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Fetal male lineage determination by analysis of Y-chromosome STR haplotype in maternal plasma

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ABSTRACT

The aim of this study is to determine the fetus Y-STR haplotype in maternal plasma during pregnancy and estimate, non-invasively, if the alleged father and fetus belong to the same male lineage. The study enrolled couples with singleton pregnancies and known paternity. All participants signed informed consent and the local ethics committee approved the study. Peripheral blood was collected in EDTA tubes (mother) and in FTA paper (father). Maternal plasma DNA was extracted by using NucliSens EasyMAG. Fetal gender was determined by qPCR targeting DYS-14 in maternal plasma and it was also confirmed after the delivery. From all included volunteers, the first consecutive 20 mothers bearing male fetuses and 10 mothers bearing female fetuses were selected for the Y-STR analysis. The median gestational age was 12 weeks (range 12–36). All DNA samples were subjected to PCR amplification by PowerPlex Y23, ampFLSTR Yfiler, and two in-house multiplexes, which together accounts for 27 different Y-STR. The PCR products were detected with 3500 Genetic Analyzer and they were analyzed using GeneMapper-IDX. Fetuses' haplotypes (Yfiler format) were compared to other 5328 Brazilian haplotypes available on Y-chromosome haplotypes reference database (YHRD). As a result, between 22 and 27 loci were successfully amplified from maternal plasma in all 20 cases of male fetuses. None of the women bearing female fetuses had a falsely amplified Y-STR haplotype. The haplotype detected in maternal plasma completely matched the alleged father haplotype in 16 out of the 20 cases. Four cases showed single mismatches and they did not configure exclusions; 1 case showed a mutation in the DYS 458 locus due to the loss of one repeat unit and 3 cases showed one DYS 385I/II locus dropout. All mismatches were confirmed after the delivery. Seventeen fetuses' haplotypes were not found in YHRD and one of them had a mutation, which corresponded to the paternity probability of 99.9812% and 95.7028%, respectively. Three fetuses' haplotypes occurred twice in YHRD, which corresponded to paternity probability of 99.9437%. In conclusion, high discriminatory fetal Y-STR haplotype could be determined from maternal plasma during pregnancy starting at 12 weeks of gestation. All male fetuses could be attributed to the alleged father male lineage early in pregnancy. The high probability of paternity associated with each case suggests that the relationship is not random and this strategy can be use as an alternative for male fetal kinship analysis.

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1. Introduction

The identification of cell-free fetal DNA (cfDNA) in maternal circulation [1] has made non-invasive prenatal testing possible [2]. Since its discovery, the cfDNA has drawn much attention because its analysis provides genetic information about the fetus with reduced risk associated with fetal material obtainment. The amniocentesis and chorionic villus sampling carry a small but clear risk of miscarriage [3]. Currently, several applications of

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non-invasive fetal genetic analysis are available at clinical services, they include detection of fetal sex [4], rhesus D blood type [5], fetal aneuploidy [6], paternal-derived mutations [7] and, also, paternity [8].

The cfDNA originates from the placenta cells and apoptosis appears to be the main mechanisms controlling its releases to the mother circulation [9]. At 10 weeks of gestation, the median cfDNA fraction in the maternal plasma is 10.2% and its levels increases throughout the pregnancy, with an initial rise of 0.1% per week from 10 to 20 weeks of gestation, followed by a sharper increase of 1% per week after 21 weeks to term [9,10]. The fetal DNA sequences in maternal plasma are present at a larger proportion in sizes of <150 bp and are rarely longer than 250 bp [11], and their final disappearance from maternal circulation occurred after 1–2 days postpartum [12].

The major challenge for cfDNA assays is to distinguish the fetal sequences in the background of the highly homologous maternal DNA. Many investigators have based their detection strategy on targeting the genetics differences between mother and fetus. The most widely used genetic difference in cfDNA studies was the Y-chromosome [13,14]. Indeed, the plasma DNA from a pregnant woman bearing a male fetus is a male:female specimen admixture.

In forensic science, the analysis of male/female DNA admixture is quite common e.g., sexual assault cases. The Y-chromosome short tandem repeats (Y-STR) haplotyping is a method of choice that unambiguous detects and differentiates the male component in DNA mixtures with a high female background [15]. Indeed, Mayntz-Press et al. reported that full Y-STR profiles are obtained from samples with 1:1000 male:female DNA ratio [16]. Furthermore, the Y-STR technology has proved useful in reconstructing paternal relationship [17] and there are many commercial kits available for Y-STR haplotyping.

Today, in our complex society, there are many situations where it would be desirable to perform the male fetal kinship analysis during pregnancy. Thus, the aim of this study is to determine the male fetal Y-STR haplotype in maternal plasma during pregnancy and estimate, non-invasively, if the fetus and alleged father belongs to the same paternal lineage.

2. Materials and methods

2.1. Ethics statement

The health science faculty ethical committee of the University of Brasilia approved this study (IRB protocol # 188/12) and written informed consent was obtained from each participant.

2.2. Subjects

This experimental study enrolled 30 couples with singleton pregnancies and no doubt about the paternity. From all volunteers who agreed to participate, the first twenty mothers bearing a male fetus (cases) and the first 10 mothers bearing a female fetus (controls) were selected for Y-STR analysis. The median (min–max) gestational age was 12 (12–36) weeks.

2.3. Alleged father and child's reference samples

The alleged father's reference sample was obtained during his first visit and the child's reference sample was collected after birth during the occasion of the fetal neonatal screening for inborn errors of metabolism. Blood samples were collected from the tip of the ring finger (father) and from the heel (child) on FTA™ paper card (Whatman). The DNA was purified from the blood spots following the protocol of the manufacturer.

2.4. Maternal blood collection, processing and transporting

Maternal blood was collected by arm venipuncture in three 4 mL Vacuette K2 EDTA Sep tubes (Greiner Bio-one). Then, the tubes were centrifuged at 2000 × *g* for 10 min at room temperature for maternal plasma separation. After that, they were transported to processing center at 22 ± 4 °C and stored at −20 °C until further use.

2.5. Maternal plasma DNA extraction

After thawing, 1100 μL of the maternal plasma were transferred into polypropylene tubes and centrifuged at 14,000 × *g* for 10 min at room temperature. The DNA was extracted from 1000 μL of each sample by using the generic protocol 2.0.1 of the NucliSENS easyMAG system (bioMerieux), 100 μL of magnetic silica particle suspension and elution in 25 μL.

2.6. Fetal gender determination (DYS-14 assay)

A multiplex qPCR reaction targeting the Y-chromosome specific sequence (DYS-14) [18] and RNAse P (internal control) were used for fetal gender determination. The DYS-14 primer and probe sequences were DYS14-F (5′-CCATGACCCCAGAGTCTGC-3′), DYS14-R (5′-CTTCCTGGCTTGGGCATTAAC-3′) and DYS14 probe (5′-6-FAM-CTCCAGCTC/ZEN/CACCTGAACGGCC-IABFQ-3′). The RNAse P primer and probe sequences were RNAse P–F (5′-AGATTTG-GACCTGCGAGCG-3′), RNAse P–R (5′-GAGCGGCTGTCTCCACAAGT-3′) and RNAse P probe (5′-HEX-TTCTGACCT/ZEN/GAAGGCTCTG CGCG-IABFQ-3′). Both were purchased as PrimeTime qPCR assays from Integrated DNA Technologies.

Briefly, qPCR assay consisted of 2 μL of 10× DSY-14 Prime Time Assay, 1 μL 10× RNAse P Prime Time Assay, 12.5 μL Maxima Probe qPCR master mix (Fermentas) and 9 μL of extracted DNA in a 25 μL volume adjusted with DNase/RNase-free water (Fermentas). It was performed on a ABI Step-One qPCR System (life technologies). The PCR cycling conditions were: preincubation for 10 min at 95 °C, 60 cycles of 15 s at 95 °C, 60 s at 60 °C. All samples were run in quadruplicate and each run included one female, one male and one no template controls. The interpretation criteria were: 4 positive results for DYS-14 with Cq < 34 indicated a male fetus, 0 or 1 positive results with Cq > 40 for DYS-14 indicated a female fetus, all other results were considered indeterminate and the reaction was repeated. RNAse P should amplify in all instances, except in the no temple control, otherwise, the result was invalid. Fetal sex was also confirmed by visualization of the external genitalia after the delivery.

2.7. Y-STR amplification in the maternal plasma

2.7.1. Powerplex Y23

The first commercial kit used for Y-STR amplification was the Powerplex Y23 System kit (Promega). Its reaction was performed according to the manufacturer's instructions in a GeneAmp 9700 PCR System (Life Technologies), except by the use of 60 PCR cycles.

2.7.2. Yfiler

The second commercial kit used for Y-STR amplification was the AmpFlST Yfiler PCR amplification kit (Life Technologies). Its reaction was performed according to the manufacturer's instructions in a GeneAmp 9700 PCR System (Life Technologies), except by the use of 60 PCR cycles.

2.7.3. Mini-1 and Mini-2 Y-STR multiplex systems (Mini-1/-2)

The third (Mini-1) and fourth (Mini-2) multiplex reactions used for Y-STR amplification were previously described by Asamura

et al. [19], they included only mini Y-STR. The mini-1 Y-STR multiplex reaction (4-plex) consisted of 1.0 μL of primer mix (see below), 12.5 μL Maxima Probe qPCR master mix (Fermentas) and 10 μL of extracted DNA in a 25 μL volume adjusted with DNase/RNase-free water (Fermentas). The primer concentration were as follow: DYS522 (6FAM) 0.5 μM, DYS508 (VIC) 0.6 μM, DYS632 (NED) 0.6 μM, DYS556 (PET) 1.4 μM. The PCR cycling conditions were: preincubation for 10 min at 95 °C, 50 cycles of 15 s at 95 °C, 30 s at 60 °C and a final extension of 20 min at 60 °C.

The Mini-2 Y-STR multiplex reaction (3-plex) was identical to the mini-1, except the primer mix composition DYS570 (6FAM) 0.5 μM, DYS576 (VIC) 0.5 μM, DYS540 (PET) 1.4 μM and the PCR cycling condition (preincubation for 10 min at 95 °C, 50 cycles of 15 s at 95 °C, 30 s at 55 °C and a final extension of 20 min) at 60 °C). TC-3000 thermocycler (Techne) was used to perform both reactions. The primers for Mini-1 and Mini-2 loci were synthesized by life technologies.

2.7.4. Y-STR amplification from alleged father and child references samples

The Powerplex Y23 and mini-1/-2 systems were used to genotype the father's reference sample. The reactions were performed as described above, except the number of PCR cycles that were reduced to 30 in all instances. Moreover, a total of 0.5–1.0 ng of DNA (contained in a 1.2 mm FTA punch) was used per PCR reactions.

2.7.5. Kinship analysis with NGM

When necessary, the AmpFlSTR NGM PCR amplification kit was used to perform the kinship analysis and the reactions were performed according manufacturer's instructions.

2.8. Electrophoresis

The PCR products were separated and detected with a 3500 Genetic Analyzer. For Yfiler, NGM and mini-1/-2 reactions, 1 μL of the amplified sample was added to 8.5 μL Hi-Di Formamide and 0.5 μL of GeneScan 600 LIZ. The electrophoresis condition was 15 s injection time, 1.2 kV injection voltage, 15 kV run voltage, 60 °C, 20 min run time, Dye Set G5 (6FAM, VIC, NED, PET and LIZ). For Powerplex Y23 reaction, 1 μL of the amplified sample was added to 10 μL Hi-Di Formamide and 1 μL of CC5 ILS Y23 (Promega). The electrophoresis condition was identical as described for Yfiler, except for the Dye Set G5 (FL, JOE, TMR-ET, CXR-ET and CC5 from Promega). Raw data was analyzed with the GeneMapper® ID-X Software V.1.2 (all the equipment and reagents were from Life Technologies, except otherwise indicated). A peak detection threshold of 200 RFUs was used for marker identification calls.

2.9. Statistical analysis

The haplotype frequencies were determined by surveying the maternal plasma Y-STR haplotype at the Brazilian national database ($n = 5328$) on the Y-Chromosome haplotype reference database (YHRD). The 17 loci included in the Yfiler were considered for this analysis (haplotype in the Yfiler format), because of the low number of Powerplex Y23 haplotypes in the database for the considered population and the absence of data for some loci included in the Mini-1 (DYS522, DYS508, DYS632, DYS556) and Mini-2 (DYS540) reactions.

The paternity index for each case was calculated as previously described [20]. In short, in cases without mutation, the paternity index is the one divided by the haplotype frequency; in cases with mutation/exclusion, the paternity index is $(0.5 \times \mu)$ divided by the haplotype frequency, where μ is the overall mutation rate of the locus, showing a single mutation/exclusion due to contraction/expansion of one repeat unit [20]. The probability of paternity was calculated by the following formula: paternity index $\times$ 100/(paternity index − 1) [21].

2.10. External quality control

Sabin laboratory is ISO9001/2008 certified, participates in th e GHEP/ISFG proficiency testing and contributes by sending haplotypes to the YHRD.

3. Results and discussion

The DYS-14 assay was used to determine the fetal sex during pregnancy and guided the volunteers' selection for the Y-STR analysis. The first consecutive 20 and 10 mothers bearing male and female fetuses, respectively, were selected for Y-STR analysis. After the delivery, we observed a complete concordance between the fetal sex attributed by the DYS-14 assay and the newborn gender.

Considering all multiplex systems (Powerplex Y23, Yfiler and Mini-1/-2), between 22 and 27 loci (25 on median) were successfully amplified from maternal plasma in all 20 cases of male fetuses and either no or neglected Y-STR amplification was observed in women bearing female fetuses (Table 1 and Table S1). Representative electropherograms obtained from maternal plasma by using the Powerplex Y23 and Yfiler in a male and in a female samples are illustrated in Figs. S1 and S2, respectively. In addition, representative electropherograms obtained by using the Mini-1/-2 can be found in Fig. S3.

Clearly, the fetal Y-STR detection success was amplicon size dependent and it ranged from 100% to 5% in Powerplex Y23, from 100% to 50% in Yfiler and it was 100% for all loci included in mini-1/-2. Indeed, all Y-STR loci with detection success of 55% or less have amplicons with size greater than 250 bp (Table S2).

The specific contribution of each multiplex for the Y-STR loci detection success is detailed in Table 2. Powerplex Y23 and the mini-1/-2 were the only contributors of 3 and 5 loci, respectively; since they are unique to these systems (Table 2, 1st and 3rd columns), moreover, they overlap in 2 successfully detected loci (Table 2, 4th column). Furthermore, Yfiler and Powerplex Y23 overlap in 9 successfully detected loci (Table 2, 5th column) and, despite the redundancy between both systems, Yfiler was the major contributor for the detection of 7 Y-STR (Table 2, 2nd column), because the amplicon size for these loci were smaller in the former one compared with Powerplex Y23 (Table S2), e.g. the amplicon size of DYS19 ranges from 167 bp to 218 bp in Yfiler and in Powerplex Y23 from 312 bp to 352 bp. However, we cannot exclude that this result is secondary to the fact that in 8 out of 20 cases two Yfiler reactions were performed.

The Y-STR haplotype detected in maternal plasma completely matched the alleged father in 16 out of the 20 cases and 4 cases showed singles mismatches (Table 1). Fig. S4 showed a representative example of matching analysis between maternal plasma and alleged father Y-STR haplotypes. In short, the extensive haplotypes retrieved from the maternal plasma resulted in an overall concordance at Y-STR loci level of 99.2%.

In regard of the mismatches, they were:

(a) The case 1 that showed a single exclusion/mutation pattern at DYS458 due to the loss of one repeat unit. The kinship analysis of the case 1 (trio), performed after the delivery by using the autosomal STR markers included in the NGM kit (life Technologies) and local allele frequency population data [22], confirmed the paternity (paternity index of 3,472,249, 188.76 and probability of paternity of 99.999999971). The mutation was also confirmed after the delivery by using the

Table 1
Qualitative results for fetal Y-STR loci detection in the plasma of mothers bearing male ($n = 20$) and female ($n = 10$) fetuses and their matching to the alleged father loci.

Case no.	Gestational age (weeks)	DYS-14 assay (maternal plasma)	Newborn's gender at delivery	Y-STR loci (maternal plasma)	Matching to alleged father	Matching to newborn
1	12	Male	Male	26	25[a]	26
2	12	Male	Male	25	25	25
3	12	Male	Male	22	21[b]	21[b]
4	12	Male	Male	26	26	26
5	24	Male	Male	23	23	23
6	36	Male	Male	27	27	27
7	12	Male	Male	24	24	24
8	17	Male	Male	24	24	24
9	12	Male	Male	25	25	25
10	12	Male	Male	26	26	26
11	16	Male	Male	26	26	26
12	12	Male	Male	26	26	26
13	12	Male	Male	25	25	25
14	12	Male	Male	24	24	24
15	12	Male	Male	23	23	23
16	12	Male	Male	25	25	25
17	12	Male	Male	25	24[b]	24[b]
18	24	Male	Male	25	24[b]	24[b]
19	19	Male	Male	25	25	25
20	17	Male	Male	24	24	24
21	13	Female	Female	0	0	NA
22	12	Female	Female	0	0	NA
23	12	Female	Female	0	0	NA
24	12	Female	Female	0	0	NA
25	12	Female	Female	0	0	NA
26	12	Female	Female	0	0	NA
27	12	Female	Female	0	0	NA
28	12	Female	Female	0	0	NA
29	12	Female	Female	0	0	NA
30	12	Female	Female	0	0	NA

NA – not applicable.
[a] Case with a mutation in the DYS 458 locus.
[b] Cases with dropout of one DYS 385I/II locus in maternal plasma.

Table 2
Major multiplex system contributors for each Y-STR detection success in maternal plasma.

Powerplex Y23	Yfiler[a]	Mini-1/-2	Mini-1/-2 + Powerplex Y23	Powerplex Y23 + Yfiler[a]
DYS 481	DYS 19	DYS 522	DYS 576	DYS 389I
DYS 549	DYS 438	DYS 508	DYS 570	DYS 448
DYS 533	DYS 437	DYS 632		DYS 389II
	DYS 439	DYS 556		DYS 391
	DYS 392	DYS 540		DYS 635
	DYS 456			DYS 390
	YGATA H4			DYS 393
				DYS 458
				DYS 385I/II

[a] Two Yfiler reactions was performed in 8 out of 20 cases.

Powerplex Y23 (Fig. S5). Indeed, the probability of find at least one mutation between two Y-STR haplotypes one generation apart, if 22 and 26 loci were genotyped, is relatively high, 6% and 7%, respectively [23].

(b) The cases 3, 17 and 18 that showed a dropout pattern in one of two DYS 385I/II locus in the fetal Y-STR profile when compared to the alleged father. The locus dropout at the DYS385 I/II was confirmed by using Powerplex Y23 in the newborn sample (Fig. S6). The observed drop-out of one locus could be secondary to the peculiarity associated to this multi-copy Y-STR [23] along with its larger amplicon-size (223–307 bp for Powerplex Y23 and 234–324 bp for Yfiler).

The number of Y-STR locus surveyed in the YHRD in each case ranged from 13 to 16, and the median was 16 (Table S3). Quantitatively, a paternity index and a probability of paternity were attributed to each case. These estimations were based on the fetus haplotype frequency retrieved from Brazilian national database found in YHRD.

In 16 out of the 20 cases, the fetal haplotype did not match any of the 5328 Brazilian haplotypes available at the YHRD, that resulted in a haplotype frequency of 0.0001877 (1/5328), in a paternity index of 5328 (1/0.0001877), exactly the database sizes, and in a probability of paternity of 99.9812%.

In 1 out of the 20 cases, the fetal haplotype did not match to any Brazilian haplotypes available at the YHRD, but has a mutation in DYS 458 locus. In this case, the haplotype frequency was 0.0001877 (1/5328), paternity index was calculated by the specific formula described in methods [$(0.5 \times 0.00836)/0.0001877 = 22.271$], which included a penalization that accounted the mutation rate of the DYS458 locus (0.00836) [24], and this paternity index resulted in a probability of paternity of 95.7028%.

In 3 out of the 20 cases, the fetal haplotype match two Brazilian haplotypes available at the YHRD, what resulted in a haplotype frequency of 0.0005631 (3/5328), in a paternity index of 1776 (1/0.0005631) and in a probability of paternity of 99.9437%.

The DYS385 locus was excluded from the quantitative analysis in the cases with dropout (3, 17, and 18) and it did not change the number of matches in the database. There was total match between the newborn Y-STR haplotype and the Y-STR loci detected in the maternal plasma in all 20 cases with male fetuses (Table S1).

Previous studies have successfully amplified Y-STR from maternal plasma by using commercial kits, howsoever, the haplotypes retrieved was not consistently extensive enough with 6–16 Y-STRs, 12 on median [25] or 5–12 Y-STRs, 8 on median [26] to be high discriminatory. Consequently, they would have higher frequency compared to haplotypes found in the present study,

which are associated with lower paternity index and probability of paternity.

The consistent obtainment of such extensive haplotypes was possible due to different reasons: (a) there were substantial overlap between the loci included in the multiplex systems; (b) the high amplification cycle number compared to previous studies [25,26]; (c) the 3500 Genetic Analyzer had several significant changes from the previous 31xx generation instruments [27]; and (d) the high input of maternal plasma (1 mL) used for DNA extraction.

The use of high amplification cycle number is a standard procedure in the non-invasive pre-natal diagnostic. Previous studies in the field have described PCR amplification step with 60–50 PCR cycles [1,28–30]. Nonetheless, this procedure together with the capillary electrophoresis analysis is prone to artifacts like nonspecific amplification and color pull-up that results in drop in (see Figs. S1 and S2). Therefore, great care should be taken in the profiles interpretation (see DYS 549 locus of the Powerplex Y23 profile at Fig. S1, it was excluded from the analysis due to the allele 12 drop in, despite the allele 13 match the alleged father profile). Furthermore, the high amplification cycles number is also prone to PCR contamination; the known procedures to avoid amplicon carryover should be applied strictly. The use of only mini Y-STR, which allows the use of less amplification cycle number should eliminate this problem.

Today, in our complex society, there are many situations where it would be desirable to perform the non-invasively prenatal paternity testing by the analysis of the circulating cell-free fetal DNA (e.g. ambiguous paternity in case of women with more than one sexual partner who are unsure of the actual father) [8,31,32]. The fetal male lineage determination by analysis of Y-chromosome STR haplotype in maternal plasma described in this study can be use as an alternative for this purpose. The main limitation of the strategy is that it is only applied for mothers bearing a male fetus and its conclusion will be if alleged father and fetus belongs the same paternal lineage or not. Consequently, the test should not be applied in population with a high rate of endogamy.

Non-invasive prenatal testing to establishing paternity, which is currently commercially offered, has been criticized due to its ethical issues [33–35]. Some authors states that a pregnant women would intente on testing to determine whether she will continue the pregnancy [33]. It has been suggested to counsel the women involved about the relative significance of biological kinship [33]. At the same time, some authors classify this approach as morally problematic [31]. On the other hand, women could feel compelled to terminate the pregnancy anyhow without paternity testing or women could feel compelled to continue the pregnancy with the consequence of having a child fathered by the wrong man. Prenatal paternity testing may, therefore, lead to the least harm for the woman involved and be morally justified [31,36].

In conclusion, here we described that male fetal Y-STR can be retrieved from maternal plasma by using complementary multiplex system (Powerplex Y23, Yfiler and two in-house mini Y-STR systems), and it can be used to link the child to the alleged father male lineage early in pregnancy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2014.11.006.

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