (Qiagen) with the "DNA Purification from Blood or Body Fluids (Spin Protocol)" [11]. We used 500 µl plasma sample per column, and eluted the DNA (in the final step) in 50 µl of deionized water. After completing the extraction procedure, the DNA eluate was concentrated by the use of a Concentrator plus (Eppendorf) by putting the samples at V-AQ (vacuum-aqueous) mode for 18 min, obtaining a final volume of concentrated DNA equals 20 µl. Extracted DNA was stored at 4 °C for less than 2 weeks until further processing.

DNA was extracted from bloodstain samples using a QIAamp DNA Micro kit (Qiagen) with the "Isolation of Genomic DNA from Dried Blood Spots" protocol [12]. Extracted DNA was stored at 4 °C for less than 2 weeks until further processing.

Two microliters of each extracted sample was quantitated utilizing a Quantifiler Duo kit (Life Technologies) according to manufacturer's protocol [13] using the Real-time PCR 7500 (Applied Biosystems).

The AmpFLSTR® Yfiler® PCR Amplification Kit was utilized to amplify the following Y-STR loci: DYS19, DYS385a/b, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS635, and Y GATA H4 located on the Y-Chromosome of male fetuses. Amplifications were conducted according to manufacturer's recommendations using a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems). AmpFLSTR® Control DNA 9947A and 007 (Applied Biosystems) were amplified. PCR amplification was performed in a final volume of 25 µl containing PCR reaction mix 9.2 µl, Taq Gold DNA Polymerase (5 U/ml) 0.8 µl, Y-Filer primer set 5 µl, and extracted DNA template 10 µl. Initial incubation at 95 °C for 11 min was followed by denaturation (94 °C for 1 min), annealing (61 °C for 1 min) and extension (72 °C for 1 min) for 29 cycles when processing plasma samples and for 28 cycles when processing bloodstain samples. At the end of PCR there was incubation at 60 °C for 80 min followed by a final hold at 4 °C [14]. Amplified samples were stored at 4 °C for less than 2 weeks until further processing.

One microliter of PCR products of each sample was mixed with 8.6 µl of Hi-Di formamide (Sigma–Aldrich, St. Louis, MO, USA) and 0.4 µl of Genescan-500 LIZ size standard (Applied Biosystems, Warrington, Great Britain). The mixture was denatured at 95 °C for 3 min and cooled to 4 °C for 3 min. Electrophoresis was performed on an ABI 3130 Genetic Analyzer by using POP4 polymer (Applied Biosystems, Fosters City, CA, USA). Samples were injected for 5 s at 15 kV, and then run at 15 kV for 25 min at a constant temperature of 60 °C. The raw data were collected by the software (3130 Data Collection, version 3.0) and analyzed by the software (GeneMapper ID-X Software, version 1.0). Genotypes were determined by comparing the size of the unknown fragments with the allelic ladders provided by the manufacturer [14].

3. Results and discussion

Real-time PCR was done two times using a Quantifiler Duo kit; in the first time it was done for the DNA extracted from maternal plasma samples from the 30 women included in the study during pregnancy, and in the second time it was done for the DNA extracted from the 12 bloodstain samples from the husbands of the women who were pregnant with male fetuses after delivery. Table 2 shows the concentrations of total human DNA (maternal and fetal) and male DNA detected in maternal plasma samples of women pregnant with male fetuses. According to the results shown in Table 2; by comparing the mean human DNA concentration (198 pg/µl), with the mean male DNA concentration (21.29 pg/µl), we found that the concentration of male DNA in maternal plasma of pregnant women with male fetuses reaches about 10.75% from the concentration of human DNA, which indicates the presence of maternal DNA in the maternal plasma with a percentage approximately reaching 10-folds that of fetal DNA. Taking into consideration ignoring the sample "ID 2" in the calculation of the mean male DNA concentration, because it possesses a very high male DNA concentration (1120 pg/µl) compared with the rest of the samples, may be because that

Table 2 Human and male DNA concentrations in maternal plasma samples for women pregnant with male fetuses.

Sample identity	Human DNA concentration (pg/µl)	Male DNA concentration (pg/µl)
ID 1	100.0	17.9
ID 2	304.0	1120.0
ID 3	155.0	10.2
ID 4	19.5	11.6
ID 5	57.6	26.3
ID 6	1300.0	82.0
ID 7	283.0	31.2
ID 8	34.8	5.9
ID 9	44.9	21.3
ID 10	20.0	17.2
ID 11	49.2	6.9
ID 12	8.8	3.7

Table 3 Human and male DNA concentrations in maternal plasma samples for women pregnant with female fetuses.

Sample identity	Human DNA concentration (pg/µl)	Male DNA concentration (pg/µl)
ID 13	11.1	–
ID 14	34.0	18.1
ID 15	126.0	18.1
ID 16	69.0	–
ID 17	219.0	–
ID 18	145.0	50.1
ID 19	37.4	–
ID 20	181.0	–
ID 21	21.3	3.79
ID 22	44.0	–
ID 23	21.0	–
ID 24	10.4	–
ID 25	4.7	–
ID 26	103.0	–
ID 27	61.2	3.3
ID 28	3.3	–
ID 29	8.7	13.5
ID 30	2.1	–

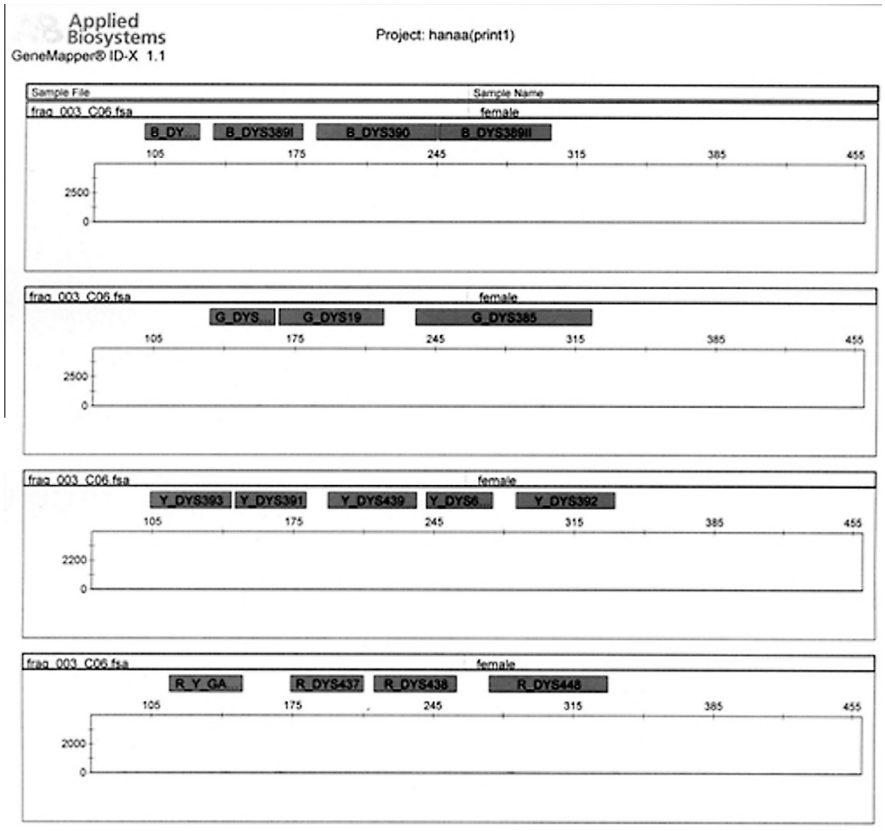

Figure 1 Electropherogram showing a maternal plasma sample for a woman pregnant with a female fetus.

woman was pregnant in a twin of 2 males and delivered 2 boys. Also, it is quite obvious from the results shown in Table 2 that Y-Filer kit can be used successfully to amplify male DNA with concentrations as low as 3.7 pg/μl.

Table 3 shows the concentrations of total human DNA (maternal and fetal) and male DNA detected in maternal plasma samples of women pregnant with female fetuses. According to the results shown in Table 3, it is clear that the male DNA concentration in maternal plasma of 12 out of 18 samples equals zero, this is logically because there is no male fetuses inside the uterus of any of those women. The rest of samples (6 out of 18) contains minute quantities of male DNA range from 3.3 pg/μl to 50.1 pg/μl. It was found from the clinical history reports of those 6 women, that only those 6 women have had a previous pregnancy with male fetuses in the past, where 5 of them delivered a boy and 1 had an abortion and did not complete pregnancy. It is proven scientifically that women who were pregnant with male fetus in the past whether the pregnancy completed and delivery occurred or the pregnancy did not complete remain possessing minute quantities of male DNA in their maternal circulation which may persists for decades [15]. Despite the presence of these detected minute quantities of male DNA in the 6 maternal plasma samples, these minute concentrations of male DNA did not have any effect on the Y-STR typing of these samples and all had given a negative result in all Y-STR loci, indicating that the gender of the fetus is female (Fig 1).

Table 4 shows the male DNA concentrations detected in bloodstain samples from the husbands of women pregnant with male fetuses. According to the results shown in Table 4, it is clear that the mean male DNA concentration (7.295 ng/μl), when compared to the mean male DNA concentration in maternal plasma samples of pregnant women with male fetuses (21.29 pg/μl), shows a huge difference which reflects the variation between the concentration of male fetal DNA present in maternal circulation and the concentration of male DNA in adult male circulation, where this difference may exceed 300-folds. This fact confirms the meagerness of fetal DNA molecules present in maternal circulation, which necessitates the performance of the concentration step which was done at the end of the extraction procedure to get the most benefit with DNA molecules in maternal plasma.

Table 4 Male DNA concentrations in bloodstain samples from husbands.

Sample identity	Male DNA concentration (ng/μl)
ID 31	8.5
ID 32	4.7
ID 33	12.36
ID 34	4.75
ID 35	9.21
ID 36	4.72
ID 37	4.77
ID 38	4.62
ID 39	11.5
ID 40	9.94
ID 41	8.22
ID 42	4.26

Table 5 Y-STR typing of maternal plasma samples for women pregnant within male fetuses. (ND: not detected).

	ID 1	ID 2	ID 3	ID 4	ID 5	ID 6	ID 7	ID 8	ID 9	ID 10	ID 11	ID 12
DYS456	14	15	14	14	15	14	14	14	15	14	14	14
DYS389I	13	13	13	14	13	13	13	13	13	13	13	13
DYS390	23	23	23	ND	23	23	23	23	24	23	22	23
DYS389II	30	29	ND	ND	30	ND	30	30	29	29	ND	30
DYS458	18	17	19	ND	19	17	18	18	17	18	18	18
DYS19	14	14	14	ND	14	14	14	14	15	14	14	14
DYS385a/b	14, ND	13, ND	13, ND	ND	13, 15	13, 13	12, ND	12, 19	18, 19	13, 18	ND	14, 17
DYS393	12	12	12	12	12	12	12	12	12	12	12	12
DYS391	11	10	10	11	11	11	11	11	10	10	11	12
DYS439	11	12	11	ND	11	11	12	11	14	11	11	11
DYS635	21	20	ND	ND	21	ND	21	ND	21	21	21	22
DYS392	11	11	ND	ND	ND	ND	11	ND	11	11	ND	ND
Y GATA H4	11	11	12	11	11	11	11	11	13	11	11	11
DYS437	14	14	13	ND	14	14	14	14	14	14	14	14
DYS438	10	10	10	10	10	10	10	10	10	10	10	10
DYS448	ND	19	ND	ND	ND	20	20	ND	19	20	20	19

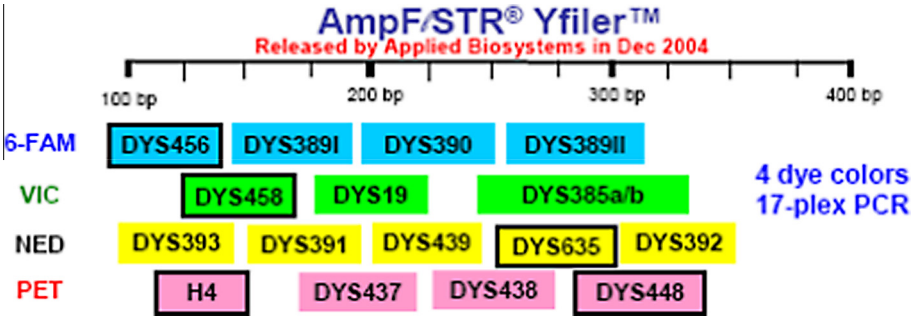

Figure 2 Sizes of amplified Y-STRs included in the Y-Filer system [16].

Multiplex fluorescent PCR with polymorphic Y-STR loci, used to analyze maternal plasma samples collected from women during the third trimester of pregnancy, revealed the presence of male fetal DNA expressed in a varied number of Y-STR loci in all women who delivered a boy afterward. The accuracy of multiplex PCR assay to detect male fetal DNA was 100%. At the same time, negative results were obtained in all cases who delivered a girl afterward showing no allele peaks at any of the analyzed Y-STR loci. All blood-stain samples from husbands show complete Y-STR profiles.

Table 5 shows the results of Y-STR typing of maternal plasma samples for women pregnant with male fetuses. From the results shown in Table 5, we notice that some plasma samples get full DNA profiles while others get partial DNA profiles due to the absence of some alleles in some Y-STR loci in their typing. In order to summarize the results of DNA typing shown in Table 5 with respect to each Y-STR locus included in the Y-Filer kit (Fig. 2), the number and percentage of maternal plasma samples detected in each Y-STR locus are listed in Table 6. According to the results shown in Table 6, we notice that the 6 Y-STR loci: DYS456, DYS389I, DYS393, DYS391, Y GATA H4, and DYS438 are detected in all maternal plasma samples with a percentage of detection reaching 100%, the 5 Y-STR loci: DYS390, DYS458, DYS19, DYS439, and DYS437 are detected in 11 out of 12 samples with a percentage

Table 6 Number and percentage of maternal plasma samples from pregnant women with male fetuses detected in each Y-STR Locus.

Y-STR Loci	Number of samples detected	Percentage of samples detected (%)
DYS456	12/12	100
DYS389I	12/12	100
DYS390	11/12	91.6
DYS389II	8/12	66.6
DYS458	11/12	91.6
DYS19	11/12	91.6
DYS385a	10/12	83.3
DYS385b	6/12	50
DYS393	12/12	100
DYS391	12/12	100
DYS439	11/12	91.6
DYS635	8/12	66.6
DYS392	5/12	41.6
Y GATA H4	12/12	100
DYS437	11/12	91.6
DYS438	12/12	100
DYS448	7/12	58.3
Mean	171/204	83.8

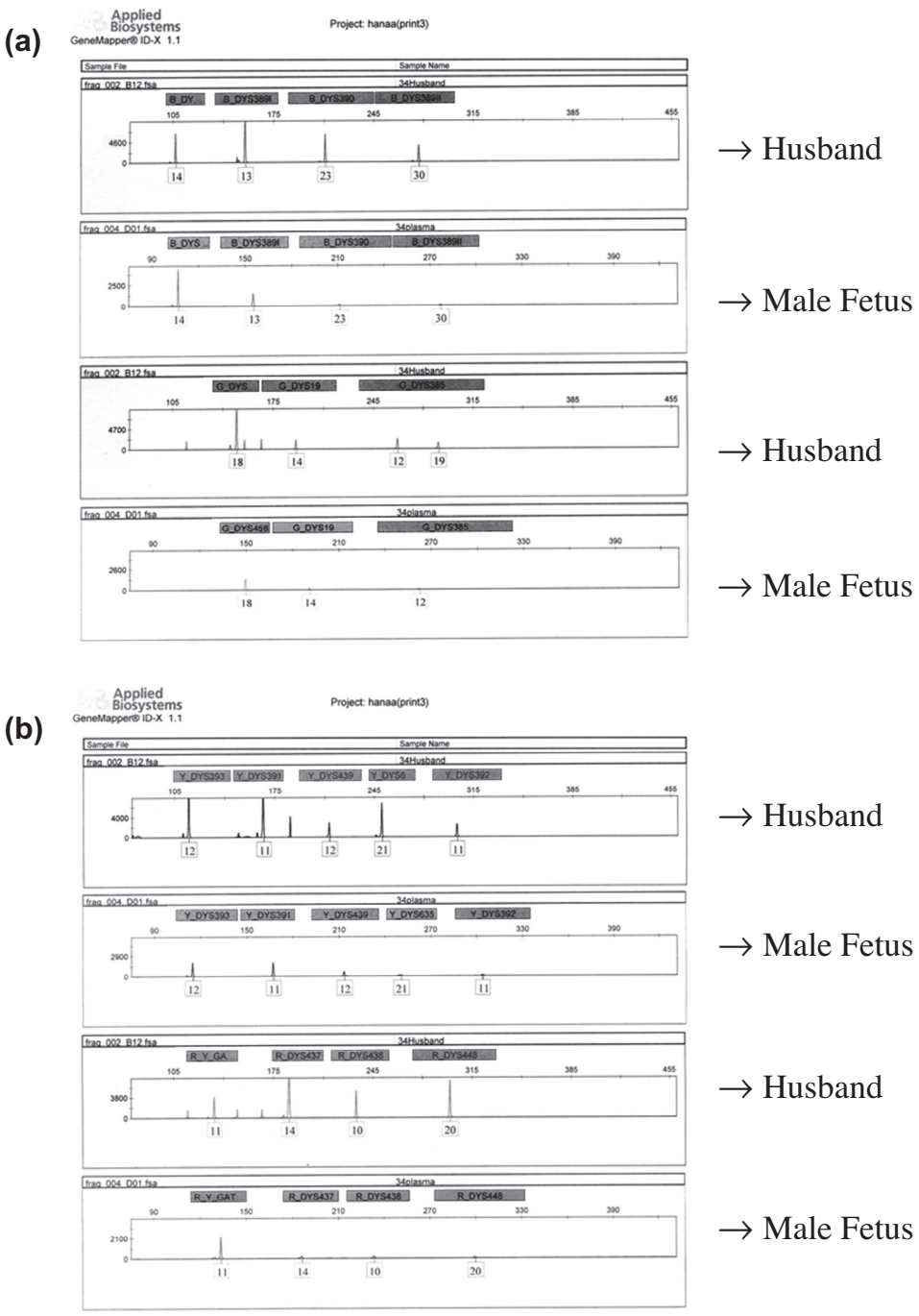

Figure 3 (a) Electropherogram showing a comparison between a maternal plasma sample for a woman pregnant with a male fetus and a husband sample. (b) Electropherogram showing a comparison between a maternal plasma sample for a woman pregnant with a male fetus and a husband sample (continued).

of detection reaching 91.6%, the 3 Y-STR loci: DYS389II, DYS635, and DYS385a are detected in 8, 8, and 10 samples respectively with a percentage of detection ranging from 66.6% to 83.3%, and the 3 Y-STR loci: DYS392, DYS385b, and DYS448 are detected in 5, 6, and 7 samples respectively with a percentage of detection ranging from 41.6% to 58.3%.

From the previous analysis, we notice that; as the size of the Y-STR locus is increased (Fig. 3), the chance of its detection in maternal plasma is decreased. There are 5 out of 6 Y-STR loci detected in all maternal plasma samples, their sizes are less than 200 base pairs, and the sixth locus is less than 275 base pairs. The 5 Y-STR loci detected in 11 out of 12 maternal plasma samples have their sizes less than 250 base pairs. The 3 Y-STR loci (DYS389II, DYS635, and DYS385a) detected in 8, 8, and 10 plasma samples have their sizes fall in the region between 250 and 315 bp. And the 3 Y-STR loci (DYS392, DYS385b, and DYS448) detected in 5, 6, and 7 plasma samples have their sizes fall in the region between 275 and 350 bp.

On the other side, Fig. 3 shows a comparison between a Y-STR profile for a bloodstain sample from a husband and

a Y-STR profile for a maternal plasma sample from the wife. In the figure, there is a complete concordance between the alleles in the fetal male DNA profile and the alleles in the husband's DNA profile in all Y-STR loci, which means that the male fetus of the wife possesses the same Y-STR typing as the husband, i.e.: the male fetus is attributed to the males of the family of the husband. In the same figure, we observe the differences between the rfu values between both samples in each Y-STR locus, determining a lower male DNA concentration in case of the maternal plasma sample compared with the high DNA concentration of male DNA in the bloodstain sample of the husband.

4. Conclusions

The success in detecting the number of Y-STRs in the result of Y-STR profiling analysis of maternal plasma of pregnant women, means that the gender of the fetus is male, and at the same time the negative result in the Y-STR profiling analysis in all Y-STR loci, means that the gender of the fetus is female. Therefore; the results of the study prove the feasibility of applying Y-STR typing as a gender determining test for the detection of the gender of the fetus during the third trimester of pregnancy with a percentage of accuracy reaching 100%.

Moreover, Y-STR profiling analysis can be done during pregnancy to decide whether the male fetus is attributed to the family of the alleged father or not. This can be achieved, in case of denying, in all paternity cases where there is a difference between the Y-STR typing of the fetus and that of the husband even in one Y-STR locus, and in case of proving, in all paternity cases where the number of detected Y-STR loci is greater than 8 [17].

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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