ARTICLES

Quantitative Analysis of Male Fetal DNA in Maternal Serum of Gravid Rhesus Monkeys (*Macaca mulatta*)

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ABSTRACT

The isolation of human fetal DNA from the maternal circulation has provided a source of fetal material for prenatal diagnosis. The objective of this study was to investigate whether a similar pattern could be observed in the maternal circulation of male-bearing gravid rhesus monkeys. A real-time PCR TaqMan system for the rhesus Y-chromosome sex determining region was used to determine fetal sex and to quantify fetal DNA concentrations. Results in 14 healthy pregnancies indicated that fetal male DNA could be routinely detected in maternal serum by 50 d of gestation (late first trimester; term 165 ± 10 d). Fetal DNA concentrations increased with advancing gestation, reaching a mean of 341 genome equivalents/mL of serum (range 11–1570 copies/mL) in the last trimester of gestation, similar to findings in

humans. The fetal DNA concentration corresponded to 2.7% of the total maternal serum DNA in the third trimester. Similar to findings in humans, male fetal DNA sequences were not detected postpartum (through 4 wk postpartum) or in animals with a previous history of delivering male offspring. These data indicate that fetal male DNA is present in the maternal circulation of gravid rhesus monkeys comparable to findings in humans and further support the use of this nonhuman primate species as a model to investigate fetomaternal cell trafficking and microchimerism. (*Pediatr Res* 53: 18–23, 2003)

Abbreviation

SRY, Y-chromosome sex-determining region

The discovery of high levels of fetal nucleic acids in the maternal circulation has opened up new areas of investigation and provided a potential new approach for prenatal molecular diagnosis (1). The isolation of cell-free fetal DNA has been shown to be useful material for fetal sex determination and screening for pregnancy-related complications and fetal disease (2, 3). Furthermore, this technique can potentially be applied to investigate other developmental questions associated with the level and degree of fetomaternal trafficking, maintenance of the fetal allograft and tolerance induction, and maternal autoimmune diseases such as scleroderma (4–14). For exploring such fields of study, appropriate animal models that closely simulate human development and disease are needed.

Studies in nonhuman primates, specifically macaques, have shown similarities in anatomy, developmental biology, hematology, and immunology that have prompted the use of this species as a model in fetal and pediatric research (15–20). Monkeys and humans share many characteristic features because of their close phylogenetic relationship. Developmental similarities include spatial and temporal pattern of organ development, placental structure, and length of gestation. Unlike rodents and sheep, human and nonhuman primates (monkeys) have a similar placental structure, which highlights the importance of this model for addressing questions related to transplacental trafficking of fetal cells and DNA.

In this study, we investigated the presence of male fetal rhesus DNA in maternal serum during the length of gestation. In addition, the ϵ -globin gene was used to quantify total (i.e. fetal and maternal) DNA concentrations in maternal serum. We addressed these questions by developing specific, highly sensitive and reproducible methods to quantify rhesus genomic DNA by real-time TaqMan PCR assays (21). The results of this study demonstrate that fetal rhesus DNA sequences can be detected reliably in early gestation and throughout the second

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and third trimesters of pregnancy. In addition, similarities in the kinetics of fetal male DNA were found when compared with the data obtained from human studies (22).

METHODS

Animals. All animal procedures conformed to the requirements of the Animal Welfare Act, and protocols were approved before implementation by the Institutional Animal Use and Care Administrative Advisory Committee at the University of California at Davis. Twenty normal, adult female rhesus monkeys (*Macaca mulatta*) ranging in age from 6 to 12 y and body weights of 5.4–9.6 kg with a history of previous pregnancies (between 1 and 9) were bred and identified as pregnant, using established methods (23). Fourteen of these animals were pregnant with male fetuses, and six animals were pregnant with female fetuses, of which three had a previous pregnancy with a male fetus (range of previous male pregnancies 2–4). Pregnancy in rhesus monkeys is divided into trimesters by 55-d increments: 0–55 d of gestation represents the first trimester, 56–110 d of gestation represents the second trimester, and 111–165 d of gestation represents the third trimester (term 165 ± 10 d) (24). All animals were examined sonographically during gestation (23), and healthy newborns were delivered by cesarean section at term. Sex was identified prenatally by ultrasound (23) and confirmed at delivery.

Blood sample collection and nucleic acid isolation. Blood samples (2–12 mL) were collected from gravid animals every 10 d from 20 d of gestation until term (160 d). Of these samples, eight aliquots per animal (200 µL serum/aliquot) were chosen spanning 20–160 d and each of the trimesters for use in the TaqMan assay. A subset of animals with male fetuses ($n = 4$) also had samples collected weekly from the day of delivery until 1 mo postpartum. Samples were obtained from a peripheral vessel and placed into anticoagulant-free Vacutainers. Serum was collected using established techniques and after samples were allowed to clot, then immediately placed on ice until centrifugation. All specimens were centrifuged at 1500 rpm for 10 min. The serum was then transferred to a microcentrifuge tube and centrifuged at 8000 rpm for 5 min. The supernatants were collected into new microcentrifuge tubes, then stored at –70°C until further processing. All samples were processed immediately after collection and frozen once the serum was aliquoted. DNA was isolated from 200 µL of serum using the QIAamp DNA Blood Kit (Qiagen, Valencia, CA), as recommended by the manufacturer.

Genomic DNA was also isolated from control male and female rhesus liver samples collected at necropsy as part of other published studies (25) and human male peripheral blood mononuclear cells. The genomic DNA was extracted by using the Gentra System DNA isolation kit (Gentra, Minneapolis, MN), as previously reported (25). DNA samples from the rhesus monkey were used as positive (male) and negative (female) controls in all PCR reactions. Additional negative controls run in each assay consisted of water in the reaction mix. Human genomic DNA was used to confirm that there was no cross-reactivity in the assay (see below).

Real-time quantitative PCR. Real-time quantitative PCR was performed using an Applied Biosystems 7700 System Detector (Foster City, CA) (21). The primers and probe sequences were designed with Primer Express software (Perkin-Elmer, Foster City, CA). Sequence data were obtained from the GenBank Sequence Database [accession numbers AF284310, *Macaca mulatta* sex determining region Y (SRY) and M81364 (ϵ -globin gene)]. To avoid false positives by cross-contamination from human male operators, the TaqMan system for the rhesus monkey was designed such that it would not amplify the corresponding human Y-chromosome region. This was accomplished by obtaining the human Y-chromosome region from the GenBank Sequence Database (accession numbers L10102; *Homo sapiens* SRY gene). Differences in the sequence between the human and rhesus monkey Y-chromosome regions for the primer and probe design were obtained after alignment of both sequences using BLAST (Basic Local Alignment Search Tool). The system was designed with two mismatched nucleotides in the sequence for the reverse primer and five nucleotides in the probe (see below).

The SRY TaqMan system was synthesized and purified by PE Biosystems (Foster City, CA), and the ϵ -globin TaqMan system was synthesized and purified by Synthetic Genetics (San Diego, CA). The internal probe was labeled at the 5' end with the reporter dye FAM (6-carboxyfluorescein) and at the 3' end with the quencher dye TAMRA (6-carboxytetramethylrhodamine) and was phosphate-blocked at the 3' end to prevent extension by the DNA polymerase. The primers for the rhesus male-specific sequence were as follows: forward SRY-569F: 5'-CGGAAGGCGAAGATGCTG-3'; reverse SRY-634R: 5'-GCAGGTACCGAAGAGGGA-3'. The probe in this system was as follows: SRY-589T: 5'-FAM-AAACAGTTGCA-GTTTGCTTCCGGCA-3'.

The ϵ -globin TaqMan system consisted of the amplification primers as follows: forward ϵ -globin-1494F: 5'-TGGCAAG-GAGTTCACCCCT-3'; reverse ϵ -globin-1557R: 5'-AATGGC-GACAGCAGACACC-3'; and a probe for ϵ -globin-1517T: 5'-TGCAGGCTGCCTGGCAGAAGC-3'.

Amplification conditions were identical for all reactions. The thermal profile was as follows: 2 min at 50°C, 10 min at 95°C, 45 cycles of 15 s at 95°C, and 60 s at 60°C. The amplification reactions were set in a final volume of 25 µL, containing 20 µL of 2× TaqMan Universal Mastermix (PE Biosystems) with 10 mM Tris (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 2.5 mM deoxynucleotide triphosphates, 0.625 U of AmpliTaq Gold per reaction, and 5 µL of the diluted DNA sample. For all TaqMan assays, primer and probe concentrations were 400 and 100 nM, respectively.

For each sample, parallel reactions were run in duplicate in separate wells for the detection of male rhesus Y sequence and ϵ -globin genes. The mean result was used for further analysis. Amplification data were analyzed by the sequence detection system software (version 1.6.3; Applied Biosystems). The concentrations of SRY and ϵ -globin sequences were expressed in genome equivalents or copies per milliliter and calculated, as previously described (22). For preventing contamination during DNA extraction, PCR amplification, and the detection

of PCR products, procedures were performed in accordance with standards previously described (26, 27). The TaqMan assay also included anticontamination measures in the form of preamplification treatment with uracil N-glycosylase (28).

Linear range and amplification efficiencies of TaqMan primers and probes. For determining the linearity and amplification efficiencies, five 10-fold dilution steps of male rhesus genomic DNA were amplified in triplicate. The average threshold cycle values (C_T) were plotted against the log quantities of target genomic DNA and the regression line was calculated (Fig. 1). The resulting standard curve was linear for five logarithmic decades in the ϵ -globin and SRY systems. The efficiency of the reaction (E) was calculated from the slope of the standard dilution series, as previously described (29). For reducing the variability in the assay, a constant amount of carrier DNA polydeoxyadenylic-thymidylic acid (Sigma Chemical Co, St. Louis, MO) was added to the diluted samples to minimize the amount of genomic DNA absorbed to the walls of the tube.

Statistical analysis. Statistical analysis was performed using statistical software (StatView 512+, version 4.5, Brainpower Inc, Calabasas, CA). The mean copy number of SRY and ϵ -globin sequences was compared between first (20–50 d), second (60–100 d), and third trimesters (110–160 d) using the

Mann-Whitney rank sum test (30). Statistical significance was assigned to all values where $p < 0.05$.

RESULTS

Linear range and amplification efficiencies of TaqMan primers and probes. Quantitation of SRY and ϵ -globin sequences showed linearity over 5 orders of magnitude. Both real-time SRY and ϵ -globin systems were sufficiently sensitive to detect the DNA equivalent to one cell (data not shown). The SRY assay was insensitive to the existence of background female rhesus DNA from 0 to 12,800 genome equivalents, and no PCR amplification was detected with human male DNA samples.

Precision determination. To determine the precision of both real-time PCR TaqMan assays, we determined the coefficient of variability within run and between runs, as previously described (31) (see Table 1). To assess the precision within run, we measured eight times within one run the target amount of SRY and ϵ -globin sequences present in the genomic DNA sample equivalent to 2.5, 25, 250, and 2500 cells and to 5, 50, 500, and 5000 cells, respectively. The cell equivalent was calculated from optical density (OD) measurements collected at 260 nm and by the use of a conversion factor of 6.6 pg of DNA per cell, as previously described (32). All samples were positive with both real-time PCR systems. Coefficients of variation (CV) were calculated on the basis of the C_T values [$CV(C_T)$] and of absolute values [$CV(Abs)$] after transformation with the standard curve. The $CV(C_T)$ for the SRY and ϵ -globin of the within-run precision systems was in the range of 0.4–2.1%, the $CV(Abs)$ was in the range of 4.5–41.9% (see Table 1).

To assess the precision between runs, we evaluated the four dilutions of genomic DNA (equivalent to 5, 50, 500, and 5000 cells) by using TaqMan PCR on five separate runs. The $CV(C_T)$ between-run experiments for both systems was in the range of 0.5–16.0%, and the $CV(Abs)$ was in the range of 10.8–48.9%. In general, the results from the within-run and the between-run experiments showed increased precision with higher amounts of starting genomic DNA.

Quantitative analysis of ϵ -globin using the TaqMan system. For each animal, DNA was extracted from maternal serum at eight time points between 20 and 160 d of gestation, with a subset of animals evaluated for 1 mo postpartum (see “Methods”). The ϵ -globin system was used as an internal control for the isolation of DNA from maternal serum samples and to calculate the total amount of cell-free circulating DNA (both maternal and fetal). Data analysis showed a consistent increase with each subsequent trimester in the concentration of fetal ϵ -globin sequences (see Table 2). This increase is statistically significant ($p < 0.007$) only when the first and third trimesters of gestation are compared.

Quantitative analysis of fetal SRY in maternal serum. Real-time quantitative PCR analysis using the SRY TaqMan system was carried out to calculate the amount of circulating fetal male DNA. Quantitative data on the concentration of SRY sequences during gestation are summarized in Table 3. Isolation of DNA from 200 μ L of serum consistently produced

Figure 1. Standard curves for the real-time PCR TaqMan assays. Isolated male genomic DNA was used as a standard for the estimation of the linear range and amplification efficiencies of the SRY and ϵ -globin real-time PCR systems. The copy number was calculated after optical density (OD) measurements were collected at 260 nm. Five 10-fold dilutions were made in PCR-grade water containing 10 μ g of polydeoxyadenylic-thymidylic acid per sample as carrier DNA. Each sample dilution was measured in triplicate. The linear regression equation, correlation (R^2), and reaction efficiency (E) are illustrated.

Table 1. Coefficients of variability in within-run and between-run precision for SRY and ϵ -globin TaqMan assays using serum from gravid rhesus monkeys (*Macaca mulatta*) (values represent percentages)

Copy #/DNA	SRY (%)				ϵ -globin (%)			
	2500	250	25	2.5	5000	500	50	5
CV (C_T)								
Within-run	0.36	0.38	1.16	1.97	0.38	0.51	1.23	2.14
Between-runs	0.50	0.48	1.26	2.66	0.55	0.63	1.36	16.00
CV (Abs)								
Within-run	4.50	8.32	28.37	41.89	6.20	10.39	24.45	41.75
Between-runs	10.80	10.52	29.35	52.61	12.30	15.30	30.47	48.89

Table 2. Comparison of the mean copy numbers of the ϵ -globin sequences in the circulation of gravid monkeys during gestation.

	Mean $\pm$ SEM	Median	Range
1st trimester			
20–50 days	3,103.6 $\pm$ 853.1	1,862	342–17,379
2nd trimester			
60–100 days	6,844.4 $\pm$ 1,799.5	3,535	141–55,818
3rd trimester*			
110–120 days	10,022.0 $\pm$ 3,590.7	5,852	553–76,109
130–160 days	13,959.3 $\pm$ 3,020.2	8,317	2,092–80,333
110–160 days	12,580.9 $\pm$ 2,349.7	7,220	553–80,333

All data are represented as copies/mL

SEM, standard error of the mean.

* Statistically significant difference when compared to other gestational time points ($p < 0.01$).

Table 3. Comparison of the mean copy numbers of Y chromosome sequences (SRY) in the circulation of gravid monkeys with a male fetus during gestation

	Mean $\pm$ SEM	Median	Range
1st trimester:			
20–50 days	10.2 $\pm$ 2.8	7.8	0–46
2nd trimester:			
60–100 days	91.4 $\pm$ 15.0	59.9	12–487
3rd trimester*:			
110–120 days	102.9 $\pm$ 20.9	87.5	11–249
130–160 days	422.4 $\pm$ 56.3	317.3	38–1,570
110–160 days	340.9 $\pm$ 46.2	212.2	11–1,570

All data represented as copies/mL.

SEM, standard error of the mean.

* Statistically significant difference when compared to other gestational time points ($p < 0.01$).

amplification of SRY sequences by 50 d of gestation in all animals with male fetuses. In a subset of animals (approximately 40%), SRY sequences were also detected by 30 d of gestation. These data also indicated that the mean concentrations of fetal male DNA in maternal serum were statistically increased with each subsequent trimester ($p < 0.001$). The greatest developmental rise in male rhesus DNA was observed in the last month of pregnancy (130–160 d of gestation; Fig. 2).

The fractional concentration of fetal DNA in maternal serum in the second and third trimesters of pregnancy was shown to be 0.9% and 2.7%, respectively. Male fetal DNA sequences were not detected in the subset of animals evaluated postpartum. In addition, none of the dams that bore female fetuses in the current pregnancy and that had previously delivered male offspring (between two and four previous male fetuses) were found to have male fetal DNA in collected serum.

Figure 2. Fetal rhesus ϵ -globin and male DNA in the maternal circulation. Fourteen animals with male fetuses were evaluated during gestation (first trimester to term; see text) (A). ϵ -globin was used as an internal control. ϵ -globin sequences in the maternal circulation were detected in all animals during all trimesters of gestation, with an approximate doubling with each subsequent trimester ($*p < 0.005$). During the first trimester (20–50 d of gestation), all animals showed male DNA sequences in the maternal circulation but at low copy numbers (B). The greatest levels of fetal Y-chromosome DNA in the maternal circulation were observed in the third trimester, with the most significant elevations observed after 130 d of gestation, or approximately 1 mo before delivery ($*p < 0.001$; $**p < 0.005$).

DISCUSSION

The results of this study show that fetal male DNA sequences can be found in the circulation of gravid rhesus monkeys during the course of gestation. Previous studies in humans have demonstrated that fetal DNA is present at high concentrations in the maternal circulation, particularly in the third trimester (1, 2, 22). In the study described here, we used a specific and quantitative assay for the Y-chromosome se-

quences of the rhesus monkey that do not cross-react with human Y. This technique is similar to one we previously reported using routine PCR techniques (33). We also developed a real-time TaqMan PCR system to calculate the quantity of total DNA in the circulation of gravid monkeys using the ϵ -globin gene. The fluorogenic TaqMan system has been shown to be a fast, simple, and reliable method to quantify genomic DNA (31). Both the SRY and ϵ -globin systems have ranges of linear quantification over 5–6 log decades (21, 34), and the results on the amplification efficiency and precision determination are in agreement with other TaqMan systems validated in previous studies (29, 35). The specificity of the SRY assay was preserved, as we did not observe amplification signals in animals from a previous pregnancy or in gravid monkeys carrying a female fetus or genomic DNA from human male samples.

Studies in humans have shown that fetal male sequences can be detected as early as weeks 5–7 of gestation (22), and recent reviews indicate the molecular analysis of fetal DNA is feasible at the beginning of the second trimester (5). In our study, amplification of SRY sequences was possible in a subset of animals at 35 ± 5 d of gestation (data not shown). However, using the techniques described, we consistently detected SRY sequences by the end of the first trimester (50 d of gestation). Preliminary results in our laboratory suggest that increasing the volume of maternal serum can increase the sensitivity of the TaqMan assay and the ability to detect reliably male offspring at earlier gestational time points (25 ± 5 d of gestation) (Jimenez and Tarantal, submitted). In the current study, we used 200 μ L of maternal serum, whereas in human studies up to 800 μ L was used. Another possible alternative to increase assay sensitivity is to design a PCR system that targets the repetitive regions of the Y chromosome. However, it has been shown that, in some cases, this strategy can produce false-positive results (36, 37).

The concentration of fetal male DNA (SRY sequences) was shown to increase significantly with the progression of pregnancy. The kinetics of male fetal rhesus DNA during gestation are similar to those previously reported in human subjects by Lo *et al.* (22). Other studies have also shown higher transplacental trafficking of fetal DNA toward the end of gestation (7). Although the biologic basis by which fetal DNA is released with increasing quantity into the maternal circulation remains to be elucidated, it has been hypothesized that fetal cell-free DNA may be released after cell lysis by immunologic processes, physical damage, or developmentally associated apoptosis within the placenta (1, 22, 27, 38).

Several studies have evaluated the presence of human fetal DNA in the maternal circulation, although none have addressed whether there is a parallel increase in total DNA (fetal and maternal) with advancing gestation. In the study described here, we observed an increase in total DNA over the course of pregnancy. However, recent data have shown that concentrations of cell-free genomic DNA can be up to 20- to 100-fold higher when evaluated in serum and compared with plasma (39). This difference has been attributed to the cell-free DNA generated during the process of clotting and as a result of lysis of nucleated cells present in whole blood. To address this issue,

we previously calculated the quantity of fetal and total DNA in serum *versus* plasma in two animals during each trimester of gestation (data not shown). This study showed a 0.4-fold increase (range 0.002–1.3) in Y-chromosome copies when comparing serum with plasma, whereas a 10-fold increase (range 2–16) in total DNA was observed. The slight differences in values when comparing Y-chromosome copies obtained from serum *versus* plasma are most likely due to the scarcity of fetal cells in the maternal circulation, which has been reported to be one fetal cell in 10^7 maternal cells (40, 41). Furthermore, it has been shown that the concentrations of cell-free fetal DNA and total DNA in maternal plasma can fluctuate between 2.2- and 13.5-fold, respectively. These results suggest that care should be taken when considering the total DNA cell-free fraction, particularly when using serum samples (42). Thus, we cannot definitively conclude that an increase in total DNA occurs with each subsequent trimester; additional investigations will be required to confirm this possibility.

Our studies have also shown that there are differences in the quantity of circulating male fetal DNA when comparing individual nonhuman primates in the last trimester (11–1570 genome equivalents/mL). Similarly, among 25 human pregnancies evaluated, a range of 34–900 male genome equivalents/mL was reported in late pregnancy (22). Whereas some reports have suggested that high levels of fetal DNA in the maternal circulation may be associated with pregnancy-associated abnormalities (43–45), other studies suggest that a 2-fold variation in free fetal DNA can be found in healthy pregnancies over a period of three successive days (46). This further emphasizes that care should be taken when using free fetal DNA elevations as an indicator of pregnancy-related problems. Our observations parallel those reported in human subjects because a comparable 2-fold difference in the amount of male fetal rhesus DNA was found when comparing individual animals with viable, healthy pregnancies and subsequent offspring (data not shown).

In conclusion, we have demonstrated that fetal rhesus DNA can be readily detected in the maternal circulation during gestation and that the kinetics of cell-free DNA are similar to findings in humans. The results of these studies support the importance of the rhesus monkey model for investigations that focus on enhancing our understanding of fetomaternal trafficking and microchimerism and can be applied to explore the potential ramifications of fetal cell and gene transfer treatment strategies from the maternal perspective.

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