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TECHNICAL NOTE

Non-invasive prenatal paternity testing from maternal blood

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Abstract Prenatal paternity analysis can be performed only after invasive sampling of chorionic villi or amnionic fluid. Aiming to enable noninvasive paternity testing, we attempted to amplify fetal alleles from maternal plasma. Cell-free DNA was isolated from plasma of 20 pregnant women and amplified with ampFLSTR Identifiler and ampFLSTR Yfiler kits. Unfortunately, autosomal fetal alleles were heavily suppressed by maternal DNA, and the only locus that was reliably amplified with AmpFLSTR Identifiler kit was amelogenin, which revealed only fetal gender. Much better success was obtained with AmpFLSTR Yfiler kit, which, in the case of male fetuses, successfully amplified between six and 16 fetal loci. All amplified fetal alleles matched the alleles of their putative fathers, confirming the tested paternity. To the best of our knowledge, this is a first report of noninvasive prenatal paternity testing.

Keywords Prenatal paternity testing · Cell-free fetal DNA · STR loci

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Introduction

DNA typing is a powerful tool for forensic analysis, and today, it is being routinely used in casework, paternity analysis, and the identification of victims of mass fatality events [5, 8, 12, 15]. At least 15 short tandem repeat (STR) loci are being analyzed in nearly all laboratories, and this system is now generally considered sufficient to determine identity or paternity with very high probability of inclusion or exclusion [4, 13]

In some cases of rape that may have resulted in pregnancy, it is necessary to perform early prenatal paternity analysis. This is most frequently done by testing of chorionic villi or amnionic fluid, but this invasive technique cannot be performed before the 13th week of pregnancy; it is stressful and also bears a small but existing risk for both mother and child. Since the first report of its existence in 1997 [10], cell-free fetal DNA in maternal plasma has become an important target for noninvasive prenatal diagnosis [14]. Circulating fetal-associated DNA is of a higher abundance than fetal cells in the maternal circulation [1], and the post-partum clearance was shown to be rapid [11]. Prenatal determination of fetal gender by the analysis of cell-free fetal DNA in maternal plasma has been reported in numerous publications [9], and this approach will soon be routinely used in many laboratories [7].

Aiming to verify whether fetal polymorphic STR loci covered by ampFLSTR Identifiler™ and ampFLSTR Yfiler™ kits can also be successfully amplified from maternal plasma and used for paternity testing, we analyzed DNA from plasma samples of 20 pregnant women and compared them to DNA from blood samples of putative fathers of their children.

Table 1 Fetal alleles amplified from maternal plasma samples

Sample no.	ampFLSTR Identifiler			ampFLSTR Yfiler	
	Amel.	STR loci	Matching to paternal	STR loci	Matching to paternal
1	Y	1	1	12	12
2	X	4	4	0	N/A
3	Y	3	3	8	8
4	Y	3	3	8	8
5	X	3	3	0	N/A
6	Y	5	5	11	11
7	X	2	2	0	N/A
8	Y	4	4	10	10
9	Y	4	4	8	8
10	X	0	N/A	0	N/A
11	Y	0	N/A	6	6
12	Y	4	4	12	12
13	Y	5	5	16	16
14	Y	6	6	16	16
15	Y	3	3	15	15
16	Y	3	3	12	12
17	Y	2	2	13	13
18	X	4	4	0	N/A
19	X	4	4	0	N/A
20	X	2	2	0	N/A

Materials and methods

Peripheral blood samples (n=20) were collected from pregnant women (length of gestation was between 9 and 29 weeks) attending the Clinical Hospital Osijek (Croatia) between 2006 and 2007. Blood samples were also taken from putative fathers. Informed consent was obtained from all studied individuals and approved by the Ethics Committee of the Clinical Hospital Osijek. Blood was collected into ethylenediaminetetraacetic acid tubes, and all samples were taken before performance of any invasive procedure.

Plasma DNA preparation

The peripheral blood samples (V=9 ml) of pregnant women were treated by centrifugation at 1,400×g for 10 min using a bench-top centrifuge. The supernatant plasma was removed very carefully and recentrifuged at the same speed for another 10 min. The plasma (V=4 ml) was then collected in a fresh tube. DNA was extracted from plasma using a QIAamp blood kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. After purification, DNA was concentrated by ultrafiltration on Centricon YM-100 tubes (Millipore; FisherScientific, Montreal, Canada) to final volume of approximately 60 μl, and 6 μl of this solution was used as a template for polymerase chain reaction (PCR). The amount of total human and male DNA was determined by real-time PCR using two DNA quantification assays: Quantifiler™ Human DNA Quantification kit and Quantifiler™ Y Human Male DNA Quantification kit (Applied Biosystems, Foster City, CA).

Fig. 1 Analysis of polymorphic Y chromosome STR loci. **a** Electropherogram of polymorphic Y chromosome STR loci from plasma of a mother bearing a male fetus (12th week of pregnancy). Cell-free DNA was isolated from maternal plasma and analyzed as described in "Materials and methods." In this example, 15 out of 16 STR loci were successfully amplified. The numbers shown next to each peak are allele names that were determined by comparison to allelic ladder and correspond to the number of repeats of basic STR sequence. **b** Comparison of polymorphic Y chromosome STR loci between father and fetus. Genotype of a fetus from **a** is shown in a tabular form and compared to a father's genotype. All Y chromosome STR alleles that were amplified from maternal plasma were identical to paternal alleles supporting the proposed paternity

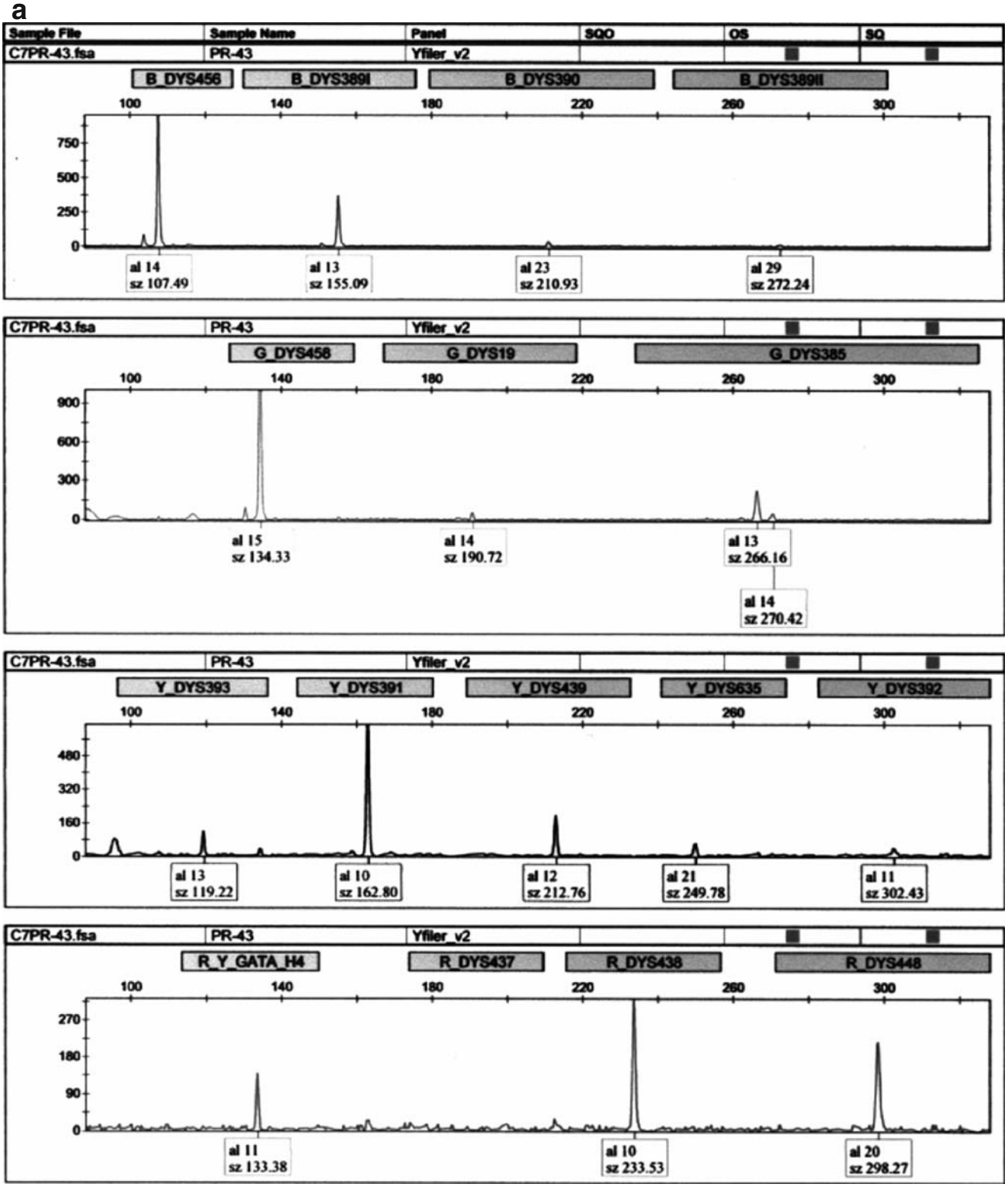
Multiplex PCR

To make the original manufacturer's protocol more suitable for amplification of low amounts of DNA, the following changes were made: (1) total reaction volume was reduced to 15 μl (6.2 μl reaction mix, 3.1 μl primer set), (2) enzyme concentration was increased by 60%, and (3) annealing and elongation times were increased to 80 s (from original 60 s). Each sample was analyzed in triplicates, with each of the triplicates being amplified for 30 cycles. Autosomal STR loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, and FGA) and amelogenin were amplified using the ampFLSTR Identifiler™ kit (Applied Biosystems). Y chromosome STR loci (DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS385, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS635, and GATA H4) were amplified using the ampFLSTR Yfiler™ kit (Applied Biosystems). Amplified fragments were analyzed on ABI Prism 310 Genetic Analyzer (Applied Biosystems). The identity of each allele was determined by comparison to allelic ladder.

Results and discussion

Cell-free fetal DNA exists in the maternal circulation, and amplification of Y-chromosome specific loci from maternal plasma has been reported by numerous groups [9]. However, to our knowledge, there is only a single report of the use of a validated STR multiplex system for the analysis of fetal DNA in maternal plasma. Birch et al. reported in [2] an attempt to amplify Y-chromosome DNA from maternal plasma using AmpFLSTR SGM Plus™ Kit (Applied Biosystems) but without any success [2]. Sup-

a



b

	DYS 19	DYS 389I	DYS 389II	DYS 390	DYS 391	DYS 392	DYS 393	DYS 385	DYS 437	DYS 438	DYS 439	DYS 448	DYS 456	DYS 458	DYS 635	GATA H4
Maternal plasma	14	13	29	23	10	11	13	13,14	-	10	12	20	14	15	21	11
Paternal blood	14	13	29	23	10	11	13	13,14	16	10	12	20	14	15	21	11

Table 2 The number of successfully amplified alleles from mixtures of male/female and male/male human genomic cell-free DNA

	Main component is female DNA					Main component is male DNA				
	50%	10%	4%	2%	1%	50%	10%	4%	2%	1%
ampFLSTR Identifiler	19	13	8	4	1	17	15	3	4	0
ampFLSTR Yfiler	17	14	12	9	6	10	8	6	5	2

Male and female cell-free genomic DNA was isolated from plasma samples, quantified using Quantifiler kit, and mixed in 1:1, 1:9, 1:24, 1:49, and 1:99 ratios of male/female and male/male DNA. Mixed samples were analyzed using AmpFLSTR Identifiler and AmpFLSTR Yfiler kits, and the number of successfully amplified informative alleles (alleles that are different between the two individuals) from minor DNA component is reported. Original electropherograms are provided in the Electronic supplementary material.

pression of minor alleles is a common complication in the analysis of low-copy DNA samples. In our work with difficult samples (degraded or burned bones analyzed for the identification of war victims), we were regularly challenged with this problem [3, 6] and have consequently developed a modified procedure to deal with this type of samples (as described in "Materials and methods").

It has been reported that up to 10% (3–6% on average) of cell-free DNA in maternal plasma can be of fetal origin [2], and this amount of minor component can be more or less routinely detected in mixed DNA samples. Aiming to perform prenatal paternity testing from maternal blood, we attempted to amplify fetal (paternal) alleles from plasma of 20 pregnant women. Unfortunately, using AmpFLSTR Identifiler kit amelogenin was the only fetal locus that we were able to reliably amplify.

The amount of fetal DNA in maternal plasma varies significantly. In general, there is an increase with the progression of pregnancy, but this is very variable, and in some late pregnancies, the concentration of fetal DNA in maternal plasma can be lower than in some early pregnancies [16]. We extracted DNA from 4 ml plasma and successfully amplified Y-chromosome specific amelogenin from all plasma samples where fetuses were male, confirming the presence of fetal DNA. However, autosomal loci amplified only sporadically (Table 1), most probably because of suppressed primer binding due to the presence of excess maternal DNA. The reduction of starting plasma volume did not improve the results (data not shown).

To eliminate the problem of allelic suppression by maternal DNA, we applied ampFLSTR Yfiler system, which amplifies 16 loci located on Y-chromosome. Since primers from this system do not bind to maternal DNA, we obtained significantly better results. Between six and 16 fetal loci were successfully amplified from plasma of mothers bearing male fetuses (Fig. 1, Table 1). All amplified alleles matched to alleles detected in samples of paternal blood, confirming that all 13 putative fathers of male fetuses were indeed the biological fathers.

Aiming to compare the ability of AmpFLSTR Identifiler and AmpFLSTR Yfiler kits to amplify minor components in a mixture, we performed the following experiment. Male and female cell-free DNA was isolated from plasma samples, quantified using Quantifiler Human DNAQuantification kit (Applied Biosystems), and mixed in 1:1, 1:9, 1:24, 1:49, and 1:99 ratios. Mixed DNA samples were amplified using AmpFLSTR Identifiler and AmpFLSTR Yfiler kits and analyzed as described in "Materials and methods." Mixtures of two different male genomic DNAs were also prepared and analyzed in the same way. Results presented in Table 2 (original electropherograms are provided in the Electronic supplementary material) clearly demonstrate that the presence of homologous DNA (autosomal or other male) significantly suppresses amplification of a minor DNA component and that the amount of cell-free fetal DNA that is present in maternal DNA is not sufficient for reliable identification of autosomal fetal alleles. However, since females do not have Y-chromosome, Y-chromosome specific loci can be successfully amplified even in the presence of 25-fold surplus of female DNA.

Conclusions

We were not able to successfully amplify autosomal fetal alleles from maternal plasma. The only locus that reliably amplified with AmpFLSTR Identifiler kit was amelogenin, which revealed only fetal gender, while the amplification of other autosomal loci was only sporadic and was not sufficient for reliable paternity testing. Much better success was obtained with AmpFLSTR Yfiler kit, which, in case of male fetuses, successfully amplified between six and 16 fetal loci, thus enabling early non-invasive prenatal paternity testing of male children from maternal blood.

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Financial interest None declared.

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