GENETIC TESTING AND MOLECULAR BIOMARKERS
Volume 13, Number 6, 2009

Pp. 803–808
DOI: 10.1089/gtmb.2009.0058

Comparative Study of Three PCR-Based Copy Number Variant Approaches, CFMSA, M-PCR, and MLPA, in 22q11.2 Deletion Syndrome

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Small submicroscopic DNA copy number variants represent an important source of variation in the human genome, human phenotypic diversity, and disease susceptibility. Consequently, there is a pressing need for the development of methods allowing the efficient, accurate, and cheap measurement of genomic copy number polymorphisms in clinical cohorts. The PCR-based strategies, being cost-effective and sensitive, are considered important in the development of screening techniques. PCR-based techniques such as multiplex PCR; multiplex ligation-dependent probe amplification; and a new single-tube assay technique, the competitive fluorescent multiplex STRP assay, have been applied to 22q11.2 detection, a typical example of deletion syndromes. In this study, we compared the reliability and application of these three techniques in a cohort of 17 patients affected with 22q11.2 deletion and 300 normal controls. All three techniques shared 100% sensitivity; however, the competitive fluorescent multiplex STRP assay had the lowest possibility of concurrent false-positive signals from two adjoining probes in a genomic region. Moreover, it is a relatively fast and low-cost procedure to detect the deletion of 22q11.2 in numerous patients with several minor symptoms of deletion syndromes. Multiplex PCR, a rapidly developing and cheap technique, allows detection of atypical deletions.

Introduction

An increasing number of human genetic diseases are being identified as resulting from genomic rearrangements, ranging from presence of extra copies of entire chromosomes to deletions or duplications of single exons (Lupski, 2007). The genomic rearrangements are relatively frequent, representing 7.3% of the reported mutations in the May 2006 release of the Human Gene Mutation database (Stenson *et al.*, 2003). This prevalence indicates that testing would be justified in appropriate referral groups, making screening of genomic rearrangements a fundamental task in diagnostic settings.

Several methods have been employed for detection of gene rearrangements, including cytogenetic approaches, for example, fluorescent *in situ* hybridization (FISH) and Southern blot. However, these methods are time consuming and expensive (Rooms *et al.*, 2005). New alternative techniques that make use of molecular methods to screen for many genomic rearrangements are being developed, and semi-quantitative PCR techniques are considered a major advance in this direction (Kobrynski and Sullivan, 2007).

Semi-quantitative PCR protocols such as multiplex fluorescent PCR (M-PCR) and multiplex ligation-dependent probe amplification (MLPA) can estimate the number of allele copies by the product ratio between the checked samples and appropriate controls independent of the deletion. Only small amounts of DNA are required, and the methods are faster and cheaper than FISH and Southern blot analysis (Shaffer and Bui, 2007).

But the reliability of these tools depends on the standard deviations (SD) of the dosage quotients of their probes, which may be quite large leading to uncertain diagnosis (Vorstman *et al.*, 2006). More recently, a new tool, the competitive fluorescent multiplex STRP assay (CFMSA), has been introduced in DNA diagnostic laboratories for the detection of deletions. This is based on PCR amplification of the tetranucleotide STRP multiplex, which typically has only a single main allele peak, allowing polymorphic and gene dosage information to be processed simultaneously. This method could improve the resolution and sensitivity of deletion detection (Yang *et al.*, 2009).

However, the reliability of these three tools is still not known. This is of particular importance if diagnostic

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laboratories wish to replace FISH with these semi-quantitative PCR protocols. The false-negative rate will depend on the robustness of the semi-quantitative technique itself and the method of analysis used, either of which could result in miscalled negative results. The experimental approach, which depends on the number of probes analysis, may thus fail to identify a region of imbalance (Ahn *et al.*, 2007).

Copy number aberrations of the 22q11.2 region are among the most common genomic rearrangements. The resulting varied and complex phenotypes make these aberrations a significant health problem (Kobrynski and Sullivan, 2007). Further, a sizable number of samples need screening according to testing strategy for 22q11.2 deletion in CHD patients as suggested by the American Heart Association (AHA) (Pierpont *et al.*, 2007).

The main objective of this study is to use different semi-quantitative PCR techniques (M-PCR, CFMSA, and MLPA) to detect chromosomal and gene alterations in 17 patients with 22q11 deletion and 300 control individuals. The information obtained would allow us to select the appropriate technique in routine analysis for clinic and research.

Materials and Methods

Subjects

Over the past 6 years, commercially available FISH and haplotype analysis with five highly polymorphic STRPs developed in our laboratory have been performed on numerous patients and their parents from Nanjing Children Hospital, Drum Tower Hospital, and Nanjing Maternity and Child Health Hospital. The selection criteria for test samples follow the 22q11.2 deletion testing strategy suggested by AHA (Pierpont *et al.*, 2007). Seventeen well-characterized deletions, including 1 truncus arteriosus, 10 TOF, and 6 VSD, spanning from 1 month to 11 years old, were selected to create a cohort for the purpose of this study. All deletions got confirmatory diagnosis on more than one STRP. Six of these deletions with bacterial artificial chromosome clones available were confirmed by FISH, the rest were confirmed by haplotype analysis. Additionally, 300 samples without a known rearrangement, spanning from 1 to 21 months, were selected to create a cohort for the purpose of this study.

The study was approved by the ethical committee of the Nanjing University School of Medicine. Informed consent was obtained from controls, patients, and their parents. All DNA samples were extracted from peripheral whole blood using UltraPure™ Genomic DNA Isolation Kit (SBS Genetech, Shanghai, China) according to the manufacturer's instructions.

Application of M-PCR

A cohort of 17 deletion and 300 normal DNA samples blinded to their molecular genetic status were analyzed for assessing the application and reliability of gene dosage of M-PCR. Two groups of M-PCR, containing five and six pairs of primers, respectively, were developed. Primer quantities were adjusted in the M-PCR mix to meet the melting temperature and amplification rate suggested by Butler (2005). In the first group, one pair of primers (ARVCF) was picked for amplifying the ARVCF in the 22q11.2 region and the other four primer pairs (HMGN1, DIP2A, RUNX1, and CDH7) for amplifying the control gene in other chromosomes. From the second group, one primer pair (MRPL40) was picked for amplifying the MRPL40 in the 22q11.2 region. The other five primer pairs (CHD7, SOX17, PLAG1, SIM2, and PKNOX1) were picked for amplifying the control gene in other chromosomes. All primers (TAKARA, Shanghai, China) are shown in Table 1, with forward primers labeled with FAM at the 5′ end. M-PCR was performed in the final volume of 25 mL

Table 1. Primers and Conditions Used for Multiplex PCR

Primer name	*Sequence (5′-3′)*	*PCR product length (bp)*	*Primer concentration (μM)*
HMGN1	F CGACAACGTGTTAAATGCTA R TGTACAAGAAGGGAGACAGG	144	0.4
ARVCF	F GCCTATGAGAGCACCTAATG R CACACTAGGCTCTCCACTTC	155	0.2
DIP2A	F TACAGCGGAAAGCTAAAGAC R TCCTTCTCTTTCTGGACTCA	172	0.2
RUNX1	F CTGTTAGGACAGCAACCTTC R TCAGAAAAGCTAGGCAAGTC	222	0.5
CDH7	F CAGGATCCCTGAGAAAGCTG R CGCACTTTAGGGAAGCAAAG	248	0.3
CHD7	F GGTTTGTCCTGTTATCCTCA R ATGGTTCTGTCTCAGAATGG	187	0.08
MRPL40	F TGGATAAAGCAAGGTAAGGA R AGAGCCATTCAAGAGTACCA	202	0.28
SOX17	F CCTGGGTTTTTGTTGTTGCT R GAGGAAGCTGTTTTGGGACA	212	0.1
PLAG 1	F TGTGTGTCTTCGTGAAGTGT R ATTAGGAGCTTTGTTGGTGA	237	0.2
SIM2	F TTTCCTAAGAAGCACAGCTC R ATCTGGTAGCAGGAGTCGTA	266	0.4
PKNOX1	F GCTTGAAATCAGGTGGTGGT R AGTCTGGGGGTTAGCACCTT	310	0.24

containing 100 ng of genomic DNA, 200 mM of each dNTP, primers, and 1 unit Taq DNA polymerase (Promega, Madison, WI).

PCR was performed in an MBS Satellite Thermal Cycler (Thermo-Hybaid, Waltham, MA) with an initial denaturation step at 95°C for 10 min, followed by 25 cycles consisting of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, elongation at 72°C for 45 s, and a final extension at 72°C for 10 min.

M-PCR products were separated on an ABI PRISM_3130 Genetic Analyzer and interpreted using Genotyper (v3.1) software (Applied Biosystems, Foster City, CA). Copy number of target sequences was determined by exporting peak area into an Excel spreadsheet designed to assess the ratio of area of each test peak relative to all other peaks for the given individual.

Further, the ratio of area of test peak to reference peaks in the patients was divided by the corresponding ratio in normal individuals, which were included in each run (Butler, 2005). For a normal individual a dosage quotient of around 1.0 was interpreted as having two copies of target sequence, a value of about 0.5 as a heterozygous deletion, and 1.5 as a one-copy increase (three copies) of the target sequence.

CFMSA

To assess the sensitivity, specificity, and application of CFMSA, 17 deletions and 300 controls blinded to their molecular genetic status were analyzed by this technique, which we previously developed. Its protocol is as described by Yang *et al.* (2009). Briefly, we used competitive M-PCR to co-amplify the five STRPs and reference genes in a single reaction for 24 cycles in a 25 uL reaction mixture. Then, 2 μL of PCR product were analyzed by capillary electrophoresis (CE) and the GeneMapper software used to size the PCR products and obtain peak areas.

Application of MLPA

All the 17 deletion samples and 120 norm controls blinded to their molecular genetic status were included in MLPA analysis with a set of commercially available P-250 MLPA probemix (MRC-Holland, Amsterdam, The Netherlands). This probemix contains 48 MLPA probes with amplification products between 130 and 481 nt, 30 of them covering the 22q11 region. The assay conditions were essentially according to manufacturer's recommendations (MRC-Holland). We used the commercially available software Coffalyser (MRC-Holland) to analyze our data.

Statistical analysis

Mean SD of each probe was calculated for normal samples. The average of mean probe SD was also calculated for each technique by Excel software (Microsoft, Seattle, WA).

Results

Sensitivity for M-PCR, MLPA, and CFMSA

In this study we developed three PCR-based copy number variant approaches, CFMSA, M-PCR, and MLPA, in our laboratory to detect microdeletions in chromosome 22q11.2. In 17 patients previously detected to have 22q11.2 deletion by haplotype analysis, experimental M-PCR, CFMSA, and MLPA analyses was performed for validation of these tools. Abnormalities in all 17 patients were confirmed by two groups of M-PCR probes between LCR-A and LCR-B, five CFMSA probes between LCR-A and LCR-B, nine MLPA probes between LCR-A and LCR-B, three MLPA probes between LCR-B and LCR-C, and two MLPA probes between LCR-C and LCR-D.

For M-PCR and MLPA, each deletion was detected as a $\leq$0.7-fold decrease in relative peak areas of both of the amplified probes compared with those of the control sample. For CFMSA results from each deletion sample, all five consecutive STRPs showed single-peak patterns and $\leq$0.7-fold decreases in relative peak areas. Figure 1 shows a representative electrophoretogram of M-PCR, MLPA, and CFMSA product from a control and a deletion individual.

No false-negative results were obtained, in accordance with cytogenetic results or haplotype analysis. All known deletion regions were correctly identified by M-PCR, CFMSA, and MLPA. Combining the results of all 22q11.2 probes, M-PCR, CFMSA, and MLPA are concordant with each other and all have a sensitivity of 100% in a 17-sample cohort.

Specificity for M-PCR, MLPA, and CFMSA

A control group consisting of 300 individuals with normal chromosome status was used to assess the specificity of these techniques. Thus, all dosage quotients for all probes in them were supposed to be 1. Overall, there were 2448 signals for M-PCR, 5760 for MLPA, and 1500 for CFMSA.

The diagnostic interpretation of multiplex for a given sample is usually performed by examining a combination of multiprobe signals, especially the signals from adjoining probes in a genomic region. Two spurious adjoining probe signals may be easily misjudged as false-positive for the whole results. Thus, we calculated the possibility for both single spurious probe signals and two concurrent spurious adjoining probe signals.

For M-PCR, results from 300 multiplex 1 and 158 multiplex 2 were available. Each probe signal in these results with dosage quotient above 1.3 or below 0.7 in normal controls was considered as spurious. The average possibility for a single spurious probe in our two multiplexes was 0.11 (270 spurious signals/2448 signals); correspondingly, the possibility for two adjoining spurious probe signals was the square of it, that is, 0.012.

For MLPA, data from 120 results were available. The average possibility for a single spurious probe out of the 45 probes in our multiplex was 0.026 (150 spurious signals/5760 signals); correspondingly, the possibility for two adjoining spurious probe signals was 0.0007.

The 300 available CFMSA results could provide quantitative as well as polymorphic information. Thus, the possibility for false-positive results for CFMSA is the product of the uninformative possibility for two adjoining probes and the spurious quantitative possibility for them. The average heterozygosity for a single STRP in our CFMSA assay was 0.7. This means that the uninformative possibility for a single STRP was 0.3, and the uninformative possibility for two adjoining probes was 0.09. Besides, the average quantitative possibility for a single spurious probe out of the five probes in our multiplex was 0.071 (107/1500); correspondingly, the possibility for two spurious adjoining probe signals was 0.005. The total possibility for false-positive results for CFMSA would be 0.005 multiplied by 0.09, which is equal to 0.00045.

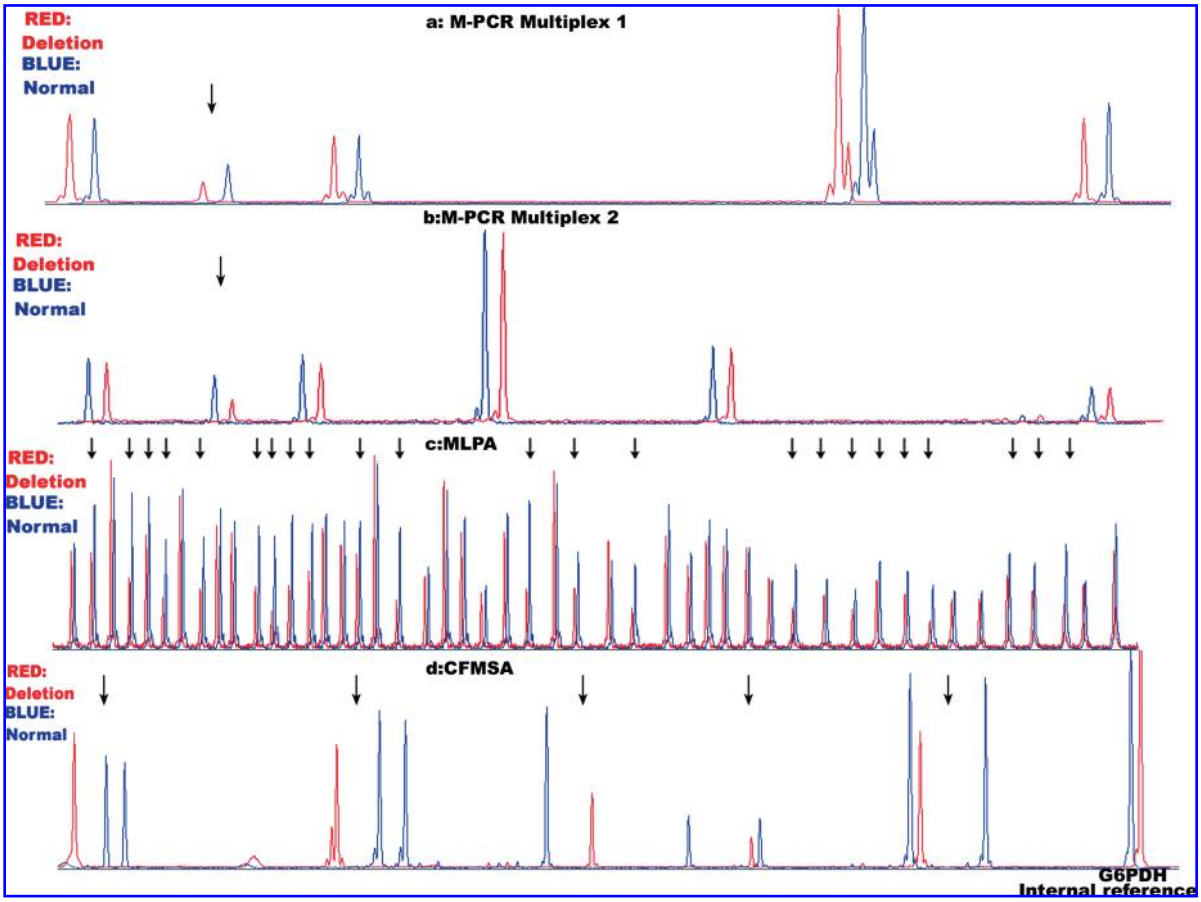

FIG. 1. Detection of 22q11.2 deletion by multiplex fluorescent PCR (M-PCR), multiplex ligation-dependent probe amplification (MLPA), and competitive fluorescent multiplex STRP assay (CFMSA). The deletion is shown in red and the normal controls in blue. (**a**) and (**b**) M-PCR showing deletions of 22q11.2, the peak area of the 22q11.2 is decreased in the patient compared with the control sample. (**c**) MLPA peak profile showing a deletion of 22q11.2. (**d**) CFMSA peak profile showing a deletion of 22q11.2, where all STRPs are single-peak patterns with $\leq$0.7-fold decreases in relative peak areas compared with the internal reference, whose dosage quotient is around 1.0.

Compared with the corresponding rates for M-PCR and MLPA, it is the smallest among the three techniques.

The mean probe SD, an expression of the extent of variability of individual probes, strongly correlates with the number of spurious results generated by the probe (Vorstman *et al.*, 2006). The mean probe SD for a given locus in each technique was calculated to measure the specificity of that technique. A comparison of the probe SD for M-PCR, CFMSA, and MLPA is illustrated in a box-plot diagram (Fig. 2).

The average of mean probe SD for each tool was calculated from the total of 300 control samples included in the study. CFMSA and M-PCR have a higher average mean probe SD (0.14 and 0.17, respectively) and MLPA has 0.10, showing that MLPA could have more specificity than CFMSA and M-PCR in the analysis of quantities in a 300-sample cohort.

Application of M-PCR, MLPA, and CFMSA

A cost analysis and comparison of M-PCR, CFMSA, and MLPA are given in Table 2. The cost analysis includes relative estimated costs, turnaround time, and relative difficulty for establishment of technique.

When 317 samples were analyzed together, M-PCR could be easily established in a week, allowed rapid diagnosis of a large number of samples in 6 hours, and was also economical. CFMSA was also considerably cheaper and fast but relatively hard to establish. This method was developed in our laboratory over half a year. MLPA could not be established in an ordinary lab and is relatively expensive; also, it would take more than 14 hours for a turnaround.

Discussion

Recently, our view of human genetic variation has been extended by the abundant and widespread variations in the copy number (Carter, 2007), generating a high interest in the development of cost-effective and sensitive screening methods (Monfort *et al.*, 2006). The M-PCR technique was easily established in our study. It took only 1 week to establish an M-PCR with a new group of primers. However, the presence of multiple pairs of primers in a multiplex reaction could reduce the robustness of PCRs and the reliability of the quantification. CFMSA, though hard to establish, is however home designed. To get distinguishable peaks, robust amplification, and reasonable spatial layout, time is needed to select suitable primers and adjust their concentrations in multiplex. Some of the primers used in this work had been corrected for more than 10 times. Moreover, this approach cannot be readily

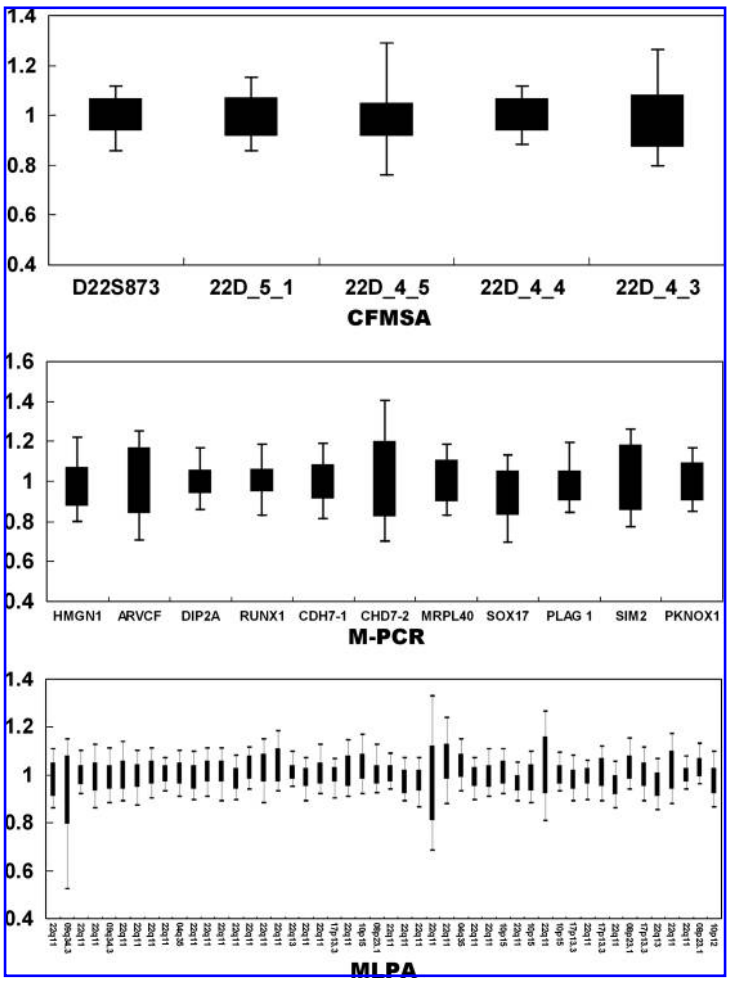

FIG. 2. Box plot of the variability of dosage quotient for each probe from CFMSA, M-PCR, and MLPA in the control samples. The dosage quotients are plotted on the Y-axis. The expected dosage quotient for each probe is 1.0, since they are exclusively derived from disomic. The top horizontal line of the box represents the 75th percentile; the bottom line is the 25th percentile. The horizontal line in the middle of the box is the median. Vertical bars outside the box represent the 10th and 90th percentiles.

adapted for deletion analysis of any region of human chromosomes because of the limited number of the highly polymorphic tetranucleotide STRPs in the genome. MLPA is not a home-designed assay, and its establishment relies on the complicated and time-consuming probe generation procedure. Application of MLPA kits except for ready-to-use commercial kits has seldom been reported.

One of the major problems of the PCR-based techniques is their reliability, including sensitivity and specificity. The variability in the performance of probes in these techniques may be a result of suboptimal probe characteristics, such as the kinetics of hemi-probe hybridization, hemi-probe ligation, and fully ligated probe amplification (Vorstman *et al.,* 2006). The mean probe SD value for a given locus provides a measure of that probe's reliability, and the average probe SD for the technique represents that tool's reliability. The three PCR-based techniques used in this study all have 100% sensitivity. MLPA has the least average mean probe SD and it can also provide more than 40 signals in one multiplex, which ensure its high specificity. CFMSA, because of its polymorphic and quantitative information, provides the lowest possibility for concurrent false-positive signals from two adjoining probes. These consecutive spurious signals may create serious confusion in the integration of results.

Table 2. Cost Analysis for Detection of Copy Number in 22q11.2 Region Using Three Quantitative PCR Methods

	M-PCR	*MLPA*	*CFMSA*
Relative estimated costs ($)	5	15	5
Turnaround time (hours)	6	24	6
Development in lab	easy	unavailable	hard

Diagnostic testing for 22q11.2 deletions is commonly performed by FISH, which is accurate but time consuming and expensive. These disadvantages become a barrier for its use in large-scale screening (Jalali *et al.,* 2008). MLPA is less expensive, less labor intensive, and has a quicker turnaround time. This study showed that CFMSA has significant advantages over other PCR-based methods such as MLPA, because it is much less expensive, less labor intensive, and faster. This is

very important because many patients referred for deletion remain without a clinical diagnosis even after a long period of unsuccessful examinations. The lower cost for the screening of deletions by CFMSA would allow its application to more patients while using less restrictive selection criteria. M-PCR is a rapidly developing and cheap technique that allows detection of atypical deletions in regions not covered by the commercially available probes and therefore not routinely tested by FISH or MLPA. These uncommon deletions are constantly defined (Frohling *et al.,* 2002). But the low reliability of M-PCR could limit its use in screening for copy number change in clinical patients.

In conclusion, we propose that CFMSA is a rapid, relatively inexpensive assay compared to MLPA and M-PCR. It shares high sensitivity with other PCR-based techniques and has higher specificity. Given the advantages of cost effectiveness, efficiency, accuracy, high throughput, and ease of an in-house protocol, our future work will include the screening by CFMSA of numerous patients with minor symptoms of 22q11.2 deletion syndromes.

Acknowledgments

This work was supported by the Health Foundation of Jiangsu Province (grant number: H2006) and the Jiangsu Foundation for Development (grant number: BS2006012), the key project of the Medical Science and Technology Development Foundation, Nanjing Department of Health (grant number: ZKX06018), and the Foundation for the Jiangsu's Outstanding Fellow in Medical Science (grant number: LJ200628).

Disclosure Statement

No competing financial interests exist.

References

Ahn JW, Ogilvie CM, Welch A, *et al.* (2007) Detection of subtelomere imbalance using MLPA: validation, development of an analysis protocol, and application in a diagnostic centre. BMC Med Genet 8:9–21.

Butler JM (2005) Constructing STR multiplex assays. Methods Mol Biol 297:53–66.

Carter NP (2007) Methods and strategies for analyzing copy number variation using DNA microarrays. Nat Genet 39:S16–S21.

Frohling S, Skelin S, Liebisch C, *et al.* (2002) Comparison of cytogenetic and molecular cytogenetic detection of chromosome abnormalities in 240 consecutive adult patients with acute myeloid leukemia. J Clin Oncol 20:2480–2485.

Jalali GR, Vorstman JA, Errami A, *et al.* (2008) Detailed analysis of 22q11.2 with a high density MLPA probe set. Hum Mutat 29:433–440.

Kobrynski LJ, Sullivan KE (2007) Velocardiofacial syndrome, DiGeorge syndrome: the chromosome 22q11.2 deletion syndromes. Lancet 370:1443–1452.

Lupski JR (2007) Genomic rearrangements and sporadic disease. Nat Genet 39:S43–S47.

Monfort S, Orellana C, Oltra S, *et al.* (2006) Evaluation of MLPA for the detection of cryptic subtelomeric rearrangements. J Lab Clin Med 147:295–300.

Pierpont ME, Basson CT, Benson DW Jr., *et al.* (2007) Genetic basis for congenital heart defects: current knowledge: a scientific statement from the American Heart Association Congenital Cardiac Defects Committee, Council on Cardiovascular Disease in the Young: endorsed by the American Academy of Pediatrics. Circulation 115:3015–3038.

Rooms L, Reyniers E, Kooy RF (2005) Subtelomeric rearrangements in the mentally retarded: a comparison of detection methods. Hum Mutat 25:513–524.

Shaffer LG, Bui TH (2007) Molecular cytogenetic and rapid aneuploidy detection methods in prenatal diagnosis. Am J Med Genet C Semin Med Genet 145C:87–98.

Stenson PD, Ball EV, Mort M, *et al.* (2003) Human Gene Mutation Database (HGMD): 2003 update. Hum Mutat 21:577–581.

Vorstman JA, Jalali GR, Rappaport EF, *et al.* (2006) MLPA: a rapid, reliable, and sensitive method for detection and analysis of abnormalities of 22q. Hum Mutat 27:814–821.

Yang C, Shen L, Xu Z, *et al.* (2009) A novel competitive fluorescent multiplex STR polymorphism assay for rapid, reliable and single-tube screening of 22q11.2 copy-number aberrations. Electrophoresis 30:465–471.

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