

Fetal aneuploidy diagnosis through rapid fluorescence in situ hybridization (FISH) on uncultured amniocytes

Diagnosticul aneuploidiilor fetale prin hibridizarea fluorescență *in situ* (FISH) rapidă a amniocitelor necultivate

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Abstract

Rapid prenatal diagnostic tests are now increasingly popular, either as a stand-alone test or associated with conventional karyotype. Their main advantages are maternal anxiety relief and low cost. However, the results are available in more **than** 48 hours from amniocentesis. We present a rapid fluorescence in situ hybridization (FISH) technique, modified to give prenatal diagnostic results in the same day as amniocentesis, using probes for chromosomes 13, 18, 21, X and Y. We tested this technique on 30 amniotic fluid samples in a blinded study. The results were released in maximum 4 hours from the sample collection, including time between laboratories. The method was also validated on cases with trisomy 21 and 18, triploidy and one XYY syndrome. Results of this fast FISH technique were 100% concordant with karyotype. By this method, the results can be released on the same day from amniocentesis, and thus this is a useful method for a one-day stop prenatal diagnosis service. As a stand-alone approach, fast FISH method could be used in a subgroup of patients, where invasive prenatal diagnosis is performed only for an increased risk for aneuploidy, from a positive screening test, but needs karyotype association especially in cases with fetal ultrasound anomalies.

Keywords: fluorescence in situ hybridization (FISH), aneuploidy, amniocyte, amniocentesis, karyotype

Rezumat

Testele rapide de diagnostic prenatal sunt tot mai populare, atât ca teste de sine stătătoare, cât și asociate cu cariotiparea convențională. Principalele lor avantaje sunt îndepărtarea rapidă a anxietății pacienților cât și costul redus. Totuși, rezultatele sunt disponibile în peste 48 de ore de la amniocenteză. Prezentăm o metodă rapidă de hibridizare fluorescență *in situ* (FISH), modificată pentru a avea rezultatele diagnosticului prenatal în aceeași zi cu amniocenteza, folosind probe pentru cromozomii 13, 18, 21, X și Y. Am testat această tehnică pe

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30 de probe de lichid amniotic într-un studiu orb. Rezultatele au fost eliberate în maximum 4 ore de la colectarea probei, inclusiv timpul dintre laboratoare. Metoda a fost de asemenea validată pe cazuri cu trisomie 21, 18, triploidie și sindrom XYY. Rezultatele acestei tehnici FISH rapide au fost 100% concordante cu cariotipul. Prin această metodă, rezultatele pot fi eliberate în aceeași zi cu amniocenteza și astfel aceasta este o metodă utilă pentru un serviciu de diagnostic prenatal de o zi. Ca și test de sine-stătător, metoda FISH rapidă poate fi utilizată într-un subgrup de paciente, la care testele invazive de diagnostic prenatal sunt recomandate numai pentru risc crescut de aneuploidie, în urma unui test de screening pozitiv, dar necesită asociere cu cariotiparea în caz de anomalii fetale ecografice.

Cuvinte cheie: diagnostic prenatal rapid, hibridizare fluorescentă în situ (FISH), aneuploidie, amniocenteza, cariotipare

Introduction

Cytogenetic prenatal diagnosis has been available for several years in Romania too. It is based on an invasive test to obtain amniotic fluid or chorionic villi and is usually recommended for pregnant women who, after screening, are at an increased risk of fetal chromosomal abnormality, particularly Down syndrome.

Historically, the selection was initially done only maternal age-related risk for trisomy (1). Currently, screening programs use different techniques such as biochemical markers in maternal serum (2), nuchal translucency in the first trimester of pregnancy (3) and biochemical tests combined with ultrasound markers in the first and second trimester (4, 5).

Karyotyping is still the gold standard for diagnosis of chromosomal abnormalities, effectively verified and recognized in the past 20 years. As the amniotic fluid or chorionic villi cells require cultivation before analysis, this method is expensive, laborious and requires 7 to 14 days for the results (6). The last one represents the main drawback of the method, because maternal anxiety remains high until the normal outcome (7, 8).

Thus, faster and cheaper molecular methods have been developed in order to identify the most common chromosomal aneuploidy (trisomy 21, 18 and 13 and numerical sex chromosome abnormalities). They are mainly represented by fluorescent in situ hybridization (FISH) and quantitative fluorescence PCR (QF-PCR) and this is done on Cori villous cells or uncultivated amniocytes. Both methods are in-

expensive and are much faster, providing results within 24-48 hours.

However, although many centers worldwide offer patients routine FISH test for patients performing mid-trimester amniocentesis, the typical release time of the results is still over 48 hours (9, 10). Ideally, a specific diagnosis or exclusion of aneuploidy should be available on the day of invasive procedure.

This prospective study presents a rapid and efficient method for FISH aneuploidy diagnosis using uncultured amniotic cells. This can be accomplished in about 4 hours from the amniocentesis, so the patient can know the result in the same day.

Materials and methods

A surplus of 3 ml amniotic fluid has been used, collected from conventional karyotyping or FISH test from 30 patients with gestational age between 16-25 weeks. Amniocentesis was performed for the following indications: maternal age ≥ 35 years, positive screening tests for trisomy 21 (nuchal translucency, nasal bone, tricuspid regurgitation, venous duct flow, serum biochemistry, soft ultrasound markers in the second trimester) or ultrasound revealed structural abnormalities. Amniocentesis was done under local protocols (according to the Helsinki Declaration) and all patients gave informed consent.

The technique was optimized on cells obtained from amniotic fluid in normal and complicated pregnancies with trisomy 18 and 21. Slides preparation and fast FISH was rapidly done on uncultivated amniocyte by chan-

ging the current protocol. So, after centrifugation at 500g for 5 minutes, the amniocyte pellet was re-suspended and incubated in 3 ml pre-warmed KCL 0.075 M at 37 ° C for 15-20 minutes. 2 ml of Carnoy fixative (methanol: glacial acetic acid [3:1]) held in freezer were subsequently added, drop by drop. This was followed by centrifugation at 500g, then again for 5 minutes and after the removal of the supernatant, the sediment was re-suspended in 200 ml from the remaining supernatant.

The cell suspension thus obtained was applied onto two areas, on two cold microscope slides, which were subsequently heated to 60° C on a hotplate. Dried slides were initially treated in 2 x SSC / 0.5% igepal solution, pH 7.0 at 37 C for 2 minutes, as recommended by the manufacturer. Final drying was done at room temperature.

FISH analysis was performed using Kreatech Diagnostics cell samples (Amsterdam, Netherlands). On a slide there were applied centromeric samples, specific to chromosomes X, Y and 18. They were designed to record and count satellite chromosomes from uncultured amniocytes and detect highly repetitive DNA sequences, located in the pericentric heterochromatin of these chromosomes. On the second slide probes specific to chromosomes 13 and 21 were applied. They have also been optimized for uncultured amniocyte and detect specific regions of the chromosomes (13q14.2, 21q22.1 respectively).

DNA probes are supplied as already prepared, in a hybridization solution containing formamide, dextran sulfate sodium in citrate saline. For each cell of the slide there were applied 10 µl of probe mix, for an area of 22 x 22 mm (or 5 µl for a smaller area obtained by cytocentrifugation).

Each area was covered with a glass cover-slip and was sealed with Fixogum. The DNA target was denatured on an in situ hybridization block (Eppendorf) placed in a PCR system (Mastercycler Pro, Eppendorf AG, Hamburg, Germany) at 85 ° C for 10 minutes. Hybridization was performed immediately following denaturation at 42 ° C for 30 minutes.

Post-hybridization washes were done according to manufacturer's protocol. Hence excess probes were removed in a washing buffer (0.4 x SSC / 0.3% igepal) for 2 minutes at a temperature of 72 ° C (± 1 ° C) without agitation. Subsequently, the slides were washed in the washing buffer number 2 (2 x SSC / 0.1% igepal) for 1 minute at room temperature without agitation. Slides with samples are washable for two minutes, others gave better signals if washed 1 minute without agitation. Slides were air dried at room temperature, in the dark. Counter-stain with 15µl solution DAPI / antifade and cover with glass cover-slip. Slides may be kept in the dark at 4°C for approximately one week before examination. Fluorescent signals were analyzed using a 1.0 Axioscop microscope (Zeiss, Jena, Germany) with x100 objective with appropriate filters. The images were photographed using a digital camera and Tissue Fax software.

Fifty nuclei for each FISH sample were scored directly and the slide was considered informative if more than 80% of the nuclei showed the same type of hybridization (normal or abnormal) for each specific probe.

Fast FISH technique was performed for chromosomes X, Y, 21, 18 and 13, to check if it is effective for all FISH samples (centromeric or specific locus). In this blinded study, conventional karyotype or FISH results were disclosed to the investigators performing fast FISH technique only after registration of all cases included in the study. Similarly, rapid FISH test results were not known by the laboratory that performed karyotyping.

Results

Representative results from the optimization phase with normal or abnormal aspects are given in *Figure 1*. Quality of the FISH signal was not affected by the new protocol.

All amniotic fluid samples included in this study were free of macroscopic contamination. Average maternal age was 35 years, meaning

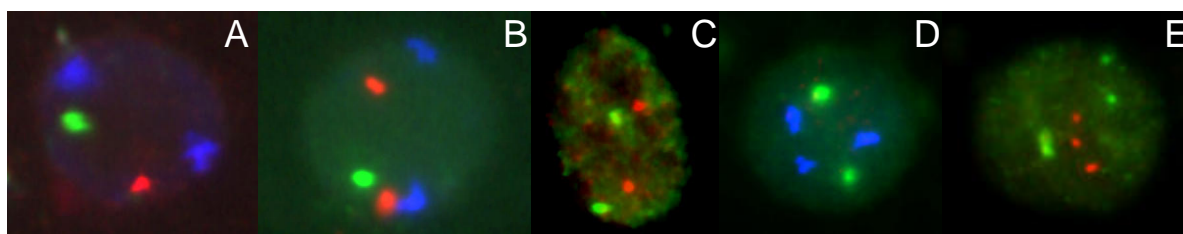


Figure 1. Rapid FISH on uncultured amniocytes: representative results from normal or aneuploidy cases, original pictures: (A) XY, diploid chromosome 18; (B) XYY, diploid chromosome 18; (C) diploid chromosome 21 and 13; (D) trisomy 18, XX; (E) triploidy. The colors were digitally enhanced for easier recognition. CEP X (red), CEP Y (green), LSI 21 (red), CEP 18 (blue), LSI 13 (green).

gestational age 22 gestational weeks and maximum time needed to analyze each case was 4 hours. 30 samples of amniotic fluid, 15 female cases, 14 male and one case with XYY syndrome were analyzed. All were correctly identified by rapid FISH technique. For the fetus contaminated with XYY syndrome, 95% of nuclei with fluorescent signal demonstrated sex chromosome trisomy.

Two cases had trisomy 21, two trisomy 18 and one triploidy, subsequently confirmed by conventional karyotyping. In all cases there were over 50 nuclei with fluorescent signals available for evaluation, of which $\geq 80\%$ had three specific signals for the particular chromosome (82% and 89% for chromosome 21, respectively 87% and 92% for chromosome 18). All the other 25 cases were also available for evaluating more than 50 nuclei, showing two signals for each chromosome in over 80%. All other 25 fetuses were normal in terms of number of chromosomes 21, 18 and 13.

In all the cases, throughout rapid FISH, fetal sex and trisomy could be correctly identified (100% accuracy, sensitivity and specificity of 100%, without false positive or false negative cases). Hybridization quality was satisfactory in all cases without uninformative cases. The time of taking the sample to release results ranged between 2 and 4 hours (2 hours - 4 cases, 2.5 hours - 9 cases, 3 hours - 11 cases, 3.5 hours - four cases and 4 hours - 2 cases). All slides were processed by the same biologist. Thus, a single technician can easily handle up to four consecutive cases per day.

Discussions

Rapid aneuploidy diagnosis tests are becoming increasingly popular. They are offered as a single test or associated to karyotyping, for patients who performed amniocentesis for increased risk in the second quarter. Their major advantage is to reduce the anxiety caused by the long wait until the outcome. However, even in this situation, the average communication (for FISH) is over two days (9, 10). In this study we describe a rapid and feasible FISH method on uncultivated amniocytes, which can be made 3 hours after amniocentesis, providing the results almost in the same day.

Median duration of the fast FISH test, from the pre-treatment time to the complete analysis of slides was 3 hours per patient. This allows up to two sets of samples to be completed by one technician, within the same day. Thus, if samples are collected in the morning, through the rapid FISH method, the result can be issued at the end of working hours.

Fast FISH procedure involves a series of changes in the conventional protocol (9, 11). Thus, this study found that effective results can be achieved by only using 3 ml of amniotic fluid, unlike the 10 ml required by the majority of laboratories. Using of special coated slides does not confer a significant advantage, so that the mere use of blades is sufficient for a good hybridization and quality of the signal.

The disposal of protein digestion steps involving pepsin or trypsin not only shortens

the procedure, but also reduces excessive dispersion of nuclei. This, together with the option of applying nuclear solution on cold slides and drying later on a hot surface, provides a group of nuclei without noticing, instead, stacked or overlapping signals.

Also, the frequency and brightness of hybridization signals will vary depending on pre-treatment conditions. On fresh blades, no treatment can drastically reduce the frequency and intensity of hybridization signals, so that this time has been preserved.

Dehydration in ethanol is optional, because even without it, most of FISH signals can be observed in the same plane of focus. However, if the use of automated image analysis is taken into consideration, this time can be used. We noticed that aging slides (cell) have not a special significance; this step has also been omitted.

In terms of denaturation, although the published techniques (12) use a smaller temperature and a shorter time (70° C, 2-4 minutes), incomplete target distortion can lead to lack of signal. Also, in the case of cycle co-denaturation- it is essential that temperature should drop very quickly, with 3-4° C/s to conserve target DNA as single strands. The use of new FISH evidence allows a drastic reduction in hybridization time. The disposal of repetitive sequences eliminates the need for Cot-1 or blocking DNA and provides a good signal to noise ratio. Hybridization flexibility and the already prepared format also allow reduction in working time.

Thus, by incorporating these changes, fast FISH can be done quickly on amniotic cells cultivated in about 3 hours by a single technician.

Some authors consider that scoring 30 nuclei per sample, per slide, is sufficient for a correct diagnosis (9). However, we have examined our slides in accordance with international guidelines, which require evaluation of 50 nuclei of each sample per slide (13). To reduce the likelihood of false positive (14) or false negative (9) the cut-off for euploidy / aneuploidy diagnosis was set at $\geq 80\%$. All cases

were informative, in no case were $>20\%$ non-modal signals per probe per slide.

Regarding this small study, the sensitivity and specificity for detecting aneuploidy was 100% (two cases with trisomy 21, two cases with trisomy 18 and one case of triploid XYY syndrome), without false positive or false negative cases. The experimental results are comparable with other large clinical studies using the standard FISH, of 48 hours, on uncultivated amniocytes (15, 16).

Sample contamination with maternal cells is strongly associated with the accuracy of the amniotic fluid collection procedure. Thus, to reduce contamination as much as possible, amniocentesis was performed by an experienced specialist in fetal medicine. None of the 30 consecutive amniotic fluid samples were macroscopically contaminated with blood. No maternal cells (XX) were observed among male nuclei (XX or XYY) examined. Thus, we found that an adequate sampling of amniotic fluid reduces the impact of this problem, unlike other similar studies, standard FISH, on uncultivated amniocytes, suggesting that maternal cell contamination is an important limitation of diagnostic reliability (17, 18).

The emergence of new techniques for prenatal diagnosis of molecular diagnosis (FISH or QF-PCR) changed the role of traditional karyotyping (19). Rapid tests are cheaper than karyotyping and allow the detection of common aneuploidy using uncultivated amniocytes, providing results within 24-48 hours. The main argument against these new technologies is that they only detect aneuploidies (abnormal number of chromosomes 21, 18, 13, X and Y) which have been specifically developed, while structural chromosomal abnormalities such as translocations, inversions and others will not be detected. Thus, a meta-analysis of 12 studies of prenatal diagnosis by amniocentesis or, estimated the risk of chromosomal abnormalities missed by these rapid diagnostic techniques at 0.9% and this figure dropped to 0.4% if only those with clinically significant anomalies were taken into consideration (19).

To reduce the false negative results, a major role was assigned to ultrasound detection of fetal structural abnormalities, 69 % clinically relevant chromosomal abnormalities, not detectable by FISH or QF-PCR had fetal abnormalities detected by ultrasound (20). Thus, the current papers (6, 21- 24) recommend using a rapid prenatal diagnosis test (FISH, QF-PCR) in cases where the indication for amniocentesis is advanced maternal age, soft ultrasound markers second trimester or positive maternal serum screening. Standard karyotyping should be used selectively in cases in which the nuchal translucency is ≥ 3.5 mm, one or more structural fetal abnormalities were seen on ultrasound, or where one parent was known to carry a balanced translocation.

The results of this approach to prenatal diagnosis of fetal aneuploidy are currently supplied only by two studies (21, 22), which found a reduction of risk for chromosomal abnormalities undetected by FISH or QF-PCR to 0.5-0.9% and clinically significant abnormalities to 0.11-0.16%.

These chromosomal abnormalities that are not detectable by a methodology based solely on FISH or QF-PCR, and especially no anomalies detected by ultrasound, have special clinical significance, very different from that of trisomy 21, 18 and 13. The risk for an unfavorable clinical outcome, such as impaired intellectual development, learning disabilities and mental abnormalities varies between 5-15% (25). Moreover, prenatal identification of this group of chromosomal abnormalities, which are largely de-novo balanced translocations and marker chromosomes, provides advice and often difficult issues and therefore their identification may not be in the interests of parents or the fetus because of the possibility of unnecessary untimely termination of pregnancy (26).

The presented results are based on ideal situations, where it is assumed that all clinically important aneuploidy (trisomy 21, 18, 13) and sexual chromosome aneuploidies detected by full karyotype would have been detectable using rapid diagnostic tests. However, this assumption is consistent with published data, documenting the spe-

cificity and sensitivity of these rapid methods. The second limitation is given by the detection rate for fetal abnormalities with ultrasound, which is currently unknown and varies upon the operator, equipment and viewing conditions.

Thus, despite the attractiveness of rapid prenatal diagnosis tests, couples should be appropriately counseled before the procedure with information on the possibilities and limits of the FISH test on uncultured amniotic fluid cells. Moreover, because of the possibility of the occurrence of a false-positive test (very low) (9, 14, 17), it is recommended that clinical decisions should be taken in the presence of at least two of the following criteria: positive FISH results, confirmatory karyotype or consistent clinical information (American College of Medical Genetics / American Society of Human Genetics Guidelines).

Conclusions

This study describes a cheap, fast and robust FISH method, verified on amniotic fluid cells obtained at the beginning of second trimester but also at later gestations. It can be used successfully within a subgroup of patients undergoing invasive prenatal diagnosis exclusively because of an increased risk for aneuploidy, but requires a combination with karyotyping, particularly in situations with ultrasound revealed structural abnormalities. However, although this approach is cost-effective solution, it cannot replace the conventional karyotyping, and is strongly dependent on the efficiency of ultrasound screening.

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