

Rapid One-day Fluorescence *In Situ* Hybridisation in Prenatal Diagnosis Using Uncultured Amniocytes and Chorionic Villi

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Abstract

Rapid assays for the detection of the common chromosomal aneuploidies using DNA probes to chromosomes 13, 18, 21, X and Y have been developed in recent years. In this study, we report our experience using fluorescence in situ hybridisation (FISH) with uncultured amniocytes from amniotic fluids (AF) and cells from chorionic villus sampling (CVS) for prenatal diagnosis in 239 assays. Only 2 ml of AF or 5mg of chorionic villi were required for FISH analysis using region-specific probes to chromosome 13, 18, 21, X and Y. Results were informative in all the assays conducted in this study. The average time to obtain a result was 24 hours. However, the results for chromosome X, Y and 18 were ready within four hours. The criteria employed for result interpretation were stringent. In a normal finding, at least 80% of all nuclei observed must have 2 discrete signals per cell. In a suspected abnormal case, a minimum of 60% of all nuclei observed must have one (monosomy X) or 3 signals (trisomy) per cell. One trisomy 13 and two trisomy 21 cases were detected. All FISH results were confirmed by karyotyping. Subsequent karyotyping of the resultant abortuses gave further confirmation of the results. The results obtained showed 100% positive and negative predictive values. This study shows that FISH can be used as a rapid routine assay in conjunction with full karyotyping analysis. Its prognostic value is especially important for patients with late referrals, parental anxiety, high-risk pregnancies identified by maternal serological screening and suspected foetal abnormalities detected by ultrasonographic examination.

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Introduction

The most common chromosomal abnormalities in newborns are trisomy 21, trisomy 18, trisomy 13, and monosomy X, with incidences of 1/800, 1/8,000, 1/20,000, and 1/10,000, respectively.¹ Conventional cytogenetic approaches traditionally employ foetal cells from amniotic fluid (AF) or chorionic villus (CV) grown *in vitro* and metaphase banding for chromosome analysis to allow detection of both numerical and structural rearrangements.²⁻⁸ However, these methods require long-term cell culture of two to three weeks.⁵⁻⁸ With improvements in tissue culture techniques, it is now possible to obtain a result in 7 days.^{9,10} Nevertheless, this may even be considered too long a wait in cases of abnormalities detected by ultrasonography and in high-risk pregnancies identified by maternal serum screening.^{5,11,12} In such cases, a rapid diagnosis for the presence or absence of certain foetal aneuploidies can provide invaluable information for better pregnancy management.

Methods developed during the last decade for rapid detection of numerical chromosomal abnormalities

(aneuploidy) using chromosome-specific probes on interphase cells have made FISH a good option.¹³⁻¹⁵ With significant clinical potential, there have been increasing numbers of investigations on its application for prenatal diagnosis.¹⁶⁻²⁰ The techniques and specific chromosome probes used for FISH on uncultured AF or CV in prenatal diagnosis have proved to be convincing.²¹⁻²⁵ We report here a rapid one-day FISH assay for prenatal detection of chromosome aneuploidy on uncultured AF and CV in this study for chromosome 13, 18, 21, X and Y in 239 assays. We believe that this is the first publication of its kind in the ASEAN region.

Material and Methods

Patients

All the patients in this study were referred to the Department of Obstetrics & Gynaecology, Singapore General Hospital, for amniocentesis or chorionic villus sampling (CVS) for karyotyping between August 1996 and September 1998. The main reasons for the referrals were abnormal ultrasound findings in the fetuses, posi-

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tive serological findings for Down syndrome (DS) from maternal screening tests, advanced maternal age of older than 35 years, patients with a known family history of DS and parental anxiety. The reasons for referral are listed in Table I. Amniocentesis and CVS were performed with the hospital's Ethics Committee's approval and consent from the patients.

TABLE I: MAIN REASONS FOR FISH

Reasons for FISH	Number of assays	% of assay
Advanced maternal age	176	74
Positive maternal serological screening	30	12
Abnormal foetal ultrasonographic findings	23	10
Others	10	
Total	239	100

Samples

All the AF and CV samples were obtained between 14 and 23 weeks gestation. A total of 20 ml of AF was obtained from each patient. 2 ml of AF aspirate was used for FISH and the remaining 18 ml was cultured for conventional karyotyping. The AF sample was classified as "clear", "blood-stained" and "bloody". A bloody AF was tinted red. A blood-stained AF was visible only after centrifugation. Otherwise samples were designated "clear". FISH was only performed in the clear and blood-stained samples. One specimen came from an anembryonic gestational sac at 5 weeks gestation. 1 ml of aspirate were used for FISH analysis.

CV samples were obtained at 9 weeks gestation in all patients. 20 mg of CV samples was obtained from each patient of which 5 mg was used for FISH and 15 mg of the remaining tissue used for culture and conventional chromosome analysis. Extreme care was exercised to separate foetal tissues from suspected maternal tissue which were fully discarded.

Fluorescence In Situ Hybridisation (FISH)

1. Probes

The probe used for the detection of chromosomes 13 and 21 was the two-colour LSI 13q14(Spectrum Green) / LSI 21q22.13-q22.2(Spectrum Orange) from Vysis (Vysis Inc, Downers Grove, Illinois, USA). The centromere probes for chromosomes X, Y and 18 were purchased from Cytocell (Cytocell Ltd, UK). Initially, a two colour X/Y (FITC/Cy3) protocol was used along with a single 18 (FITC) probe. This was later changed to a three-colour X/Y/18 (FITC/Cy3/FITC-Cy3) system. All probes used were direct-labelled probes.

2. Sample Preparation: AF

Interphase nuclei from uncultured AF were used for FISH. Slide and cell preparations were performed as

previously described with some modifications.¹⁸ Briefly, 2 ml of the cell suspension was centrifuged at 400 g for 10 minutes and the supernatant was discarded. The cell pellet was resuspended in 1 ml of phosphate buffered saline (PBS, pH 6.8), pre-warmed to 37°C. The cells were centrifuged again and resuspended in 80 to 160 µl PBS. 40 µl of this suspension was placed on a pre-cleaned glass slide and incubated at 37°C for 20 minutes. Two such slides were made from each sample. The cells were next subjected to hypotonic treatment with 100 µl of 50 mM KC1 pre-warmed to 37°C and incubated for 20 minutes. The cells were then pre-fixed with 3:7 methanol:acetic acid (3:1):75mM KC1 for 2 minutes at room temperature. The pre-fixed was carefully removed and replaced with 100 µl of fresh methanol:acetic acid fixative (3:1). The slides were then rapidly dried and dehydrated through an ethanol series of 70%, 80% and 100% for 1 minute each respectively. The slides were then ready for FISH.

3. Sample Preparation: CVS

Interphase nuclei from uncultured CVS were used. Slides and cell preparation were made according to the methods previously described by the others.^{26,27} Briefly, the villi were washed twice with serum free Ham's F10 medium (Biological Industries, Israel) and then digested with 0.05% trypsin-EDTA (Gibco, New York, USA) for 20 minutes at 37°C and were made into a single-cell suspension. The cell suspension was then centrifuged at 400 g for 10 minutes and the supernatant was decanted. The cell pellet was resuspended in 1 ml of phosphate buffered saline (PBS) pre-warmed to 37°C. The cells were centrifuged again and resuspended in 80 to 160 µl PBS. Subsequent slide preparation was the same as for amniotic fluids (above).

4. FISH Protocol

Hybridisation and post-hybridisation wash protocols followed the protocols described by the manufacturers of the probes. If a sample showed more than 20% of hybridised nuclei with only 1 signal, a re-hybridisation was carried out. With the X/Y probe, if more than 20% of hybridised nuclei had a mixture of XX and XY nuclei, a re-hybridisation step was carried out.

Scoring for FISH Signals and Criteria for Diagnosis

The scoring for aneuploidy using FISH was based on the fluorescence signals generated from interphase nuclei. FISH signals were visualised under a fluorescence microscope (Olympus BH2, Japan) with a 100x oil objective lens and a triple filter cube for DAPI/FITC/TRITC (Chroma Technology, Brattleboro, VT, USA). Only intact nuclei without overlapping adjacent nuclei or debris were evaluated. The patterns of signals were categorised as discrete, patchy, or diffuse. Split signals were scored as one if the distance between the signals was less

than one signal domain. The signals were otherwise scored as two if the gap between the split signals was greater or at least equal to one signal domain.²⁸ The number of fluorescence signals that were not equal to two for the autosomal chromosomes was considered as abnormal. For sex chromosomes, the number of nuclei with different combinations of X and Y signals was recorded as X, XX, XY, XXX, XXY and XYY. Each probe was evaluated on a minimum of 100 nuclei by two independent observers. Results were considered normal when 2 discrete signals (Figs. 1A & 1B) were seen in 80% or more of the interphase nuclei by both observers. In suspected abnormal cases, at least 60% of the interphase nuclei had one or three signals (Figs. 1C & 1D).^{21,23} In other words, a dual 80% to 60% cut-off value was used

to determine normal and abnormal cases, respectively. Results of assays that failed to meet these criteria were deemed uninformative and a repeat re-hybridisation procedure was warranted.

Conventional Chromosomal Analysis

Conventional chromosomal analysis was performed in conjunction with FISH. Three primary cultures of AF or CV samples were established for each case. Cytogenetic analysis of the chromosomes using G-banding was performed as previously described.²⁹ At least 20 metaphases were scored and 5 metaphases were analysed per individual. Classification was made according to the International System of Cytogenetic Nomenclature (ISCN).³⁰

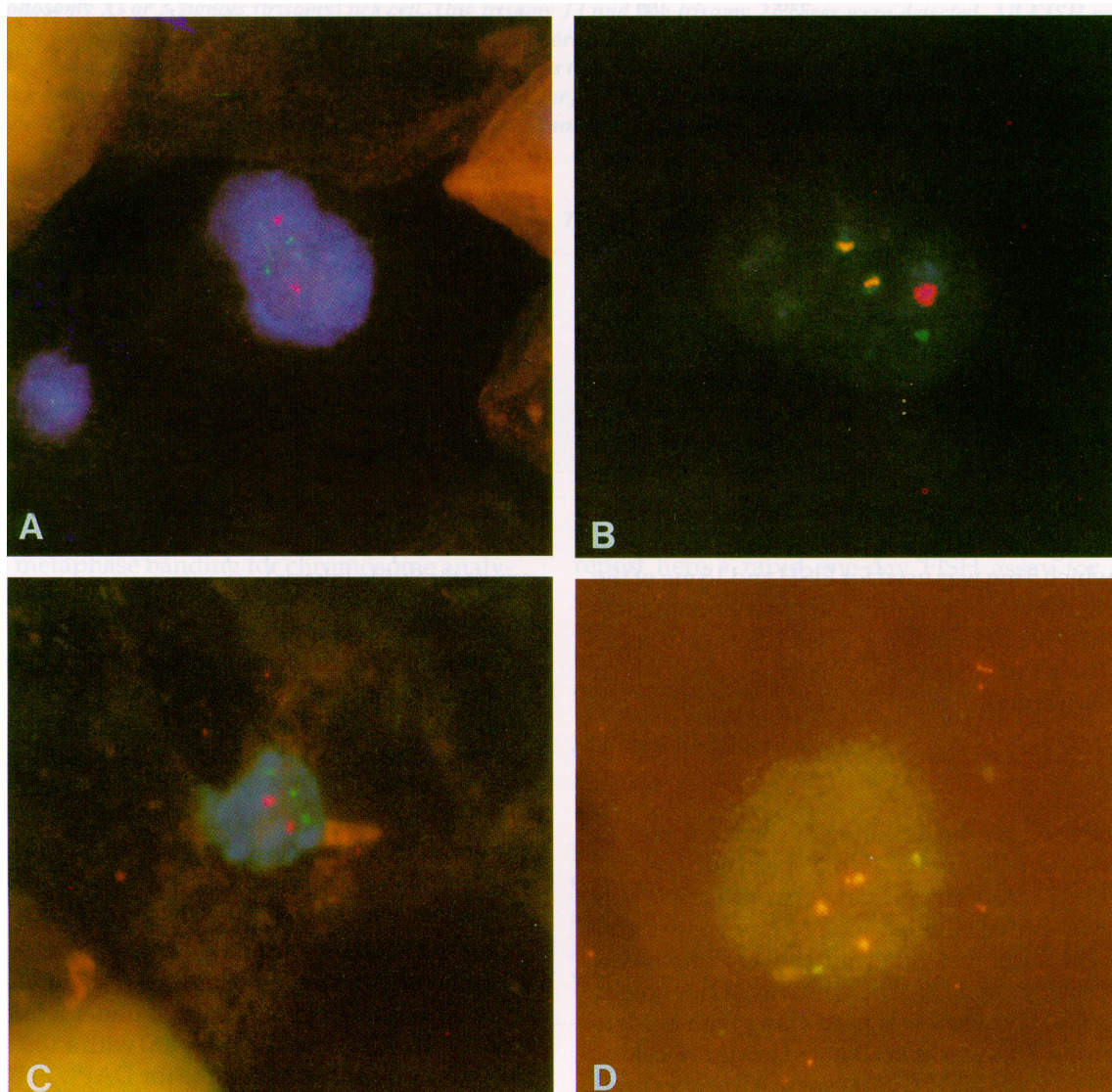


Fig. 1. Amniocyte nuclei labelled with FISH probes 13, 18, 21, X and Y.

Fig. 1A. Normal cell showing two signals of chromosomes 13 (green) and 21 (red) each.

Fig. 1B. Normal male cell showing two signals of chromosome 18 (yellow) and one signal for X (green) and Y (red) each.

Fig. 1C. Trisomy 13 cell with three signals of chromosome 13 (green) and two signals of chromosome 21 (red).

Fig. 1D. Trisomy 21 cell with three signals of chromosome 21 (orange) and two signals of chromosome 13 (green).

Statistical Analysis

The data were analysed by t-test. A *P* value of <0.05 was considered significant.

Results

FISH was performed in a total of 239 assays using uncultured AF and CV cells. Of these, one had predominantly three signals for chromosome 13 and in 2 samples, three signals were observed for chromosome 21. The reasons for FISH assays are shown in Table I. The mean gestational age for amniocentesis was 16 weeks, ranging from 14 to 23 weeks; CV sampling was exclusively performed at 9 weeks of gestation. FISH was carried out in the anembryonic case, a biochemical IVF pregnancy at 5 weeks gestation. This was later revealed to have a male Down syndrome karyotype by FISH analysis. The mean time to obtain a result was 24 hours with a range of 4 to 48 hours, compared an average of 15 days (range 8 to 19 days) for conventional karyotyping. FISH results were significantly shorter than conventional karyotyping (mean 1 day versus 15.3 days, $P < 0.001$). The time taken for a result depended on the probes used but did not vary with gestational age or with the presence of a foetal abnormality. The mean frequency of disomic signals for chromosomes 13, 18, 21, XX and XY were 82%, 82%, 86%, 95% and 97%, respectively. All 239 FISH assays were fully informative. One trisomy 13 (Fig. 1c) and two trisomy 21 (Fig. 1d) cases were detected. Seventy-one per cent of interphase nuclei displayed 3 fluorescence signals in the trisomy 13 cases, whilst 69% of hybridised interphase nuclei displayed 3 fluorescence signals in the trisomy 21 cases. All FISH results including sex determination were confirmed by subsequent full karyotyping of the prenatal samples as well as karyotyping of the abortuses. The results of the FISH assays in comparison with conventional karyotyping are summarised in Table II.

Discussion

Prenatal diagnosis for expectant mothers above 35

TABLE II: CHROMOSOMAL ABNORMALITIES DETECTED BY FISH AND KARYOTYPE ANALYSIS

	Karyotype n = 238*	FISH n = 239
Normal	235	236
Trisomy 13	1	1
Trisomy 18	0	0
Trisomy 21	1	2
Turner syndrome	0	0
Klinefelter syndrome	0	0
Triploidy	0	0
Others (47,XY,+mar/46,XY)	1	0
Total	238	239

* The anembryonic case was not karyotyped due to insufficient material.

years of age is recommended by WHO because of the increased risk for DS. In addition, women who are below this age but who have been tested positive for serological markers for DS are also urged to go for such screening. Routine karyotyping procedures usually take two to three weeks. However, sometimes results are urgently required due to late clinical referrals near the 24th week of gestation or with difficulties in the determination of foetal abnormalities by ultrasonographic examinations until a late gestational stage.

FISH yields signals that are visualised as hybridised targets with probes directed at chromosomes 13, 18, 21, X and Y. Aneuploidies (monosomy and trisomy) are easily identified by analysis of signal number. Based on our diagnostic criteria, a normal outcome is considered when at least 80% of hybridised nuclei show 2 signals, while in the case of a monosomy or trisomy, a minimum of 60% hybridised nuclei should show 1 and 3 signals, respectively. Of the 239 FISH assays, 236 were diagnosed as normal while 1 trisomy 13 and 2 trisomy 21 cases were detected. These FISH results were confirmed with conventional chromosome analysis of the prenatal samples as well as with karyotyping of the products of conception. The results by conventional chromosome analysis of the 239 cases were obtained between 9 and 16 days, with a 0% failure rate and no misdiagnosis. All karyotypes were consistent with FISH results. The mean time for FISH was significantly shorter than that for conventional karyotype (mean 1 day versus 15.3 days, $P < 0.001$).

The cut-off levels of 60% and 80% for abnormal and normal cases used in this study for the diagnosis of foetal aneuploidy were similar to that reported by other investigators.^{18,21,22,24,25} An XX-signal pattern was observed in 85% to 95% of karyotypically female samples. Although our sample size was relatively small, the FISH signal patterns were consistent, and analytic sensitivities and specificities were high for both normal and trisomic samples (100%).

FISH assays usually require 5 ml of AF samples and the average number of analysable nuclei is 50.^{18,21} With the method used in this study, up to 200 analysable interphase nuclei was obtained in most cases from just 2 ml of AF samples. Successful detection of a trisomy 21 male foetus by FISH was obtained from an anembryonic sac using only 1.5 ml aspirate.

FISH can also provide additional information of DS resulting from Robertsonian translocations. The locus-specific chromosome 13 and 21 probes we use detect the extra long arms of these two chromosomes involved in Robertsonian translocations as extra FISH signals.²⁵ In the case of a failed AF culture, FISH can still be used to determine the status of aneuploidy. This is especially so in the case of a suspected abnormal sample

where tissue cultures are known to have failed.³¹ FISH is helpful in this situation if a repeated sample is not available.

The efficiency of detection of mosaicism by FISH on interphase cells remains to be tested. Jalal et al²⁵ showed that mosaicism involving trisomies can be detected by FISH on interphase nuclei. They reported an 8% to 12% incidence of chromosomal trisomy 21 mosaicism in a newborn detected by FISH. We previously reported the case of a mosaic trisomy 18 individual with a 76% trisomy cell line karyotyped from his peripheral lymphocytes.³² This was confirmed by FISH. However, in the absence of a known karyotype, diagnosis of mosaicism by FISH alone remains questionable. The disparity between FISH results and conventional chromosome analysis of mosaic cases has also been reported.^{22,24} However, FISH can be used to distinguish between true and pseudo mosaicism in prenatal diagnosis.³³ FISH also may be used to screen for the most common aneuploidies when a marker chromosome of unknown origin is present. Recently, prenatal diagnosis revealed a foetus with a 47,XY,+mar/46,XY karyotype. The parents were anxious but did not agree to foetal blood sampling. Subsequently, locus-specific probes for chromosomes 13/21, 14/22, 16/18, and X/Y were used to determine the nature of the marker. None of these chromosomes appeared to be involved. The foetus was fortunately born normal, without the marker chromosome.

Erroneous FISH results because of contaminated AF with maternal cells have been reported. Contaminating maternal cells carry an increased risk for false-negative results and gender misclassification.^{21,34,35} The current methods for rapid and sensitive separation of contaminated maternal cells from foetal samples are time consuming and are still under research.^{36,37} As such, red-tinted AF samples were excluded from this study because of this reason. Maternal cell contamination in amniotic fluid is a problem frequently encountered during clinical practice and the contamination rate has been reportedly associated with increased gestational age.^{23,38,39} D'Alton et al²³ recently reported that FISH on AF samples at above 24 weeks gestation resulted in a 36.4% inaccuracy rate.

In conclusion, FISH, with its high correlation rate with conventional karyotyping, can be used as an adjuvant routine procedure for rapid prenatal detection of aneuploidy. Due to the low incidence of aneuploidy in the population, the sensitivity of FISH needs to be further investigated. FISH is not a replacement for conventional karyotyping. Full karyotyping analysis is still required, as it is presently the most reliable method of chromosome analysis. However, FISH assays are clearly desirable, especially in cases of late referrals, parental anxiety, abnormal maternal screening examinations and structural abnormalities detected on ultrasound.

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