



Importance of Application of Appropriate Genetic Tests in Day Today Fetal Medicine Clinical Practice: Illustration with Cases

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ABSTRACT

It is important to apply appropriate genetic tests to have the best yield and for complete final diagnosis. Here we would like to illustrate the importance by demonstrating of cases.

First is a case with major lethal anomalies. Fetus had holoprosencephaly, umbilical cord cyst, and facial dysmorphic features. After termination, QFPCR was done on fetal tissue. Fetus had trisomy 18: Edward syndrome.

Second case was 12 weeks fetus with cystic hygroma. FISH was done and fetus had turner syndrome. Third was an interesting one. Fetus had right aortic arch with polyhydramnios. We had dilemma whether to apply MLPA or CMA. As there was polyhydramnios, we did MLPA first, which was positive for 22q deletion.

The last case was the most interesting one which showed us karyotyping is still the best one and needed for everyone case was referred for amniocentesis in view of high risk for trisomy 21. Fish report was normal. However, at karyotyping there was monosomy 12, demonstrating importance of karyotyping and the need for all cases.

Keywords: FISH; QFPCR; MLPA; Karyotyping; Ultrasound

INTRODUCTION

Trisomies 21, 18 and 13 are the most common chromosomal aneuploidy. We usually don't miss trisomy 18 and 13 by ultrasound as they have in major malformations of either heart, kidney and as well as brain in majority of times [1]. But it is important to confirm diagnosis by genetic testing as this helps in genetic counseling. Karyotype is the confirmatory diagnostic test. Although the advantage of Karyotype is, time tested with 99.9% accuracy, it is time consuming and cell culture failure is the limitation. Hence rapid aneuploidy tests are useful. These tests are faster, accurate and also take care

of cell culture failure. But it is questionable whether this can replace karyotyping. Hence we felt to write this write up to illustrate the importance of each tests.

CASE PRESENTATION

Case 1

Fetus at 20 weeks had alobar holoprosencephaly, congenital diaphragmatic hernia and umbilical cord cyst at ultrasound. Fetal autopsy confirmed these findings. QF PCR was trisomy 18 (**Figure 1**). Karyotyping was not possible from fetal tissue.

Received: 23- April -22

Manuscript No: IPGOCR-22-13113

Editor assigned: 25- April -22

PreQC No: IPGOCR-22-13113 (PQ)

Reviewed: 06- May -22

QC No: IPGOCR-22-13113 (Q)

Revised: 10- May -22

Manuscript No: IPGOCR-22-13113 (R)

Published: 16- May -22

DOI: 10.36648/2471-8165.8.5.21

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Citation: Kodandapani S, Rao S (2022) Importance of Application of Appropriate Genetic Tests in Day Today Fetal Medicine Clinical Practice: Illustration with Cases. Gynecol Obstet Case Rep. Vol.8 No.5:21.

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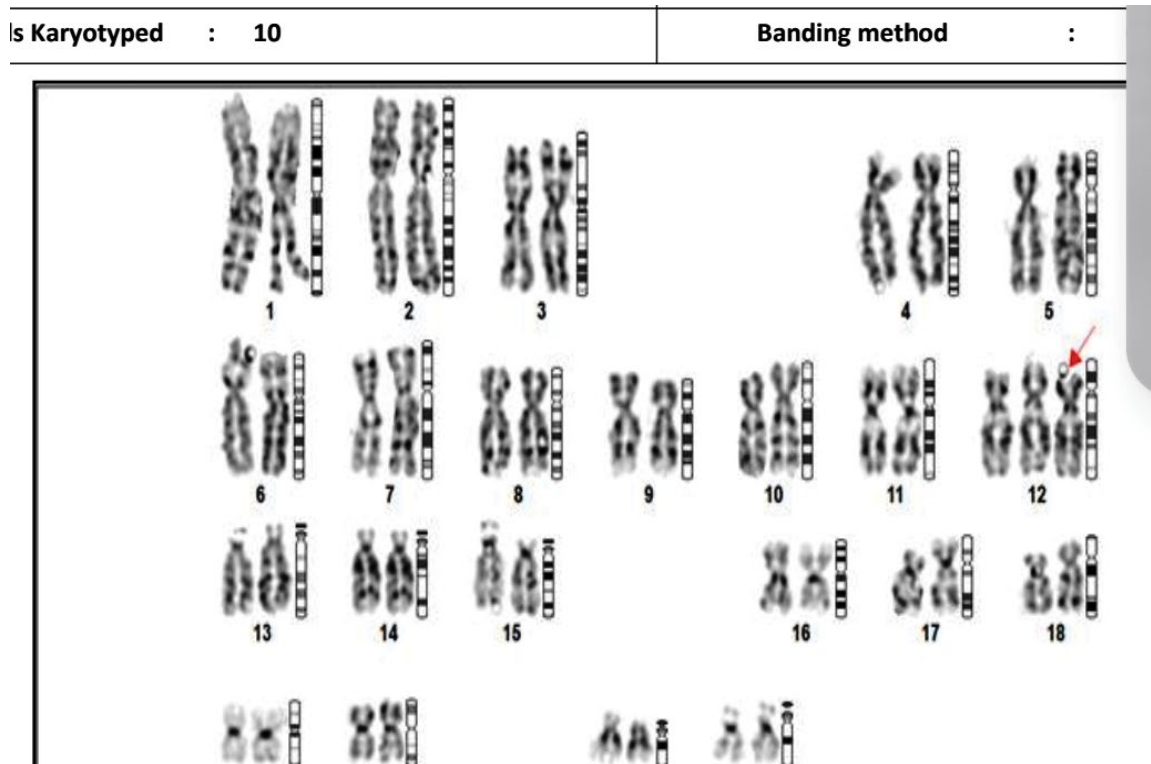


Figure 4: Karyotyping report showing mosaic trisomy 12.

DISCUSSION

It is important to select appropriate genetic testing for correct result. An ideal genetic test should be highly sensitive, specific, cost effective and fast. Although a near ideal test is available and affordable especially in the past ten years, it takes lot of intelligent input to choose the best appropriate genetic test. Hence the author felt there is a need to know various genetic tests, their uses: advantages, disadvantages and limitations with illustration of cases.

Karyotyping is the gold standard test for nearly half a century. This is highly reliable test in detection of numeric chromosomal and major structural abnormalities. These include unbalanced translocations, balanced translocations (familial and *de novo*), mosaicism, supernumerary chromosomes, triploidy and sex chromosome abnormalities. Karyotyping is a labor intensive and takes an average of 14 to 21 days. As karyotyping reveals all chromosomal abnormalities that can be microscopically detected, it may lead to other findings than the aneuploidy targeted in prenatal screening. Among those 'incidental' (or with a misnomer: 'unexpected') findings, there can be severe or mild abnormalities or abnormalities of which the impact on the health of the child is unsure. Case 4 is the perfect example for this statement. Patient was referred for amniocentesis, as there was high risk for trisomy 21 in quadruple test. Karyotyping was positive for trisomy 12 [2]. Mild fetal growth restriction was present at follow up ultrasound; however there were no structural abnormalities. Let us discuss clinically relevant points of each test.

Rapid Aneuploidy Detection Tests

Quantitative Fluorescence PCR (QF-PCR): It is a reliable molecular method for rapid aneuploidy diagnosis. DNA will be isolated from the given sample. Multiplex PCR amplification of Short Tandem Repeat (STR) markers using fluorescently tagged primer will be carried out [3]. The resulting fragments will be analyzed on the genetic analyzer for visualization and quantification. The copy number of each chromosome is quantified by calculating the relative allele ratio. Analyzed region includes: D13S252, D13S305, D13S634, D13S800, D13S628, D18S819, D18S535, D18S978, D18S386, D18S390, D21S11, D21S1437, D21S1409, D21S1442, D21S1435, and D21S144. Steps, advantages and limitations are written in detail in **Table 1**.

Multiplex Ligation Dependent Probe Amplification (MLPA): Micro deletion and micro duplication syndromes are defined as a group of clinically recognizable disorders characterized by a small (<5 Mb) deletion or duplication of a chromosomal segment spanning multiple disease genes. The phenotype results from haploinsufficiency for specific genes in the critical interval. MLPA is used for detection of micro deletion and duplication.

MLPA is a multiplex reaction, meaning one reaction provides information on up to sixty targets. For most applications, a single MLPA reaction is sufficient to answer the specific questions asked by a physician or researcher. Each MLPA reaction requires only 50 ng of human DNA. Each MLPA probe detects a sequence of 60-80 nucleotides, meaning that single exon deletions and duplications can be seen. Even when MLPA does not detect any aberrations, the possibility remains that

Table1: Steps, advantages and limitations of genetic tests.

Genetic Tests	Steps	Advantages	Limitations
QFPCR	DNA extraction PCR amplification Capillary electrophoresis Export data and analysis	<ul style="list-style-type: none"> Large number of samples can be done simultaneously Reproducible, easy to perform, and sensitive 	<ul style="list-style-type: none"> Balanced and unbalanced translocations not possible by sensitivity & specificity of the assay may be influenced by the quality of the specimens QF-PCR. Samples with significant mosaicism and maternal cell contamination may impact the diagnostic accuracy of QF-PCR
FISH	Denaturation Hybridization Probe detection Analysis	<ul style="list-style-type: none"> Rapid technique and large numbers of cells can be stored quickly. The efficiency of hybridization and detection is high Sensitivity and specificity are high 	<ul style="list-style-type: none"> FISH can only detect deletions or duplications of regions targeted explicitly by the probe used and more significant than the probe used. It is possible that FISH may not detect rare, tiny deletions.
MLPA	Denaturation Hybridization Ligation Amplification Fragment separation and data analysis	<ul style="list-style-type: none"> Cheap, accurate, known micro deletion and micro duplication can be detected 	<ul style="list-style-type: none"> Cannot detect copy number neutral inversions, translocations, and methylation changes. Not a suitable method to detect unknown point mutations. Any mismatch in the probe's target site can theoretically affect the probe's signal.
Karyotyping	Culture initiation Harvest Hypotonic treatment Slide preparation, banding and analysis	<ul style="list-style-type: none"> Time tested Accurate 	<ul style="list-style-type: none"> Requires fresh tissue Although direct preparations can be performed, cell culture is typically required (1–10 days). May encounter complex Karyotype with suboptimal morphology. Submicroscopic or cryptic rearrangements may result in a false-negative result. Normal Karyotype may be observed following therapy-induced tumor necrosis or overgrowth of normal supporting stromal cells. Difficulties encountered with bone tumors include low cell density and the release of cells from bone matrix. Results are based on the samples received at the laboratory. Interpretation is only in correlation with the demographic data provided in the Test Requisition Form. Submicroscopic and cryptic chromosome rearrangements cannot be ruled out. Partial reproduction of this report is not permitted. However, the contents of this report may be used for research purposes without revealing the personal information of the subject. The excess procedure-related risk above the baseline risk for miscarriage is 0.2 % (1:100)

biological changes in that gene or chromosomal region do exist but remain undetected.

When designing MLPA probes, we exclude any sequences where known SNVs or other polymorphisms are found using the latest genetic search engines. This means that a non-pathogenic polymorphism can also result in a decreased probe signal, leading to an ambiguous result or, in the worst case, mimicking a deletion. However, this isn't always possible, and new polymorphisms are continuously discovered. We recommend confirming all MLPA findings with another method, especially in the case of single probe deletions [4-7].

Fluorescence *In-Situ* Hybridization (FISH): FISH is a rapid diagnostic test and detects targeted chromosomes; 18, 13, 21 and sex chromosomes. It is as accurate as QFPCR but more laborious. However maternal cell contamination can be tackled with this test [4,8]. FISH test does not provide information about any chromosome other than the loci mentioned in this report. A negative result does not exclude the presence of chromosome alterations other than the one screened for and imposes the need for a Karyotype. The clinical interpretation of any test result should be evaluated within the context of the patient's medical history and other diagnostic laboratory test results [9].

Karyotyping: It is the first genetic test and still has a pivotal role in the genetic diagnosis. The culture failure rate is approximately 3%. In these cases, repeat sampling may be required. Results may get delayed due to slow cultures. In 3% of cases, placental mosaicism may be observed. The resolution of the Karyotype is 5 Mb. Hence genetic abnormalities more minor than 5 Mb cannot be detected. Normal investigation results do not ensure a 100% exclusion of genetic abnormalities. Karyotyping relies on G-band quality and resolution. In general, blood samples give the best quality chromosomes and, therefore, the best chance of detecting small subtle chromosome abnormalities. Chromosomes from other tissues (e.g. amniotic fluid, chorionic villus and products of conception) give poorer quality chromosomes; hence the risk of missing a subtle abnormality increases. It should be noted that on rare occasions, a subtle abnormality may be missed at prenatal diagnosis, only to be diagnosed later a postnatal blood sample. The Association of Clinical Cytogeneticists (ACC) has issued a policy statement to this effect [10,11].

Caution: It should also be understood that even a G-band blood Karyotype can never exclude extremely subtle chromosome abnormalities at the limit of resolution of light microscopy. Microarray testing should be performed in these cases where patient meets the appropriate criteria.

The Laboratory adheres to national professional standards for the minimum acceptable banding resolution for specified types of clinical referral. If a repeat sample is required due to analysis failure to meet the minimum standard, the report will state this.

Although mosaicism may be detected by routine karyotyping, it can never be 100% excluded. However, if there is an indication of suspected mosaicism, additional cells will be examined to exclude 10% mosaicism at a 95% confidence level—interpretation of mosaicism in prenatal diagnosis.

True mosaicism, when detected prenatally, can be difficult to interpret, and a further invasive diagnostic test may be required. Mosaic cell lines may be unevenly distributed between the fetuses and there is possibility of extra-fetal tissues leading to false positive and false negative results in the most extreme cases. Confined placental mosaicism' (CPM) is observed in approximately 1-2% of CVS samples. Pseudomosaicism can arise as cultural artifact and does not represent the fetal Karyotype. This is usually present in only one or three independently established cultures and can therefore be interpreted accordingly. In most cases, no further invasive testing is required. Maternal cell contamination of chorionic villous and amniotic fluid occurs in approximately 1/250 samples and may occasionally complicate the interpretation of results.

Normal variation: Each chromosome pair has a specific and identical G-banding pattern in all individuals. However, variation of no clinical significance may occur around the centromeric regions and short arms of some chromosomes. These variations are known as 'polymorphic variants', 'polymorphisms' or 'normal variants'

CONCLUSION

RAD techniques are reliable, cheap, fast and cost effective. As of now these tests don't need reconfirmation by karyotyping.

However, karyotyping is needed for all prenatal samples, irrespective of the indication for the invasive procedure. RAD techniques don't need reconfirmation from karyotyping and each other as well

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