

# Fetal cell detection for chromosome analysis from leaking amniotic fluid in pregnancies with rupture of membranes

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## Abstract

**Objective:** In this study, our goal was to assess the feasibility of using vaginally obtained amniotic fluid samples for prenatal chromosome analysis by fluorescence in situ hybridization (FISH) in pregnancies with ruptured amniotic membranes.

**Methods:** Twenty-four pregnant women with known male fetal gender were retrieved for the study. All had ruptured membranes either artificially (AROM) or spontaneous (SROM) at term or at preterm gestations (PPROM). Samples from leaking amniotic fluid were collected during speculum examinations and slides were prepared for FISH using probes specific for chromosomes X and Y. Fetal cell detection rate was calculated as percentage of XY nuclei. Specimen volume, presence of mucus, presence of blood, gestational age, artificial versus spontaneous rupture of membranes and time elapsed until specimen processing were compared with regard to fetal cell detection rate.

**Results:** There were 12 patients with AROM (50%) and 12 with SROM (50%). Only two of those were preterm (8.3%). Six of the specimens were bloody (25%) and 16 (66.6%) were macroscopically with mucous. The proportion of male fetuses identifiable by FISH was 100% (95% CI: 86%, 100%) after exclusion of technical failures (n=4). Overall, fetal cell detection rate was 6.4%. Samples collected after AROM had borderline higher percentage of fetal cells compared with SROM after adjusting for presence of blood in the sample (p=0.07). In addition, bloody samples had a significantly higher percentage of fetal cells than those that were not bloody (p=0.01).

**Conclusion:** Amniotic fluid collection for prenatal chromosome analysis by interphase FISH is a feasible, non-invasive and reasonable approach on rupture of membranes patients and may be accomplished on preterm premature rupture of membranes with known male fetus pregnancies when indicated. Further studies are needed to assess the value of molecular analysis to differentiate fetal cells with higher specificity for female fetuses.

**Keywords:** Fluorescence in situ hybridization, membrane rupture, vaginal, pregnancy.

## Özet: Membran rüptürlü gebeliklerde amniyotik sıvı sızıntısından kromozom analizi için fetal hücre tespiti

**Amaç:** Bu çalışmada amacımız, amniyotik membran rüptürlü gebeliklerde floresan in situ hibridizasyon (FISH) ile prenatal kromozom analizi için vajinal olarak elde edilen amniyotik sıvı örneği kullanımının fizibilitesini değerlendirmektir.

**Yöntem:** Fetal cinsiyetin erkek olduğunun bilindiği 24 gebe çalışmaya dahil edildi. Tüm gebeler, miadında veya preterm gebelikte yapay (AROM) veya spontan (SROM) rüptüre membrana sahipti. Örnekler spekulum muayenesi sırasında amniyotik sıvı sızıntısından alındı ve lamalar, kromozom X ve Y'ye özel probalar kullanılarak FISH için hazırlandı. Fetal hücre tespiti oranı, XY çekirdekleri yüzdesi olarak hesaplandı. Örnek hacmi, mukus varlığı, kan varlığı, gestasyonel yaş, yapaya karşı spontan membran rüptürü ve örnek işlemeye kadar geçen süre, fetal hücre tespiti oranı bakımından karşılaştırıldı.

**Bulgular:** On iki AROM (%50) ve 12 SROM (%50) hastası mevcuttu. Bunlardan yalnızca ikisi (%8.3) preterm idi. Örneklerin altısı (%25) kanlıydı ve 16'sı (%66.6) makroskopik olarak müközdü. Teknik hataların (n=4) hariç tutulması sonrasında FISH ile tespit edilebilen erkek fetüslerin oranı %100 idi (%95 GA: %86, %100). Genel olarak fetal hücre tespiti oranı %6.4'tü. AROM sonrasında alınan örnekler, örnekte kan varlığı için düzenleme yapılmasının ardından SROM'a kıyasla daha yüksek sınırdaki fetal hücre oranına sahipti (p=0.07). Ayrıca kanlı örnekler, kanlı olmayan örneklerden anlamlı derecede daha yüksek fetal hücre yüzdesine sahipti (p=0.01).

**Sonuç:** İnterfaz FISH ile prenatal kromozom analizi için amniyotik sıvı alımı, membran rüptürlü hastalarda elverişli, invaziv olmayan ve makul bir yaklaşımdır ve endike olduğunda, fetüsün erkek olduğu bilinen gebeliklerde preterm erken membran rüptürü üzerinde başarılı olabilir. Dişi fetüslerde daha yüksek özgüllük ile fetal hücreleri ayırt etmeye yönelik moleküler analizin değerini değerlendirmek için daha fazla çalışmaya ihtiyaç vardır.

**Anahtar sözcükler:** Floresan in situ hibridizasyon, membran rüptürü, gebelik, vajinal.

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## Introduction

Prenatal diagnosis of a genetic disorder is valuable information at any gestational age. Many available techniques require invasive procedures such as amniocentesis, chorionic villi biopsy or cordocentesis. Although cell-free fetal DNA testing in maternal blood has reduced the need for invasive approaches significantly, it is still regarded as a good screening test for aneuploidies. Some other methods are still under investigation for their routine use as in transcervical cell sampling (TCC) by intrauterine lavage (IUL) and by mucus sampling.<sup>[1-3]</sup> These earlier studies reported that fetal chromosome analysis is possible through recovered trophoblasts in early gestations with intact membranes. However, after early years of 2000's, interest in this topic has faded in the medical literature.<sup>[2]</sup>

Amniotic fluid contains desquamated fetal cells; they can be reached via amniocentesis when genetic, metabolic or immunologic testing of fetus is indicated. With the rupture of membranes at preterm gestations, amniotic fluid becomes available through vaginal examination, just like it is used in routine clinical practice for confirmation of membrane rupture. To the best of our knowledge, there is no data for feasibility of using vaginally obtained leaking amniotic fluid for prenatal diagnosis so far. Fetal aneuploidy analysis on amniotic fluid is accomplished by conventional karyotyping, a technique that needs rapidly dividing cells to capture as many as possible metaphase plates. Therefore, it always requires a sterile sample, since bacterial and fungal growth may inhibit fetal fibroblast culture. However, by molecular techniques such as fluorescence in situ hybridization (FISH) and quantitative-fluorescent polymerase chain reaction (QF-PCR), the need for sterile sampling and culturing can be bypassed while analyzing great number of cells/molecules in shorter period. Therefore in this study, we aimed to test the feasibility of using leaking amniotic fluid samples for fetal chromosome analysis by FISH. Primary objective was to assess the feasibility of using vaginally obtained amniotic fluid samples for prenatal chromosome analysis by FISH on ruptured amniotic membrane patients. Our secondary objective was to observe fetal cell detection rate, specimen/patient related variables and their effects on fetal cell detection rate.

## Methods

### Patient population

This was a two-center observational study that was conducted at Georgetown University Hospital in Washington, DC and Virginia Hospital Center in Arlington, VA, USA. The study was approved by the Institutional Review Board of Georgetown University (IRB 2008-43) and written consents were obtained from each participant. Pregnant women who came to labor and delivery clinics of these centers with complaints of ruptured membranes were evaluated for their eligibility. Inclusion criteria were male fetal gender (detected by ultrasonography or by amniocentesis/chorion villi sampling), spontaneous rupture of membranes at term ( $>37$ wks; SROM), preterm premature rupture of membranes (PPROM) at any gestational age after viability  $[(26+0)-(36+6)]$  or artificially rupture of membranes (AROM) ( $>37$ wks) at term. None of the artificial rupture of membranes was performed for the study; all had obstetric indications. All fetal genders were later confirmed by postpartum inspection of neonatal genders. Only cases with overt membrane rupture as determined by active cervical discharge and fluid pooling were recruited. Exclusion criteria were multifetal pregnancy, female fetal gender, active vaginal bleeding, recent sexual intercourse (last 7 days), premature rupture of membranes before 24 weeks, maternal age under 18 and any maternal/fetal condition that requires prompt delivery such as placental abruption, chorioamnionitis and non-reassuring fetal heart rate.

### Sample collections

Samples were collected during the diagnostic speculum evaluation of PPRM or SROM or right after membranes were ruptured by the operator (in AROM cases). All diagnoses for membrane rupture were confirmed by positive inspection of pooling, nitrazine test and ferning pattern under microscope. Patients those having all of the three findings were regarded as having ruptured amniotic membranes. Sterile syringes without needle were used to aspirate the amniotic fluid accumulations on speculums. Samples were immediately transferred into a tube containing phosphate-buffered saline solution (PBS). Specimen amount, presence of mucus, presence of blood, gestational age (term vs preterm), time elapsed until specimen processing were compared with regard to fetal cell detection rate. All specimens were processed in the first 5 days of their collection.

## Fluorescence in situ hybridization

### Slide preparations

Slide preparations and FISH analysis for chromosomes X and Y were accomplished by using the protocols previously described in the literature.<sup>[4]</sup> Briefly, PBS was added to samples and centrifuged at 1000 RPM for 10 minutes. Supernatants were discarded and the precipitant cells were mixed gently. Sample mixes were dropped on a clean and dry slide. Hypotonic solution (50 mM potassium chloride) was added to each slide and incubated at 370°C for 20 minutes. Excess hypotonic was decanted and this step was repeated. Slides were then dried at 60°C for 5 minutes. After dehydration steps with ethanol series, slides were stored at -200°C until FISH procedure.

### Pretreatment of slides

Slides were treated in 50% acetic acid and 50% methanol fixative solution for 10 minutes and then in 5 µl pepsin stock (10%) /HCl solution for 2.5 minutes. Formamide denaturation step was completed after another series of ethanol dehydration.

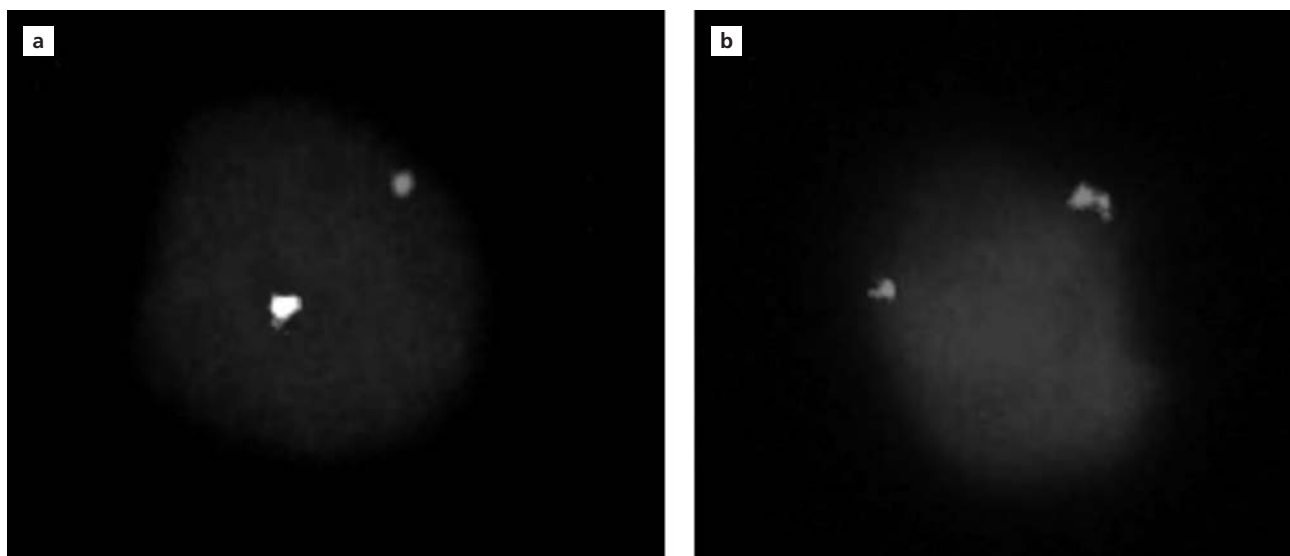
### Pretreatment of probes and detection steps

For each slide a total of 20 µl hybridization mix containing 1 µl centromeric probe for each sex chromosome (X and Y) was prepared and denatured at 800°C for 10 min-

utes. After denaturation, probe mixes were incubated at 370°C overnight in humidifying chamber. The next day, slides were treated with formamide for stringency wash and rinsed with saline-sodium citrate (SSC) buffer. A volume of 100 µl blocking solution was added to each slides and covered with coverslips. After 30 minutes of incubation at 370°C, cover slips were removed. For each slide, 100 µl detection solution containing 1 µl avidin-FITC + 1 µl mouse anti-digoxin was added and slides were incubated at 370°C for 45 minutes.

### Fluorescence microscopy

Slides that were hybridized with centromeric probes for chromosomes X and Y were analyzed under fluorescence microscope in dark room settings. For each slide (patient), 200 interphase nuclei were counted. A pattern of one green and one red signal in an interphase nucleus was recorded as male gender (XY, Fig. 1a). Nuclei with two red signals were counted as female nuclei (XX, Fig. 1b). Since maternal cell contamination (XX) was expected in all samples, male and female signal patterns were counted per slides and percentages were recorded as “fetal cell detection rate”. For example, a slide of a patient with 200 counted nuclei having XX[194]/XY[6] signals, was recorded as having 3% fetal cells in that specimen. To minimize the possible effect of signal artifacts, at least three interphase nuclei with XY signal pattern were sought per patient to call a specimen as con-



**Fig. 1.** FISH image of an XY nucleus carrying one red and one green signal (a) and other XX nucleus carrying two red signals (b).

taining male fetal cells. Signal properties of FISH probes were tested previously on male and female control blood samples.

### Statistics

The proportion of male children identifiable as male by FISH was estimated with an exact 95% confidence interval. In addition, an exact one-sided binomial test was performed to test if this proportion was greater than 80%. The mean percentage of fetal cells was compared for each sample characteristic (type of membrane rupture, type of delivery, volume, presence of mucus, presence of blood, and time until processing) using t-tests and ANOVA. Linear regression was used to determine characteristics associated with percentage of fetal cells.

### Results

A total of 28 pregnant women at gestational ages between 240/7 weeks and 410/7 were recruited. FISH analysis could not be done in four patients due to technical problems. Therefore remaining 24 patients were included in the final analysis. In 12 patients, samples were obtained after artificial membrane rupture (50%); the rest of the pregnancies had spontaneously ruptured membranes (50%). Only two patients were preterm (8.3%), one had PPROM at 31st weeks of gestation and the other had preterm labor with PPROM at 30th weeks of gestation. Six of the specimens were bloody (25%) and, 16 (66.6%) were with mucous macroscopically.

The proportion of male children identifiable by FISH was 100% (95% CI: 86%, 100%) ( $p=0.005$ ) after exclusion of technical failures ( $n=4$ ). When the percentage of fetal cells was calculated (XY signals), overall fetal cell detection rate was 6.4% (2.7–21.8%). Table 1 displays the mean percentage of fetal cells for each sample characteristic (gestational age, specimen volume, presence of mucus, presence of blood, artificial versus spontaneous rupture of membranes, time elapsed until specimens processing). Bloody samples had a significantly higher percentage of fetal cells than those that were not bloody ( $p=0.01$ ). Also, samples collected after an AROM had a borderline significantly higher percentage of fetal cells than SROM ( $p=0.07$ ). No other significant differences were observed in the percentage of fetal cells with regard to recorded characteristics. The final linear regression model only included type of membrane rup-

**Table 1.** Mean values and 95% confidence intervals of fetal cells by sample characteristics ( $n=24$ ).

|                                 | n  | Mean | 95% CI     | p-value |
|---------------------------------|----|------|------------|---------|
| <b>Type of membrane rupture</b> |    |      |            | 0.07    |
| AROM                            | 12 | 8.3  | (4.3, 12)  |         |
| SROM                            | 12 | 4.6  | (3.0, 6.3) |         |
| <b>Type of delivery</b>         |    | 0.60 |            |         |
| Term                            | 22 | 6.7  | (4.3, 9.0) |         |
| Preterm                         | 2  | 4.6  | (-6.6, 16) |         |
| <b>Volume</b>                   |    |      |            | 0.16    |
| <2 ml                           | 10 | 6.2  | (2.4, 9.9) |         |
| 2–4 ml                          | 10 | 5.1  | (4.0, 6.2) |         |
| >4 ml                           | 4  | 11   | (-3.1, 25) |         |
| <b>Mucus</b>                    |    |      |            | 0.93    |
| Absent                          | 10 | 6.4  | (3.0, 9.7) |         |
| Present                         | 14 | 6.6  | (3.4, 9.8) |         |
| <b>Blood</b>                    |    |      |            | 0.01    |
| Absent                          | 13 | 4.2  | (3.4, 4.9) |         |
| Present                         | 11 | 9.2  | (4.9, 14)  |         |
| <b>Time until processing</b>    |    |      |            | 0.36    |
| >24 hours                       | 17 | 4.7  | (3.2, 6.1) |         |
| <24 hours                       | 7  | 7.2  | (4.2, 10)  |         |

AROM: artificially rupture of membranes; SROM: spontaneous rupture of membranes

ture and blood. The adjusted mean values and 95% confidence intervals are shown in Table 2. After adjusting for the type of membrane rupture, bloody samples had a significantly higher mean percentage of fetal cells than samples with no blood ( $p=0.01$ ).

### Discussion

In this study, our results indicated that fetal chromosome analysis by FISH is a feasible approach for prenatal cytogenetic diagnosis on leaking amniotic fluid in pregnancies with membrane rupture. We also observed that overall percentage for fetal cells in leaking amniotic fluid is sufficient for FISH analysis.

**Table 2.** Adjusted mean values and 95% confidence intervals estimated from the linear regression model ( $n=24$ ).

|                                 | Mean | 95% CI     | p-value |
|---------------------------------|------|------------|---------|
| <b>Type of membrane rupture</b> |      |            | 0.07    |
| AROM                            | 8.3  | (5.8, 11)  |         |
| SROM                            | 5.0  | (2.5, 7.6) |         |
| <b>Blood</b>                    |      |            | 0.01    |
| Absent                          | 4.3  | (1.8, 6.7) |         |
| Present                         | 9.1  | (6.4, 12)  |         |

AROM: artificially rupture of membranes; SROM: spontaneous rupture of membranes

Prenatal diagnosis to detect fetal genetic disorders is desired to make informed decisions at any time during pregnancy. While unsatisfactory sensitivity/specificity levels are inherent drawback for screening tests, procedure related complications can be a problem in invasive diagnostic tests. Recent advances in testing for cell-free fetal DNA in maternal blood is considered as a good but an expensive screening test for now.<sup>[5]</sup> Observation of trophoblast presence in cervical canal during first trimester has inspired researchers for alternative prenatal diagnostic sampling methods.<sup>[6-8]</sup> Consequently, studies have demonstrated the feasibility of FISH and molecular analysis on trophoblasts obtained by two TCC sampling techniques.<sup>[6]</sup> In IUL technique, investigators were able to retrieve trophoblasts for genetic analysis by advancing a sterile flexible catheter (Pipelle) up to the point of internal cervical os, and rinsing with a small amount of sterile saline solution, and collecting it back. IUL is possible only in the first trimester and it has been tested on patients prior to their planned termination of pregnancies.<sup>[9,10]</sup> Other technique is cervical mucus collection (usually by cytobrush) and identification of trophoblasts under inverted microscope for prenatal genetic testing.<sup>[3,11-13]</sup> In a study where TCC by IUL was compared to mucus sampling in pregnant women between 7th to 12nd gestational weeks, fetal cell detection rate was 2–90% (mean 40%) and correct sex determination in male embryos was 90.2% by IUL. On the other hand, fetal cell detection rate was 1–4% and correct sex determination in male embryos was 56% by cervical mucus sampling.<sup>[14]</sup> Another study has used immunohistochemistry and demonstrated that trophoblasts were easily detectable by HLA-G staining in 35 out of 37 cervical mucus samples of first trimester intrauterine pregnancies.<sup>[15]</sup>

In this study, target population was pregnant patients with ruptured membranes. Since we did not come across a similar report in the medical literature (PubMed database), it was not possible to compare our results to others: TCC by IUL or mucus sampling methods have tested the prenatal fetal cell detection and diagnosis feasibility on patients with intact membranes in the first trimester.

One interesting finding of this study was that bloody samples had a significantly higher percentage of fetal cells than those were not bloody ( $p=0.01$ ). Vaginally obtained leaking amniotic fluid may contain maternally derived cells such as leukocytes, macrophages, squamous and columnar epithelial, as well as fetal-derived

cells. The reason behind this finding is not clear; however, it can be speculated that the origin of blood was more fetal than maternal. In a study where IUL was used in the first trimester, blood contamination was reported to correlate with trophoblast presence.<sup>[10]</sup> Although we did not test the rate of trophoblast presence in leaking amniotic fluid samples, we infer that their percentage is negligible since the shedding of trophoblasts ceases after the fusion of decidua basalis and parietalis by the end of the first trimester.<sup>[9]</sup> Therefore, utilizing fetal cells directly rather than trophoblasts (as in TCC and chorion villi sampling), minimizes the risk of confined placental mosaicism.

Our results showed that, samples collected after AROM had borderline higher percentage of fetal cells compared with SROM after adjusting for presence of blood in the sample ( $p=0.07$ ). This finding is justifiable since membrane rupture is imminent and collection of sample is recent in AROM. However, results also indicated that fetal cells are detectable by 100% in patients with SROM, thus eliminating the need of higher percentage of fetal cells.

In this study, specimen collection and FISH analysis steps were achieved by the same operator (corresponding author), which could be a source of bias. However, this was a feasibility study and there were no group of patients to compare each other that would necessitate blinding. Sperm contamination possibility may be another question, as addressed by previous studies that evaluated cervical mucus samples under microscope to rule out sperm cell presence.<sup>[10]</sup> Instead, we asked patients their last vaginal intercourse time and did not recruit if they have had intercourse recently. Besides, a haploid nucleus of a sperm cell would give only one signal, easily identifiable from fetal cells with two signals in one nucleus (red and green). We also assumed that all fetuses were non-mosaic XY males and they did not have sex chromosome aneuploidies.

In the current approach, FISH analysis alone or conventional karyotyping cannot be applied to patients with female fetal gender due to maternal cell contamination. However, this obstacle is easy to overcome by short tandem repeat (STR) analysis as it is performed in QF-PCR assays.<sup>[16]</sup> Maternal cell contamination should not be considered as a disadvantage; rather it was our goal to assess fetal cell (chromosome) detection rates in amniotic fluid samples contaminated with maternal cells, which was already expected. We were able to observe fetal cells

(XY carrying interphase nuclei) in all of these samples with overall cell detection rate 6.4% (2.7–21.8%), after excluding technical failures (n=4). This seems like a considerable amount of maternal cell contamination. However, for every SROM patient, detected fetal cells were more than adequate for molecular cytogenetic (FISH) and for PCR techniques that could be used in patients with female fetal gender.

As mentioned, in this study vaginally obtained leaking amniotic fluid samples were employed for fetal cell sampling. Non-invasive nature of the approach is an advantage. Clearly, it could only be applied to patients with ruptured membranes. Management of PPROM requires an assessment of risks and benefits of continued pregnancy or expeditious delivery. In certain clinical settings when prenatal diagnosis is indicated and invasive techniques are not feasible such as PPROM with oligo-anhydramnios or PPROM and patient preference, this method might be of value.

## Conclusion

Amniotic fluid collection for prenatal chromosome analysis by interphase FISH is a feasible and reasonable approach on rupture of membranes patients and may be accomplished on preterm premature rupture of membranes with known male fetus pregnancies. Future studies are needed to test the utility of molecular analysis to differentiate female fetal cells with accuracy in patients carrying female fetuses.

**Conflicts of Interest:** No conflicts declared.

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