Rapid Prenatal Diagnosis of Chromosome Abnormalities

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ABSTRACT:

The aim of the present work was to examine the efficacy of using FISH for the rapid prenatal diagnosis of common chromosome aneuploidies. A total of 100 analyses over a six month period were included in the study. Diagnosis was possible in all cases. A mosaic for trisomy 21 proved, by comparison with an extensive analysis of long term cultures, to be an apparent false positive. Otherwise the technique was reliable, accurate and relatively straightforward to perform. Results could be available within 24 hrs. In most cases an additional long term full analysis was also done, so as to exclude rarer aneuploidies and structural rearrangements. This methodology is seen as a useful addition to the prenatal diagnostic repertoire.

Keywords: Chromosome, Aneuploid, FISH, Prenatal, Probe

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INTRODUCTION

The prenatal diagnosis of foetal chromosome abnormality is an essential facet of the clinical management of pregnancy. The usual source of foetal cells is either amniocytes or chorionic villi. However, the period necessary for culturing of either tissue is usually around 10 -14 days. For a variety of reasons this can sometimes prove to be an unacceptably long time for both patient and clinician.

Mary Lou Pardue first demonstrated in the 1970's that radioactively labeled DNA fragments (probes) could be used to identify corresponding regions on chromosome preparations by means of *in-situ* hybridization (ISH) Since then the technique has been refined by using non-isotopic fluorescent labels (FISH) and longer probe sequences. This in turn has resulted in the introduction of the use of uncultured interphase cells (Reid et al, 1992). Recently, commercially available probe sets (Vysis) have become available for the most commonly encountered chromosome abnormalities, enabling results to be obtained within 24 hours. Our experience in the clinical application of these probes for the so-called prenatal aneuploidy screening is the subject of this report.

MATERIALS AND METHODS

Pre-hybridization

Generally 2-5ml of amniotic fluid (AF) provides sufficient uncultured cells for analysis, whilst a total of 15-20ml also allows for the normal additional long term cultures. It is essential that amniotic fluid is free from maternal cell contamination such as fresh blood. (Although we have successfully processed three specimens which were heavily brown stained possibly the result of previous foetal/placental bleeding episodes). Pre-hybridization preparative methods are as the protocols described by Vysis. For chorionic villi samples (CVS) we have used the quantity that normally would be necessary for a direct preparation, but again whilst ensuring enough material remains for long term culture. In this case the specimen must be free of maternal decidua as well as blood. We have found that subsequent cell separation can best be achieved by means of collagenase (100µL for 1.5hrs at 37ºC, after fine dissection of the villi). Slide preparation is as standard for a direct CVS. In our experience the described pre treatments (used for AF) are not necessary for CVS. Both AF and CVS cultured cells may also be used and again standard methodology is from this stage to slide preparation. Once the slides have been made, denaturation of the nuclear DNA is achieved by means of 70% formamide in 2xSSC), followed by immediate ethanol washes (70% to absolute).

Probes

The probes used are those able to detect the most commonly encountered chromosome abnormalities. These are the aneuploid states for chromosomes 21; 18; 13 and the sex chromosomes X and Y. They are used in combination such that a mixture of probes for the 18 and X and Y make up one set, whilst those for 21 and 13 make up the other. This latter combination consists of a series of unique sequences that hybridize to the long arms of these chromosomes (13q14 and 21q22.13-q22.2). The chromosome 13 probe is approximately 410 - 470 kilobase(kb) in length and contains the entire Retinoblastoma 1 gene. The 21 probe is smaller but is nonetheless about 200kb in length. The 18/X/Y

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combination of probes are each alpha satellite, centromeric in origin (18p11.1-q11.1;Xp11.1 q11.1;Yp11.1-q11.1). Although these alphoid sequences and the 13/21 unique sequence probes are chromosome specific, unlabelled blocking DNA is added to the probe mixture to suppress DNA sequences common to both the target and other chromosomes.

Hybridization

The probe combinations are provided in a single stranded state, suspended in hybridization solution. We routinely hybridize two separate areas of a slide, one for each probe combination. Immediately the probes are added, each is covered with a 22 x 22mm coverslip and sealed using rubber cement. Hybridization takes place at 37ºC usually overnight but for at least 6 hours. After the post hybridization washes the slides are air dried in the dark. A counter stain (below) is then added and a coverslip applied. The slides are kept in the dark until analysis.

Fluorophore label

Computer imaging : the probes are pre-labeled with fluorochromes which are proprietary compositions with peak excitation characteristics of 433; 509 and 559nm for the blue (aqua) green and orange colors respectively.

Diamidinophenylindole (DAPI) is used as the counter stain throughout. Although triple or dual bandpass excitation filter sets can be used, we have routinely used a single filter appropriate for the maximum emission peak of the particular fluorochrome. Thus Olympus filter cubes U-MWU;U-MWIB;U-MWG and Vysis 19460 808 are used for DAPI; Green ; orange and blue fluorochromes respectively. (Probes for chromosomes 13 and the X fluoresce green; 21 and the Y, orange/red whilst the 18 fluoresces blue). Each filter is housed in a rotating turret in an Olympus BX50 fluorescent microscope using UPLAPO x10 and x100 universal objectives. The acquisition, processing, and analysis software for FISH is the MacProbe (version 4.0) multi-color imaging with a PowerMac 8600 computer.

Analysis

Initially slides are assessed for the signal intensity and their general shape. Preferably, they should be bright, distinct and oval, although less compact shapes are also acceptable when necessary. More than 98% of cells should demonstrate suitable signals with the background free of fluorescence. Signals are recorded in 50 nuclei per probe per sample. Those samples demonstrating below 10% aneuploidy are either disregarded or at best treated with extreme caution. We have found that in cases of complete aneuploidy usually more than 90% of nuclei will be positive.

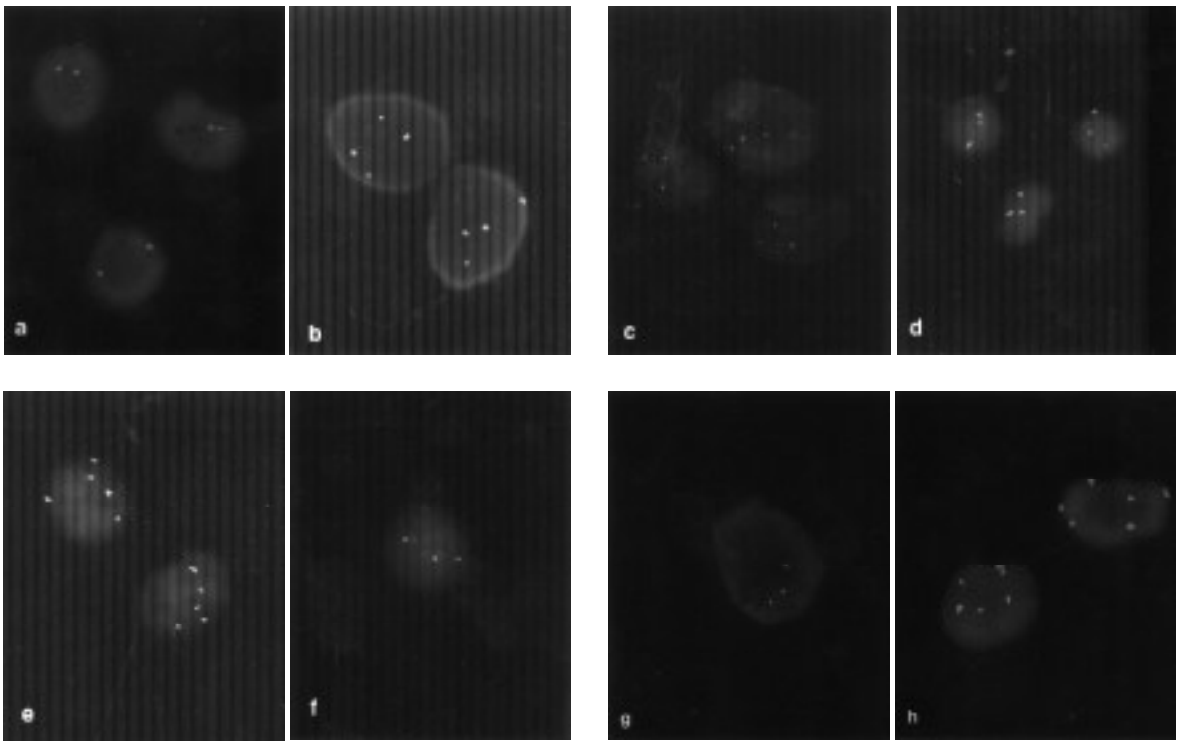
RESULTS

Prior to offering this technique as a diagnostic service, ten AF specimens were used in an albeit limited trial to assess the level of accuracy. The aneuploidy screening results were confirmed by the subsequent long term analysis in all these cases. Elsewhere more extensive multi-center trials involving a total of 2238 AF's (Romana et al, 1993) have allowed a 99.9% accuracy level. As a diagnostic service we have received over a six month period 100 specimens. They were mainly AF's, together with a lesser number of CVS and a minority of products of conception. The mean reporting time was 72 hrs. (When possible results were given within 24 hrs. However the constraints imposed by test cost, required that specimens arriving at or close to the weekend will have a delayed reporting time). The reasons for referral with percentages are given in Table I. These have not changed and remain as initially anticipated for such a test in its present form.

Table I. Reason for Referral.

Reason	Percentage
Maternal Age	33%
Triple Test Pos.	26%
Mat,Anxiety	14%
Abnormal @ Scan	13%
Fam,hist: Chrom.	8%
POC	4%
Fam,hist: Gene	1%
Other	1%

The abnormality rate was 6%, and each type of aneuploidy for the probes used have now been seen by us (Fig. 1). In addition all abnormals were later confirmed by means of long term culture. There was no significant difference between the observed and expected gender ratio with (X^2 0.01(1) = 6.635 > 1.4), and hence maternal contamination was not apparent. It was not possible to assess the false negative rate in this cohort, since approximately one third were not followed by a long term culture. However, in those which did have a subsequent full analysis no false negatives were seen and no other chromosome abnormalities were present. There was one apparent false positive, which presented as a 10% mosaic for trisomy 21. However, this was not confirmed in the following long term culture despite extensive examination by means of an appropriate number of cells (in this case 72, which allows a 7% or above level of mosaicism to be detected within 99% confidence limits). Except for this false positive mosaic, no other mosaics were detected. However, one sample was further investigated using FISH since the long term



Legends:

Figure 1. a - c: Trisomy 21;18 and 13 indicated by three red; blue and green signals respectively. Since the 21 probe is applied with that of the 13, two green signals are seen with the three red for trisomy 21 (Down's Syndrome). Similarly, trisomy 18 (three blue) is seen with a red and green for the Y and X respectively, indicating a male Edward's foetus. Trisomy 13 (Patau's Syndrome) is demonstrated by three green signals and two red for a normal 21 compliment.

d - f: Turner's Syndrome is indicated by only one X (green) signal with two 18 (blue) signals and no Y. Alternatively, three such green signals demonstrate a triple X female (with again two blue for chromosome 18). An XYY male has two red for the Y (instead of the normal situation of one) with one green for the X and again two blue for the 18.

g - h: Here all the probe signals are seen as three copies in the same cell. No Y signal was seen indicating a female triploid foetus (partial hydatidiform mole).

prenatal result was a 10% mosaic for a supernumerary Y. Here we were interested to compare the two techniques in terms of their sensitivity. In this case the aneuploidy result agreed with the long term result but at a slightly increased level (12%). All of our samples have been successfully processed and except for the one described above were fully informative. (We have had one episode of an assaying error which resulted in three specimens - one CVS and two AF's - requiring extra slides to be made. The source of this error was incorrectly constituted denaturation solution. The assays were repeated and were then successful but the subsequent reporting time was delayed by 24hrs). Thus far then our results are within those of the multicenter trial of 97.5% informativeness and where there was : maternal cell contamination[3]; assay error[4] and insufficient nuclei for analysis[48].

DISCUSSION

Aneuploidy involving the chromosomes examined here account for 67% of all liveborn chromosome abnormalities accompanied by birth defects. Thus approximately one third will remain undetected by the FISH probes used here. This clearly indicates the continuing need for a subsequent complete chromosome analysis via a long term culture. Nonetheless, although culture times can occasionally be reduced given the best circumstances, traditional prenatal diagnosis usually takes 10-14 days. FISH offers a rapid and relatively extensive screen prior to the slower long term cultures. It is also extremely accurate and in the case of mosaicism may have a sensitivity superior to a routine long term culture. Thus Nazarenko et al (1999) have demonstrated that 29% of Turner's Syndrome with an apparently pure form of monosomy X are in fact tissue mosaics. Similarly Zaslav et al (1998) were able to identify a low level mosaic trisomy 15 in various tissue types in an aborted second trimester aborted foetus when ultrasound had revealed a hypo plastic right ventricle and intrauterine growth retardation.

The probe specificity appears to allow chromosome detection without cross hybridization creating ambiguity. This has been achieved both by increasing the probe length and chromosomal in-situ suppression. Such high detection efficiency is important since as the signal number increases, this would be expected to decrease. Thus if the probability of detecting one signal is 0.9, then two will be 0.81 and three 0.73. Therefore in 50 cells 36 could be expected to show three signals

in the presence of aneuploidy. But by analyzing 50 cells mosaicism of as little as 5 to 9% can be detected within 90 to 99% confidence limits respectively suggesting that 73% may be an underestimate even for the required three signals. Indeed our experience is that better than 90% of cells will demonstrate aneuploidy if present. Furthermore, it is recognized that each interphase chromosome has a distinct focal domain, which also may help to increase the probability of recognizing an individual signal. Ruangvutilert et al (2000) have extensively examined the probability of detecting three signals in known trisomic interphase fibroblasts. They demonstrated that for chromosomes 21 and 13, 88% of such cells would show three signals. However, chromosome 18 appeared somewhat lower at 79%. Although these authors thought signal overlay may be responsible in all chromosomes, they did not explain the disparity between 18 and the rest. We will suggest elsewhere that cell cycle time may be responsible and that this may be important when considering the phenotypic impact of mosaicism at prenatal diagnosis.

The use of positive controls ensures that the specimen processing is successful, eliminating this as a source of false negative error. A false positive rate of 5% (76/1516) and 0% false negative rate has been reported as the result of the multicenter trial. At present our false positive rate is 1%, due to one borderline mosaic which presented at our cut off level of 10% for positivity. Maternal cell contamination does not appear to be a problem since there is no evidence of skewing in favour of female results and in any case the long-term results match exactly. These figures compare favourably with traditional long term cultures where biological error rates may be 1%, mainly due to maternal cell contamination (0.34% in AF) and where mosaicism can go undetected because of the limited number of cells examined in routine analyses. We have not been able to accurately assess the false negative rate since approximately one third of the patients in this sample opted for FISH analysis alone. In those who did proceed to a full chromosome analysis no false negatives were found, nor were there any other chromosome abnormalities not covered by the preceding FISH screen.

In our sample the majority reason for referral divided into three main categories. A triple test result above the cut-off level of 1:250 accounted for 26%; whilst a detected abnormality at ultrasound was 13%. However, maternal age above 35 yrs gave rise to the majority of referrals at 33%. Surprisingly maternal anxiety did not figure as a large reason (14%), but perhaps some of the maternal age group belonged in this category. Family history, not surprisingly, made up a small group (9%). This probably reflects the small percentage of inherited chromosome abnormalities and that those single gene defects requiring sexing - as in our case of Haemophilia A - are also relatively rare. Furthermore, for those familial chromosome abnormalities such as balanced rearrangements in a carrier parent, FISH would be inappropriate in an at risk pregnancy.

As well as these reasons for referral we have been able to provide results on one failed AF culture that would otherwise have moved beyond the legal limit for elective abortion had the sample been repeated; and four products of conception (POC) at the request of the mother. In these, all the results were negative using FISH except for one POC which later demonstrated trisomy 15 by means of long term culture. Again this emphasizes the need for patients to clearly understand that not all aneuploidy and no structural rearrangements can be identified by this technique.

Since the introduction of hybridization based interphase aneuploidy screening there has followed a modification based on the use of semi-automated DNA sequencers and PCR. Here DNA extracted from AF or CVS cells and oligonucleiotide sequences (primers) specific for a chromosome are used in a PCR reaction. Several sets of these fluorescently labeled primer pairs per chromosome are used in one reaction (multiplexing). The amplified sequences are automatically detected quantitatively. Nonetheless, the sensitivity and accuracy is the same as non PCR based methods, as is the reporting time. Furthermore, unless very large numbers of specimens are to be analyzed, such methodology is not cost effective since automated DNA sequencers are expensive. This, together with the relative rarity of chromosome abnormalities, other than those described above, does not augur well for such developments. Finally, the detection of rearrangements such as reciprocal translocations are still not be possible unless there is prior knowledge and only then if primers can be synthesized for the relevant breakpoints.

The level of specificity described in this paper may allow the introduction of less or non-invasive prenatal diagnosis. Chang et al (1997) have shown that transcervical cells obtained by uterine lavage could be successfully used for FISH foetal sexing and aneuploidy screening. Also urgent newborn uncultured blood samples have been screened with 100% efficiency by means of FISH (Jalal and Law, 1997) but buccal mucosa may provide an alternative less invasive cell source and have successfully used it in the diagnosis of a case of mixed gonadol dysgenesis in a newborn child (Quaife et al, 2000).

Two other major areas of research are of interest. The first is pre-implantation diagnosis using the first

or second polar bodies. This work has progressed such that commercially available probes (the same as those described above) are now available. Since the cells examined are limited, all five probes are used at once. In one study (Verlinsky et al, 1998), 3651 oocytes were examined, with 2952 giving interpretable results (80.9%).

The second major area of development is the analysis of foetal cells in maternal circulation. In Professor Nicolaides unit for Foetal Medicine (London), 230 at risk pregnancies were analyzed at 10-14wks gestation using maternal peripheral blood. For a 13% false positive rate the sensitivity for trisomy 21 was 97% (Al-Mufti et al. 1999) and similar for 13 and 18.

Such developments may herald the demise of invasive procedures for prenatal diagnosis. But whatever techniques are used in the future, the present allows rapid prenatal detection of chromosomal abnormality. A comprehensive analysis via long term cultures will remain necessary as a means of detecting structural rearrangements, at least for the time being. Nonetheless as an appropriate adjunct, aneuploidy screening seems to be a valuable addition to the prenatal diagnostic repertoire.

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