Quantitative Fluorescent-PCR: Current Scenario and Future Approach for the Investigations of Spontaneous Miscarriages in Karachi

Misbah Iqbal Hanif¹, Afsheen Arif², Sitwat Zehra², Erum Shoeb¹, Ayesha Khan³

¹University of Karachi, Faculty of Science, Department of Genetics, Karachi, Pakistan
²University of Karachi, Faculty of Science, The Karachi Institute of Biotechnology & Genetic Engineering, Karachi, Pakistan
³Dow Medical College, Dow University of Health Sciences, Department of Gynecology & Obstetrician, Karachi, Pakistan

Corresponding Author: Misbah Iqbal Hanif
misbahhanif88@outlook.com

ABSTRACT

Chromosomal numerical aberration is one of the leading reasons behind spontaneous pregnancy loss. The probability of spontaneous miscarriage increases with advanced parental age. After miscarriage patients and their families feel desperate, which consequently create psychological illness. The review evaluates current techniques being used in the diagnosis and focuses toward a recently introduced molecular technique Quantitative Fluorescent-PCR (QF-PCR) which based on the use of fluorescent primers (probes). Several published articles were selected for the review regarding the frequency of spontaneous miscarriages, its reasons, and outcomes. Studies that focus on QF-PCR, karyotyping and their comparison were included. Current situation of management of spontaneous miscarriages was also observed through surveys and consultant views in Karachi. On the basis of the above-mentioned piece of information, QF-PCR would be a good addition in the field of prenatal diagnosis. However, karyotyping is also a reliable method of prenatal diagnosis with little limitations.

Keywords: Spontaneous miscarriage, chromosomal numerical aberration, QF-PCR, karyotyping, fluorescent primers, aneuploidies

1. INTRODUCTION

Miscarriage is defined as “the unexpected and unplanned, spontaneous loss of a pregnancy before the fetus becomes capable of extra-uterine survival.” Spontaneous miscarriage is one of the most frequently encountered problems of pregnancy. Due to its high occurrence in population and associated costs of clinical test and subsequent treatment, it causes substantial load on society. For patients and their spouses, it is a hard task to bear this loss that really causes hostile effects on their communal and psychological health. After the spontaneous pregnancy loss, the gap is difficult to fulfill for patients and their partners, especially when it happens repeatedly. Recurrent miscarriage is more clearly understand as two or more miscarriages, either consecutive or non-consecutive(1). Recurrent spontaneous miscarriages also called recurrent pregnancy loss described as two or more consecutive pregnancy losses with ≥ 20 weeks of gestation. It occurs in 10-15% of all known pregnancies(2). If consider first-trimester pregnancy loss, the most common cause is a chromosomal abnormality.

Almost 99% pregnancies with chromosomal abnormality result in miscarriages, in which 86% arise from numerical chromosomal abnormality, such as trisomies, monosomies, polyploidies (triploidy or tetraploidy). Approximately 60% cases of recurrent miscarriages are related with a chromosomal aberration in the fetus and about 3-5% of couples with recurrent miscarriages have one partner with reciprocal translocation or balanced chromosomal rearrangements\(^3\). There are variable chromosomal numbers and several appearances, which can results in early fetal death, negligible to significant hereditary defects and infertility or sterility. Unfortunately, these conditions cannot be cured, so it is necessary to find chromosomal abnormalities before birth\(^1,4\).

Embryonic chromosomal abnormalities may be the consequence of errors during gametogenesis, fertilization or initial cellular division. Generally the risk of fetal loss increases with the increase in maternal age. In 35-year-old women, one-fifth pregnancies results in miscarriage, and at 42 years of maternal age more than half of the planned pregnancies (54.5%) results in a fetal loss\(^5\). Paternal chromosomal aberration is also common in many spontaneous miscarriages, and the probability of first-trimester spontaneous miscarriage is 1.26 times more with advanced paternal age\(^6\).

Other than chromosomal aberrations, there could be many other causes of recurrent pregnancy loss, including, hormonal and metabolic factors such as hypothyroidism, diabetes mellitus (unrestrained), some of the uterine anatomic abnormalities, anti-phospholipid antibody syndrome (APS), genetic and/or non-genetic thrombophilia, immunological disorders, untreated infections, and other environmental factors, including, lifestyle such as smoking, excessive use of caffeine, consumption of alcohol or obesity.

The objective of this review is to evaluate the causes of miscarriages and its possible consequences on the patients and their family’s communal and psychological health and to assess current diagnostic methods in Karachi. This review is more focused on Quantitative Fluorescent-PCR (QF-PCR), a newer molecular diagnostic technique to explore its advantages and limitations as a standalone technique in prenatal diagnosis in Karachi.

**Psychological Issues after Spontaneous Abortion**

Women and their partners feel hopeless often after a miscarriage, even in early gestation it is a loss of a baby. These grief feelings are common but generally self-limiting. The anxiety and depression are quite common in women suffered a miscarriage. They may get psychological relief by receiving additional counseling. General practitioners should be familiar with the psychological problems those are frequent with patients of spontaneous miscarriages. In patients who had a miscarriage, a significantly high percentage of anxiety and depression is found during pregnancy as compared to the pregnant women with no miscarriage history. Especially in initial weeks of incident psychological symptoms are more common.

Women who are more susceptible to these symptoms have lost a wanted pregnancy or have no child. The percentage of psychiatric problems is approximately 11 times higher among women who had miscarriages than infertile women\(^9\). Therefore, this issue is really challenging for the gynecologist; they should address the matter with these women and relieve them from self-blame and feeling of guilt. In the case of recurrent miscarriages, this anxiety may reach its highest level.

The prevalence rate of psychiatric disturbance is generally high among early pregnancy miscarriages. Delay in diagnosis may cause an increase in the depression level. The newer prenatal diagnostic techniques can support to resolve this issue.

**Conventional Diagnostic Techniques**

The prenatal investigation can be initiated with non-invasive and invasive techniques. A non-invasive technique which is the prime test used to evaluate pregnancy viability includes ultrasonography and biochemical tests from maternal blood. Usually, spontaneous vaginal bleeding is indicative of pregnancy loss. Now a day’s first-trimester screening tests are also available that gives a choice of former analysis for fetal aneuploidy. Prenatal invasive diagnosis is recommended to women with prior history of individual chromosomal aberration or history of such disorder in the family, with progressive maternal age, abnormal serum metabolites, or unusual ultrasound findings (abnormalities in the fetus).

Invasive testing includes amniocentesis and chorionic villus sampling (CVS). Chorionic villus sampling is performed between 10-13 weeks of gestation, while amniocentesis is suggested from 15 weeks of gestation and onward.

Cytogenetic analysis is crucial for fetus abortuses to study the cause of spontaneous abortion. There are many types of chromosomal abnormalities common among spontaneous miscarriages which are completely absent or rarely found in the alive...
population. Abortuses can be helpful in the exploration of hidden chromosomal abnormalities and to find the parental origin of the chromosomal abnormalities. Multiple cytogenetic abnormalities are observed in fetal wastages including autosomal trisomies which is 27%, polyploidies 10%, sex chromosome monosomy 9%, and structural rearrangements about 2%\(^{(11,13)}\).

Parental karyotyping must be included in the appropriate evaluation of recurrent pregnancy loss that can provide a valuable understanding of causes and probability of recurrence. Cytogenetic analysis of abortuses is highly recommended even in the case of first spontaneous miscarriage parallel to other test should also be performed simultaneously.

Karyotyping is the standard practice in many laboratories to recognize all aneuploidies and balanced translocations and structural abnormalities with great accuracy (99.4-99.8%) since the late 1960s or early 1970s (9). In Karachi, karyotyping is being performed in five diagnostic centers at the cost of $100-150 per sample, most of the times patient, are unable to bear this cost. Physicians have pressure to initiate analysis of the causes of recurrent miscarriages because the loss of a desired pregnancy is unacceptable for patients. Detailed knowledge of the causes of spontaneous miscarriages is greatly important for the exclusion of additional diagnosis and further assistance. Karyotyping is a laborious and expensive technique; usually, it takes 14 days for the whole procedure with maximum accuracy even in the detection of minor abnormalities, but the success rate of karyotyping is low with up to 40% due to culture failure and overgrowth of maternally derived cells\(^{(5,10)}\).

Karyotyping requires viable cell while cell culturing and metaphase cell gathering is a long term process. The period between the collection of sample and final report can greatly influence the consequence and the anxiety level of patient particularly if non-invasive test have suggested the presence of an abnormal pregnancy.

Molecular cytogenetic techniques can help in reducing these problems through non-cultured embryo cells analysis. Present development in molecular cytogenetic techniques significantly enhances our knowledge regarding the prenatal genetics of fetuses. There are few techniques that can be used in association with traditional metaphase karyotyping to reduce the waiting time such as; fluorescence in situ hybridization (FISH), Southern hybridization, loss of heterozygosity (LOH) assays, microarray technology, comparative genomic hybridization (CGH), automated nuclear DNA cytometry, several modifications of polymerase chain reaction (PCR) and quantitative fluorescence polymerase chain reaction (QF-PCR)\(^{(11)}\).

**Quantitative Fluorescent Polymerase Chain Reaction (QF-PCR)**

QF-PCR is a molecular diagnostic technique which is used in the UK for over ten years. It has been proved as a robust technique\(^{(9)}\), which appears as a potential technique to evade the need of culturing the fetal cells. QF-PCR was primarily presented in the USA in 1993\(^{(12)}\), then it was familiarized into the UK National Health Service (NHS) as a certified diagnostic test in 2000\(^{(9)}\). QF-PCR is an automated procedure; it has proven to be very useful in the diagnosis of the most frequent aneuploidies (presently deletion and duplication of sub-chromosomal regions are also included in aneuploidy)\(^{(11,13)}\). Through QF-PCR the existence of different alleles are examined by polymorphic markers (microsatellites) of sex chromosomes and autosomal chromosomes such as 13, 18, and 21\(^{(10,14)}\). QF-PCR consumes parental DNA and some of the molecular markers which are di, tri, tetra, and pentanucleotides. These nucleotides are short tandem repeats (STRs) which are specific to chromosomes and having an extraordinary polymorphism in any population; due to these characteristic STRs are very useful in identification and diagnosis of major chromosomal aneuploidies\(^{(15)}\). QF-PCR can also determine the origin of non-disjunction in parental meiosis that is actually the cause of the aneuploidy. On an electropherogram of multiplex PCR reaction aneuploidy shows triallelic or diallelic peak pattern\(^{(11)}\). QF-PCR can produce test report in 6-12 hours from the collection of the sample\(^{(15)}\). In the UK QF-PCR has been widely used for prenatal investigation of sex chromosomal aneuploidies, and some common autosomal aneuploidies and this technique is also followed by numerous other European countries for rapid prenatal aneuploidies screening.

In Pakistan, currently, karyotyping is done at the cost of $150 and QF-PCR is not established as the proper diagnostic procedure in labs. However, the research-based estimated cost of QF-PCR is $100 approximately.

**Technique of QF-PCR**

QF-PCR is a firm molecular technique based on amplification of chromosome specific selected markers (STRs). In QF-PCR technique STRs specific
primers are designed for amplification purpose. DNA is extracted from uncultured amniocytes or chorionic villus samples\(^\text{(10)}\) and studied for unique STRs through QF-PCR using specific DNA primers. During the amplification primers are labeled with a fluorescent tag that binds to each target sequence. The fluorescent bound to target sequences can be measured precisely as peaks of fluorescent light on automated DNA analyzer. Measurement of the fluorescent signal of the product determines the copy number of each target sequence and consequently each chromosome. Normal heterozygous subject with the normal heterozygous condition is likely to have two peaks for a specific chromosome 1:1 ratio. Subjects with trisomies are imagined as three peaks with a 1:1:1 ratio (trisomic tri-allelic subjects) or it may be visualized as two peaks with a 2:1 ratio (trisomic di-allelic subjects)\(^\text{(16)}\).

**Advantages of QF-PCR**

QF-PCR is a good addition to traditional prenatal diagnostic methods. There are several advantages of QF-PCR:

- A major advantage of QF-PCR is its flexibility to automation and low cost as compare to karyotyping.
- It is a rapid, reliable and precise molecular biological method in aneuploidies detection, as FISH and karyotyping with high sensitivity up to 95% \(^\text{(17)}\).
- In most of the cases it can determine the origin of supernumerary chromosomes.
- In some familial cases, it can also evaluate the origin between meiotic and postzygotic mitotic aneuploidies.
- It yields only clear test results and facilitates post-test counseling and decision-making.
- It can be performed with very less, or contaminated sample with a maternal cell which is very frequent\(^\text{(11,15)}\).
- A high incident rate of spontaneous abortion is estimated due to aneuploidies, and those can be preferably excluded by QF-PCR\(^\text{(10)}\).

**Limitations of QF-PCR**

There are few drawbacks of substituting karyotyping with QF-CR technique. QF-PCR is not capable of eliminating all significant health problems which can be hidden in the fetus, resulting parents denial to terminate such pregnancy. In some cases, inaccurate QF-PCR results obtain due to placental mosaicism at some level, with normal fetal karyotype. If parents are carriers of a balanced rearrangement offspring can remain undetected with the unbalanced chromosomal abnormality\(^\text{(18)}\).

QF-PCR is not able to recognize chromosomal structural abnormalities\(^\text{(19)}\), although many chromosomal rearrangements have little or no clinical significance. Moreover, the incidence rate of numerical chromosomal abnormalities is 10.2%, which is much higher than 1.9% of structural chromosomal abnormalities\(^\text{(20)}\).

QF-PCR is able to detect aneuploidies on selected chromosomes, X, 13, 18 and 21. There is a need to designed further primers for other chromosomes\(^\text{(15)}\). Therefore, early test result has minor importance. However, QF-PCR is cheaper than karyotyping, but this cannot resolve the matter completely\(^\text{(11,19)}\).

### 2. DISCUSSION

Through the analysis of different studies, QF-PCR is proved as a reliable and precise technique in prenatal diagnostics of trisomies. It can detect aneuploidies with 98.6% accuracy (autosomal chromosomes 13, 18, and 21 and the sex chromosomes)\(^\text{(17)}\). In 17 month’s parallel investigation of QF-PCR and karyotyping no divergence were found\(^\text{(21)}\). QF-PCR results found to be consistent with the cytogenetic analyses\(^\text{(16)}\). Failure in getting a genotype is reported in <1/1000 prenatal samples\(^\text{(9)}\). In another study, QF-PCR correctly recognized fetal sex in all normal samples with 99.7% accuracy without any false positive result\(^\text{(22)}\). In another recent study, QF-PCR is found to have a low sensitivity in identifying sex chromosomes mosaics or mosaic of different trisomies\(^\text{(23)}\).

On the other hand karyotyping is still a standard procedure in a complete prenatal diagnosis, but it is not necessary to perform on all samples. QF-PCR can stand alone in prenatal diagnosis, and it is performed on demand when cell growth failure occurs. In a German study of QF-PCR not a single test of a single chromosome is found uninformative\(^\text{(24)}\). Therefore QF-PCR is found to be very important test whenever culture is failed to grow. Hence it is improving the achievement rate in clinically significant chromosomal aberration.

QF-PCR could be an appropriate technique in the field of prenatal diagnosis, and it could be the first choice for detection of aneuploidies of the women experiencing invasive testing. A proper evaluation of biochemical tests results in combination with an accurate examination of ultrasound can also help in suggesting QF-PCR. The decision of preferring QF-
PCR on full karyotyping can also be based upon the prior history of patients and their families of having any chromosomal abnormality. QF-PCR must be recommended after having a detailed conversation between physician and patients. If there is any history of structural chromosomal abnormality, karyotyping must be performed to avoid all the chances of adverse outcomes, which can remain unknown in QF-PCR. More expansion in primers of specific chromosomes can permit better detection of hidden chromosomal disorders at a lesser price through QF-PCR(25).

3. CONCLUSION

After reviewing the benefits and limitations of QF-PCR technique, it is now clear that QF-PCR is a good addition to molecular genetics in the field of prenatal diagnosis of chromosomal disorders. It is useful for detection of aneuploidies (for chromosomes 13, 18, and 21 and the sex chromosomes). QF-PCR is well-known for its high sensitivity and specificity, without any false positive result, and because of automation can be performed with lesser quantity(17). It is a cost-effective technique with a lesser turnaround time(25). Using QF-PCR one can reduce parental anxiety by reducing the waiting time. Through the rapid detection of aneuploidies with a combination of ultrasound and biochemical analysis, QF-PCR can help in decision making for keeping the pregnancy if results are normal or permit the disruption of pregnancy if any abnormal results are found. Only one expert can run up to 5000 samples annually. Hence QF-PCR can reduce the load of karyotyping greatly(16). Spontaneous miscarriages during the first trimester are caused by aneuploidies very common that can be readily diagnosed through QF-PCR.

These facts ground the perception that QF-PCR is reliable, accurate, and robust, it could be a supreme technique for those societies where the rate of consanguineous marriages is high, like in Pakistan. On the basis of these facts, future application of this technique is inevitable in the field of prenatal diagnosis. In Karachi, Pakistan QF-PCR technique is not established yet, but considering the advantages of this technique, we are looking forward to applying this modern molecular biology technique in our diagnostic centers as well.

REFERENCES