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Detection of Aneuploidies in Products of Conception and Neonatal Deaths in Iranian Patients Using the Multiplex Ligation-Dependent Probe Amplification (MLPA)

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Abstract

Background: Around 70% of all pregnancies (Including 15% of clinically-recognized ones) are lost due to various fetal or maternal disorders. Chromosomal aneuploidies are among the most common causes of pregnancy loss. Standard chromosome analysis using G-banding technique (Karyotype) is the technique of choice in studying such abnormalities; however, this technique is time-consuming and sensitive, and limited by vulnerabilities such as cell culture failure. The use of molecular cytogenetic techniques, including array-based techniques and Multiplex Ligation-Dependent Probe Amplification (MLPA), has been proposed to overcome the limitations of this method to study the products of conception. This study has been designed to investigate the feasibility of using MLPA technique as a standalone genetic testing, with histopathologic examinations and genetic counseling to detect aneuploidies in products of conception and neonatal deaths.

Methods: Forty-two verified fetal and neonatal samples were studies and genetic counseling was scheduled for all parents. Histopathologic examinations were carried out on the products of conception, and appropriate fetal tissues were separated for genetic studies. Following DNA extraction and purification, MLPA was carried out to investigate chromosomal aneuploidies.

Results: Nine samples (21.42%) were diagnosed to be affected with aneuploidy. Detected aneuploidies were trisomy 22 (n=3), trisomy 21(n=1), trisomy 18 (n=2), trisomy 16 (n=1), trisomy 13 (n=1), and monosomy of chromosome X (n=1). The MLPA analysis results were conclusive for all of the fetal samples (Success rate: 100%).

Conclusion: These results suggest that MLPA, as a standalone genetic testing, is an accurate, rapid, and reliable method in overcoming the limitations of standard cytogenetic techniques in genetic investigation of products of conception.

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Introduction

Approximately 70% of all pregnancies including 15% of clinically detected ones are lost due to various fetal or maternal disorders [1,2]. Parents often want to know the reasons for the pregnancy loss. Identifying causes can help determine the aneuploidy recurrence risk in future pregnancies, which is particularly relevant in cases of pregnancy loss. Chromosomal aneuploidy is one of the most common causes of pregnancy loss [3,4].

The metaphase karyotyping is the technique of choice to study common genetic causes of pregnancy loss and neonatal deaths. However, metaphase karyotyping requires cell culture, which leads to long turnaround time for results. Also, there is a risk of gaining no results due to high culture failure rates of 10-40% and the potential for selective overgrowth of maternally derived cells leading to a 46, XX karyotype despite underlying fetal abnormality in 29-58% of cases [5-10].

However, molecular cytogenetic techniques such as Fluorescence In Situ Hybridization (FISH), Quantitative Fluorescence PCR (QF-PCR), Comparative Genomic Hybridization (CGH), and array CGH can detect copy number variations, but they have some limitations. QF-PCR, CGH, and FISH are restricted by the number of target chromosomes that can be analyzed for each assay. Although array CGH as a more comprehensive technique can overcome the limitations, it remains an expensive technique which is not available in several genetic laboratories in Iran. Regarding these facts, perhaps in some instances, Multiplex Ligation-dependent Probe Amplification (MLPA) would be a better alternative to the mentioned techniques for detecting chromosome aneuploidies [9,11-15]. This research was carried out to investigate the feasibility of using MLPA technique as a standalone genetic testing, together with histopathologic findings and genetic counseling data, to detect aneuploidies in products of conception and neonatal deaths.

Materials and Methods

Totally 50 samples of products of conception and neonatal deaths from patients referred to Department of Genetics and Pathology of Hope Generation Foundation and Avicenna Fertility Center were collected for genetic analysis regarding the cause of pregnancy loss or neonatal death. Medical records of each case including screened records were reviewed by a certified genetic counselor. A pathologist conducted internal and external examinations of neonatal deaths; chest and abdominal cavities, the central nervous system, and in some cases, the position of placenta were inspected. Abnormal pathological findings were later cross-referenced with genetic findings. Histopathologic examinations were also carried out on products of conception and neonatal deaths. Eight samples had no embryonic or neonatal tissue, therefore, the remaining forty-two samples were considered for genetic study by a pathologist. This study was performed on thirty-three fetal and nine newborn samples. Collected data set from genetic counseling and histopathologic examinations were entered into the genetic data management system.

A pathologist examined the samples and collected suitable fetal or neonatal tissues for genetic analysis into a sterile falcon tube. Some samples which were paraffin-embedded (2/42) or had a long interval between miscarriage or fetal death until genetic testing (40/42) were sent for MLPA testing as they were not suitable for culture. DNA extraction was performed on cells from 20 mg of tissues using phenol-chloroform DNA extraction method. The concentration and quality of recovered DNA was estimated by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA).

MLPA allows the relative quantification of more than 40 sequences in a single multiplex assay using only 20 ng of sample DNA. Denatured genomic DNA was hybridized with a set of two probes, which consisted of a target-specific sequence and a universal forward or reverse PCR primer binding site. One probe had a stuffer sequence to generate various PCR products with different sizes. SALSA MLPA probemix P036B and P070 contained different probes for each chromosome's subtelomeric region and two probes for non-telomeric Y chromosome-specific sequences. After ligation, products were amplified by PCR using only one fluorescent-labeled primer pair. The multiplex-fluorescent products were separated by capillary electrophoresis. The relative amounts of amplified products depended on the quantity of target DNA present in the sample. Therefore, aneuploidy was suspected if probes for both arms of chromosome appeared deleted (Monosomy) or duplicated (Trisomy).

MLPA reaction was performed in a GeneAmp PCR System 9700 (Applied Biosystems, USA) according to manufacturer's instructions. PCR products were electrophoresed in an ABI Prism 3130 Genetic Analyzer and analyzed with GeneMapper software v.4. MLPA peak areas were exported to a Microsoft Excel datasheet to calculate relative peak areas of the amplified probes as a fraction of total sum of peak areas by Coffalyser software v. 9.4.

All procedures were carried out in accordance with the ethical standards of the local committee and the revised version of Declaration of Helsinki in 2013.

Results

MLPA detected chromosome aneuploidies in 21.42% (9/42) of cases. Typical profiles for normal and abnormal samples are shown in figure 1. All forty-two verified fetal and neonatal samples, which could not be cultured, were analyzed. In general, 95.23% (40/42) of samples were marred by time (Not fresh), and 4.76% (2/42) were paraffin-embedded samples. MLPA gave a conclusive result in all 42 samples. MLPA detected chromosome aneuploidies in 9/42 (21.42%) of cases. These included trisomy 21, 1/9 (11.1%), monosomy X, 1/9 (11.1%), trisomy 18, 2/9 (22.2%), trisomy 13, 1/9 (11.1%), trisomy 16, 1/9 (11.1%), and trisomy 22, 3/9 (33.3%).

Among male fetuses, 79.17% were normal, and among female fetuses, 77.78% were normal. According to Fisher's exact test, there was no difference between normal girls and boys (Table 1).

Distributions of gestational age in total samples were 12/42 (28.57%), from the first trimester, 15/42 (35.71%) the second trimester, 6/42 (14.28%) the third trimester, and 9/42 (21.42%) neonatal samples. When chromosomal abnormalities were analyzed according to the gestational age of the samples, 5/9 (55.55%) of first trimester losses, 3/9 (33.33%) of second trimester losses, and 1/9 (11.11%) of all neonatal deaths with preterm labor in 30 weeks occurred in embryonic specimens with chromosomal abnormalities (Table 2). Data showed no significant difference between gestational

A

Gene	Chr Pos	Length	MV70	Recommended order	Ratio
TNFRSF18 probe 2270-L1762	01p36.3	306	01-001.0 TNFRSF18	1	Normal (0.97)
SH3BP5L probe 4084-L3605	01q44	132	01-247.1 SH3BP5L	2	Normal (0.76)
ACP1 probe 2709-L2856	02p25	315	02-000.3 ACP1	3	Normal (1.02)
ATG4B probe 2781-L3168	02q37.2	139	02-236.3 ATG4B	4	Normal (0.93)
CHL1 probe 2896-L2363	03p26.1	323	03-000.3 CHL1	5	Normal (1.04)
KIAA0226 probe 2690-L2842	03q29	145	03-198.8 KIAA0226	6	Normal (0.84)
PIGG probe 14440-L16146	04p16.3	329	04-000.5 PIGG	7	Normal (0.91)
FRG1 probe 2691-L2843	04q35	152	04-191.1 FRG1	8	Normal (1.07)
LOC133957 probe 2791-L2233	05p15.33	337	05-000.3 LOC133957	9	Normal (0.86)
GNB2L1 probe 2790-L2232	05q35.3	160	05-180.6 GNE2L1	10	Normal (0.84)
IRF4 probe 4077-L3462	06p25-p23	346	06-000.3 IRF4	11	Normal (0.9)
TBP probe 2694-L2844	06q27	166	06-170.7 TBP	12	Normal (0.94)
UNC84A probe 2780-L2857	07p22.3	355	07-000.9 UNC84A	13	Normal (0.92)
VIPR2 probe 2793-L3167	07q36.3	172	07-158.6 VIPR2	14	Normal (1.04)
FBXO25 probe 2715-L0973	08p23.3	362	08-000.4 FBXO25	15	Normal (0.94)
RECQL4 probe 2655-L0610	08q24.3	179	08-145.7 RECQL4	16	Normal (0.9)
DOCK8 probe 2716-L0688	09p24.3	370	09-000.4 DOCK8	17	Normal (0.97)
EHMT1 probe 2792-L2846	09q34.3	186	09-139.8 EHMT1	18	Normal (1.06)
ZMYND11 probe 5180-L16343	10p14	379	10-000.2 ZMYND11	19	Normal (1.04)
ECHS1 probe 2696-L2847	10q26.2-q26.3	193	10-135.0 ECHS1	20	Normal (1)
BET1L probe 2784-L2226	11p15.5	387	11-000.2 BET1L	21	Normal (0.88)
IGSF9B probe 2697-L2848	11q25	202	11-133.3 IGSF9B	22	Normal (1.07)
JARID1A probe 2787-L2229	12p11	393	12-000.3 JARID1A	23	Normal (0.96)
ZNF10 probe 2686-L2849	12q24.33	211	12-132.2 ZNF10	24	Normal (1.05)
PSPC1 probe 2717-L3608	13q12.11	402	13-019.2 PSPC1	25	Normal (1)
CDC16 probe 2698-L0753	13q34	218	13-114.0 CDC16	26	Normal (1.21)
PARP2 probe 2718-L0732	14c11.2-q12	409	14-019.9 PARP2	27	Normal (1)
MTA1 probe 2699-L2850	14q32.3	226	14-105.0 MTA1	28	Normal (1.06)
NDN probe 4026-L1542	15c11.2-q12	418	15-021.5 NDN	29	Normal (0.9)
TM2D3 probe 2701-L2851	15q26.3	233	15-100.0 TM2D3	30	Normal (1.2)
DECR2 probe 2720-L0648	16p13.3	427	16-000.4 DECR2	31	Normal (0.89)
GAS8 probe 2702-L0734	16q24.3	241	16-088.6 GAS8	32	Normal (1.04)
RPH3AL probe 4081-L3465	17p13.3	436	17-000.1 RPH3AL	33	Normal (1.03)
SECTM1 probe 2703-L3169	17q25	250	17-077.9 SECTM1	34	Normal (1)
THOC1 probe 2789-L2231	18p11.32	444	18-000.2 THOC1	35	Normal (1.1)
CTDP1 probe 2704-L3607	18q23	258	18-075.6 CTDP1	36	Normal (0.94)
PPAP2C probe 3501-L2880	19p13	450	19-000.2 PPAP2C	37	Normal (0.89)
CHMP2A probe 2705-L2853	19q13.43	265	19-063.8 CHMP2A	38	Normal (1.03)
ZCCHC3 probe 2723-L0641	20p13-p12.2	459	20-000.2 ZCCHC3	39	Normal (1.03)
UCKL1 probe 2706-L0642	20q13.33	274	20-052.1 UCKL1	40	Normal (1.26)
STCH probe 2724-L0334	21q11	466	21-014.7 STCH	41	Normal (1.12)
S100B probe 2587-L2854	21q22.3	281	21-046.8 S100B	42	Normal (1.04)
IL17RA probe 2725-L16344	22q11.1	478	22-016.0 IL17RA	43	Normal (1.02)
ARSA probe 2707-L0661	22q13.33	290	22-049.4 ARSA	44	Normal (1.13)
SHOX probe 3714-L16345	Xpter-p22.32	484	X-000.5 SHOX	45	Normal (0.91)
SYBL1 probe 2708-L2855	Xq28	298	X-154.8 SYEL1	46	Normal (0.96)

B

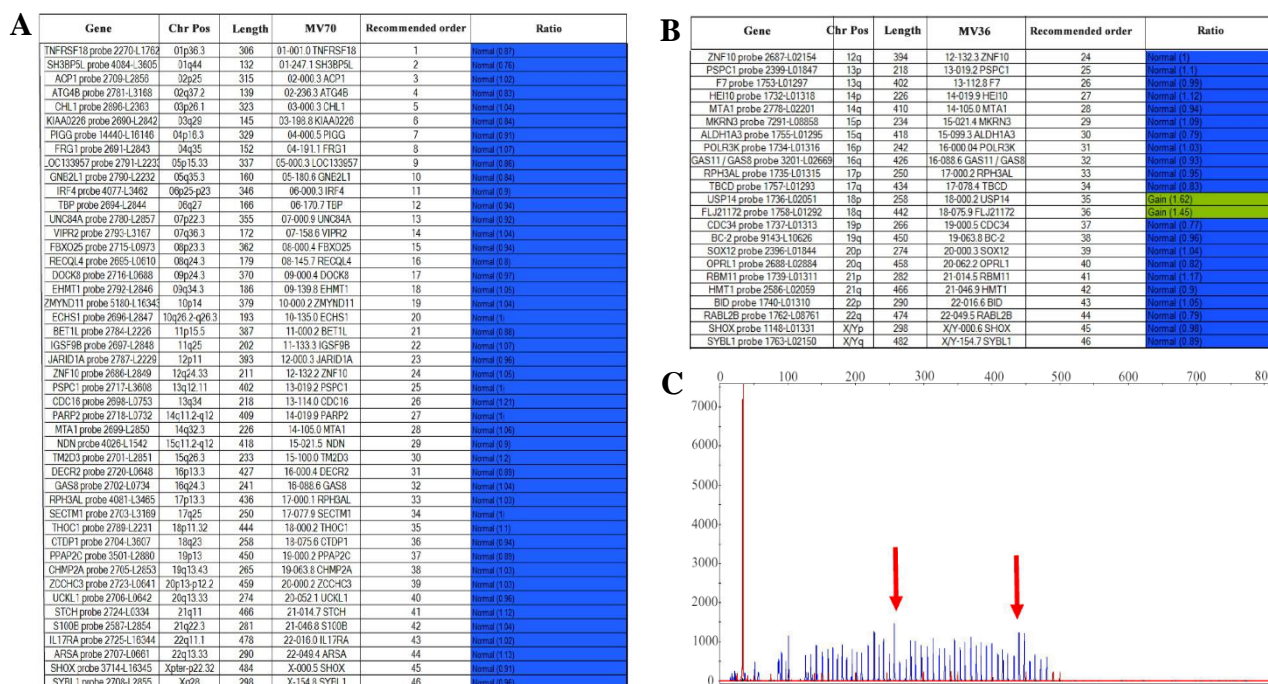| Gene	Chr Pos	Length	MV36	Recommended order	Ratio
ZNF10 probe 2687-L02154	12q	394	12-132.3 ZNF10	24	Normal (1)
PSPC1 probe 2399-L01847	13p	218	13-019.2 PSPC1	25	Normal (1.1)
F7 probe 1753-L01297	13q	402	13-112.8 F7	26	Normal (0.99)
HEI10 probe 1732-L01318	14p	226	14-019.9 HEI10	27	Normal (1.12)
MTA1 probe 2778-L02201	14q	410	14-105.0 MTA1	28	Normal (0.94)
MKRN3 probe 7291-L08858	15p	234	15-021.4 MKRN3	29	Normal (1.09)
ALDH1A3 probe 1755-L01295	15q	418	15-099.3 ALDH1A3	30	Normal (0.79)
POLR3K probe 1734-L01316	16p	242	16-000.04 POLR3K	31	Normal (1.03)
GAS11 / GAS8 probe 3201-L02669	16q	426	16-088.6 GAS11 / GAS8	32	Normal (0.93)
RPH3AL probe 1735-L01315	17p	250	17-000.2 RPH3AL	33	Normal (0.95)
TBCD probe 1757-L01293	17q	434	17-078.4 TBCD	34	Normal (0.83)
USP14 probe 1736-L02051	18p	258	18-000.2 USP14	35	Gain (1.62)
FLJ21172 probe 1758-L01292	18q	442	18-075.9 FLJ21172	36	Gain (1.45)
CDC34 probe 1737-L01313	19p	266	19-000.5 CDC34	37	Normal (0.77)
BC-2 probe 9143-L10626	19q	450	19-063.8 BC-2	38	Normal (0.96)
SOX12 probe 2396-L01844	20p	274	20-000.3 SOX12	39	Normal (1.04)
OPRL1 probe 2688-L02884	20q	458	20-062.2 OPRL1	40	Normal (0.82)
RBM11 probe 1739-L01311	21p	282	21-014.5 RBM11	41	Normal (1.17)
HMT1 probe 2586-L02059	21q	466	21-046.9 HMT1	42	Normal (0.9)
BID probe 1740-L01310	22p	290	22-016.6 BID	43	Normal (1.05)
RABL2B probe 1738-L08761	22q	474	22-049.5 RABL2B	44	Normal (0.79)
SHOX probe 1146-L01331	X/Yp	298	X/Y-000.6 SHOX	45	Normal (0.98)
SYBL1 probe 1763-L02150	X/Yq	482	X/Y-154.7 SYBL1	46	Normal (0.89)

C

Figure 1. Typical profiles for normal and abnormal samples. A) Table A shows the normal female data analysis of MLPA products for SALSA P070 using Coffalyser software. B) Table B represents the male, trisomy 18 data analysis of MLPA product for SALSA P036 using Coffalyser software. C) Electropherogram showing MLPA product for SALSA P036 in Gene Mapper software, arrows marking 18p, and 18q peaks, respectively (47, male, trisomy 18).

Table 1. Distribution of gender in samples

	Normal	Abnormal	Total	p-value
Male	19 (79.17)	5 (20.83)	24 (100.00)	
Female	14 (77.78)	4 (22.22)	18 (100.00)	1.00
Total	33 (78.57)	9 (21.43)	42 (100.00)	

Fisher's exact test

Table 2. Distribution of the gestational age in total samples

Gestational age	Normal	Abnormal	Total
First trimester	7 (58.33)	5 (41.67)	12 (100.00)
Second trimester	12 (80.00)	3 (20.00)	15 (100.00)
Third trimester	6 (100.00)	0 (0.00)	6 (100.00)
Neonatal	8 (88.89)	1 (11.11)	9 (100.00)
Total	33 (78.57)	9 (21.43)	42 (100.00)

Table 3. Distribution of normal and abnormal pathological reports (Internal and external exam) in total samples

	Normal	Abnormal	Total
Normal cases			
External	6 (50.00)	6 (50.00)	12 (100.00)
Internal	4 (40.00)	6 (60.00)	10 (100.00)
Total	10 (45.45)	12 (54.55)	22 (100.00)
Abnormal cases			
External	0 (0.00)	3 (100.00)	3 (100.00)
Internal	0 (0.00)	2 (100.00)	2 (100.00)
Total	0 (0.00)	5 (100.00)	5 (100.00)

age of normal and chromosomally abnormal cases (p= 0.090).

The pathological study results in 64.28% of samples (27/42) were available. As shown in table 3, slightly more than half of samples of pregnancy products with normal genetic reports had abnormal findings in the internal or external pathological examination. Of abnormal internal pathologic examinations, 16.66% (7/42) were related to placenta, 7/42 (16.66%) the thoracic cavity, 5/42 (11.90%) the abdominal cavity and 1/42 (2.38%) were associated with central nervous system. Histopathologic exam results included abnormalities in heart tissue 1/42 (2.38%), thymus tissue 1/42 (2.38%), renal tissue 4/42 (9.52%), adrenal tissue 2/42 (4.67%), and liver tissue 2/42 (4.67%). Moreover, all samples with abnormal genetic reports had also been reported as abnormal in histopathology.

In total, 33 and 40% of the patients subjected to first and second trimester screenings, respectively were categorized as cases with high risk of aneuploidy. MLPA method revealed that 40% of cases that underwent screening tests had aneuploidy. Furthermore, abnormal chromosome findings were obtained in a sample with a low risk of aneuploidy.

Discussion

Prevalence of chromosomal abnormalities in pregnancy has changed the attitude in health systems toward using rapid, accurate, and cost-effective detection methods. This approach could have a significant im-

pact on family health and, subsequently, community's health. Finding causes of fetal or neonatal loss reduces the feelings of self-blame, anxiety, depression, and grief in women with miscarriages or neonatal deaths. Moreover, knowing the exact origin of fetal loss allows us to give improved genetic counseling in future pregnancies to predict the recurrence frequency in each engaged family. Based on these considerations, this study was designed to investigate the feasibility of using MLPA, histopathologic, and genetic counseling data to detect aneuploidies in products of conception and neonatal deaths.

In this study, 50 samples of products of conception and neonatal deaths that could not be cultured, *i.e.*, paraffin-embedded samples or had a long interval between miscarriage or fetal death until genetic testing were evaluated. These samples were collected from Hope Generation Foundation and Avicenna Fertility Center for one year. Based on pathological findings, only forty-two samples contained the fetal or neonatal tissues. Verified samples were analyzed by genetic counseling and MLPA method. Genetic examination was successful in all samples (Failure rate equal to zero), and aneuploidy was detected in 21.42% of samples (Table 4). Aneuploidies included trisomy 18 (22.22%), trisomy 21 (11.11%), trisomy 22 (33.33%), monosomy X (11.11%), trisomy 13 (11.11%), and trisomy 16 (11.11%).

Since 1984, researchers have considered cytogenetic evaluation of products of conception. A review of these studies reveals that culture failure was present in 10-40% of cases. Since 2007, MLPA has been used as a culture-independent method in evaluating those products of conception and neonatal deaths that had no thriving culture [11,12]. The results of some studies indicated that MLPA subtelomeric probemix for all chromosomes is the most useful method, especially during the first trimester when numerical aberrations involving all chromosomes may occur [13].

The success rate of MLPA technique in this study is higher than in some similar studies, which could be attributed to smaller sample size [14-16]. However, Smits *et al* conducted a meta-analysis on eight studies that focused on using MLPA in pregnancy loss samples; they reported a 5% failure rate of MLPA [12]. Reported aneuploidies in this study have more varieties than similar studies as most of these studies used the SAL-

SA MLPA probemix P095, which is only capable of detecting aneuploidies in chromosomes 13, 18, 21, X, and Y [17-19]. However, SALSA MLPA probemix P036 and P070 not only detect subtelomeric rearrangements but also help to study aneuploidies in all chromosomes. However, another study obtained successful MLPA results in only 74.4% of 90 abortus samples while using the SALSA MLPA probemix P036 and P070 [20].

Nagashi *et al* reported 85.3% success rate for karyotype on 407 samples of aborted fetuses in which 61.2% of chromosomal abnormalities were trisomy (Mostly trisomies 16, 21 and 22). They showed that trisomies 7, 8, 14, 15, 16, and 22 were found mostly among the first trimester abortions while trisomies 4, 13, 18, 21 were prevalent in the second trimester [20]. In this study, the order of frequency of trisomies was found to be 22: 3/9 (33.3%), 18:2/9 (22.2%), and then 21, 13, 16 each 1/9 (11.1%). Furthermore, in our study, analysis of chromosomal abnormalities (Table 2) shows that there is no significant difference between the gestational age of normal and chromosomally abnormal cases (p= 0.090). A larger sample size is required to make a more reliable conclusion.

In 2011, McClelland *et al* evaluated 382 samples of aborted fetuses and neonatal losses with both MLPA, using SALSA MLPA probemix P069 and P036, and QF-PCR. Seventy-seven reported defects were diagnosed by QF-PCR and the remaining by MLPA. They concluded that with this approach, only 4% of chromosomal aberrations were not detectable, compared to 20-30% failure rate of karyotype analysis. Considering the fact that QF-PCR can only identify common aneuploidies (Trisomy 13, 18, 21, X, Y), MLPA seems a more reasonable approach for the concurrent investigation of such abnormalities together with other aneuploids [21]. In this study, 88.88% of aneuploidies were identified as trisomy and 11.11% as monosomy. Ttrisomy 22 was the most frequent aneuploidy detected.

In line with this study, Zimowski *et al* in 2016 carried out 181 MLPA analyses using subtelomeric and subcentromeric probe kits (SALSA P070 and SALSA P181) on products of first trimester spontaneous miscarriage. As expected, they reported that the differentiation between simple trisomies and trisomies caused by Robertsonian translocation is not feasible [22]. In our study, all parents had genetic counseling before the test, and if they had a history of miscarriage, parents' karyotypes were also investigated. The rate of consanguinity between families in this study was 45%, which is in line with the 38.6% consanguinity rate within Iranian families reported by Saadat *et al* [22].

Careful pathologic examination of the products of conception can help the process of clinical diagnosis, particularly unexpected anomalies and may influence the clinical care, family planning, genetic counseling, and grief management [23]. Stuppia *et al* demonstrated the broad spectrum of MLPA applications in different fields such as detection of neuromuscular disorders,

Table 4. Distribution of aneuploidy in total samples

Aneuploidy	Quantity ratio	Percentage
Trisomy 22	3/9	33.3
Trisomy 18	2/9	22.2
Trisomy 21	1/9	11.1
Trisomy 13	1/9	11.1
Trisomy 16	1/9	11.1
Monosomy X	1/9	11.1
Total	9/42	21.42

analysis of SHOX gene, prenatal diagnosis, and cancer. Since through MLPA, a large number of genes can be analyzed by a single technique, they argued that MLPA can even be the gold standard for analysis of copy number variation pathologies [24]. About 54% of samples with normal genetic report had an abnormal report in the internal or external pathological examination, which were not strictly associated with aneuploidies. On the other hand, all samples with the abnormal genetic report were also reported as abnormal cases pathologically.

Conclusion

A comprehensive approach using genetic counseling, histopathologic examination, and genetic testing is mandatory to address the possible genetic causes for abortion and neonatal death. In this regard, MLPA as a standalone genetic testing is an accurate, rapid, and reliable method with a low failure rate to overcome the limitations of standard cytogenetic techniques. Moreover, in certain and well selected conditions, this technique may be considered as an alternative to array-based techniques when resources are limited.

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