LETTER TO JMG

Rapid, high throughput prenatal detection of aneuploidy using a novel quantitative method (MLPA)

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Prenatal diagnosis of syndromes caused by chromosomal abnormality is a long established part of obstetric care in developed countries. In this area, there have been recent significant advances in the identification of high risk pregnancies using sophisticated serum analyte and ultrasound screening methods.^{1–2} For follow up diagnostic testing, karyotyping has provided the gold standard. This technology has remained essentially unchanged over 30 years, as no new technology has yet proven superior in terms of being able to detect such a wide range of abnormalities with the necessary precision. Nevertheless, molecular tests, such as fluorescent in situ hybridisation (FISH)³ and short tandem repeat analysis,⁴ are now in common practice for the diagnosis of specific abnormalities. These adjunctive tests importantly decrease turnaround times from 1–2 weeks to 1–2 days.

We assessed the performance of multiplex ligation dependent probe amplification (MLPA) as an alternative method for the detection of aneuploidy, which is by far the most common prenatal chromosome abnormality. This novel technique⁵ detects sequence dosage differences in a semi-quantitative manner and has many potential applications in diagnostic molecular genetics and cytogenetics. For example, a recent report describes its use for detection of large genomic deletions and duplications in the *BRCA1* gene.⁶ This technology appears to have significant advantages in that it is extremely versatile in its applications, flexible in its target loci, highly automated, suitable for high throughput testing, efficient, and cost effective. Its application for aneuploidy detection has not been reported in a clinical setting. In order to assess the precision and robustness of the test, we conducted a prospective blind trial on 492 consecutive amniocentesis samples referred to our cytogenetics laboratory.

MATERIALS AND METHODS

Study design

This study was designed to blind test amniocentesis samples prospectively. The samples were collected over a 4 month period as they were referred to the cytogenetics laboratory of Genetic Health Services. The referral reasons covered the whole range from low risk anxiety to very high risk cases with known clinical abnormalities assessed by ultrasound and/or serum screening. With the exception of 30 blood contaminated samples, none was excluded from the study. Usually 15–20 ml of amniotic fluid was received and 1 ml was removed for MLPA testing. Tests were processed in batches of 16–96 samples. Data processing of the MLPA tests was performed without knowledge of the karyotype/FISH results.

Sample preparation

Lysates were made by centrifuging 1 ml of amniotic fluid for 20 minutes at 3500 rpm in a benchtop centrifuge. The supernatant was discarded and the remaining cell pellet lysed with 25 µl of 50 mmol/l NaOH and heated at 95°C for

Key points

- The use of multiplex ligation dependent probe amplification (MLPA) for rapid, high throughput prenatal detection of common aneuploidies (13, 18, 21, X, and Y) was assessed.
- A blind, prospective trial in a clinical diagnostic setting was conducted using 492 amniotic samples referred for routine testing. The assay was designed as a single tube procedure using 1 ml lysates of amniotic fluid.
- There were no failed tests. The difference in distributions of normal and aneuploid samples clearly identified all 17 autosomal aneuploid cases. Sex determination was also 100% accurate and included a single case of monosomy X. This dataset, together with measurement of intra- and inter-assay variation (both SD <7%), has been used to determine threshold values for screening with a false negative rate of 0 and a false positive rate of 5% within our own laboratory.
- MLPA is a rapid, flexible, sensitive, and robust test for prenatal aneuploidy detection.

15 minutes. Each lysate was neutralised with 5 µl of 1 mol/l Tris-HCl buffer (pH 7.4, 25°C) giving a total volume of 30 µl, of which 5 µl was used in the MLPA reaction. When required, lysate DNA was concentrated by ethanol precipitation and redissolved in 5 µl of TE buffer.

MLPA reaction

The MLPA-Trisomy test kit (P001) was obtained from MRC Holland, Amsterdam, the Netherlands. The principle of MLPA has been described in detail previously.⁵ Briefly, for each specific genomic target, a set of two probes is designed to hybridise immediately adjacent to each other on the same target strand. Both probes consist of a short (22–43 nt) target specific sequence and a universal forward or reverse PCR primer binding site. In addition, one of the probes contains a so-called "stuffer" sequence. For each probe in the multiplex, the stuffer part has a specific length (19–364 nt) and sequence. After overnight hybridisation to the target DNA, each pair of adjacent probes is joined by a ligation reaction. Next, PCR is performed with a single fluorescent labelled primer pair, which ensures that the relative yield of each of the PCR products is proportional to the amount of each of the

Abbreviations: FISH, fluorescent in situ hybridisation; MLPA, multiplex ligation dependent probe amplification; QF, quantitative fluorescence; STR, short tandem repeats

target sequences. The different lengths of products are separated on an automated capillary sequencer and the peak areas quantified.

The P001 probe mix for aneuploidy detection contains four probes each for the human chromosome X and Y target sequences, and eight probes that are specific for each of the chromosome 13, 18 and 21 sequences. In addition, eight probes specific for other chromosomes are included. Details on probe sequences, gene loci and chromosome locations can be found at <http://www.mrc-holland.com>.

MLPA was performed essentially as described by Schouten *et al.*⁵ Briefly, 5 µl of lysate were denatured for 5 minutes at 98°C. Next, 3 µl probe mix were added and the mix was heated at 95°C for 1 minute and incubated at 60°C for 16 hours (overnight). All incubations were performed in a PCR thermocycler with heated lid.

Ligation was performed using the heat stable ligase-65 enzyme (MRC Holland) at 54°C for 15 minutes, followed by ligase inactivation at 98°C for 2 minutes, after which 10 µl of this ligation mix was added to 40 µl of PCR buffer containing dNTPs, *Taq* polymerase and PCR primers. One primer is unlabelled and the other is labelled with FAM [N-(3-fluoranthyl)maleimide]. The reaction mixture was preheated at 95°C for 1 minute, followed by 32 cycles (30 seconds at 95°C, 30 seconds at 60°C, and 60 seconds at 72°C).

Fragment and data analyses

Fragment analysis was performed using a MegaBACE 1000 capillary sequencer with ROX-550 (both Amersham Biosciences, Amersham, Buckinghamshire, UK) as size standards. A profile of 40 peaks ranging in size from 130 to 456 nt was obtained.

The multiplex contains five internal DNA quantity control fragments. Four of these are ligation independent and indicate sufficient template DNA (>50 ng) for the entire multiplex, when amplified (64, 70, 76, and 82 nt). The fifth fragment (94 nt) indicates successful ligation by producing a peak of comparable size to that of the other chromosome specific probes in the multiplex.

Data analysis was performed using Fragment Profiler software (Amersham Biosciences). To automate the interpretation of fragment analysis, the relative peak areas of the amplified probes in each sample was determined using a Microsoft Excel template. For quantification purposes, the relative peak area for each probe was calculated as a fraction of the total sum of peak areas in a given sample, (that is, it was normalised). Subsequently, the fraction of each autosomal peak was divided by the median peak fractions of the corresponding locus for all samples in that run. In normal individuals, these calculations will result in a value of 1.0, representing two copies of the target sequence in the sample. Male samples were compared with male samples, and female samples with female samples for quantitation of X and Y linked probes. Relative peak areas for these probes in normal individuals will therefore also give values of 1.0. All data calculations were performed on samples processed within an assay run.

As the distributions were often skewed, median rather than mean values for each chromosome set were used for all calculations. All statistical comparisons with the normal dataset determined *p* values using means and standard deviations with confidence limits greater than 99%, using two-sample *t* tests.

RESULTS

The test protocol was modified to analyse amniotic fluid lysates directly rather than purified DNA. Initial studies showed that tests performed using lysates were just as robust

and accurate as those performed using purified DNA samples. The trial therefore used lysates that were run in batches of samples including purified DNA controls. Samples that were visibly blood contaminated (5.7% of the total), either before or after centrifuging, were excluded from the study. The standard volume of amniotic fluid used for lysate preparation was 1.0 ml. In 8% of samples, the internal control data for the multiplex indicated that inadequate DNA was present, and a repeat assay using a more concentrated sample was required. In all cases where a repeat assay was performed, only a single repeat was necessary.

Typical profiles for normal and aneuploid cases are shown in fig 1. All 40 locus specific products were well and consistently resolved by capillary electrophoresis. Peak profiles were uniform, enabling accurate, automatic quantification of peak areas. Abnormalities in aneuploid samples are qualitatively apparent by comparing the relative heights of peaks in normal control and test sample profiles (see fig 1 legend for examples). Notably, most but not all peaks within a chromosome specific set of probes were found to be abnormal in any aneuploid case. Furthermore, there was no consistency among different cases in the specific probes, which did not show the dosage difference, although there was a suggestion of consistency within replicates from the same case.

Quantitative analysis used the average of the results for each chromosome specific set of probes. The performance of the individual probes in each of the chromosome 13, 18, and 21 sets is shown in fig 2. The mean values for each chromosome specific set are very close to 1, but there is some individual probe variation (standard variation is 0.13 to 0.3).

Intra- and inter-assay variation was measured in randomly selected normal and aneuploid samples. The standard deviation of intra-assay replicates ranged from 2.4–6.7% of the means (calculated from table 1 (A)). The normal replicates (*n* = 3) were statistically indistinguishable from the normal control set (*n* = 474); *p* values were determined using two sample *t* tests (table 1 (A)). The mean relative copy numbers for aneuploid replicates (*n* = 3) were all significantly higher (*p* < 0.01) than normal samples (*n* = 474). Aneuploid samples run in three separate assays showed standard deviations of 3.6 to 5.8% of the means (calculated from table 1 (B)). The mean copy numbers for these aneuploid replicates (*n* = 3) were all significantly higher (*p* < 0.01) than normal samples (*n* = 474).

A summary of the test results on all 492 samples is shown in fig 3. The medians for all chromosome specific probe sets in normal samples are shown in fig 3A, and all approximate very closely to a value of 1.0. The variation in relative copy number is slightly higher for chromosomes X and Y. The medians for all chromosome specific probe sets in each aneuploid sample (*n* = 18) are shown. Some of the aneuploid samples were re-assayed to provide data for statistical comparison of the normal and aneuploid distributions (fig 3B). In all cases they were significantly different (*p* < 0.01; two sample *t* test). This dataset enables threshold values to be set for rigorous assignment of test results into normal, abnormal, and inconclusive (repeat assay) categories. The threshold values calculated with 99% confidence limits are shown in table 2.

In total, 18 aneuploidies were detected; 2 with trisomy 13, 4 with trisomy 18, 11 with trisomy 21, and 1 with monosomy X. There was complete concordance with results obtained by karyotyping and FISH, where the latter test was performed. This included correct assignment of sex in all 492 cases. There were no false positive or false negative results using this data calculation.

Figure 2 Quantitative MLPA analysis of normal samples. The quantitative results are represented by box plots with median relative copy numbers; 50% of samples are within the box, 95% within the error bars. The box plots show the distribution for individual probes specific for chromosomes 13, 18, and 21 in our normal set ($n=474$). The SD is 0.13–0.3 depending on the probe.

DISCUSSION

Modification of the MLPA assay based on the direct use of small volumes of amniotic fluid lysates instead of purified DNA samples was important because elimination of the DNA extraction step reduced time and costs. There was no indication that the modified method was compromised, either in initial comparisons or in the actual sample runs, where extracted DNA controls were included. Test failure using the maximum 5 μ l of lysate (from a total of 30 μ l, derived from 1 ml of amniotic fluid) was 8%. All failed tests were repeated successfully with more concentrated lysate samples. As the multiplex requires a minimum of 50 ng of template DNA, use of 2 ml amniotic fluid samples would virtually eliminate test failures resulting from inadequate DNA.

Processing of samples was highly flexible using the 96 well formats of both the thermocycler and the capillary sequencer. The simplicity of the single tube hybridisation, ligation, and amplification stages combined with automatic product separation and fragment analysis lent itself to processing batches of any sample size up to 96 in a single run. The maximum time required to process samples was 48 hrs. This included a convenient overnight hybridisation step that can be reduced to a minimum of 3 hours if a same day result is required.

The procedure was shown to be robust, which is of obvious importance in a clinical diagnostic setting. The internal ligation and amplification DNA quantity control fragments proved extremely informative in identifying inadequate template DNA. Separation of products on the capillary sequencer was consistent between runs. The peaks were easily identified using the internal size standards, allowing reliable automatic labelling using the Amersham Fragment Profiler software.

MLPA detected all 17 autosomal aneuploidy cases in the trial. There were no false positive or false negative results. This was also the case for sex determination, which showed complete concordance with the karyotype results and

included a single case of monosomy X. The sample set also contained 15 other chromosome abnormalities not detectable by this MLPA. There were eight balanced translocations/inversions, two Robertsonian translocations, two autosomal aneuploidies other than 13, 18, and 21, one unbalanced translocation, one deletion, and one triploidy.

The trial was designed as a single test procedure. Selected abnormal samples were repeated to determine intra- and inter-assay variation. This variation showed a standard deviation of less than 7% of the mean, which in comparison with the difference in normal and abnormal distributions (fig 3), indicates that there would be no gain in running replicates other than as a check for sample mixups.

The relationship between chromosome dosage and mean peak ratios for chromosome specific sets is not completely linear (fig 3A). Whether this is due to the MLPA itself or the data analysis is unclear. Discrimination can be improved by comparing individual peaks with near neighbours rather than all peaks within a sample, but this requires more sophisticated spreadsheet analysis.

The specificity and quantitative nature of ligation dependent probe amplification resides in the use of adjacent probes to hybridise to the target locus. Pre-trial studies using known aneuploid samples indicated that most, but not all, loci within a chromosome specific set would show the dosage abnormality (data not shown). This finding has been consistent for all the abnormal test results in the trial. This variation in probe performance is due in part to the nature of the specific sequences targeted—that is, possible polymorphic differences, and the limitations imposed by optimising conditions for the multiplex.

The sensitivity of this multiplex assay therefore relies on the targeting and averaging of the dosages for an entire chromosome specific probe set. Aneuploid results are assumed to involve entire chromosomes, which is reasonable for chromosomes 13, 18, and 21. Segmental aneuploidies for the X chromosome, however, represent a substantial proportion of karyotypes in Turner syndrome, for example

Figure 1 (From previous page) Aneuploidy can be detected by qualitative inspection of the profiles. For example, comparison of relative heights of the chromosome specific peaks within the boxes clearly indicates dosage differences consistent with the aneuploidies or sexes indicated. Calculation of a consensus diagnostic outcome for each sample will take into account the median peak area of a probe set for a chromosome relative to the total sum of peak areas for that sample (see Materials and methods). Each peak represents a specific probe amplification product. The exact gene and sequence recognised by each probe of the P001 probe mix can be found at www.mrc-holland.com.

Table 1 Determination of intra- and inter-assay variations

Probe set	Samples (n = 3)	Replicates			Mean	SD	p value*
		Median relative copy number					
(A) Intra-assay variation							
Chromosome 13	47, XY, +13	1.34	1.31	1.41	1.35	0.051	<0.01
	46, XX	1.00	1.06	1.07	1.04	0.037	0.20
Chromosome 18	47, XY, +18	1.33	1.34	1.42	1.36	0.049	<0.01
	46, XX	1.05	1.03	1.08	1.05	0.025	0.14
Chromosome 21	47, XY, +21	1.42	1.39	1.54	1.45	0.079	<0.01
	46, XX	1.11	0.99	0.99	1.03	0.069	0.26
(B) Inter-assay variation							
Chromosome 13	47, XY, +13	1.46	1.35	1.32	1.38	0.073	<0.01
	47, XY, +18	1.50	1.40	1.40	1.43	0.057	<0.01
Chromosome 18	47, XY, +18	1.36	1.32	1.44	1.37	0.061	<0.01
	47, XY, +21	1.44	1.47	1.37	1.43	0.051	<0.01
Chromosome 21	47, XY, +21	1.43	1.46	1.36	1.42	0.051	<0.01
	47, XY, +21	1.49	1.33	1.38	1.40	0.081	<0.01

*Two sample *t* test comparing replicate samples to normal set (n = 474).

isochromosome Xq. Caution would therefore have to be exercised in interpreting any inconsistency in results within the X chromosome probe set. Performance is already impressive in this first generation, 40-plex probe set, but the variation within chromosome specific probe sets (fig 2) suggests that improvements in sensitivity at the individual locus level within a multiplex could be achieved through formulation of even better matched probe/locus sets.

To achieve a test with no false negatives, it is necessary to set appropriate thresholds based on knowledge of the distribution of aneuploid results. The number of aneuploid results derived from this study is too small to set thresholds accurately. Acceptance of a 5% false positive rate would appear to be consistent with a zero false negative rate given our limited data, which suggest that the normal and aneuploid distributions are almost non-overlapping (table 2, fig 3). In practical terms, it is important at this stage to view

MLPA as a screening test and to delay any clinical action until results are confirmed with a FISH or karyotype result. MLPA tests can further be designed to detect deletions or duplications at any gene locus. However, as quantification is based on normalising the peak area of each locus by expressing it as a proportion of the total of all the other peaks in the same profile, it is unable to detect triploidy. Triploidy is almost always ascertained through ultrasound examination, and represents in our experience approximately 7% of abnormalities detected at second trimester chromosome testing.

The introduction of sophisticated serum analyte and ultrasound screening for detection of pregnancies at increased risk of aneuploidy^{1,2} has prompted the need for rapid follow up diagnostic testing. Several molecular methods have become available and these have been comprehensively reviewed by Armour *et al.*⁷ By far the most established in

Figure 3 Quantitative MLPA analysis of normal samples and 18 cases of previously unknown aneuploidy. (A) The quantitative results are represented by box plots for each autosomal chromosome specific probe set for the normal samples (n = 474) with median relative copy numbers; 50% of samples are within the box, 95% within the error bars. The sex chromosome probe sets are separated into two populations, with sample sizes of 239 and 235 for females and males, respectively. The SD is 0.05–0.11, depending on the chromosome set. The results for the 18 cases of aneuploidy are shown in the dotted inset boxes, where the straight lines represent the median relative copy number for the chromosome specific probe sets indicated. (B) Comparison of box plots for the replicate experiments for selected cases of trisomy 13 (n = 5), trisomy 18 (n = 10), and trisomy 21 (n = 22) samples (shown in the dotted inset box), with those for each of the chromosome specific probe sets for the normal samples (n = 474).

Table 2 Threshold values for chromosome specific probe sets

Probe set	Sample set	n	Mean	SD	Threshold mean+3SD
Chromosome 13	Trisomy 13	5*	1.34	0.076	1.11 (1.57)
	Normal	474	1.016	0.054	0.85 (1.18)
Chromosome 18	Trisomy 18	10*	1.36	0.073	1.14 (1.58)
	Normal	474	1.023	0.049	0.87 (1.17)
Chromosome 21	Trisomy 21	22*	1.38	0.081	1.13 (1.62)
	Normal	474	1.011	0.048	0.86 (1.16)
Chromosome X (female)	Normal	239	1.00	0.075	0.77 (1.23)
Chromosome X (male)	Normal	235	1.00	0.11	0.67 (1.33)
Chromosome Y	Normal	235	1.01	0.11	0.68 (1.34)

*As inter- and intra-assay variation was the same, data from replicate assays have been used to determine means/SDs for abnormal populations.

current prenatal diagnostic practice is FISH,³ followed by STR/microsatellite based assay,⁴ and quantitative fluorescence (QF)-PCR.⁸ Newer techniques such as multiplex amplifiable probe hybridisation,⁹ and MLPA (this study) are being assessed. Table 3 compares MLPA with other molecular methods that are currently in use. All these methods are reported to be highly accurate and robust. They all reduce turnaround time for an aneuploidy test from 7–14 days for karyotype testing to less than 24–48 hours. All except FISH are suited to high throughput processing using 96 well formats per assay. MLPA, QF-PCR, and STR assays can, however, be used flexibly with smaller throughputs, which are more likely to be needed in the typical cytogenetics laboratory processing 1000–2000 samples per year. None of the techniques can process samples contaminated with maternal blood. Interphase FISH, however, as the only single cell based technique, can be used to test such a sample if the fetus is male. Mosaicism can be detected by FISH and QF-PCR, but with limited sensitivity. Interphase FISH has two disadvantages. Firstly, it is labour intensive and therefore is limited in the number of samples that can be processed simultaneously. The second disadvantage, which is also shared by QF-PCR and STR assays, is that the number of loci that can be tested in a multiplex is limited to approximately 12 or less. In comparison, MLPA is particularly attractive in that 40 loci per multiplex can be selected in a very flexible manner to target combinations of common abnormalities such as aneuploidies and microdeletions. MLPA can potentially even distinguish sequences that differ only in a single nucleotide. It is suited to tailor made strategies for different clinical settings. For instance, a prenatal multiplex could include common aneuploidies, microdeletion targets, and the more common single gene mutations such as in cystic fibrosis. A mental retardation multiplex could include microdeletion and telomere targets.

QF-PCR and STR assays use polymorphic loci, which show variable allele frequencies in different populations and furthermore are not informative for all samples. MLPA avoids these issues by using non-polymorphic, intragenic targets. The estimated cost of consumables for all these techniques is of the order of US\$15–80 per sample for a common aneuploidies test. Although still in the developmental stage, microarray comparative genomic hybridisation

Table 3 Comparison of currently available tests for chromosomal abnormalities

Technique	Turnaround time	Locus/allele target (size)	Targets/ assay	Samples/ assay
MLPA	<48 h	Non-polymorphic (60 nt)	40	≤96
Interphase FISH	~24 h	Non-polymorphic (>2 kb)	<7	~10
QF-PCR	<48 h	Polymorphic (25 nt)	~12	≤96
STR	<48 h	Polymorphic (25 nt)	~12	≤96

promises great improvements in multiplexing, with the possibility of investigating thousands of loci. Given the range of mutation types and clinical presentations, it is unlikely that any one technique will be adequate for all situations. MLPA appears to be an attractive choice as part of the diagnostic laboratory's repertoire of techniques, perhaps even by itself in a microarray format.

The results of this study therefore highlight the important advantages of MLPA over existing methods for aneuploidy testing. Further improvements on existing MLPA probe set performance and design of new probe sets for other chromosomal loci should see the useful diagnostic potential of this technique being substantially expanded.

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