Original Article

The Differentiation Of Chromosomal Analysis By Karyotype And Fluorescent In Situ Hybridization (Fish) In The Product Of Conception (Poc) With Recurrent Pregnancy Loss (Rpl)

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Abstract

Introduction: About 1–2% of women experience recurrent pregnancy loss (RPL). Chromosome analysis is the current gold standard for genetic evaluation of products of conception (POC) but failure occurs in 10–40% of cases due to microbial contamination or lack of viable dividing cells. We implemented a reflex fluorescent in situ hybridization (FISH) assay to detect numeric chromosome abnormalities for unsuccessful cultures

Materials and Methods: All POC samples received in the laboratory were first processed for chromosome analysis (adherent cell cultured). To improve the success rate of POC analysis in our laboratory, we implemented a reflex FISH assay to detect common numeric chromosome abnormalities from unsuccessful cultures. On an unsuccessful chromosome analysis, an interphase FISH assay consisting of probes for chromosomes 13, 18, 21, X, and Y was performed on the uncultured fixed-cell pellet.

Results: Out of 300 POC samples, chromosomal analysis results were obtained for 190 (63.3%) samples, 120 (63.2%) samples were found to have normal and 70 (37%) samples were found to have chromosomal abnormalities. Of the remaining failed 110 samples studied by FISH, an abnormality was identified in 30 (27.3%) samples. When the results for chromosome analysis and FISH were combined, an abnormality was detected in 100 of 300 (33.3%) analyzed samples. The most common chromosomal abnormality detected by FISH was Trisomy [Trisomy 13 (9, 9.7%), Trisomy18 (15, 16.1%), Trisomy 21 (29, 31.1%)] Monosomy X (10, 10.8%), and Kline filter syndrome - 47, XXY (5, 5.4%).

Conclusions: Chromosomal investigation in POC using first conventional Karyotype and then FISH in resolving results have the advantage to reduce the failure rate of reporting chromosomal aberrations. We analyzed the prevalence of chromosomal abnormalities in POC involved in women with recurrent pregnancy loss.

Keywords: FISH, Karyotyping, Recurrent pregnancy loss (RPL), Trisomy

INTRODUCTION:

First-trimester miscarriages are common with up to 20% of clinically recognized pregnancies ending in miscarriage. [1–3] Recurrent pregnancy loss (RPL) is defined as three consecutive pregnancy losses before 20 weeks of gestation. Epidemiological studies have revealed that 1–2% of women experience RPL, representing

approximately 1 in 300 pregnancies. [1] There are numerous etiologies for recurrent miscarriage, including structural uterine anomalies; endocrine disorders; prothrombotic conditions, such as antiphospholipid syndrome; and balanced translocation involving one of the parents. Despite a multitude of maternal factors, the majority (50–60%) of first-trimester miscarriages are due to fetal chromosomal abnormalities.[4] Among these, autosomal trisomies constitute the major group (50%), followed by monosomy X (18%), triploidy (17%), tetraploidy (6%), and others.[5] (Sankaranarayanan, 1979). Recent studies observed an increase in the detection of autosomal trisomies (63%-64%) and multiple aneuploidies (6%), and a decrease in monosomy X (10% -11%) and polyploidies (12% -17%).[4,6]

Chromosome analysis, which requires dividing cells, is the current gold standard for genetic evaluation of products of conception (POC) but has three limitations. First, a successful cell culture is required but failure occurs in 10–40% of cases due to microbial contamination or lack of viable dividing cells.[7] Second, the results take approximately 4–6 weeks. And third, if the results suggest normal female karyotype (46,XX), a result that happens 55–80% of the time, it is unknown whether the tested sample was fetal or maternal in origin.[8]

Several alternative assays have been used to evaluate POC genetics, including comparative genomic hybridization (CGH), array CGH, quantitative fluorescent polymerase chain reaction (PCR), multiplex ligation-dependent probe amplification (MLPA), and fluorescent in situ hybridization (FISH). These techniques circumvent the need for dividing tissue and can be readily integrated into the clinical laboratory setting.[9]

Materials and Methods:

Type of study: Cross-sectional study

Sample collection: Products of Conception (POC) samples were collected from different hospitals after medically terminated pregnancies and pregnancies that ended in spontaneous fetal death with a history of RPL. Written consent was taken during sample collection and POC samples were collected in a sterile container with normal saline and antibiotics. Human ethical committee approval for this study was obtained from Gujarat University, Ahmedabad, Gujarat, India vide approval no. GU/IEC/01/2018.

All POC samples received in the laboratory were first processed for chromosome analysis using the adherent cell culture method. To improve the success rate of POC analysis in our laboratory, we implemented a reflex FISH assay to detect common numeric chromosome abnormalities from unsuccessful cultures. On an unsuccessful chromosome analysis, an interphase FISH assay consisting of probes for chromosomes 13, 18, 21, X, and Y was performed on the uncultured fixed-cell pellet.

Chromosome Analysis: Conventional karyotyping from POC samples using Giemsa-Trypsin-Giemsa (GTG) banding was performed by standard protocol (Levy, B., et al. 2009)· Nutrient Mixture F-10 Ham complete medium (HiMedia, AL083A) used for POC sample culture with sodium bicarbonate, 25mM HEPES buffer, L Glutamine, 20mL Fetal Calf Serum (HiMedia, RM9955), and 1mL antibiotics (Penicillin-Streptomycin) (HiMedia, A001). Cultured cells were harvested using 30µL colchicine (HiMedia, TCL062) for 3 hours, followed by hypotonic treatment of 0.56% KCl solution for 15 minutes and fixation using standard 3:1 methanol: acetic acid fixative. Standard GTG banding technique (Francke, U. & Oliver, N. 1978) was performed with slight modification. Microscopic examination of 20 metaphases for each case was carried out using the software Applied Spectral Imaging (ASI) in an upright fluorescent Olympus BX53 microscope.

FISH: The FISH slides were aged for 30 minutes in a 65°C oven. The slides were then immersed in a 2X Saline Sodium Citrate (SSC) solution at 37°C for 30 minutes, digested in a 0.9% NaCl pepsin working solution (160 mg pepsin/40 mL 0.9% NaCl) (pH 1.5) at 37°Cfor 13 minutes, and immediately rinsed in phosphate-buffered solution for 5 minutes at room temperature. Next, the slides were immersed in 1% formaldehyde and phosphate buffered solution at room temperature for 5 minutes each. Slides were dehydrated in 70%, 85%, and 100% ethanol for 2 minutes each at room temperature and air dried. Commercial probes (Metassystem aneusomy probe kit) were used for common aneuploidy detection. The aneusomy kit consisted of centromere probes for chromosomes X, Y, and

18, as well locus-specific probe sets, X;Y;18, and 13;21 were applied to two separate hybridization targets, covered by cover-slips, and rubber cemented. The slides and probes were co-denatured at 73°C for 5 minutes and hybridized overnight in a 37°C humidified oven. After hybridization, the slides were subjected to a post-hybridization wash in 2XSSC for 2 minutes at 74°C and then rinsed in 2XSSC/0.1% NP-40 for 2 minutes at room temperature. Slides were stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), and anti-fade compound (Vectashield) and were coverslipped. The FISH slides were evaluated using a BX53 fluorescent microscope equipped with an appropriate filter wheel and cubes to visualize the Spectrum Orange™, Spectrum Green™, Spectrum Green/Spectrum Orange™, and Spectrum Aqua™ fluorophores. Two technologists each scored 100 consecutive nonoverlapping nuclei (200 total). After the analysis, the results for both scorers were combined, and the percentage of abnormal nuclei was calculated. Normal cut-offs, which were previously established in our laboratory, were then used to determine whether the sample was abnormal. The normal cut-offs for trisomy of chromosomes 13, 18, 21, X, and Y were 3%, 6%, 4.5%, 10%, and 10%, respectively. Whereas, sex chromosomes, monosomy X and Triple-XXX, and XXY had normal cut-offs of 20% and 5%, respectively.

Statistical analysis: The data were entered in Microsoft Excel 2010. Categorical data were presented with frequency and percentage and compared using the Chi-square test. P value less than 0.05 is considered as significant.

Ethical consideration: The study was conducted after getting ethical approval from the Institutional Ethical Committee of Gujarat University

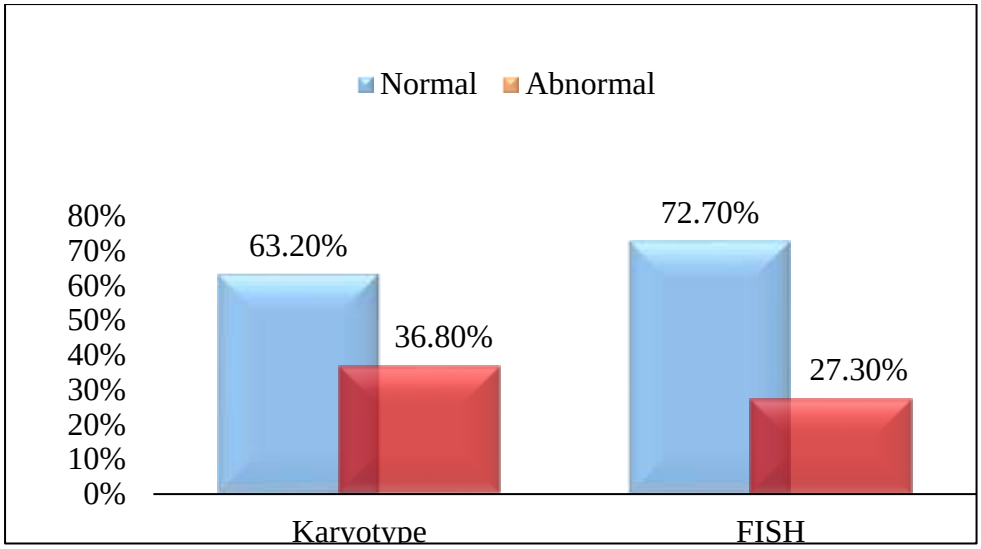
RESULTS

In this study, a total of 300 samples of Product Of Conception (POC) were included with a history of Recurrent Pregnancy Loss (RPL). Chromosomal analysis results were obtained for 190 (63.3%) samples, 120 (63.2%) samples were found to have normal results of chromosomal analysis and 70 (36.8%) samples were found to have chromosomal abnormalities. The remaining 110 (36.7%) samples failed due to unviable tissue received or microbial contamination. However, these 110 samples were further studied for Fluorescent in situ Hybridization (FISH). Of the 110 samples studied by FISH, normal results were found in 80 (72.7%), and abnormality was identified in 30 (27.3%) samples. When the results for chromosome analysis and FISH were combined, an abnormality was detected in 100 of 300 (33.3%) analyzed samples (Table 1; Figure 1).

Table 1: Cytogenetic abnormalities found in POCs

Cytogenetic Techniques	No. of samples (%)	No. of samples with normal results (%)	No. of samples with abnormal results (%)
Karyotype	190 (63.3%)	120 (63.2%)	70 (36.8%)
FISH	110 (36.7%)	80 (72.7%)	30 (27.3%)
Total	300 (100%)	200 (66%)	100 (33.3%)

Figure 1: Cytogenetic analysis of POC



A total of 100 (33.3%) chromosomal anomalies were detected by both techniques including 93 (31%) numerical as well as 7 (4%) structural chromosomal abnormalities (Table 2).

Table 2: Chromosomal abnormalities detected in POCs

Chromosomal anomalies	**No. of abnormal samples (%)**
Numerical anomalies	93 (31%)
Structural anomalies	7 (4%)
Total	100 (33.3%)

Chromosomal aneuploidy (63, 21.0%) was the most common numerical chromosomal abnormality detected in POC by conventional karyotyping followed by Robertsonian translocation (2, 1.0%) and Polymorphic variants (2, 1.0%), reciprocal translocation (1, 0.5%), inversion (1, 0.5%), satellite association (1, 0.5%), and inversion (1; 0.5%) (Table 3 and Table 4). Aneuploidy results were obtained in 80 (26.6%) samples with normal FISH and 30 (10%) samples with abnormal FISH. The most common aneuploidy was observed for chromosomes 13, 18, 21, X, and Y by karyotype and FISH in POC (Table 5).

Table 3: Chromosomal abnormalities detected by Karyotyping (70) in POC.

Chromosomal pattern	**No. of abnormal Karyotyped %**
Aneuploidy	63 (33.1%)
Robertsonian translocation	2 (1.0%)
Polymorphic variants	2 (1.0%)
Reciprocal translocation	1 (0.5%)
Inversion	1 (0.5%)
Satellite association	1 (0.5%)
Total	70 (36.8%)

The most common numerical chromosomal abnormality detected by conventional karyotyping was trisomy including, a total of 42 (66.7%) with trisomy 13 (4; 6.3%), trisomy 18 (9; 14.3%), trisomy 21 (18; 28.6%). In addition, sex chromosome abnormalities, include monosomy X (5; 8.0%), and, 47,XXY (3; 4.8%). Other abnormal results were found in two cases with 69,XXX triploidy, and five cases (8.0%) with mosaicism containing two cell lines. In three samples (4.8%), mos,47,XX,13/47,XX,21, and in two samples (3.2%), mos,47,XY,13/47,XY,21. Likewise, the most common chromosomal abnormalities detected by FISH was trisomy including, a total of 22, (73.3%) with trisomy 13 (5; 16.7%), trisomy 18 (6; 20.0%), and trisomy 21 (11; 36.7%). One case (3.3%) was found with monosomy 21, and various sex chromosomal abnormalities [monosomy X (4; 13.3%), XXX (1; 3.3%), XXY (2; 6.6%)] were also found using FISH analysis. When the results for conventional

karyotype and FISH were combined, the most frequent chromosomal abnormalities were trisomy 21 (28; 30.1%), trisomy 18 (15; 16.1%), trisomy 13 (9; 9.6%), and monosomy X (9; 9.6%) observed in POC with recurrent pregnancy loss (Table 5 Figure 2).

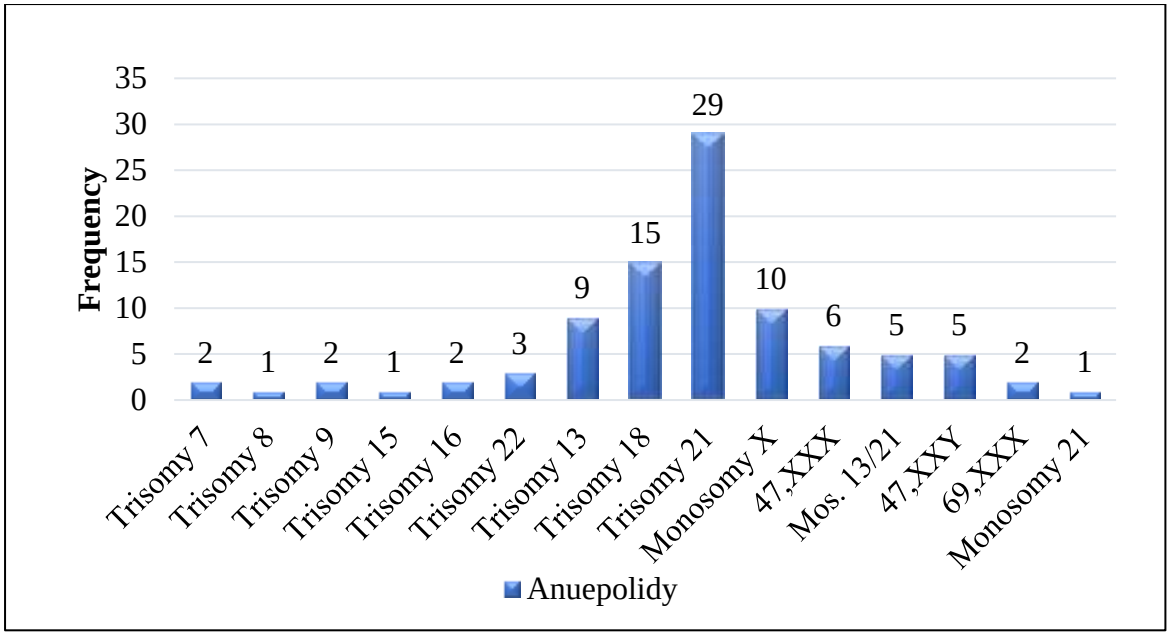
Table 4: Cases with structural chromosomal anomalies by Karyotyping (7) in POC

Structural chromosomal abnormalities	Frequency
46,XX,der(13;14)(q10;q10)+13	1
46,XX,t(7;8)(p22p14;24.2q24.3)	1
46,XX,inv9(p12;q13)	1
46,XX,9qh+	2
46,XX,15ps+	1
46,XX,22ps+	1
Total	7

Table 5: Comparison of numerical chromosomal aneuploidies between Karyotyping and FISH

Numerical chromosomal abnormalities	Karyotyping (63 Samples)	FISH (30 Samples)	Total (93 Samples)
Trisomy	**42 (66.7%)**	**22 (73.3%)**	**64 (68.8%)**
– Trisomy 7	2 (3.1%)	0 (0%)	2 (2.2%)
– Trisomy 8	1 (1.6%)	0 (0%)	1 (1.1%)
– Trisomy 9	2 (3.2%)	0 (0%)	2 (2.2%)
– Trisomy 15	1 (1.6%)	0 (0%)	1 (1.1%)
– Trisomy 16	2 (3.2%)	0 (0%)	2 (2.2%)
– Trisomy 22	3 (4.8%)	0 (0%)	3 (3.2%)
– Trisomy 13	4 (6.3%)	5 (16.7%)	9 (9.7%)
– Trisomy 18	9 (14.3%)	6 (20.0%)	15 (16.1%)
– Trisomy 21	18 (28.6%)	11 (36.7%)	29 (31.1%)
Monosomy	**6 (9.5%)**	**5 (16.7%)**	**11 (11.8%)**
– Monosomy 21	0 (0%)	1 (3.3%)	1 (1.1%)
– Monosomy X	6 (9.5%)	4 (13.3%)	10 (10.8%)
Mosaic	**5 (8.0%)**	**0 (0%)**	**5 (5.4%)**
– Mosaic (47,XX,Trisomy 13 & 21)	3 (4.8%)	0 (0%)	3 (3.2%)
– Mosaic (47,XY,Trisomy 13 & 21)	2 (3.2%)	0 (0%)	2 (2.2%)
Triple X syndrome (47,XXX)	**5 (7.9%)**	**1 (3.3%)**	**6 (6.5%)**
Kline filter syndrome (47,XXY)	**3 (4.8%)**	**2 (6.6%)**	**5 (5.4%)**
69,XXX	**2 (3.2%)**	**0 (0%)**	**2 (2.2%)**

Figure 2 Chromosomal aneupoidies detected in POCs



DISCUSSION

The cause of recurrent pregnancy loss is very complicated. In addition to anatomy, endocrine, thrombophilic, immune, and other factors, fetal chromosomal abnormalities are often considered an important cause of pregnancy loss. The fetal chromosomal abnormalities rate in the general population is 60%. While the rate of recurrent pregnancy loss is varying between 29-60%, generally attributed to gamete problems.[11] Gamete problems are often considered to be abnormal parental or fetal chromosomes. Studies showed that the incidence of chromosomal abnormalities may decrease as the number of pregnancy losses increases.[12]

This study, showed a relatively low incidence of POC chromosomal abnormalities (33.3%) by conventional karyotype and FISH. By contrast, typical culture failure rates of 36.7% were detected. Failed samples were further studied for FISH analysis, in which abnormalities were identified in 27.3% of samples. The analysis of results of both techniques depicted that chromosomal aneuploidies are more likely detected in POC with a comparison of other chromosomal abnormalities, lower than the 60% expected in spontaneous miscarriage, similar to the incident reported by Carp H et al.[13]. Lower than the incidence reported by Stern JJ et al.[14] and Ogasawara M et al.[12] for recurrent pregnancy loss. The low incidence may be due to our study only including POC with three or more pregnancy losses. In comparison, both Kaji T et al.[15] and Ogasawara M et al.[12] included patients with two pregnancy losses.

The overall results showed that numerical abnormalities constitute 93% of all those POC with abnormal results, closer than that in other studies were observed in numerical abnormalities 94%.[13] Structural chromosomal abnormalities consist of translocation, polymorphic variants, inversion, and satellite association is a major etiology of recurrent pregnancy loss.[11] This study reported structural abnormalities at 4%, lower than the reported 6.5% by Zhang T et al.[16] However, this study cannot exclude all chromosomal causes of recurrent pregnancy loss. Conventional chromosome abnormalities include structural and numerical rearrangement; however, approximately 20% of POC samples fail to harvest a good karyotype due to culture failure, often attributable to microbial contamination or nonviable tissue. Other more sophisticated techniques such as FISH can be the dignified solution and overcome this problem the procedure is fast and does not require live fetal tissue as compared with the difficulty of conventional karyotyping.

Karyotyping can detect abnormalities throughout the entire genome and is therefore used as the standard for detecting chromosome abnormalities in recurrent pregnancy loss. The incidence of chromosomal aneuploidies observed in these studies was 43% of the entire chromosome, resembling frequencies observed in previous studies (41.8%).[17] However, there are some differences in frequency and difference maternal age.

Although the FISH detected an overall abnormality rate of 27.3% for the FISH panel (13, 18, 21, X, and Y) used in the present study can be explained by the limited view of the genome provided lower than that reported in women with the risk of aneuploidy with an increasing number of prior miscarriages. In the study of Bianco L et al.[18], they reported women with three prior miscarriages would increase that risk by 51%. In addition, the incidence of the common trisomies (13, 18, and 21) increased with increasing with the number of prior miscarriages, these studies reported a higher rate in 57% of common trisomies with recurrent pregnancy loss. In addition, FISH was identified as monosomy 21 in one sample with autosomal monosomy, while triploidy was found in two samples. Similar results were reported by Carp H et al.[13] and Fejgin MD et al.[19] who used conventional karyotype and FISH techniques in abortus. These results indicated that the limitation of chromosomal aneuploidies was detected by the FISH panel.

Sex chromosomes also showed the unusual occurrence of sex chromosomes trisomy of 47,XXX (6; 6.5%), and 47,XYY (5; 5.4%) in conventional karyotype and FISH. Though the causes of sex chromosomal abnormalities in recurrent pregnancy loss are still unclear, still the previous study by Gu C et al.[17] was also reported sex chromosome abnormalities in abortion which support the findings of the present study. Current study is based on a dual cytogenetic technique (conventional Karyotype and FISH) for the chromosomal analysis of the POC samples with RPL decreased the failure rate of reporting of chromosomal aberrations in POCs. Our study reported several possible chromosomal abnormalities were most frequently appear in recurrent pregnancy loss. Among them, aneuploidies are more often associated with pregnancy loss as compared with other structural chromosomal abnormalities.

CONCLUSION

This study suggested different possibilities of chromosomal aberrations responsible for recurrent pregnancy loss, including aneuploidy, deletions, inversions, duplications, reciprocal translocation, Robertsonian translocation, polymorphic variants, and satellite association, which cause errors leading to disturbance of conception. Chromosomal investigation in POCs using first conventional Karyotype and then FISH provides results that have the advantage to reduce the failure rate of reporting of chromosomal aberrations. We analyzed the prevalence of chromosomal abnormalities in POC involved as major causes of fetal loss in women with recurrent pregnancy loss. Chromosomal study of POCs will help to evaluate the association of fetal chromosomal abnormalities with fetal loss or recurrent pregnancy loss.

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