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Analysis of multiple loci can increase reliability of detection of fetal Y-chromosome DNA in maternal plasma

Jasenka Wagner[1], Snježana Džijan[1], Doroteja Pavan-Jukić[2], Jadranka Wagner[1] and Gordan Lauc[1,3]*

[1]*DNA Laboratory, University of Osijek School of Medicine, J. Huttlera 4, 31000 Osijek, Croatia*
[2]*General Hospital Karlovac, Karlovac, Croatia*
[3]*Genos d.o.o., Gajev trg 6, 31000 Osijek, Croatia*

Objective Aiming to develop more reliable methods for determination of fetal gender from maternal plasma we compared three different systems of polymerase chain reaction (PCR) detection of Y-chromosome DNA.

Methods Cell-free DNA was isolated from 96 samples of maternal plasma and (1) amplified using AmpFLSTR-Identifiler (15 autosomal STR loci and amelogenin) or AmpFLSTR-Yfiler (16 Y-chromosome STR loci) kits and subsequently analyzed on ABI-PRISM 310 Genetic Analyzer, or (2) analyzed using Quantifiler-Y DNA-Quantification kit. Gender of fetuses was confirmed by cytogenetic analysis or phenotypically at birth.

Results and Conclusions AmpFLSTR-Identifiler and Quantifiler-Y Human-Quantification kits were rather reliable in determining fetal gender (92.5 and 98.1%, respectively), but false negatives were still present in both systems. AmpFLSTR-Yfiler was found to be fully reliable as it amplified Y-chromosome in all cases of male fetuses, and was thus 100% correct in determining fetal gender. In addition, it enabled comparison of polymorphic Y-chromosome loci between father and a child, thus further supporting specificity of obtained results.

KEY WORDS: prenatal gender determination; cell-free fetal DNA; Y-chromosome DNA; polymorphic STR loci

INTRODUCTION

For a number of X-linked genetic diseases, early determination of fetal gender is of utmost importance. Since the first report of its existence in 1997 (Lo *et al*., 1997), cell-free fetal DNA in maternal plasma become a primary target for noninvasive prenatal diagnosis (Tong and Lo, 2006). Circulating fetal-associated DNA is of a higher abundance than fetal cells in the maternal circulation (Bianchi, 1999), and the postpartum clearance was shown to be rapid (Lo *et al*., 1999). Successful prenatal determination of fetal gender by the analysis of cell-free fetal DNA in maternal plasma has been reported in numerous publications (for a review (Lo, 2005)), and this approach will soon be routinely used in many laboratories (Guetta, 2006). By detecting Y-chromosome signals in maternal plasma with real-time PCR, Honda *et al*. reported a 100% sensitivity from the fifth week of gestation (Honda *et al*., 2002), but recent interlaboratory comparison reported much lower success rates for the detection of Y-chromosome DNA (Johnson *et al*., 2004). While analyzing plasma samples from 35 women carrying male fetuses, five different laboratories identified Y-chromosome DNA in 11, 15, 27, 33, and 34 samples, respectively; indicating that this type of analysis is still far from being reliable and robust enough for routine use.

The main problem in the determination of fetal gender by PCR analysis is the lack of positive control for the amplification of fetal DNA in maternal plasma. Thus when there is no amplification of Y-chromosome DNA, this could either mean that the fetus is a female, or alternatively that there is simply not enough fetal DNA in maternal plasma for successful amplification. The existence of an intraassay positive control for the amplification of fetal DNA would resolve the problem, but unfortunately it is not possible to devise real positive controls for this type of assays. An approach that could enable some kind of a positive control would be the use of a multiplex system that analyzes polymorphic loci (such as AmpFLSTR-Identifiler, or AmpFLSTR-Yfiler kits developed for the determination of human identity). In these systems a number of polymorphic loci are being simultaneously amplified, thus enabling a comparison of differences and identities of parental and fetal genotypes. The presence of paternal alleles in maternal plasma would confirm successful amplification of fetal DNA, and in addition, the ability to differentiate real paternal alleles amplified from fetal DNA from randomly amplified fragments could also enable higher number of application cycles, thus increasing sensitivity of the method.

Aiming to verify whether ampFLSTR-Identifiler and ampFLSTR-Yfiler kits can be used for the determination of fetal gender from maternal plasma, we have determined gender of 96 fetuses using both multiplex systems and compared them to real-time PCR system for quantification of Y chromosome.

*Correspondence to: Gordan Lauc, DNA Laboratory, University of Osijek School of Medicine, J. Huttlera 4, 31000 Osijek, Croatia.
E-mail: glauc@mefos.hr

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MATERIALS AND METHODS

Peripheral blood samples ($n = 96$) were collected from pregnant women (length of gestation was between 10 and 36 weeks) attending Clinical Hospital Osijek or Clinical Hospital Zagreb between 2005 and 2007. Blood samples were also taken from fathers. Informed consent was obtained from all studied individuals, as approved by the Ethics Committee of both hospitals.

Where certain, gestational age was based on menstrual dates and was confirmed by ultrasound before 20 week of gestation; where gestational age was not certain, it was confirmed by ultrasound before 15 weeks of gestation. Blood was collected into ethylenediaminetetraacetate (EDTA) tubes, and all samples were taken before performance of any invasive procedure. Fetal gender of each sample was confirmed phenotypically at birth or by cytogenetic analysis of fetal material.

Plasma DNA preparation

The peripheral blood samples of pregnant women were treated by centrifugation at $1400 \times g$ 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 was then collected in a fresh tube. DNA was extracted from plasma using a QIAamp blood kit (Qiagen Company) 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 and 6 μL of this solution were used as template for PCR.

Multiplex PCR

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, Foster City, CA, USA) and analyzed on ABI-PRISM 310 Genetic Analyzer (Applied Biosystems).

Original manufacturer's protocol was followed with following modifications which made it more suitable for amplification of low amounts of DNA: (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% (2.5 units of Ampli Taq Gold DNA Polymerase), 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. The identity of each amplified allele was determined by comparison to allelic ladder.

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, Foster City, CA, USA) according to manufacturer's instructions and analyzed on ABI-PRISM 310 Genetic Analyzer (Applied Biosystems). The identity of each amplified allele was determined by comparison to allelic ladder.

Real-time PCR

Real-time PCR was performed using two DNA-quantification assays: Quantifiler Human DNA-Quantification kit and Quantifiler-Y Human Male DNA-Quantification kit (Applied Biosystems). Each quantification assay combines two 5′-nuclease assays: a target specific assay and an internal PCR control (IPC) assay. The target-specific assay consists of two primers for amplifying human DNA or human male DNA and one TaqMan minor-groove binder (MGB) probe labeled with FAM dye for detecting the amplified sequence. The IPC assay consists of IPC template DNA (a unique synthetic sequence not found in nature), two primers for amplifying the IPC template DNA and one TaqMan MGB probe labeled with VIC dye for detecting the amplified IPC DNA. Both target and IPC detectors are designed to amplify in parallel in every reaction.

A coding region of the male-specific SRY gene (sex determining region on Y chromosome) was chosen to monitor the presence of fetal DNA and a coding region of the human telomerase reverse transcriptase gene (hTERT locus located on chromosome 5) was used to quantify total DNA. Both targets were detected independently in singleplex reactions to prevent outcompetition of the SRY amplification. Reaction conditions for both targets were as follows: 12.5 μL of Quantifiler PCR Reaction Mix (containing NTP's, buffer, AmpliTaq Gold DNA polymerase and ROX passive reference standard), 10.5 μL of Quantifiler Human Primer Mix or Quantifiler Y Human Male Primer Mix (containing target specific primers, FAM-labeled probe, IPC template, IPC primers, and VIC-labeled probe) and 6 μL of DNA extract in a final reaction volume of 29 μL. Thermal cycler conditions were: denaturation at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s.

Amplification data were collected and analyzed with an ABI-Prism 7000 sequence detection system (SDS) instrument (Applied Biosystems). The cycle threshold value (Ct) was measured in all cases. Each sample was analyzed in triplicate, and multiple negative reaction blanks were included in every analysis for both sample extraction and amplification stages. Calibration curves (duplicate samples) were analyzed on the same reaction plate for each run. Quantification standard dilutions were made by serially diluting the 200 ng/μL stock solution from the kits (human male genomic DNA) to the following concentrations: 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, 0.023, 0.0076, 0.0025 ng/μL.

RESULTS AND DISCUSSION

To our knowledge there is only a single report of the use of STR multiplex systems for the analysis of

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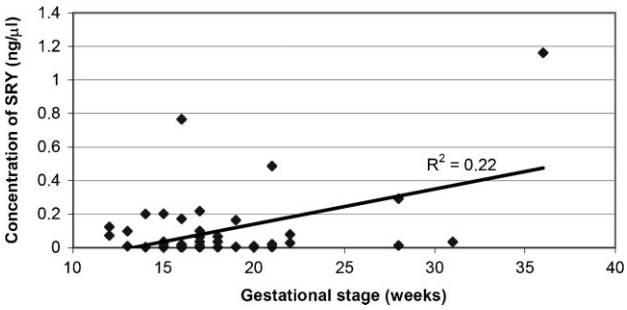

Figure 1—Concentration of SRY DNA in plasma of mothers bearing male fetuses. Cell-free DNA was isolated from plasma of pregnant women ($n = 96$) and SRY gene was amplified and quantified as described in the Materials and Methods Section. Results for women that were subsequently confirmed to bear female fetuses ($n = 43$) are omitted from the chart

fetal DNA in maternal plasma. Birch *et al*. reported in 2005 an attempt to amplify Y-chromosome DNA from maternal plasma using AmpFLSTR-SGM Plus kit (Applied Biosystems), but without any success (Birch *et al*., 2005). Suppression of minor alleles is a common complication in the analysis of low-copy DNA samples. In our work with difficult samples (degraded or burnt bones analyzed for the identification of war victims) we were regularly challenged with this problem (Gornik *et al*., 2002; Biruš *et al*., 2003; Džijan *et al*., 2005), and have consequently developed a modified PCR procedure to deal with this type of samples (as described in the Materials and Methods Section).

DNA was purified from plasma of 96 pregnant women using a method that was recently confirmed to reliably extract fetal DNA from maternal plasma (Legler *et al*., 2007). Fetal gender was determined either by (1) amplifying gender-specific amelogenin gene as a part of multiplex STR kit (ampFLSTR-Identifiler kit), (2) by amplifying 16 polymorphic STR loci located on Y chromosome (ampFLSTR-Yfiler kit), or (3) by amplifying coding region of the male-specific SRY with real-time PCR (Quantifiler-Y Human Male DNA-Quantification kit). Actual fetal gender was confirmed phenotypically at birth or by cytogenetic analysis of fetal material.

Using AmpFLSTR-Identifiler kit we were able to successfully amplify Y-chromosome DNA in 49 out of 53 samples from mothers bearing male fetuses. There were no cases of falsely amplified Y-chromosome in samples from mothers bearing female fetuses. Unfortunately, amelogenin locus was the only locus that was reliably amplified. Autosomal fetal STR loci were amplified only sporadically, and thus could have not

Table 1—Samples that gave false negative results using ampFLSTR-Identifiler or Quantifiler-Y kits

Sample no.	Week of gestation	Identifiler	Quantifiler-Y	concentration of SRY (ng/µL)
28	16	False negative	Positive	0.0042
38	22	False negative	Positive	0.0218
48	21	False negative	Positive	0.219
58	16	Positive	False negative	0
74	20	False negative	Positive	0.00441

Table 2—Obtained Ct values for plasma samples from woman bearing male fetuses

Sample	Week of gestation	Ct-1	Ct-2	Ct-3
1	15	38,54	37,75	Undet.
2	17	39,66	37,04	38,67
4	16	38,88	37,48	37,59
6	15	38,73	Undet.	38,75
8	17	38,69	37,09	Undet.
9	16	39,18	Undet.	38,18
12	17	32,6	33,82	34,38
13	15	34,97	34,99	34,77
14	14	38,45	37,0	37,48
16	17	39,09	38,01	38,59
17	16	35,8	35,38	35,95
21	16	38,36	36,04	37,89
22	17	36,46	35,66	36,16
23	15	36,8	39,53	39,46
25	16	37,8	35,63	36,48
28	16	39,0	37,39	Undet.
30	15	35,86	34,86	34,88
32	21	35,85	36,75	37,59
34	15	37,77	36,94	39,0
35	18	35,03	36,24	35,09
38	22	36,19	35,09	37,59
44	17	35,27	35,92	36,95
48	21	32,17	32,02	31,98
50	14	33,41	33,05	33,36
55	17	32,82	32,73	32,46
56	16	31,89	31,98	31,62
57	18	34,04	33,64	33,92
58	**16**	**Undet.**	**Undet.**	**Undet.**
59	16	33,31	33,25	33,73
60	15	32,92	33,40	33,12
61	28	39,40	39,63	Undet.
62	31	38,09	37,37	Undet.
65	21	37,64	38,77	39,8
66	18	37,40	39,35	37,29
67	13	Undet.	38,72	Undet.
68	16	Undet.	39,63	Undet.
69	20	37,16	37,95	39,39
71	16	36,49	38,79	Undet.
72	16	Undet.	38,85	Undet.
74	20	35,65	37,33	37,51
79	19	Undet.	38,81	38,16
80	12	34,33	34,19	34,8
81	17	33,94	33,26	33,71
82	22	33,42	33,03	33,43
83	19	33,31	34,14	34,36
85	28	31,38	31,33	31,25
88	36	29,45	29,37	29,73
89	12	33,28	33,25	33,78
90	13	34,29	34,00	33,65
91	15	36,75	36,97	Undet.
92	11	32,66	32,74	32,68
94	14	31,34	31,78	31,71
95	14	33,09	33,40	34,39

Undet., undetermined.

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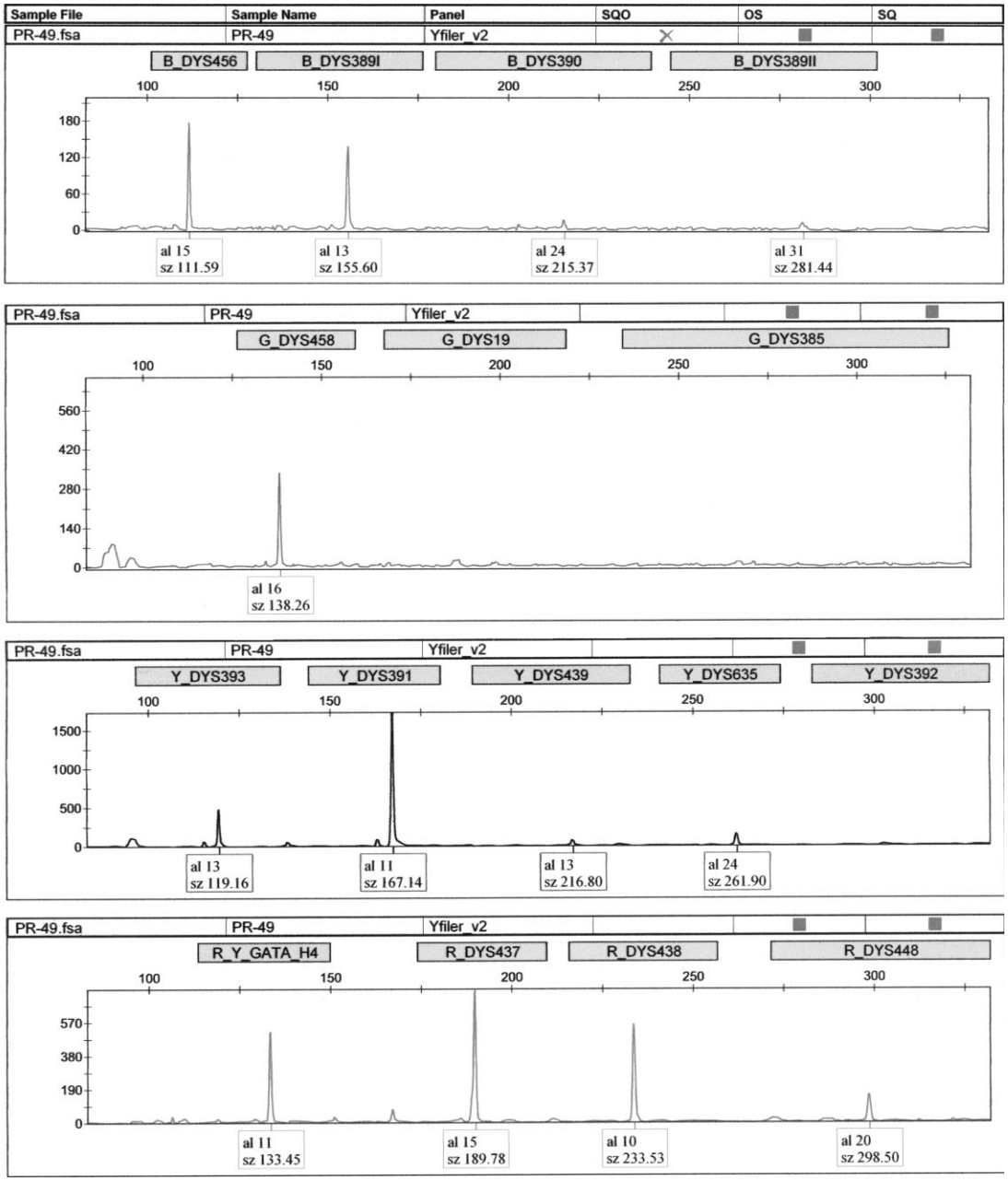
A



B

	DYS456	DYS389I	DYS390	DYS389II	DYS458	DYS19	DYS385	DYS393
maternal plasma	15	13	24	31	16	-	-	13
paternal blood	15	13	24	31	16	16	14, 15	13

	DYS391	DYS439	DYS635	DYS392	Y-GATA H4	DYS437	DYS438	DYS448
maternal plasma	11	13	24	-	11	15	10	20
paternal blood	11	13	24	11	11	15	10	20

Figure 2—Analysis of polymorphic Y-chromosome STR loci. (a) Electropherogram of polymorphic Y-chromosome STR loci from plasma of a mother bearing a male fetus (14 week of pregnancy). Cell-free DNA was isolated from maternal plasma and analyzed as described in the Materials and Methods Section. In this example 13 out of 16 STR loci was 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 panel A is shown in a tabular form and compared to a genotype obtained in an analogous way from blood of a father. All Y-chromosome STR alleles that were amplified from maternal plasma were identical to paternal alleles what further confirmed that they indeed originate from fetal Y chromosome

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been used as a control for the amplification of Y-chromosome DNA.

Quantifiler-Y Human Male DNA-Quantification kit was more successful in detecting Y chromosome in maternal plasma. Using this system we successfully determined sex of male fetuses in 52 out of 53 samples, also without any false positive results. Quantitative analysis of SRY gene revealed some positive correlation between gestational stage and the amount of SRY gene in maternal plasma ($R^2 = 0.22$), but the observed dissipation was rather high and the concentration of fetal DNA in maternal plasma is apparently more determined by other factors than gestational stage (Figure 1).

Both AmpFLSTR-Identifiler and Quantifiler-Y Human Male DNA-Quantification kits were rather reliable in determining fetal gender (92.5 and 98.1%, respectively), but false negatives were still present in both systems (Tables 1, 2). The absence of amplification of Y-chromosome DNA in these cases could have been a consequence of inadequate quantity of fetal DNA, but this was probably not the only reason, since different methods gave false negatives in different individuals. Individual false negative samples are listed in Table 1. Even some samples with rather high content of Y-chromosome DNA (e.g. sample 48 with over 0.2 ng/μL) were falsely negative, what strongly supports the hypothesis that falsely negative result were a consequence of some type of variability in primer binding regions.

To eliminate effects of individual variation in primer binding regions we applied multiplex system which concurrently amplifies 16 different Y-chromosome loci (ampFLSTR-Yfiler kit). With this system Y-chromosome was successfully detected in all 53 pregnancies bearing male fetuses. Between 6 and 16 Y-STR loci were amplified in individual samples. In addition, amplification of polymorphic Y-chromosome loci enabled comparison of fetal and paternal loci (Figure 2), what further strengthened the hypothesis that amplified fragments indeed originate from Y chromosome, and not from nonspecific amplification of another DNA fragment. Even though difference between 1 and 0 false negative results cannot be used to claim that ampFLSTR-Yfiler kit should be used instead of Quantifiler-Y for detection of Y-chromosome, the fact that 'classical' PCR detected a sequence that was not detected by multiple repetitions of a real-time PCR assay indicate that individual variability in primer binding regions can be a problem. In a recent publication by Illanes and colleagues it was recommended to analyze each sample eight times and to use 5/8 threshold for reporting the presence of Y chromosome (Illanes *et al.*, 2007). After realizing that sample #58 was false negative by real-time PCR, we analyzed it three more times, but there was still no amplification of Y-chromosome DNA, what would result in declaring this fetus as female even with 5/8 criterion. Since ampFLSTR-Yfiler detected Y-chromosome in this sample, the only plausible explanation is that this individual had some sequence variability in the primer-binding region which prevented primers from binding to SRY gene in fetal DNA. Sequence variability in primer binding regions is probably not very frequent, but it can be expected and perhaps a new real-time assay which amplifies two or more Y-chromosome regions in parallel should be developed to overcome this problem.

CONCLUSIONS

Both real-time PCR and multiplexed autosomal STR systems were found to be rather reliable in amplifying Y-chromosome DNA from maternal plasma (92.5 and 98.1%, respectively), but in our hands both systems failed to detect some male fetuses. Autosomal STR loci amplified only sporadically, thus adequate control for the amplification of fetal DNA is still missing. Multiplexed Y-chromosome STR system (ampFLSTR-Yfiler) was found to be more reliable as it amplified Y chromosome in all cases of male fetuses, and was thus 100% correct in determining fetal gender from maternal plasma. In addition it enabled comparison of polymorphic Y-chromosome loci between father and a child, thus further supporting obtained results.

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