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Short Report

Non-invasive prenatal determination of fetal sex: translating research into clinical practice

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The effectiveness and clinical utility of non-invasive prenatal diagnosis (NIPD) for fetal sex determination using cell-free fetal DNA (cffDNA) was assessed by undertaking a prospective national audit of UK testing. NIPD was performed using real-time polymerase chain reaction analysis of the *DYS14* or *SRY* gene in cffDNA extracted from maternal plasma. All cases referred for fetal sex determination from 1 April 2006 to 31 March 2009 were ascertained from two laboratories offering the test. Fetal gender determined by NIPD was compared with that based on ultrasound, invasive test or phenotype at birth. Indication and rate of invasive testing was ascertained. In the first year, results were issued in 150/161 pregnancies tested. Of the 135 with outcome data, results were concordant in 130/135 [96.3% (95% CI 91.6–98.8%)]. Reporting criteria were changed and in the subsequent 511 pregnancies the concordancy rate increased to 401/403 [99.5% (95% CI 98.2–99.9%)]. Over the 3 years only 32.9% (174/528) underwent invasive testing. NIPD for fetal sex determination using cffDNA is highly accurate when performed in National Health Service laboratories if stringent reporting criteria are applied. Parents should be advised of the small risk of discordant results and possible need for repeat testing to resolve inconclusive results.

Conflict of interest

No conflicts of interests were identified for any author.

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Early determination of fetal gender is clinically indicated for women who are carriers of X-linked genetic conditions where pregnancies with male fetuses are primarily at risk and for those at risk of conditions associated with ambiguous development of the external genitalia, e.g. congenital adrenal hyperplasia (CAH) where early maternal treatment with dexamethasone can reduce the degree of virilization of female fetuses with CAH (1, 2). Traditionally invasive testing is required using either chorionic villus sampling (CVS) or amniocentesis; both of which carry a risk of miscarriage of around 1% (3) and cannot be performed until 11 weeks of gestation.

Cell-free fetal DNA (cffDNA) in maternal plasma is an alternative source of fetal genetic material for prenatal testing (4). This emanates from the placenta (5), can be detected from 4 weeks of gestation (6) and is rapidly cleared from the maternal circulation after delivery (7, 8). As the levels of cffDNA present in maternal blood are relatively low (9, 10) current clinical applications are limited to the detection of alleles arising *de novo* or those found in the father but not the mother. Non-invasive prenatal diagnosis (NIPD) for fetal sex determination is performed by targeting Y-chromosome-specific sequences, most commonly the multicopy *DYS14* sequence located

within the *TSPY* gene or the single copy *SRY* gene, using real-time polymerase chain reaction (PCR) (11).

Research laboratories report cffDNA for fetal sex determination with sensitivities up to 100% (9, 12–14) and some countries, including the UK, now offer cffDNA testing as a clinical service (15–17). Here we report the successful implementation of fetal sex determination using cffDNA in two National Health Service (NHS) laboratories and show that these tests are highly accurate when performed after 7 weeks using stringent reporting criteria (Table 1).

Materials and methods

Auditing of pregnancy outcomes

The Prospective Register of Outcomes Free Fetal DNA Testing (PROOF) was established to audit a consecutive patient series from 1 April 2006 until 31 March 2009 in two UK laboratories; the International Blood Group Reference Laboratory (IBGRL) in Bristol and the North East Thames Regional Molecular Genetics Laboratory (NETRMGL) at Great Ormond Street Hospital in London. Referring clinicians were contacted to obtain details of fetal sex (determined by invasive testing, fetal ultrasound or at birth), whether invasive testing was performed and the pregnancy outcome.

Sample collection and DNA extraction

Maternal peripheral blood (10–20 ml) was collected into ethylene diamine tetra-acetic acid anticoagulant tubes. Samples were transferred to the laboratories, usually by first class post, and processed within 24 (82.3%) to 48 h (93.6%) (up to 4 days). Plasma was separated from venous maternal blood by double centrifugation at 3000 ***g***

Table 1. Reporting criteria for *DYS14* and *SRY* real-time polymerase chain reaction (PCR) results for assignment of fetal sex

Report	Audit Phase 1: 1 April 2006–31 March 2007	Audit Phase 2: 1 April 2007–31 March 2009
IBGRL	PCR reaction reagents (total volume 25 μl): TaqMan universal PCR master mix, 100 nM FAM-labeled probe, 300 nM primers for *DYS14* or 200 nM CCR5 primers and 5 μl of DNA Number of replicates performed: Eight *DYS14* and two CCR5 replicates per extract PCR cycling using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Life Technologies, Carlsbad, USA): 50°C for 2 min, 95°C for 10 min, then 45 cycles of 95°C for 15 s, 56°C for 1 min	
Male	At least five out of eight replicates for *DYS14* had a Ct value of 39 or less	At least seven out of eight replicates for *DYS14* had a Ct value of 37 or less
Female	All eight replicates had Ct values of 45	At least six replicates had Ct values of 45, and no more than two replicates had Ct values >39
Inconclusive	If less than five replicates had Ct values of 39 or less, or if Ct values higher than 39 but lower than 45 were seen	If less than seven replicates had Ct values of 37 or less, or if >2 Ct values higher than 39 but lower than 45 were seen
NETRMGL	PCR reaction reagents (total volume 20 μl): TaqMan universal PCR master mix, SRY or CCR5 TaqMan Gene Expression Assay mix with 250 nM FAM-labeled MGB probe, 250 nM VIC-labeled MGB probe, 900 nM primers (Applied Biosystems, Life Technologies, Carlsbad, USA) and 5 μl of DNA Number of replicates performed: Six *SRY* and three CCR5 replicates per extract PCR cycling using an ABI PRISM 7300 Real-time PCR System (Applied Biosystems, Life Technologies, Carlsbad, USA): 95°C for 10 min, then 45 cycles of 95°C for 15 s, 60°C for 1 min	
Male	At least four out of six replicates for *SRY* with a Ct value of 40 or less seen in two extracts[a]	At least five out of six replicates for *SRY* with a Ct value of 40 or less seen in at least two of four extracts[b]
Female	All six replicates with Ct values of >40 seen in two extracts[a]	All six replicates with Ct values of >40 seen in four extracts[b]
Inconclusive	If less than four replicates had Ct values of 40 or less or inconsistent results between two extracts[a]	If less than five replicates had Ct values of 40 or less or inconsistent results between four extracts[b]

[a]Two extracts from one maternal sample.

[b]Two separate maternal samples are analyzed; these can be taken 1 week apart or at the same time after 9 weeks of gestation. Two extracts from each maternal sample are analyzed, giving a total of four extracts.

for 15 min and then at 3000 ***g*** for 15 min (NETRMGL) or 1600 ***g*** for 10 min and then 4600 ***g*** for 10 min (IBGRL). The plasma was extracted immediately or aliquoted and stored at −20°C.

Cell-free DNA was extracted using the QIAamp DSP Blood kit (mini) (Qiagen, Hilden, Germany): final volume 55 μl (IBGRL) or both the manual QIAamp MinElute Virus Spin kit (Qiagen, Hilden, Germany) and the automated QIAamp EZ1 Virus Mini kit (Qiagen, Hilden, Germany): final volume 55 μl (NETRMGL). Maternal lymphocyte DNA was extracted using the QIAamp DSP Blood kit (mini) (Qiagen, Hilden, Germany) (IBGRL) or Autogen FlexiGene DNA (salting out) method (Qiagen, Hilden, Germany) (NETRMGL).

Real-time PCR

Details of the PCR reaction and reporting criteria are presented in Table 1. CCR5 was used by both laboratories as a control for the DNA extraction process. Dilution standards (100–0.1 ng/well) and controls were included in each assay and relative quantification was performed using the dedicated instrument sequence detection software.

Marker analysis

Maternal DNA was tested for the presence or absence of a bank of eight bi-allelic polymorphisms or markers (S01, -03, -04, -05, -06, -08, -10 and -11) (18). Markers found to be absent from the maternal genome were targeted in maternal plasma. DNA was extracted from fresh aliquots of maternal plasma and tested for the presence of potential fetal markers (four replicates per marker per patient). If at least 2/4 replicates of any marker were positive, cffDNA was presumed to be present in the maternal plasma.

Results

Requests were received from clinicians throughout the UK and overseas for testing in 672 pregnancies. Indications included sex-linked disorders in 546 (81.2%), of whom 140 (20.8%) were at risk of hemophilia, 76 (11.3%) for CAH, 14 (2.1%) for renal or other ultrasound anomalies, 7 (1.0%) for confirmation of gender seen on ultrasound and 2 (0.3%) for discordant genotypic and phenotypic sex. Gestations at first requests for testing ranged from 4 to 36 weeks with the majority (49%) between 7 and 9 weeks (Fig. 1).

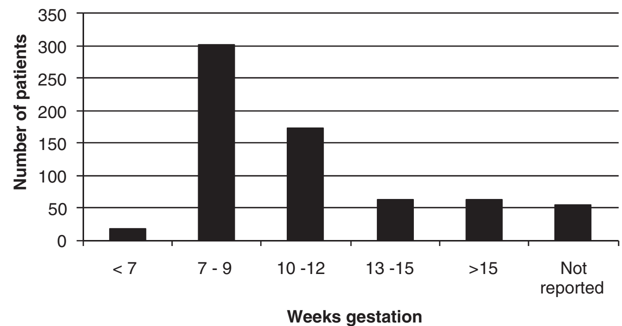

Fig. 1. Gestation at time of first request for testing.

Test accuracy Phase 1: 1 April 2006–31 March 2007

In the first year, 161 pregnancies were referred and a total of 208 tests performed (Table 2). Laboratory standards for issuing a report were not achieved in 18.5% (IBGRL: 18.8% and NETRMGL: 20.0%) and a repeat sample was requested. It was not possible to issue a report in 11 pregnancies (6.8%); in 8 cases no repeat sample was received and in 3 cases the repeat test was inconclusive.

Fetal sex, based on invasive testing, ultrasound or phenotype at birth, was ascertained in 145 (90.1%; 145/161) pregnancies. There was one miscarriage and 15 cases were lost to follow-up. At IBGRL, where a result was issued and fetal sex confirmed, 112/114 [98.2% (95% CI 93.8–99.8%)] concordant results were obtained. One falsely predicted male and one falsely predicted female occurred (Table 3). At NETRMGL, where a result was issued and fetal sex confirmed, 18/21 [85.7% (95% CI 63.7–97.0%)] concordant results were obtained. Two falsely predicted males and one falsely predicted female occurred (Table 3). Overall, accuracy was 96.3% (95% CI 91.6–98.8%) and error rate by reported gender was 4.4% (3/68) (95% CI 0.9–12.4%) for males and 2.9% (2/69) (95% CI 0.4–12.4%) for females. Of six tests performed before 7 weeks of gestation, three gave discordant results. Both laboratories subsequently stipulated that maternal blood be taken at 7 weeks or later (confirmed by ultrasound scan) and increased the stringency of reporting criteria (Table 1).

At NETRMGL 14 tests negative for *SRY* in Phase 1 were subsequently tested with bi-allelic markers to ensure the presence of fetal DNA in the sample. Marker testing was only informative in 40% of families due to low heterozygosity rates for the bi-allelic markers. In view of this and the labor-intensive nature of the test this practice was discontinued.

Table 2. Summary of audit outcomes

	Audit Phase 1 (1 April 2006–31 March 2007)			Audit Phase 2 (1 April 2007–31 March 2009)		
	IBGRL	NETRMGL	Total	IBGRL	NETRMGL	Total
Pregnancies tested	136	25	161	322	189	511
Assays performed	168	40	208	411	394	805
Assay failure rate	31/168 (18.5%)	8/40 (20.0%)	39/208 (18.8%)	63/411 (15.3%)	41/394 (10.4%)	104/805 (12.9%)
No result issued	9/136 (6.6%)	2/25 (8.0%)	11/161 (6.8%)	13/323 (4.0%)	9/189 (4.8%)	22/511 (4.3%)
Result issued and gender outcome known	114	21	135	258	145	403
Male-bearing pregnancies[a]	57	9	66	136	73	209
Female-bearing pregnancies	57	12	69	122	72	194
Falsely predicted male	1	2	3	0	0	0
Falsely predicted female	1	1	2	2	0	2
Accuracy[b]	112/114	18/21	130/135	256/258	145/145	401/403
% (95% CI)	98.2% (93.8–99.8)	85.7% (63.7–97.0)	96.3% (91.6–98.8)	99.2% (97.2–99.9)	100% (97.5–99.9)	99.5% (98.2–99.9)
Sensitivity[c]	56/57	8/9	64/66	134/136	73/73	207/209
% (95% CI)	98.2% (90.8–99.6)	88.9% (51.8–99.7)	97.0% (89.5–99.6)	98.5% (94.8–99.8)	100% (95.1–99.9)	99.0% (96.6–99.9)
Specificity[d]	56/57	10/12	66/69	122/122	72/72	194/194
% (95% CI)	98.2% (90.8–99.6)	83.3% (51.6–97.9)	95.7% (87.8–99.1)	100% (97.0–99.9)	100% (95.0–99.9)	100% (98.1–99.9)

[a]Includes twins where at least one is a male.
[b]Accuracy: total number of male- and female-bearing pregnancies correctly identified/total number of pregnancies tested.
[c]Sensitivity: number of male-bearing pregnancies correctly identified/total number of male-bearing pregnancies tested.
[d]Specificity: number of female-bearing pregnancies correctly identified/total number of female-bearing pregnancies tested.

Table 3. Clinical details of discordant results

Audit year	PCR	Gestation (weeks)	cffDNA result	Outcome	Comment
1	*SRY*	5	Female	Male	CAH; retested at 7 weeks = male
1	*DYS14*	8	Female	Male	Ectodermal dysplasia; retested at 12 weeks = male
1	*DYS14*	9	Male	Female	DMD; retested at 12 weeks = female Would have failed with current standards? vanishing twin
1	*SRY*	6	Male	Female	CAH; USS indicated female Retested at 28 weeks = female
1	*SRY*	5	Male	Female	CAH; USS indicated female Retested at 16 weeks = female
2	*DYS14*	8	Female	Male	ALD; retested at 11 weeks = male
2	*DYS14*	20	Female	Male and female	Female by amniocentesis, twin–twin transfusion syndrome, one twin looked male on USS. No repeat cffDNA test

ALD, adrenoleukodystrophy; CAH, congenital adrenal hyperplasia; cffDNA, cell-free fetal DNA; DMD, Duchene muscular dystrophy; PCR, polymerase chain reaction; USS, ultrasound scan.

Test accuracy in Phase 2: 1 April 2007–31 March 2009

In Phase 2, 511 women were referred and 805 tests performed (Table 2). Laboratory standards for issuing a report were not achieved initially in 12.9% (IBGRL: 15.3% and NETRMGL: 10.4%) of tests. Ultimately in 22 (4.3%) cases no report was issued. In 12 cases, this was because no repeat sample was sent and in 10 cases repeat testing performed once ($n = 8$), twice ($n = 1$) or thrice ($n = 1$) failed to meet reporting standards. Inconclusive results occurred in 20% (3/15) of samples collected at 5–6 weeks of gestation, 14.8% (53/358) collected at 7–9 weeks, 10.9% (26/238) collected at 9–12 weeks and 8.2% (12/146) collected after 13 weeks.

Confirmation of fetal sex was ascertained in 416 (81.4%; 416/511) pregnancies, 95 outcomes were not obtained as a result of miscarriage (16 cases) or loss to follow-up (79 cases). Concordant results, where a result was issued and fetal sex confirmed, were obtained in 256/258 (99.2% (95% CI 97.2–99.9%) pregnancies at IBGRL and 145/145 [100% (95% CI 97.5–99.9%)] pregnancies at NETRMGL (Table 2). Two falsely predicted females occurred at IBGRL (Table 3). The sensitivity of the *SRY* assay was slightly, but not significantly ($p > 0.05$) higher than the *DYS14* assay (100% *vs* 99.04%). Overall, concordant results were obtained in 401 of 403 cases, giving an accuracy of 99.5% (95% CI 98.2–99.9%) (Table 2). Error rate by predicted gender was 0% (0/207) (95% CI 0.0–1.8%) for males and 1.02% (2/196) (95% CI 0.1–3.6%) for females.

Invasive testing

In 528 pregnancies where the decision to undergo invasive testing was reported and a cffDNA result had been issued, 32.9% (174/528) subsequently had an invasive test. In referrals for risk of X-linked conditions (excluding hemophilia) invasive testing was performed in 41.1% (132/321): 15.5% (25/161) where a female was predicted by NIPD; and 66.9% (107/160) where a male was predicted by NIPD. Where the referral was for prenatal diagnosis for CAH, invasive testing was performed in 32.8% (20/61): 61.9% (13/21) where a female was predicted by NIPD; and 13.2% (5/38) where a male was predicted by NIPD. In referrals for risk of hemophilia, invasive testing was performed in 16.1% (18/112): 3.3% (2/60) where a female was predicted by NIPD; and 30.8% (16/52) where a male was predicted by NIPD.

Discussion

This 3-year audit shows that NIPD using cffDNA is highly reliable when performed in NHS laboratories after 7 weeks using stringent reporting criteria. This is the largest clinical audit of fetal sex determination using cffDNA reported to date, with testing requested in 672 pregnancies and outcomes available for more than 500. The importance of auditing laboratory services was shown as the subsequent change in practice resulted in an improvement in test accuracy for both laboratories. Discordant results do occur, *albeit* rarely, and ultrasound scanning after 12 weeks of gestation to confirm sex is recommended. Furthermore, as results were not issued in approximately 4% of pregnancies, parents should be counseled regarding the possible need for repeat testing.

A key change in practice following the first year of the audit was the stipulation that testing only be conducted after 7 weeks of gestation as three discordant results occurred in six pregnancies tested before 7 weeks. Although cffDNA can be detected

from 4 weeks in pregnancy, the levels are low (9). Bustamante-Aragones et al. (16) have also showed that the accuracy of fetal sex determination using cffDNA was greater after 7 weeks using a *SRY* real-time PCR assay. It is essential that an accurate gestational age is obtained by ultrasound scanning prior to taking maternal blood for cffDNA testing.

There were three false-positive results predicting male fetuses in the first audit year, two using *SRY* and one using *DYS14*, but none identified in the following 2 years of testing. Evidence of amplification of Y-chromosome sequences in female-bearing pregnancies has been seen in other studies using *DYS14* (17) or both *SRY* and *DYS14* (19). The cause of the amplification is not clear and it is important that reporting criteria follow stringent algorithms to prevent the reporting of false positives. In our study one false-positive case had a note on the request form stating 'empty second sac? vanishing twin'. The transmission of cffDNA continues in the absence of a fetal pole and beating fetal heart (5); thus, in twin pregnancies discordant for gender, the death of a male twin with survival of the female could result in the false report of a male fetus. Careful ultrasound scanning prior to cffDNA testing is required to exclude a multiple pregnancy.

Although fetal sex determination was highly reliable in predicting fetal gender during Phase 2 of the audit, around 13% of individual real-time PCR assays gave an inconclusive result. If repeat samples are not received, a result will not be issued for the pregnancy. Similar rates of inconclusive results have been reported by other units, one of which had a repeat testing rate of between 12% (for *DYS14*) and 16% (for *SRY*) (19). In this study, miscarriage had occurred in four cases and it may be that levels of cffDNA were low due to impending fetal demise. There was also some evidence that more inconclusive results occurred earlier in gestation, which may be due to increasing cffDNA levels during pregnancy. In many cases, however, there was no clear relationship between pregnancy outcome and the occurrence of an inconclusive result which occurred in both male- and female-bearing pregnancies at various gestations and sample processing times.

Maximizing cffDNA concentration is critical for reducing the number of inconclusive assays and avoiding false-negative results. It has been suggested that the assays may be improved with the use of larger volumes of plasma for extraction (2 ml) and larger volumes of eluted DNA (9 μl) in the real-time PCR reaction (17). Sample processing variables, such as time taken from venipuncture to sample processing, can also impact upon cffDNA quantity. Lysis of the maternal nucleated blood cells increases appreciably after 24 h affecting the volume of maternal cell-free DNA in the plasma (20–22). Where processing within 24 h is not possible due to distance from the laboratory or other issues, LoBind blood collection tubes that stabilize the nucleated blood cells could be used (22) or a local laboratory could spin the sample prior to transfer.

Comparisons between the *SRY* and *DYS14* assays have been explored in several studies (17, 19, 23, 24). In addition, testing both loci in a single assay has also been advocated as the combination reduces the likelihood of false-positive results (17). Direct comparisons between the two Y-chromosome targets have suggested that utilizing the multicopy *DYS14* results in better sensitivity and reproducibility (19, 23, 24). We made no direct comparisons in our study and so cannot use our data to confirm this. Tests were carried out using *SRY* gave slightly, but not significantly ($p > 0.05$) better results than *DYS14*, which may be a reflection of the greater number of *DYS14* tests.

When using NIPD for fetal sex determination a female fetus is predicted on the basis of a null result, which could also reflect failure of amplification of cffDNA or the absence of cffDNA. While the use of a CCR5 control confirms DNA is present in the PCR it is not fetal-specific. Sex independent markers or polymorphisms can confirm amplification of cffDNA when a female is predicted (17, 18, 25) but to be widely applicable markers must have a high degree of heterozygosity. This method was used to confirm amplification in a few female cases in our series but was not pursued as the process was very labor-intensive and the markers were not informative in >60% of cases. Epigenetic markers might provide a universal fetal DNA marker. For example, sequences within the promoter region of the tumor suppressor genes Maspin and RASSF1A are differentially methylated in maternal red blood cells and fetal placenta (26–28).

The clinical utility of NIPD was clearly shown as only 41.1% of women at risk of X-linked conditions (excluding hemophilia) and 30.5% at risk of CAH subsequently underwent invasive testing. This is consistent with previous reports of cffDNA sex determination for pregnancies at risk of X-linked disorders, where most women carrying female fetuses chose to avoid CVS, while the majority carrying male fetuses underwent invasive testing as there is still a need to confirm if the fetus had inherited the mutant allele or not (12, 15).

Conclusions

Fetal sex determination using cffDNA can be performed efficiently in NHS laboratories in samples taken after 7 weeks of gestation using stringent reporting criteria. The need for invasive diagnostic testing in women at high risk of sex-linked genetic disease is reduced. Parents should be advised of the small risk of discordant results and of the possibility that repeat testing may be required to resolve inconclusive results. Ultrasound should be used to confirm the gestation and check for twin pregnancies before taking maternal blood and should also be offered to confirm the fetal sex reported using cffDNA.

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Ethics approval

Ethical approval for the audit was obtained from the University College London Hospital Research Ethics Committee.

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