FastFISH: technique for ultrarapid fluorescence in situ hybridization on uncultured amniocytes yielding results within 2 h of amniocentesis

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Rapid aneuploidy detection methods allow prenatal diagnosis results to be released within 48 h, but not on the same day as the invasive test. We aimed to develop a rapid fluorescence in situ hybridization (FISH) method (FastFISH) that releases accurate results on the same day as amniocentesis. FastFISH was optimized to be completed within 2 h of sample collection using CEP and LSI probes for chromosomes 13, 18, 21, X, Y and DiGeorge syndrome (DGS). The technique was tested on 100 consecutive amniotic fluid samples in a blinded study. It was also validated as a 1-day molecular genetic test on three representative fetal tissue samples: chorionic villus, amniotic fluid and fetal blood. In the blinded study, FastFISH results were ready within 2 h of sample collection. Of the 100 amniotic fluid samples, 49 male and 50 female fetuses were identified. One fetus was 47, XXY (Klinefelter syndrome). Three fetuses had trisomy 21. One fetus suspected of DGS by ultrasound was identified as normal. Results of FastFISH analyses in all 100 cases were concordant with their karyotypes (100% accuracy; lower 95% CI, 97.05%). In the 1-day test validation, all results were released on the same day and were concordant with their respective karyotypes. FastFISH allows results to be released on the same day as amniocentesis. It represents the necessary development for a 1-day prenatal diagnosis service.

Keywords: Aneuploidy/Down syndrome/FISH/prenatal diagnosis/rapid aneuploidy detection

Introduction

Amniocentesis is the most common invasive prenatal-diagnosis procedure offered to pregnant women at increased risk of chromosomal abnormalities. Cytogenetic results typically take 7–14 days, because amniocytes must be cultured prior to metaphase analysis. This lengthy wait causes considerable parental anxiety (Tercyak et al., 2001). A recent UK Government white paper on Genetics (Our Inheritance, Our Future: Realising the potential of genetics in the NHS, Department of Health, London: Stationery Office, 2003) has challenged institutions to make prenatal test results available within 3 days (compared with the usual 7–14 days), underpinning the importance of parental anxiety. Accordingly, more rapid molecular methods have been developed on uncultured amniotic fluid cells to detect common aneuploidies involving chromosomes 13, 18, 21, X and Y, namely fluorescence in situ hybridization (FISH) (Klinger et al., 1992) and genomic amplification of chromosome-specific short tandem repeats (Perlt et al., 1994). FISH relies on visual counting of fluorescent signals within target fetal cells rather than comparing fetal with informative parental genotypes. The results are informative in all cases where hybridization is successful, and the technique can also be used for chromosomal rearrangements such as DiGeorge syndrome (DGS) (Jouannic et al., 2003). Abnormal results may be important in clinical decision-making, whereas normal results allay the anxiety associated with the wait for the full karyotype (Evans et al., 1988; Caccia et al., 1991; Tercyak et al., 2001). Many centres worldwide offer this service routinely to patients undergoing mid trimester amniocentesis (Bryndorf et al., 1997; Eiben et al., 1998; Jalal et al., 1998; Waters and Waters, 1999; Pergament et al., 2000; Were- mowicz et al., 2001; Jobanputra et al., 2002; Lim et al., 2002; Luquet et al., 2002; Witters et al., 2002), but the typical reporting time is still 24–48 h. Ideally, specific diagnosis or exclusion of aneuploidy should be available on the same day as the diagnostic invasive procedure.

We report a low cost method of FastFISH using only 2 ml amniotic fluid which allows accurate results to be reported within 2 h of amniocentesis. This rapid test will allow many institutions worldwide to release prenatal diagnosis results on the same day.
Materials and Methods

Samples collection
Two millilitres of amniotic fluid surplus to conventional karyotyping requirements were collected from 100 women between 14 and 24 weeks of pregnancy. The indications for amniocentesis were maternal age ≥ 35 years, positive screening test for Down syndrome (nuchal translucency or maternal serum markers), or an abnormal fetal ultrasound scan. One chorion villus sample, one amniotic fluid and one fetal blood sample (11, 15, 23 gestational weeks, respectively) were also collected for the 1-day validation study. Sample collection for research was approved by the National Healthcare Group Domain Specific Review Board in Singapore, and all women gave written informed consent.

Optimization of FastFISH
The technique was first optimized in cells obtained from amniotic fluid (n = 18), chorion villous biopsy (n = 5), and fetal, cord and neonatal blood samples (n = 8), in known normal pregnancies, and in pregnancies complicated by trisomies 13, 18 and 21. FastFISH was optimized for Turner syndrome using adult peripheral blood samples. After density gradient centrifugation, nucleated blood cells were processed in the same way as uncultured amniotic fluid cells, obviating several days of culture, metaphase preparation, overnight ageing and prolonged hybridization.

For slide preparation and FastFISH on uncultured amniotic fluid cells, we modified currently used protocols. Briefly, after centrifuging 2 ml of amniotic fluid at 500 g for 5 min, the amniocyte pellet was resuspended and incubated in 3 ml prewarmed 0.075 M KCl for 30 min at 37 °C. Carnoy’s Fixative (3:1 of Methanol: Glacial Acetic Acid) 2 ml was added drop wise, the cell suspension centrifuged at 500 g for 5 min and the pellet resuspended in Carnoy’s. The cell suspension was dropped onto two cold slides and placed on a 60 °C hot plate. On one slide, 2 μl centromeric enumeration (CEP) X/Y probe (Vysis Inc., Downers Grove, Illinois, USA) mixed with 3 μl hybridization buffer (50% formamide and 10% dextran sulphate in 2 x SSC, pH 7.0) was added to the cell spot under a coverglass sealed with Parafilm™ (American National Can Company, Chicago, IL, USA). The other cell spot was hybridized with locus-specific (LSI™) 21 probe (Vysis). In the case also tested for 22q, the LSI™ DGS/Velo-cardiofacial syndromes Dual Colour Probe (Vysis) was used. Target DNA was denatured on an in situ hybridization block (MJ Research PTC-200, Waltham, USA) at 80 °C for 90 s followed by 15 min hybridization at 42 °C. Post-hybridization washes included once in 0.4 x SSC/0.3% NP-40 at 72 °C for 2 min and once in 2 x SSC/0.1% NP-40 at room temperature for 2 min. Slides with LSI™ probes gave better signals when washes were only 1 min each with less agitation. Air-dried slides were mounted in fluorescent antifade medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) and analysed by an automated epifluorescence microscope fitted with a Xenon lamp (Olympus BX61, Centre Valley, USA). Fifty nuclei were scored directly from the microscope without image enhancement for each probe, and the sample considered informative if ≥80% of the nuclei displayed, respectively, the same normal/abnormal hybridization pattern for any specific probe. Image capture was performed using FISHview, 2.0 EXPO (ASI, Carlsbad, USA). SPSS 14.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

Blinded study
FastFISH was performed for chromosomes X, Y and 21, to verify that the technique was suitable for both CEP™ and LSI™ commercial probes. In this blinded study, FastFISH was also performed for chromosomes 13 and 18 where multiple fetal malformations were present on ultrasound scan, and for the 22q deletion where conotruncal abnormalities were noted. These were in addition to FastFISH for chromosomes 21, X and Y. Of the 100 consecutive participants, a conotruncal abnormality was found in one fetus during ultrasound. FastFISH was performed for 22q11.2 deletion to exclude DGS; this was in addition to FastFISH for chromosomes 21, X, Y, 13 and 18. In this blinded study, conventional karyotype results were disclosed to the investigators performing FastFISH only after all 100 amniotic fluid cases had been analysed and recorded, and the FastFISH results were not disclosed to the cytogeneticists performing the karyotype.

Validation of 1-day testing
FastFISH for chromosomes 21, X and Y was performed on one chorionic villus, one amniotic fluid and one fetal blood sampling sample (11, 15, 23 gestational weeks, respectively) within the same day of sample collection to validate that the results could be released on the same day.

Results

Optimization of FastFISH
Representative results from the optimization phase on normal and trisomic samples from blood, villi and amniotic fluid are shown in Fig. 1.

Blinded study
In the blinded clinical study, all amniotic fluid samples were free of macroscopic blood contamination. Median maternal age was 36 years (range 24–45), the median gestational age was 16 weeks (14–24) and the maximum time taken to analyse each of the 100 samples was 2 h. There were 49 male (XY) and 50 female (XX) fetuses and one 47, XXY (Klinefelter syndrome) fetus; all were correctly identified by the FastFISH technique. For the fetus with Klinefelter syndrome, 100% of nuclei demonstrated the sex chromosome trisomy (Fig. 1). In three cases, ≥80% of nuclei had three chromosome 21 signals (80.0%, 84.4% and 83.3%); all three fetuses were confirmed by conventional karyotyping to have trisomy 21. More than 50 nuclei were available for evaluation in the first case and 80.0% of the 50 evaluated nuclei showed trisomy 21. In the second and third cases, 38 of 45 (84.4%) and 30 of 38 (83.3%) nuclei that could be evaluated showed trisomy 21; in all other 97 cases, more than 50 nuclei were available for evaluation and 50 were examined. All 97 other fetuses were disomic for chromosome 21. One case with conotruncal abnormalities on ultrasound at time of amniocentesis was evaluated for DGS; FastFISH revealed no deletion of 22q11.2 and FastFISH for chromosomes 21, X, Y, 13 and 18 also revealed no aneuploidies. These results were later confirmed by conventional FISH and karyotyping, respectively.

In all cases gender, trisomy and chromosomal rearrangement (deletion) status were identified correctly (100% accuracy; lower 95% CI, 97.05%). In this blinded sample, the sensitivity and specificity were both 100%, with no false-positive or false-negative results. Hybridization was satisfactory in all cases, and there were no uninformative cases. The time taken from sample collection to release of FastFISH results was recorded (Table 1); all cases were processed by the same technician. One technician can readily complete up to four samples by the end of the same day.

Validation of 1-day testing
As a proof-of-principle, one sample each of chorionic villus, amniotic fluid and fetal blood were collected and FastFISH was performed. Results were released on the same day as sample collection. The chorionic villus and amniotic fluid samples were diagnosed as disomic for chromosome 21 whereas the fetal blood sample revealed trisomy 21; all three fetuses were male (Fig. 2).

All the blinded study and 1-day testing results matched the conventional testing methods.

Discussion
Interphase FISH analysis on uncultured amniocytes is increasingly popular as a routine adjunctive test at midtrimester amniocentesis (Bryndorf et al., 1997; Eiben et al., 1998; Jalal et al., 1998; Waters and Waters, 1999; Pergament et al., 2000; Weremowicz et al., 2000).
Improved FISH technique for same-day prenatal diagnosis

Figure 1: Results from the optimization phase on normal and trisomic samples (A–E): Uncultured amniocytes [(A)-XY; (B)-XX]; (C)-normal diploid 21; (D)trisomy 18; (E)trisomy 13; (F)embryoblast cells (normal diploid 13 and 21). (G, H) Mononuclear cells from fetal blood [(G)-XY; (H)-normal diploid 13 and 21]. (I) Cultured neonate peripheral blood cells in the metaphase and interphase of a DiGeorge patient showing deletion of TUPLE 1 locus on chromosome 22q11.2. (J) Mononuclear cell from fetal cord blood (trisomy 18). (K) Mononuclear cell from neonatal blood (trisomy 21). (L) Mononuclear cell of a Turner syndrome patient (45. XO). (M) Uncultured amniocyte (trisomy 21). (N) Uncultured amniocyte; Klinefelter syndrome (47, XXY). Interphase FISH of CEP and LSI probes on different cell types. Colours have been enhanced for ready recognition: CEP X (red); CEP Y (green); LSI 21 (yellow); CEP 18 (aqua); LSI 13 (purple); LSI TUPLE1 (HIRA) (5' non-coding region of TUPLE1, D22S553, D22S609 and D22S942) (red); LSI ARSA (Arylsulfatase A gene) (green).

Table 1: Total time taken from collection to release of results in 57 samples

<table>
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<tr>
<th>No. of samples/day (two slides/sample)</th>
<th>Total time from collection to release of results (hours to complete all samples; two slides per sample; 21/XY)</th>
<th>No. of days (times) tested</th>
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One technician can readily complete up to four samples by the end of the same day.

using uncultured amniotic fluid cells can be performed within 2 h of amniocentesis, so that the patient can have same day reassurance. The time taken for FastFISH from pretreatment to complete analysis of slides was 2 h per sample. As shown in Table 1, up to two samples can be reliably completed by one technician within the same day. If samples are collected in the morning, the results will be available by the evening using the FastFISH method. As proof-of-principle, FastFISH was performed in three representative cases: chorionic villus sample, amniotic fluid sample and a fetal blood sample. In all cases, the total time taken from sample collection to release of results was less than 6 h inclusive of logistics. In all three cases, results were released on the same day as the sample collection procedure.

FastFISH involves a number of changes to the conventional protocol (Klinger et al., 1992; Ward et al., 1993; Weremowicz et al., 2001; Luquet et al., 2002). First, we found no advantage in washing, sonication or coating glass slides; using clean slides was sufficient for good hybridization and signal quality. Next, eliminating the protein digestion steps involving pepsin or trypsin not only saved time, but also reduced excessive spreading of the nuclei. Together with the cold slide on hot plate modification, this ensured close proximity of the...
nuclei (clustering) without causing clumping or overlapping signals. Even without ethanol dehydration most FastFISH signals could be readily visualized on the same focal plane. Next, we found that only 2 ml of amniotic fluid was needed, in contrast to the 5 ml required by most laboratories. Further, we could not confirm any value in ageing the slides, so this step was deleted. Finally, 15 min was sufficient time for hybridization of both CEP and LSI probes on different cell types. Colours have been enhanced for ready recognition: CEP X (red); CEP Y (green); LSI 21 (yellow).

Figure 2: Results of the FastFISH technique on chronic villus, amniotic fluid and fetal blood sample (A) Trophoblasts from chorionic villus sample; (B) uncultured amniocytes; (C) mononuclear cells from fetal blood sample. (a) CEPXY probe signals; (b) LSI 21 probe signals. Interphase FISH of CEP and LSI probes on different cell types. Colours have been enhanced for ready recognition: CEP X (red); CEP Y (green); LSI 21 (yellow).

of trisomy 21 and one case of Klinefelter syndrome); there were no false-positive or false-negative results. The results of this experimental study are comparable with recent larger clinical evaluations of 48 h standard FISH on uncultured amniocytes (Ward et al., 1993; Feldman et al., 2000; Witters et al., 2002; Wyant et al., 2006).

Maternal cell contamination is a procedure-related problem: by ensuring that in each case the amniocentesis was performed by a fetal medicine specialist, none of the 100 consecutive amniotic fluid samples was blood stained. In contrast to earlier reports of standard FISH on uncultured amniocytes suggesting that maternal cell contamination was the most important limitation of diagnostic reliability (Christensen et al., 1992; Bryndorf et al., 1997; Winsor et al., 1999), we found that proper sample collection could significantly reduce the impact of this problem. No maternal (XX) cells were among all the male (XY or XXY) nuclei examined.

Reasons cited for choosing rapid molecular testing such as standard (or Fast-) FISH analysis of uncultured amniotic fluid cells include rapid results, emotional preparation where a karyotypic abnormality is strongly suspected and relief of anxiety associated with prenatal screening (Evans et al., 1988; Caccia et al., 1991; Pergament et al., 2000; Tercyak et al., 2001). All patients choosing standard FISH have reported relief of anxiety upon receipt of normal results, but the high cost has limited uptake (Pergament et al., 2000). A strategy of routinely testing for trisomy 21, but testing for trisomy 13 and 18 only if indicated by abnormal ultrasound findings, has been shown to be cost-effective (Witters et al., 2002), and our own analysis of almost 200 cases of autosomal trisomy supports such a strategy (Su et al., 2004). Together with our modified FISH protocol described above, appropriate targeted testing for trisomies 13, 18 and 21 will further lower the cost of the procedure to the patient.

Standard FISH on uncultured amniotic fluid cells is now used routinely in many centers worldwide (Bryndorf et al., 1997; Eiben et al., 1998; Jalal et al., 1998; Waters and Waters, 1999; Pergament et al., 2000; Weremowicz et al., 2001; Jobanputra et al., 2002; Lim et al., 2002; Luquet et al., 2002; Witters et al., 2002; Liehr and Ziegler, 2005). The value of rapid results is clear from the increasing popularity of this test, but patients still have to wait for 24–48 h for the results, and the test is only available at high cost. We have modified the conventional test to make it cheaper and faster. Nonetheless, it continues to share with standard FISH and karyotyping the limitation of being labour-intensive. This may change once automated scanning microscopes become more readily available.

Despite the obvious attraction of rapid molecular testing, couples need to be counselled appropriately. Genetic counselling before prenatal diagnosis is useful to explain to the patients the capabilities and also the limitations of interphase FISH on uncultured amniotic fluid cells that this technology cannot detect aneuploidy for non-tested chromosomes. Abnormal FISH results have been used in clinical decision-making (Cheong et al., 2001; Caine et al., 2005; Locatelli et al., 2005), but false-positive results have been reported (Winsor et al., 1999; Weremowicz et al., 2001; George et al., 2003). Our own practice is to follow the American College of Medical Genetics/American Society of Human Genetics guidelines, and base our clinical decisions on the presence of at least two of the three following criteria: positive FISH results, confirmatory karyotype or consistent clinical information.

The robustness of our FastFISH protocol was verified not only on amniocytes obtained in the early to midsecond trimester, but also on uncultured amniotic fluid cells obtained at later gestations, on fetal nucleated red blood cells on fetal trophoblast cells, and on mononuclear cells obtained from neonates and adults. We have not tested our protocol on blastomeres, but preimplantation genetic diagnosis results could be released earlier if the typical hybridization duration
of 4 h (Staessen et al., 2003) were shortened to 15 min as described in our protocol. Our data confirm that this novel technique could be used in many clinical circumstances where rapid results on qualitative changes in chromosome integrity and quantitative changes in chromosome number would be useful. It represents the necessary development for a potential 1-day prenatal diagnostic service.

Author Contributions

Dr Choolani, Miss Sherry Ho and Dr Sonia Baig had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Choolani, Ho, Razvi, Fisk, Biswas.

Selection and enrollment of patients: Biswas, Wong, Rauff, Ponnusamy, Razvi, Gole.

Acquisition of data: Ho, Baig, Mohammed, Zhang, Tan, Gole, Ponnusamy, Choolani.

Analysis and interpretation of data: Ho, Baig, Mohammed, Zhang, Tan, Ponnusamy, Gole, Biswas, Fisk, Choolani.

Drafting of the manuscript: Choolani, Ho, Baig, Biswas, Razvi, Ponnusamy, Gole, Fisk.

Critical revision of the manuscript for important intellectual content: Choolani, Ho, Fisk.

Statistical analysis: Chan YH.

Funding Obtained by Choolani, Biswas, Wong, Fisk.

Financial Disclosures

Choolani, Ho, Razvi and Biswas have filed for a patent covering the use of FastFISH protocol for in situ hybridization method. This patent is owned by the National University of Singapore.

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