Comparison of cytogenetics and molecular karyotyping for chromosome testing of miscarriage specimens

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Objective: To compare chromosome testing of miscarriage specimens between traditional cytogenetic analysis and molecular karyotyping using single nucleotide polymorphism microarrays (SNP) and array comparative genomic hybridization (aCGH).
Design: Prospective blinded cohort study.
Setting: University–based practice.
Patient(s): Women undergoing dilation and curettage for first-trimester miscarriage between March 2014 and December 2015.
Intervention(s): None.
Main Outcome Measure(s): Chromosome analysis from chorionic villi separated equally and submitted for cytogenetics, SNP microarray, and aCGH testing.
Result(s): Sixty samples were analyzed, of which 47 (78%) were chromosomally abnormal. A correct call was defined when a result was concordant with at least one other testing platform. The correct call rate was 85%, 93%, and 85% using cytogenetics, SNP array, and aCGH, respectively. We found a 33% overall discordance rate between results. Discordances were due to maternal cell contamination, balanced chromosome rearrangements, polyploidy, and placental mosaicism. Mosaicism was detected in 18% of all samples. Growth failure occurred in four samples sent to cytogenetics, of which three were chromosomally abnormal by molecular testing.
Conclusion(s): This study demonstrates the many technical limitations of the three testing modalities. Our rates of maternal cell contamination were low, but it is important to note that this is a commonly reported limitation of cytogenetics. Given the similar overall performance of the three testing modalities, providers may choose a method based on individual availability and consideration of limitations as it applies to each clinical scenario. The unexpected high rate of placental mosaicism warrants further investigation. (Fertil Steril® 2017;107:1028–33. ©2017 by American Society for Reproductive Medicine.)
Key Words: Chromosome analysis, cytogenetics, miscarriage, molecular karyotype analysis

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Spontaneous abortion occurs in 10% to 15% of all clinically recognized pregnancies (1). Chromosome abnormalities account for 65% to 70% of miscarriages, of which autosomal trisomy is the most common (2). Chromosome testing of products of conception (POC) offers valuable information to both the provider and couple and can aid in future clinical management. One study found only 8% of first-trimester miscarriages underwent chromosome analysis, but the majority of couples desire testing (3). These results often provide an explanation for the miscarriage and help couples cope with the emotional aspects of the pregnancy loss (4, 5). Furthermore, chromosome testing in women with recurrent pregnancy loss offers cost savings by decreasing the need for other investigational studies to identify other causes (6, 7).

The current gold standard of POC testing is with metaphase karyotype, which is typically performed by the cytogenetics department at each

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institution. However, this method has practical limitations, including the reliability of successful cell culture with failure rates ranging from 10% to 40%. A cytogenetic result of 46,XX, which occurs in 55% to 80% of cases, cannot exclude the presence of maternal cell contamination (MCC) (8). In addition, prolonged culture may lead to culture artifacts, which may produce inaccurate results. Finally, due to the band resolution of this method, submicroscopic deletions and duplications, typically less than 5 Mb, cannot be detected.

Newer molecular methods have offered novel techniques to analyze miscarriage specimens. These include single nucleotide polymorphism (SNP) microarrays and array comparative genomic hybridization (aCGH). These technologies offer faster results with higher resolution and minimal tissues requirements. Patients may also send their samples collected at home within 7 to 14 days of passing tissue. In addition, molecular platforms can detect MCC and can be applied to tissue stored in paraffin blocks from prior miscarriages. To date, there have been no studies to compare these three modalities head to head.

The objective of this study was to identify the best method for chromosome testing of miscarriage specimens by comparing results from cytogenetics, SNP array, and aCGH platforms. To achieve this, we performed a prospective, blinded comparison of these three methods.

MATERIALS AND METHODS

We performed a prospective, blinded study at an academic reproductive health and infertility practice. This study was approved by the Stanford University institutional review board. All patients undergoing dilation and curettage (D&C) for a documented intrauterine pregnancy loss in the first trimester from March 2014 to December 2015 were eligible. Verbal and written consent was obtained from all patients before the procedure.

A missed abortion was diagnosed by transvaginal ultrasound and confirmed by repeat ultrasound before the D&C. The procedure was performed under ultrasound guidance in the usual fashion using manual vacuum aspiration. The POC was separated to identify chorionic villi using a technique used to minimize MCC (9). If chorionic villi could not be clearly identified, the patient was excluded from the study. Once the chorionic villi was cleaned, it was separated into three equal parts and sent for routine cytogenetic testing, SNP array testing, and aCGH analysis. Samples were labeled with a unique deidentified number. A maternal blood sample was also obtained and sent to the molecular karyotyping laboratories for detection of MCC and parental source of aneuploidy; the latter was only obtained by SNP array methodology. Each laboratory reported their results to the study site and was blinded to the results of the other testing modalities.

Cytogenetic testing was performed by a university reference laboratory using standard cell culture and analysis by Giemsa-trypsin-wrights banding methods. Tissue was treated to arrest cells in metaphase using Giemsa to stain bands in the chromosomes, using a method referred to as G-banding. When two different karyotypes were identified by cytogenetics, mosaicism was confirmed by identifying the same karyotypes in a separate, independent culture.

Molecular testing was performed by two separate laboratories for SNP microarray testing and aCGH analysis. For SNP array testing, genotyping of maternal and POC samples was performed at a commercial reference laboratory using Illumina CytoSNP-12 genotyping microarrays, which measure approximately 300,000 SNPs across the genome (roughly one every 10 kb) according to the manufacturer's instructions. After a genomic sample has been run on a SNP array, the results must pass an in-house quality control test before further analysis is done. The Parental Support algorithm is then used to determine the number and origin of each chromosome. In short, the allele ratios are calculated for each locus across a chromosome, and the clustering of allele ratios is indicative of the copy number for that chromosome; mosaic samples have their own distinct allele ratio clustering patterns. Complex mosaic results cannot be easily defined using intensity ratios and are typically reported out as either monosomy/disomy mosaics or trisomy/disomy mosaics. Comparison of the SNP identities between the maternal and POC data is used to identify MCC, parental origin of aneuploidy and unbalanced chromosome segments. Additional details regarding this testing platform are described by Lathi et al. (10).

The aCGH was performed at a commercial reference laboratory using a BlueGnome 24Sure bacterial artificial chromosome (BAC) microarray (Illumina). This type of array contains approximately 3,000 BAC probes covering approximately 30% of the genome, with a resolution of 5 Mb to 10 Mb. The DNA was first extracted using a standard Qiagen kit. Amplification was conducted using AmpFlSTR Identifiler Plus kits (Promega) The use of short-tandem repeat markers (STRs) was added to the analysis for the second half of this study (samples 31–60) to increase the detection of maternal cell contamination and polyploidy. Short-tandem repeats are repetitive sequences of DNA that can be measured to compare specific loci of DNA from two or more samples. We included 15 autosomal STR markers (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, and FGA) and Amelogenin, the sex-determining marker (Applied Biosystems AmpFlSTR Identifiler Direct User Guide, 2012).

A correct call rate was defined as a result that was concordant with at least one other testing platform, unless the result was incorrect due to a known inherent limitation of the testing platform detected by the third platform. For example, in cases of structural abnormalities detected by cytogenetics (i.e., balanced tetraploidy), molecular testing results, although concordant, were labeled incorrect.

Discordant results were defined as any two results that were not identical. In the event of a discordant result between molecular karyotype results or in discordant results from cytogenetics not explained by technical limitations of the molecular testing methods (i.e., balanced tetraploidy, chromosome rearrangements), an aliquot of DNA from the two molecular tests was swapped to reanalyze on the other molecular platform. Tissue from the cytogenetics analysis was unavailable for this aspect of the study. During the second half of this study (samples 31–60), discordant samples

and a random sampling of concordant samples were swapped and blinded prior to reanalysis. We defined placental mosaicism by a single modality with two different results or discordance results, which were concordant when swapped and reanalyzed on the other molecular platform. When a single test detected mosaicism while the other platforms only detected one karyotype and the option of swapping samples was not available, the mosaic result was considered an incorrect call. Discordances due to technical limitations were defined as discordant swapped results that could not be explained by the inherent limitations of each testing modality. These cases were not classified as mosaic.

A kappa analysis was performed to measure the agreement between the three testing modalities. In brief, a kappa value of 0 indicates agreement simply by chance, while a value of 1 indicates perfect agreement. We used a commonly cited scale to determine the degree of agreement among the three testing modalities (11). This analysis was performed using STATA software (StataCorp).

RESULTS

Sixty miscarriage specimens were collected during the study period. Of these, 47 (78%) of 60 were chromosomally abnormal by at least one testing modality. The mean maternal age at the time of miscarriage was 37 years (range: 29–46 years) and average BMI was 24.8 (range: 18–41). The mean gestational age at the time of D&C was 8.4 weeks (range: 6.5–10) (Table 1). Most women were Caucasian (48%) and had conceived with in vitro fertilization (56%). We found that 42% of women had diminished ovarian reserve, and 48% had a history of recurrent pregnancy loss. The remainder of women were seen in our infertility clinic for a history of endometriosis (3%), male factor infertility (12%), polycystic ovary syndrome (12%), and unexplained infertility (8%) (Table 1).

Of the 60 samples analyzed in our study, 40 (66%) of 60 samples demonstrated concordant results. This included two cases of growth failure; but both molecular techniques reported concordant results. Of the 40 concordant samples, 13 were euploid and 27 aneuploid (data not shown). The kappa value comparing the agreement between these three testing modalities was 0.65, which represents "substantial agreement" (11).

The remaining 20 (33%) discordant results of 60 are presented in Table 2. Discordant samples were further categorized into mosaic, mosaic versus culture artifact, MCC, polyploidy, chromosome rearrangements, and technical differences. Mosaic versus culture artifact included samples in which molecular results were concordant but the cytogenetics revealed a different result. Correct call rates were 85% (51 of 60), 93% (56 of 60), and 85% (51 of 60) for cytogenetics, SNP array, and aCGH, respectively. The results indicating "incorrect calls" based on our definition stated here are shown in the table. Of the 47 (78%) chromosomally abnormal samples by at least one testing modality, 72% (34 of 47) were due to chromosomal trisomies, and 89% (42 of 47) were of maternal origin, as determined by the SNP array methodology. The other abnormalities included polyploidy, chromosome inversion, and a translocation. Of the 13 euploid results, the ratio of XX to XY was 4:9. The result turnover times were 23 versus 9 versus 8 days ($P<.01$) for cytogenetics, aCGH, and SNP array, respectively.

There were a total of four samples (7%) with growth failure using cytogenetics. All four cases were characterized by minimal chorionic villi and/or prolonged time from diagnosis of miscarriage to D&C. Molecular testing was able to provide results for all four cases, three of which were chromosomally abnormal. Of the cytogenetic results, 11% (6 of 56), which excludes cases of growth failure, were inconclusive due to a 46,XX result. This alone could not definitely exclude MCC, without additional information from the molecular methods.

Among discordant results, 13 (65%) of 20 were clinically significant, as defined by a discordance in which at least one result was normal while another was abnormal. The 20 discordant samples were characterized by placental mosaicism (n = 11), polyploidy (n = 4), growth failure (n = 2), unexplained discordances (n = 2), and chromosome inversion (n = 1). Of note, among the four cases of polyploidy, two discordances were prior to the addition of STR analysis to the aCGH platform.

Among the discordant samples, there were eight cases in which molecular testing results were concordant while cytogenetics revealed a discordant result. Of these cases, three were explained by inherent limitations of molecular testing, including tetraploidy and chromosome rearrangements. Two cases were euploid by both molecular methods while cytogenetics detected an autosomal trisomy. In the remaining three cases, cytogenetics detected mosaicism, which included the karyotype detected by the molecular method. In these latter five cases, we were not able to determine whether the abnormalities detected in cytogenetics were due to low level mosaicism or a testing artifact.

Due to the high rate of discrepancies and mosaicism detected in our primary analysis, we attempted to further clarify which discrepancies were due to mosaicism versus technical differences between testing modalities by swapping the samples and rerunning the DNA on the other testing platform.

TABLE 1

Demographics of patients undergoing D&C for first-trimester miscarriage.

Characteristic	Value
Maternal age, y (range)	37 (29–46)
Ethnicity, %	
Caucasian	48
East Asian	30
South Asian	20
Hispanic	2
Maternal BMI, kg/m^2 (range)	24.8 (18–41)
Mode of conception, %	
Spontaneous	17
IUI	27
IVF	56
Gestational age at time of D&C, wk (range)	8.4 (6.5–10)
Mean sac diameter (mm)	18.7

Note: BMI = body mass index; D&C = dilation and curettage; IUI = intrauterine insemination; IVF = in vitro fertilization.

Shah. Chromosome testing of miscarriage specimens. Fertil Steril 2017.

TABLE 2

Products of conception chromosomal analysis: discordant results.

Discordant result category	Cytogenetics	SNP + PSA	aCGH + STR
Mosaicism	47,XY,+17	46,XY (UPD 17)	46,XY[a]
	47,XX,+7	46,XX/45XX,−7	47,XX,+7
	47,XXX,+21 der (21;21) (q10; q10)	46XX/48,XXX, +9 OR 46,XX/ 44,X,−9	46,XX[a]
	46,XX	46,XX/45XX,−1[a]	46,XX
	45,XY, −21	46,XY/47,XY,+21 or 46,XY/ 45,XY,−21	47,XXY
	47,XY,+3	47,XY,+3	46,XY
Mosaicism vs. culture artifact	51,XXYY,+3,+5,+18[a]	46,XY	46,XY
	47XX,+2/46,XX[a]	46,XX	46,XX
	47,XY, +12[a]	46,XY	46,XY
	47,XY,+22/48,XY,+7,+22[a]	47,XY,+22	47,XY,+22
	69,XXY,del(14)(q32)/ 68,XXY,−14[a]	69,XXY	69,XXY
Maternal cell contamination, polyploidy, chromosome rearrangements	Growth failure[a]	47,XX, +8	MCC
	Growth failure[a]	68,XY	MCC
	69,XXX	69,XXX	46,XX[a,b]
	92,XXYY	46,XY[a]	46,XY[a]
	92,XXYY	46,XY[a]	46,XY[a]
	70,XXY, +15	70,XXY, +15	47,XY, +15[a,b]
	46,XX, inv(9)(p11q13)	46,XX[a]	46,XX[a]
Technical differences	47,XX, +22	47,XX,+22	46,XX[a]
	46,XX	46,XX	46,XX (+chr13 35MB)[a]

Note: Array nomenclature has been adjusted to reflect karyotype nomenclature. MCC = maternal cell contamination; PSA = parental support algorithm; STR = short tandem repeats; UPD = uniparental disomy.

[a] Results indicate incorrect calls.

[b] Results obtained before addition of STR analysis.

Shah. Chromosome testing of miscarriage specimens. Fertil Steril 2017.

This could not be done for cytogenetics because this method requires intact viable cells for culture, therefore it was only performed between the molecular platforms. Reanalysis of discordant samples was performed on 18 samples. Of these, five demonstrated identical results when the same DNA sample was run on each molecular platform. The remaining 13 discordances could be explained by inherent or technical limitations of the testing methods (data not shown).

DISCUSSION

Chromosomal abnormalities account for the majority of first-trimester miscarriages and provide important information to establish causality of a pregnancy loss. Providing accurate results is essential to both the provider, for the purposes of counseling and future pregnancy planning, and the couple facing the loss, for grieving purposes and reducing self-blame (4, 5). To our knowledge, this study is the first and largest to prospectively compare cytogenetic testing with SNP array and aCGH platforms. The results highlight the limitations of each testing modality that contribute to the high discordance rate found in our study.

Conventional karyotyping is currently considered the gold standard of POC testing and offers important advantages over molecular techniques, including the detection of balanced tetraploidy (2:2) and Robertsonian translocations. Tetraploidy is found in 2% of POC samples, consistent with the findings in our study (2). Similarly, Robertsonian translocations are found in approximately 0.5% of POC samples, and was detected in one sample in our study (12). Balanced translocations are not thought to directly contribute to the cause of miscarriage, but they can be a risk factor for future miscarriages if the chromosomal products are unbalanced. Additionally, cytogenetics can detect low-level mosaicism below the threshold detected by molecular methods.

It is possible that at least three cases listed under mosaic versus culture artifact represent true results as the cytogenetic result included similar karyotypes detected by the molecular platforms. This would improve the correct call rate of cytogenetics to 90%. While our correct call rate analysis may place cytogenetics at a disadvantage, we felt this analysis allowed for removal of any bias toward a method considered to be the gold standard, and more importantly reflects the reality of the inability to reanalyze cytogenetic specimens after processing.

Cytogenetic testing does have important pitfalls that molecular techniques can overcome. These include the inability to detect MCC, which can occur in 29% to 58% of 46,XX results and can prevent reporting these results with certainty (8). Our rates of MCC were lower than reported in the literature, owing to our separation technique (9). Another important limitation of cytogenetics is growth failure, which occurred in 7% of our cases; however, this varies widely among institutions and may be as high as 40% (8). All cases occurred when minimal chorionic villi sample was obtained. However, in all four cases, both molecular methods were able to detect the karyotype; it is interesting that, of these, 75% were chromosomally abnormal. These findings are

similar to a prior study in which 70% of all POC samples with growth failure on cytogenetic testing were found to contain a chromosomal abnormality on aCGH testing (13).

In 2013, the American College of Obstetricians and Gynecologist published a committee opinion that stated that limited data was available to support the routine use of microarray testing for first-trimester miscarriage specimens (14). Shortly thereafter, a large cohort analysis was performed on 2,389 POC samples using SNP-based microarray testing, which demonstrated a 22% MCC rate; among the 40.6% cytogenetically normal samples, they found a 2% rate of clinical abnormalities by SNP array analysis (15). These results challenged the new standard of care for POC testing. In our study, SNP-based array testing offered a 93% correct call rate, with the inability to detect balanced chromosome rearrangements; however, it outperformed the other modalities by detecting MCC, uniparental disomy, and parental source of aneuploidy.

Several studies using aCGH for POC testing have demonstrated improved diagnostic yield as compared with routine cytogenetic testing (16). In our study, aCGH performed similar to SNP array testing in its ability to detect MCC and triploidy (with the use of STR analysis). The correct call rate was 85% with similar limitations to SNP testing. In addition, placental mosaicism was not detected, which is a known limitation of this platform, and contributed to the lower correct call rate (17). We found that 3% of samples were inconclusive due to detection of MCC, but once the samples were swapped, aCGH provided concordant results with SNP testing. Inclusion of this swapped data in addition to the two missed polyploidy cases before STR analysis would improve the correct call rate of aCGH to 92%.

Both molecular testing modalities provided a significantly faster turnover time compared with cytogenetics. This is an important advantage to these methods when considering the emotional aspect of coping with a pregnancy loss. Earlier knowledge of the potential cause of a miscarriage may help couples reduce anxiety and begin healing and moving forward with their next attempt to pregnancy.

The cost of each testing modality varies based on the institution and insurance provider, but it is an important consideration in choosing a method. In our study, the amount billed to the insurance provider for cytogenetics, SNP array, and aCGH was $6,600, $6,600, and $4,000 (USD), respectively. The out-of-pocket costs can vary widely, but most insurance providers cover 90% to 100% of the total cost.

Our study design of dividing the sample into three parts and analyzing them separately revealed a higher than expected mosaicism rate (10% to 18%) among first-trimester miscarriages, as only detected by cytogenetics and/or the SNP methodology. The lower range (10%) excludes the cases of possible culture artifact. These higher rates may be related to our unique study population, which included patients with recurrent pregnancy loss and diminished ovarian reserve.

Our current findings reflect results in women with infertility and recurrent pregnancy loss and may not be generalizable to the general population of women with first-trimester miscarriages. Another important limitation of our study was the inability to reanalyze cytogenetic results on the molecular platforms and the smaller specimen provided for analysis, which may have resulted in higher MCC rates. Finally, an important consideration in this study was the early detection of miscarriage and short time interval to D&C due to the clinical practice setting in which these patients were recruited. The optimized tissue specimens likely contributed to the high accuracy of the cytogenetic results, which rely heavily on tissue quality. In most clinical practices, however, miscarriages are diagnosed after the onset of symptoms (that is, bleeding), so the ability to confidently provide results, particularly when minimal tissue is present or when tissue quality is poor, may be improved using molecular techniques.

This study has many strengths. To our knowledge, this is the first and largest study to compare three different testing modalities on a single miscarriage specimen head to head. By comparing these testing platforms without assigning a gold standard, our analysis was performed using an unbiased approach. The addition of swapping discordant samples allowed for detection of placental mosaicism as well as identification of technical differences between testing methods. The patients were provided with all the results, which was very helpful for counseling purposes and in prompting additional testing to help determine the underlying cause of their pregnancy loss.

In conclusion, no single modality detected all abnormalities, and a high discordance rate was present due to the inherent limitations of each test. Given similar detection rates between cytogenetics and molecular methods, we suggest that providers choose a method based on individual availability and consideration of the strengths and limitations of each test as it applies to each clinical scenario. All three methods provide important information regarding a possible genetic cause of miscarriage, and we encourage providers to perform chromosomal testing when possible to help counsel patients and aid in future pregnancy planning.

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