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Molecular karyotyping of 1,295 spontaneous consecutive abortions by sequential analysis with QF-PCR, HGQ-PCR and SNP-array

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ABSTRACT. Spontaneous abortions, which occur in 15-20% of all pregnancies, constitute the most common human genetic disease, since most miscarriages are caused by chromosomal anomalies. In our laboratory, we devised a protocol for sequential use of quantitative fluorescent PCR (QF-PCR), homologous gene quantitative PCR (HGQ-PCR) and SNP-arrays that allows examination of numerical and structural analyses of all chromosomes in spontaneous abortions at low cost. We describe our results with 1,295 samples of fetal tissues collected consecutively after pregnancy losses. Positive signals with QF-PCR and HGQ-PCR were always confirmed using microsatellite amplification of the specific chromosome involved. Among the exams, 64.6% were abnormal. The most common anomalies were trisomies (69.5%), triploidy (13.5%) and monosomy X (9.1%). The most frequent trisomies involved the following chromosomes: 16 (23.5%), 22 (16.0%), 21 (14.1%) and 15 (10.1%). SNP array analysis permitted the diagnoses of all trisomies. Additionally, deletions and/or duplications and chromosomal mosaicism were detected by SNP-array in 23 cases. In

conclusion, our sequential analysis of fetal tissues is a new, highly useful, rapid, and cost-effective approach for the diagnosis of chromosomal alterations in spontaneous abortions.

Key words: Spontaneous abortions; Molecular cytogenetics; SNP-arrays; Quantitative Fluorescent PCR (QF-PCR); Homologous Gene Quantitative PCR (HGQ-PCR)

INTRODUCTION

We now know that the most frequent cause of human miscarriages is genetic and more specifically cytogenetic (due to chromosomal abnormalities). Boué and Boué (1973) in Paris found chromosomal anomalies in 61% of 1500 first-trimester miscarriages studied. Likewise, Hassold (1986) found chromosome disorders in 50.5% of 2919 aborted fetuses weighing less than 500g. These two studies were based on long-term cultures of fetal tissues, a method known to be complex and subject to not allowing conclusive results in many cases in which fetal cell growth does not occur in the laboratory.

Spontaneous abortion, although common, is emotionally devastating and its impact is heightened when the cause is not established. This is because fetal loss is often not properly investigated. Although more than 50% of abortions have proven to have abnormal karyotypes and geneticists have for years emphasized the need for cytogenetic studies of fetal losses, they have not become an integral part of routine obstetrics. One of the reasons is the fact that, in the past, there was often failure of the long-term classical fetal tissue culture, then essential for chromosomal studies. Today, this is no longer true, thanks to significant advances in molecular cytogenetics, as demonstrated in this study.

In the United States, 4% of married women had two fetal losses and 3% had three or more losses (National Center for Health Statistics, 1982). In these cases of recurrence, the fetal karyotype is essential to establish whether the miscarriage is or is not related to a chromosome disorder. When there is chromosomal disease, the cause of fetal loss can be considered established and there is no need to carry out any investigation of the mother. On the other hand, in the absence of a fetal karyotype change, there is an indication for further investigations, which should include chromosomal investigations of the parents, genetic studies for thrombophilia, and exams for malformations of the uterine cavity (American College of Obstetricians and Gynecologists, 2001).

Even in the first miscarriage, there are good reasons for fetal chromosomal studies. The grieving process after fetal loss is complex and associated with anxiety and depression (Neugebauer et al., 1997). In cases where the loss goes unexplained, the patient often experiences self-destructive feelings of guilt and inadequacy, and questions whether the fetal loss occurred because she did or did not do something. Friedman and Gath (1989) interviewed 67 women four weeks after a miscarriage and noted psychiatrically significant depression in 48% of them. Some physicians and health professionals undervalue these feelings of loss, which can result in negative consequences for the doctor-patient relationship, often leading to an unjustified, and unadvised, change of doctor in the subsequent pregnancy (Schuth et al., 1992). Psychological sequelae also often have a negative impact on family relationships, including husbands and other children (Frost and Condon, 1996). Clarifying to the couple that the abortion was caused by a sporadic

chromosomal defect ("a genetic accident") and that the continuation of the pregnancy would be impossible or would culminate in the birth of an abnormal child, significantly contributes to the acceptance of fetal loss. Due to these reasons, all abortions should undergo molecular cytogenetic tests, thus allowing a relevant and quality genetic counseling for the couple, before a new pregnancy.

In this article we demonstrate that with the use of a protocol for sequential use of Quantitative Fluorescent PCR (QF-PCR), Homologous Gene Quantitative PCR (HGQ-PCR) and SNP-Array, which allows the examination of numerical and structural analyses of all chromosomes in spontaneous abortions at low cost, it is now possible to achieve rapid diagnosis of human chromosomal diseases in DNA extracted from aborted fetal tissue without the need for tissue culture. PCR is a highly sensitive and specific technique for detecting minute amounts of DNA, but it is not primarily quantitative. This is because PCR amplification is a complex exponential phenomenon and small differences in initial conditions can have a profound impact on the amount of final product. However, in recent years it has become evident that co-amplifying the target sequence along with known amounts of an internal standard with the same primers generates competitive kinetics that can make PCR an extremely useful comparative quantification tool (Zimmermann and Mannhalter, 1996).

Mansfield (1993) had the idea that in heterozygous individuals, autosomal polymorphic microsatellites could be excellent tools for the establishment of gene dosage, as one allele could serve as a competitive internal control for the other. She used an automated fluorescent DNA sequencer to perform computer-assisted laser densitometry and showed that trisomic patients had, in heterozygous microsatellites, three peaks of equal intensity or two different peaks with an average dose of 2:1. This technical approach later received the name of QF-PCR (quantitative fluorescent PCR).

Several groups of researchers used QF-PCR for the diagnosis of Down syndrome and other trisomies (Mansfield, 1993; Pertl et al., 1994; 1996; Toth et al., 1998; Verma et al., 1998) especially for the prenatal diagnosis of DNA extracted from fetal cells, amniotic fluid, or chorionic villi. The first proposals for the use of molecular cytogenetics by PCR as a unique tool in the prenatal diagnosis of trisomies were made in London by Mann et al. (2001), who published experience with prenatal diagnosis of trisomies 21, 18 and 13 exclusively by PCR. At the same time, QF-PCR was being used for the diagnosis of chromosomal aberrations in spontaneous abortions (Pena, 1998). A problem with QF-PCR was that microsatellites can be homozygous, and thus, uninformative. Hence, several microsatellites for each chromosome should be used. This was not a big problem for prenatal diagnosis since only chromosomes 13, 18 and 21 were examined. However, in the analysis of prenatal losses there was a need for appropriate methodology to diagnose trisomies of all 22 autosomes.

We decided to add to our protocol the technique of homologous gene quantitative-PCR (HGQ-PCR), a method first described by Lee et al. (1997) based on using the same primer pair in different chromosomes. Like QF-PCR, it also establishes internal genomic competition, transforming PCR into a semi-quantitative assay. One difference from QF-PCR is that for trisomies there are no patterns of three peaks, only two peaks with an average dose of 3:2.

Another problem with the diagnosis of chromosomal aberrations in spontaneous abortions is the need for the diagnosis of monosomy X, which constitutes a significant

fraction of cases (Boué and Boué, 1973; Hassold, 1986). We then decided to design HGQ-PCR pairs that could be used for determination of chromosomal sex and for detection of all alterations of number in the sexual chromosomes. We designed two pairs for chromosome X, to wit, Xq23/2p23.3 and 1q32.1/Xp11.3 (Rocha et al., 2022). One of the amplicons of the X chromosome was in the long arm and the other in the short arm. Consequently, they are capable of diagnosing deletions of both arms and isochromosomes. We also designed HGQ-PCR pairs for detection of the human Y chromosome.

In the early 2000s, the development of chromosomal microarray enabled improved detection of copy number variants (CNVs) that were not routinely detected in karyotyping. Comparative genomic hybridization (CGH) was first used to determine the chromosomal constitution of fetuses arising from recurrent spontaneous abortion in 1998 (Daniely et al., 1998). All chromosomes could be studied at the same time and the results were comparable to those obtained by conventional cytogenetic techniques. High-resolution chromosomal analysis allowed for the identification of visible and submicroscopic cytogenetic imbalances However, there were problems with comparative genomic hybridization (CGH), because it was unable to detect triploidy, tetraploidy or uniparental disomy. These problems were later solved using the similar, but more modern, technique of single-nucleotide polymorphism chromosomal microarray analysis (SNP arrays) (Lathi et al., 2012; Wapner et al., 2012; Levy et al., 2014), which could diagnose cases of triploidy, tetraploidy, and uniparental disomy. Wapner et al., (2012) showed that chromosomal microarrays, compared with karyotyping, revealed additional clinically significant CNVs in 6% of fetuses with structural anomalies. This SNP approach uses high density oligonucleotide probes to detect copy number by measuring signal intensities. It detects allele frequency data, which can be used to detect CNVs with increased sensitivity for mosaicism detection. Furthermore, the SNP-based approach will also allow detection of absence of heterozygosity and uniparental isodisomy. Variants of uncertain meaning (VUS) can be detected in about 1-2% of cases (Wapner et al., 2012).

Levy et al. (2014) described the study of a commercial SNP array (CytoSNP-12 genotyping microarray platform, that included 317,000 single-nucleotide polymorphisms across the genome) as a diagnostic test for the evaluation of products of conception. The diagnoses were consistent with previous reports based on traditional cell culture and karyotyping of products of conception. Approximately 59% of samples were cytogenetically abnormal (including 43% single trisomies, 3.5% monosomy X, 6.1% triploidy, 4.6% multiple abnormalities). Structural abnormalities such as translocations, duplications, deletions, or marker chromosomes accounted for 4.7% of cases, of which three (0.16% of all samples) were 10–13 Mb in size and would not have been detected by conventional karyotyping. They concluded that microarray analysis could be applied reliably to the analysis of products of conception with advantages traditional cytogenetic techniques. However, as pointed out by Wenstrom (2014), commenting on the article by Levy et al. (2014), the technique of SNP arrays had the disadvantage of being expensive.

To overcome this problem of elevated price, we decided to build a sequential protocol. Our protocol starts with the application of QF-PCR and HGQ-PCR, which allowed the detection of numerical abnormalities of chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13, 15, 16, 17, 18, 20, 21, 22, X and Y. Positive signals with QF-PCR and HGQ-PCR were always confirmed using microsatellite amplification of the specific chromosome involved.

Only the samples with no molecular evidence of alteration by joint QF-PCR and HGQ-PCR were additionally studied with a SNP-array. Since we expected that numerical abnormalities of chromosomes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22 and X would constitute a very high percentage of the chromosomal aberration in spontaneous abortion, the use of SNP-array is thus reserved for a minority of cases, thus significantly decreasing the average cost per patient. Here we present the results of this protocol in the molecular karyotyping of 1,295 fetal losses.

MATERIAL AND METHODS

Clinical material

Fetal placental tissues from 1,295 fetal losses, spontaneously expelled, obtained by curettage or collected by intra-uterine aspiration were dissected to eliminate maternal decidua. As long as the DNA was not excessively degraded, extraction could be done with fresh, dry, frozen, macerated, contaminated with bacteria or fungi, fixed in ethanol (excellent), or even sometimes material fixed with formalin and/or embedded in paraffin (often the DNA is degraded). Using sterile forceps and scissors, the fetal tissues were separated. If the fetal material came without a preservative, or in absolute alcohol, then lysate and DNA precipitation were performed exactly as described in detail by Pena et al. (1991). If the fetal tissues had been fixed in formalin, incubation in proteinase K was extended for 72 hours before DNA extraction. The study protocol was approved by the institutional ethics commitees and the parents provided informed consent.

Sequential protocol - part 1

The extraction and dosage of the DNA was followed by rapid initial analysis with QF-PCR and HGQ-PCR, which, together, allowed the detection of numerical abnormalities of chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13, 14, 15, 16, 17, 18, 20, 21, 22, X and Y. Eight PCR reactions were performed as follows: QF-PCR-1 amplified *AMEL* (chromosomes X and Y), *D13S317, D18S51, D21S11, PENTAD* (chromosome 21), *TPOX* (chromosome 2) and *D3S1358*; QF-PCR-2 amplified *D16S539, D16S2622, D22S686* and *PENTAE* (chromosome 15); QF-PCR-3 amplified *D9S304* and *D9S938*; QF-PCR-4 amplified *SE33* (*ACTBP2* in chromosome 6); HGQ-PCR-1 amplified the following pairs, where the numbers refer to chromosomes in the order of appearance in the gels: 7-21, 10-21, 5-13, 17-13, 1-18, 18-7 and 2-18; HGQ-PCR-2 amplified 22-3, 22-15 and 22-8; HGQ-PCR-3 amplified 20-4 and HGQ-PCR-4 amplified AMEL, X-2, 19-X, 1-X and Y-8. Using this methodology, we were able to diagnose all cases of triploidy, 45,X, 47,XXX, 47,XXY, 47,XYY and almost all autosomal trisomies.

The primers used in PCR were labeled with one of the fluorochromes Cy5, FAM, VIC, PET and NED and the products of the multiplex system were resolved by electrophoresis in the computerized laser densitometer ABI 3130 (Thermo-Fisher Scientific) or automatic fluorescent sequencer ALF-Express (GE). Positive signals with QF-PCR and HGQ-PCR were always confirmed using microsatellite amplification of the specific chromosome involved. Only the samples with no molecular evidence of alteration by joint QF-PCR and HGQ-PCR were additionally studied with a SNP Array.

Sequential protocol – part 2

The samples with no molecular alteration by joint QF-PCR and HGQ-PCR were additionally studied with the SNP Array CytoSNP-12 (Illumina, San Diego, CA, USA) that included 317,000 single-nucleotide polymorphisms at Eurofins, Denmark. The results were analyzed with the software Genome Studio (Illumina, San Diego, CA, USA) and Nexus 10 Copy Number (BioDiscovery, El Segundo, CA, USA).

RESULTS AND DISCUSSION

As shown in Table 1, among the 1,295 samples of spontaneous abortions, 837 (64.6%) presented numerical or structural chromosomal aberrations and 416 (32.1%) were normal. Thus, our study confirms aneuploidy as the leading cause of miscarriages and demonstrates that most aneuploid conceptuses die *in utero*.

Table 1. Results of the study of 1295 spontaneous abortions.

Results	Number of Cases	%
Abnormal results	837	64.6
Normal results	416	32.1
No fetal material detected	39	3.1
No amplification	3	0.2
Sum	1295	

In only 42 of our 1,295 cases (3.3%), it was not possible to reach a diagnosis because of absence or excessive degradation of fetal tissues in the samples received. This finding demonstrates that our new protocol for sequential use of Quantitative Fluorescent PCR (QF-PCR), Homologous Gene Quantitative PCR (HGQ-PCR), and SNP-arrays allows efficient examination of numerical and structural analyses of all chromosomes in spontaneous abortions.

As disclosed in Table 2, most common anomalies were trisomies (582; 69.5%), triploidy (113; 13.5%) and monosomy X (76; 9.1%). The most common trisomies involved the following chromosomes: 16 (137; 23.5%), 22 (93; 16%), 21 (82; 14.1%) and 15 (59; 10.1%). The list of all chromosomes involved in trisomies is given in Table 3.

Table 2. Abnormal results on the study of 1295 spontaneous abortions.

Abnormal Results	Number of Cases	%
Trisomy	582	69.5
Monossomy X	76	9.1
Autosomal monosomy	9	1.1
Triploidy	113	13.5
Tetraploidy	4	0.5
Hydatiform mole (complete)	2	0.2
Structural alterations	14	1.7
Loss of heterozygosity	28	3.3
Mosaics	9	1.1
Sum	**837**	

Table 3. Trisomy cases on the study of 1295 spontaneous abortions.

Trisomy	Number of Cases	% of Trisomies
47,+1	0	0.0
47,+2	7	1.2
47,+3	11	1.9
47,+4	16	2.7
47,+5	4	0.7
47,+6	9	1.5
47,+7	6	1.0
47,+8	9	1.5
47,+9	22	3.8
47,+10	6	1.0
47,+11	3	0.5
47,+12	7	1.2
47,+13	40	6.9
47,+14	11	1.9
47,+15	59	10.1
47,+16	137	23.5
47,+17	7	1.2
47,+18	32	5.5
47,+19	1	0.2
47,+20	11	1.9
47,+21	82	14.1
47,+22	93	16.0
Double trisomy	9	1.5
Sum	582	

SNP array permitted the diagnoses of trisomies 11, 12, 14 and 19, which had not been initially targeted by QF-PCR and/or HGQ-PCR. In this manner, aneuploidies were identified on all chromosomes, except chromosome 1, which is known to be associated with very early pregnancy losses that normally do not attract medical attention (Hassold and Jacobs, 1984). Additionally, deletions and/or duplications and chromosomal mosaicism were detected by SNP array in 23 cases.

In conclusion, our sequential analysis of fetal tissues is a highly useful, rapid, and cost-effective approach for the diagnosis of chromosomal alterations in spontaneous abortions.

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AUTHOR CONTRIBUTIONS

SDJP conceived the study and wrote the manuscript, CSS conducted the experiments, HBP and JR analyzed the data; all authors read and approved the final version.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

American College of Obstetricians and Gynecologists (2001). Committee on Practice Bulletins--Gynecology. ACOG Practice Bulletin. Clinical management guidelines for obstetrician-gynecologists. Medical management of abortion. *Obstet. Gynecol.* 97: 1-13.

Boué A and Boué J (1973). Evaluation des erreurs chromosomiques au moment de la conception. *Biomedicine.* 18: 372-377.

Daniely M, Aviram-Goldring A, Barkai G and Goldman B (1998). Detection of chromosomal aberration in fetuses arising from recurrent spontaneous abortion by comparative genomic hybridization. *Hum. Reprod.* 13: 805-809.

Friedman T and Gath D (1989). The psychiatric consequences of spontaneous abortion. *Br. J. Psychiatry.* 155: 810-813.

Frost M and Condon JT (1996). The psychological sequelae of miscarriage: a critical review of the literature. *Aust. NZJ Psychiatry.* 30: 54-62.

Hassold TJ (1986). Chromosome abnormalities in human reproductive wastage. *Trends Genet.* 2: 105-110.

Hassold TJ and Jacobs PA (1984). Trisomy in man. *Ann. Rev. Genet.* 18: 69-97.

Lathi RB, Massie JA, Loring M, Demko ZP, et al. (2012). Informatics enhanced SNP microarray analysis of 30 miscarriage samples compared to routine cytogenetics. *PLoS One.* 7: e31282.

Lee HH, Chang JG, Lin SP, Chao HT, et al. (1997). Rapid detection of trisomy 21 by homologous gene quantitative PCR (HGQ-PCR). *Hum. Genet.* 99: 364-367.

Levy B, Sigurjonsson S, Pettersen B, Maisenbacher MK, et al. (2014). Genomic imbalance in products of conception: single-nucleotide polymorphism chromosomal microarray analysis. *Obstet. Gynecol.* 124(2 Pt 1): 202-209.

Mann K, Fox SP, Abbs SJ, Yau SC, et al. (2001). Development and implementation of a new rapid aneuploidy diagnostic service within the UK National Health Service and implications for the future of prenatal diagnosis. *Lancet.* 358: 1057-1061.

Mansfield ES (1993). Diagnosis of Down syndrome and other aneuploidies using quantitative polymerase chain reaction and small tandem repeat polymorphisms. *Hum. Mol. Genet.* 2: 43-50.

National Center for Health Statistics (1982). Health, United States, 1982. DHHS Pub. No. (PHS) 83-1232. Public Health Service. Washington. U.S.

Pena SDJ (1998). Molecular cytogenetics I: PCR-based diagnosis of human trisomies using computer-assisted laser densitometry. *Genet. Mol. Biol.* 21: 317-322.

Pena SD, Macedo AM, Gontijo NF, Medeiros AM, et al. (1991). DNA bioprints: simple nonisotopic DNA fingerprints with biotinylated probes. *Electrophoresis*. 12: 146-152

Pertl B, Weitgasser U, Kopp S, Kroiser PM, et al. (1996). Rapid detection of trisomies 21 and 18 and sexing by quantitative fluorescent PCR. *Hum. Genet.* 98: 55-59.

Pertl B, Yau SC, Sherlock J, Davies AF, et al. (1994). Rapid molecular method for prenatal detection of Down's syndrome. *Lancet.* 343: 1197-1198.

Rocha JLF, Pena HB and Pena SDJ (2022). MQS-PCR: a simple and quick multiplex PCR molecular method for human chromosomal sex determination in DNA samples. *Genet. Mol. Res.* 21: gmr19067.

Schuth W, Posselt E and Breckwoldt M (1992). Miscarriage in the first trimester--a "non-event"? An empirical study of 40 women and their physicians of experiences and coping. *Geburtshilfe Frauenheilkd.* 52: 742-748.

Toth T, Findlay I, Papp C, Toth-Pal E, et al. (1998). Prenatal detection of trisomy 21 and 18 from amniotic fluid by quantitative fluorescent polymerase chain reaction *J. Med. Genet.* 35: 126 -129.

Verma L, Macdonald F, Leedham P, McConachie M, et al. (1998). Rapid and simple prenatal DNA diagnosis of Down's syndrome. *Lancet.* 352: 9-12.

Wapner RJ, Martin CL, Levy B, Ballif BC, et al. (2012). Chromosomal microarray versus karyotyping for prenatal diagnosis. *N. Engl. J. Med.* 367: 2175-2184.

Wenstrom KD (2014). Microarray analysis: elegant, accurate, and expensive. *Obstet. Gynecol.* 124(2 Pt 1): 199-201.

Zimmermann K and Mannhalter JW (1996). Technical aspects of quantitative competitive PCR. *Biotechniques.* 21: 268-279.