Review

Dong-Sheng Lian*, Xiang-Yuan Chen, Hua-Song Zeng* and Yan-Yi Wang

Capillary electrophoresis based on nucleic acid analysis for diagnosing inherited diseases

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Abstract: Most hereditary diseases are incurable, but their deterioration could be delayed or stopped if diagnosed timely. It is thus imperative to explore the state-of-the-art and high-efficient diagnostic techniques for precise analysis of the symptoms or early diagnosis of pre-symptoms. Diagnostics based on clinical presentations, hard to distinguish different phenotypes of the same genotype, or different genotypes displaying similar phenotypes, are incapable of pre-warning the disease status. Molecular diagnosis is ahead of harmful phenotype exhibition. However, conventional gold-standard molecular classifications, such as karyotype analysis, Southern blotting (SB) and sequencing, suffer drawbacks like low automation, low throughput, prolonged duration, being labor intensive and high cost. Also, deficiency in flexibility and diversity is observed to accommodate the development of precise and individualized diagnostics. The aforementioned pitfalls make them unadaptable to the increasing clinical demand for detecting and interpreting numerous samples in a rapid, accurate, high-throughput and cost-effective manner. Nevertheless, capillary electrophoresis based on genetic information analysis, with advantages of automation, high speed, high throughput, high efficiency, high resolution, digitization, versatility, miniature and cost-efficiency, coupled with flexible-designed PCR strategies in sample preparation (PCR-CE), exhibit an excellent power in deciphering cryptic molecular information of superficial symptoms of genetic diseases, and can analyze

in parallel a large number of samples in a single PCR-CE, thereby providing an alternative, accurate, customized and timely diagnostic tool for routine screening of clinical samples on a large scale. Thus, the present study focuses on CE-based nucleic acid analysis used for inherited disease diagnosis. Also, the limitations and challenges of this PCR-CE for diagnosing hereditary diseases are discussed.

Keywords: capillary electrophoresis; diagnosis; inherited disease; PCR; symptom.

Introduction

A majority of hereditary diseases cannot be cured after clinical features are manifested [1, 2], highlighting the indispensability of early-stage precautionary diagnosis and management in enhancing patients' quality of life or blocking inter-generational transmission. The diagnostics and classifications based on clinical manifestations, clinical features or pathophysiological parameters [3–9] have potential risks and obvious defects: for example, (1) making a late judgment in the advanced stages of the disease; (2) incompetence of differentiating the cases sharing similar or common phenotypical features among different genotypes or genotype sub-types or differentiating the cases exhibiting various clinical symptoms under the same genotype sub-type [10]. For example, spinocerebellar ataxia type 7 (SCA7) patients with the same CAG repeats exhibit a different age of onset that might correlate with inherent gene polymorphisms, epigenetic modification or somatic mosaicism [4]. A wide heterogeneity is detected in phenylalanine hydroxylase (PAH) mutations found in phenylketonuria (PKU) patients [11]. Diverse molecular heterogeneity is distributed in α -thalassemia and β -thalassemia patients [7, 8, 12]. Overlapping manifestations existed between myotonic dystrophy type 1 (DM1) and other myotonic disorders [13]. Similar clinical appearances were presented in patients with Huntington's disease (HD) vs. patients with HD-like features or HD phenocopies [14], in Diamond-Blackfan anemia (DBA) patients vs. other bone marrow failure syndrome (BMFS) patients [15, 16] and in SCA 37 patients vs. SCA7 patients

*Corresponding authors: **Dong-Sheng Lian**, Guangzhou Women and Children's Medical Center of Guangzhou Medical University, NO. 9 at Jinsui Rd., Tianhe District, Guangzhou, Guangdong, P.R. China; and **Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, P.R. China**, E-mail: papersend1@163.com; and **Hua-Song Zeng**, Guangzhou Women and Children's Medical Center of Guangzhou Medical University, NO. 9 at Jinsui Rd., Tianhe District, Guangzhou, Guangdong, P.R. China, E-mail: huasongxuqing@163.com
Xiang-Yuan Chen: Guangzhou Women and Children's Medical Center of Guangzhou Medical University, NO. 9 at Jinsui Rd., Tianhe District, Guangzhou, Guangdong, P.R. China
Yan-Yi Wang: Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, P.R. China

[4, 5, 17]; (3) unable to diagnose asymptomatic carriers such as phenotypically normal intermediate or pre-mutation patients [4, 13, 14, 18–22], and unable to uncover the underlying cryptic discrepancies between genotype and phenotype [6].

Conversely, the genetic-level diagnostics excluded the aforementioned drawbacks to reach reliable and precise diagnostic conclusions [6, 7, 23]. For instance, normal $\alpha 5$ (IV) expression is regarded as an X-linked trait, which was also found in autosomal recessive Alport syndrome (ARAS) by molecular analysis [6]. However, conventional genetic diagnostic techniques, such as karyotype analysis, Southern blotting (SB) and sequencing, despite being gold-standard approaches for diagnosing inherited disease at nucleic acid levels [13, 17, 19, 21, 22, 24–31], are time-consuming, tedious, labor-intensive, low-throughput and expensive for extensively screening populations that consist of potentially affected individuals or asymptomatic carriers [28]. Furthermore, direct sequencing, including the advanced next-generation sequencing (NGS) with an enhanced capability for detecting large-sized genes, is deficient in detecting complicated genetic abnormalities, such as aberrant splicing or overlarge genes or long-range insertion or deletion in heterozygotes [6, 10, 32]. SB analysis requires abundant DNA but is incapable of determining the exact size of DNA fragments, or analyzing the allele structure as intergenic and extragenic polymorphisms or differentiating diverse types of alleles [21, 31].

To complement the shortcomings of traditional methods, high-performance capillary electrophoresis (HPCE), with the advantages of automation, high-speed, high-throughput, high-resolution, high-efficiency, miniature, versatility and low operational costs, being capable of analyzing multiple PCR product fragments in a single run providing qualitative and quantitative results at the same time, is explored [1, 22, 33, 34]. This HPCE integrated with flexible and customized PCR designs accommodates diverse genomic detecting requirements. This preliminary approach has great potential and applicable value for diagnosing genetic diseases and might serve as a potent alternative tool for common techniques. Strikingly, the development of microchip electrophoresis (MCE) integrates PCR, separation, detection and CE electropherogram analysis within a miniaturized chip (lab on a chip) to make the diagnostic workflow automatic, fluent and streamlined, and promotes the clinical diagnostic efficiency for inherited diseases [35].

Thus, the present study focuses on the use of capillary electrophoresis based on nucleic acid analysis for

diagnosing inherited diseases. Also, the limitations, challenges and perspectives of this CE-based diagnostic technique are discussed.

The procedures of CE-based nucleic acid analysis for diagnosing hereditary diseases

The CE-based diagnostic technique is composed of two critical steps. First, a reliable and suitable PCR strategy is designed to amplify target genomic regions according to the trait of pathological mutated genes [35]. These PCR strategies consider the characteristics of mutated genes and the surrounding conserved regions (such as microsatellite sequence) [36, 37] and employ informative markers [31, 38] to allow the genotype differentiation between full mutations, permutations, intermediate and normal [14, 19, 24, 25, 27], as well as discrimination of different subtypes from the same genotype [10]. In order to detect the PCR products, capillary gel electrophoresis (CGE) with fluorescence detection (FD) or laser-induced fluorescence (LIF), or CGE-single-strand conformation polymorphism (SSCP) was utilized. Therefore, the accuracy of diagnostic techniques was based on the optimization of PCR strategies and the high efficiency of CE detection. Because PCR is the first step in the diagnostic flowchart, the quality of PCR products crucially affects the subsequent CE analysis. Vice versa, selecting the appropriate CE detection mode and optimizing the CE separation system is critical for interpreting the cryptic information of the PCR products. Next, we adapted the automation and high-throughput PCR amplifications from single to duplex PCR, and finally to multiplex PCR. CE also developed from single-tube CE to array CE, and finally to MCE [34]. Furthermore, MCE, based on the lab-on-chip/micro total analysis systems (uTAS) concept, integrated these two separate steps into one chip in the form of high automation, high speed, high efficiency and low costs to facilitate and promote clinical diagnostics [35].

Diagnosis of hereditary diseases

The hereditary disease diagnoses are classified as autosomal dominant inherited disease diagnosis, autosomal recessive inherited disease diagnosis, X-linked dominant inherited disease diagnosis, X-linked recessive inherited disease diagnosis, preimplantation genetic diagnosis

(PGD) and prenatal diagnosis (PND), and hereditary cancer diagnosis.

Autosomal dominant inherited disease

The expansion of CAG trinucleotide repeats in exon 1 of the huntingtin (*HTT*) gene causes HD [22], and thus, accurate detection of the number of CAG repeats is pivotal for HD diagnosis. Repeat-flanking-PCR-CE (RF-PCR-CE), in which primers flank the CAG repeats, is used for amplification. Then, CE analysis deciphers the information from PCR results and serves as a diagnostic approach for HD. However, RF-PCR efficiency is lowered with high CAG repeats; also, the polymorphisms of the flanking sequence lower the PCR specificity. These two defects make RF-PCR-CE incapable of determining large-sized CAG repeats (<110 CAG repeats), thus risking a false-negative result for long-range expanded alleles [14] (Figure 1).

In order to address this issue, TP-PCR-CGE-FD was developed. A flanking primer was paired with the other primer annealing randomly within the CAG repeats to produce amplicons of different sizes. Using TP-PCR, all expanded alleles were amplified robustly and reliably irrespective of the size. Subsequently, CGE-FD was used to detect these TP-PCR products of expanded alleles and generate a distinctive CE electropherogram, wherein larger expansions correspond to longer stretched peaks with decreased peak height; these CE electropherograms could

easily differentiate the affected HD and intermediate from non-expanded normal alleles by calculating the number of amplicon peaks from the first peak to the tallest peak on the right [14, 24] (Figure 2). This improved TP-PCR-CGE-FD could detect a maximum of 180 CAG repeats with 100% analytical specificity and sensitivity in blinded testing for clinical samples, thereby proving it as an accurate and robust tool for large-scale screening of inherited diseases.

Although TP-PCR-CGE-FD exhibited excellent diagnostic abilities, complicated post-PCR handling steps are necessary before CGE-FD detection of PCR products [14, 26]. Also, TP-PCR may or may not be coupled with an MCA for detecting CAG repeats (Figure 1). However, although TP-PCR-MCA could readily and rapidly separate the expansion-positive allele from the expansion-negative allele in a single step, it could not distinguish between intermediate alleles (33–35 CAGs repeats) and expansion-positive alleles. Furthermore, due to the incapability of providing an exact number of CAG repeats for expansion-positive samples indicated by TP-PCR-MCA, a post-MCA CGE-FD analysis is essential for separating intermediate from positive and for determining the accurate number of CAG repeats [14].

Therefore, despite TP-PCR-MCA-post-CGE-FD, drawing a rapid qualitative (without quantitative) conclusion regarding positive or negative seems to be superior over TP-PCR-CGE-FD, thereby being cost-effective for the whole diagnostic process with respect to reducing the post-PCR management [14]. Nonetheless, in our

Figure 1: Comparison of repeat-flanking PCR and triplet-primed-PCR [14].

Melt peaks of normal, pHTT(CAG)₂₆ and pHTT(CAG)₃₃ (controls), and HD-affected samples are plotted in blue, black and red lines, respectively. (1) Using RF-PCR-MCA (melting curve assay) of normal samples, a single melt peak with a T_m in the normal range is produced. Two melt peaks are produced by the HD-affected samples, in which one dominant melt peak is located in the normal range, and the other lies in the expanded range. The height of the melt peaks representing the expanded allele decreases with the increment of CAG repeats. If CAG repeat expansion is sufficiently large to lose the expanded allele peak, the only remaining normal allele peak might provide a false-negative report. (2) In triplet-primed-PCR-MCA (TP-PCR-MCA), only a distinct melt peak is generated for every sample. It is irrelevant to the disease status or the CAG repeat length. The location of the melt peaks, relative to the peaks of pHTT(CAG)₂₆ and pHTT(CAG)₃₃, effectively distinguishes between normal and HD-affected samples.

Figure 2: TP-PCR-CGE-FD electropherogram [16].

opinion, the accurate determination of the CAG repeat number for estimating HD severity and delicately differentiating the diverse mutated genotypes are critical. Regarding decreasing the fees for large-scale population screening, in consideration of low morbidity for the majority of the population, using TP-PCR-MCA as the first step to exclude normal samples and TP-PCR-CGE-FD as the second step to precisely distinguish positive results, is helpful. Nevertheless, the peaks in the CE electrophoretograms can be more visually distinguished than a lot of curves, especially those that are at proximity [27] (Figure 1). In the case when numerous clinical samples are simultaneously analyzed, and a clinical database needs to be established, MCA panels are difficult to differentiate, quantify and digitalize. Hence, MCA acted as an optional and supplemental tool for CE-based diagnosis.

CTG repeat expansion in the 3'UTR of the dystrophin myotonia-protein kinase (*DMPK*) gene leads to disease with DM1 disease. Regardless of the expanded size, TP-PCR was also adopted to replace the repeat-spanning PCR that was only capable of detecting small expanded *DMPK* alleles. However, the interruptions found within CTG repeats are likely to arouse the annealing failure of the TP primer, thus disabling the TP-PCR. Therefore, a modified bidirectional TP-PCR, which simultaneously targeted the 5' and 3' ends of the CTG repeats to prevent these interruption-caused false-negative results, was developed. Subsequently, this bidirectional TP-PCR coupled with MCA was combined with a five-cycle labeled-primer (LP)-PCR-CGE-LIF to screen the expanded-positive samples and accurately determine the exact size of CTG repeats in expanded allele [13] (Figure 3). An equivalent of 10 ng DNA was sufficient for one-step TP-PCR-MCA to obtain positive or negative results with 100% sensitivity and specificity,

which remarkably decreased the amount of each sample required for TP-PCR-CGE-LIF analysis, significantly enhancing the analytical throughput and reducing the budget of DM1 diagnosis. This phenomenon enables the TP-PCR-MCA-five-cycle LP-PCR-CGE-LIF approach as a robust, cost-effective and speedy tool for routine analysis of a large number of samples [13]. Furthermore, although the almost incurable hereditary DM1 discouraged the exploration of state-of-the-art diagnostic techniques, recently, affinity capillary electrophoresis (ACE) was employed to study the interactions between nucleic acid and ligand. EBAB (1,2-ethane bis-1-amino-4-benzamidine, a pentamidine analog) was screened out as an active candidate compound to bind and reverse the formation of toxic RNA-(CUG)_n repeats. The efficacy, low cellular toxicity and high affinity of EBAB to (CUG)_n RNA repeat motif provided an insight into stopping DM1 deterioration if confirmed early, thereby valuing the development of early diagnostic techniques [1].

The polymorphic ATTTT repeats in the DAB1 (reelin adaptor protein) 5' UTR region were inserted with (ATTTC)_n to form structures of [(ATTTT)_n(ATTTC)_n(ATTTT)_n], which were accounted for SCA37. The traditional diagnostic methodology using long-range PCR-based sequencing is time-consuming, laborious and expensive for analyzing a large number of samples. Thus, standard PCR methods, as a preliminary screening step before sequencing, are useful to decrease the number of samples that need to be sequenced; however, it is only able to detect samples with pentanucleotide repeats <30. Exceeding 30 ATTTT repeats (non-pathogenic alleles) from 7% of the normal population would generate missing PCR or homoallelism similar to the PCR results of the inserted mutated genotype. To solve this problem, repeat-primed (RP)-PCR-CE was designed to detect pentanucleotide repeats >30, which produced 5-bp interval peak ladders extending

Figure 3: Bidirectional TP-PCR with corresponding MCA profiles and CE electrophoretogram [13].

(A) 5' and 3'-TP-PCRs showing positions of primers; (B) 17 Coriell Cell Repository samples were subjected to bidirectional TP-PCR, followed by MCA analysis to produce melt curves or melt peaks in panels. Threshold temperatures (TTs) of melt peaks were annotated by vertical dotted lines. In MCA analytical profiles, normal and DM1-affected samples, melt curves or melt peaks were segregated to the left or the right of TTs line established from pDMPK(CTG)₃₅ and pDMPK(CTG)₄₆ (controls), respectively; (C) CE electrophoretograms of pDMPK(CTG)₃₅ and pDMPK(CTG)₄₆; -dF/dT, negative first derivative of fluorescence vs. temperature; RFU, relative fluorescence unit.

over 250 bp (≤ 250 bp indicates <30 ATTTT repeats, and thus, standard PCR is eligible). Combining RP-PCR-CE with standard PCR as the first filtering step, the number of samples requiring sequencing was reduced to 18% [17] (Figure 4), thereby cost-effectively promoting SCA37 diagnosis.

The CAG repeats in exon 3 of the *ATXN7* gene are responsible for SCA7. Multiplex PCR-CGE-LIF studied the correlations between clinical features and genetic status involving CAG repeats among the SCA7 Mexican population. The results found that CAG repeat number in SCA7 was about 37–72, and $(CAG)_{39}$ was the most common. More the CAG repeats, earlier the disease onset-age and symptoms. More than 46 CAG repeats indicated early disease onset, while <46 CAG repeats correlated with the adult-onset; paternal transmission is preferential to cause a greater CAG repeat instability [4]. However, voice impairment, one of the clinical features of SCA7, was found irrelevant to CAG repeats, which might be utilized to discriminate SCA7 from other types of SCA [5]. In addition to SCA7, CAG repeats also cause SCA 1–3, 6 as well [10, 30]. Surprisingly, their phenotypic performances were variably correlated with Parkinson's disease and amyotrophic lateral sclerosis [30],

emphasizing that it is imperative to discriminate these SCA sub-types at a genetic level. However, common molecular diagnostic approaches, such as sequencing, SB or standard PCR plus the second step of eliminating false-negative rooting from long CAG repeats that could not be amplified by common PCR, are not suitable for routine analysis. Moreover, CAT and CAA interruptions within CAG repeats impede the discrimination of SCA sub-types. In order to settle the aforementioned predicaments, a tentative TP-PCR-CGE-LIF [30], with the other improved tethering PCR-CGE-LIF comprising a forward and a reverse primer (reverse primer could anneal within and downstream of CAG repeats simultaneously) [10] (Figure 5), was developed. The reliability of the approach was evaluated by clinical samples and additional laboratory tests. The results showed that homozygous and heterozygous (>100 repeats) pathogenic CAG expansions could be discriminated concurrently in a single tethering PCR-CGE-LIF run [10]. Also, an expanded allele sample without being detected by common PCR was newly identified via TP-PCR-CGE-LIF [30], demonstrating that developing flexible PCR-CE-based novel diagnostic techniques consummated the conventional approaches.

Figure 4: ATTTT RP-PCR-CE and optimized diagnostic workflow [17].

(A) ATTTT RP-PCR design and (B) CE electropherogram showing the fluorescent ATTTT RP-PCR results; (C) Optimized diagnostic workflow by integrating standard PCR and ATTTT RP-PCR-CE with long-range PCR-based sequencing. A standard PCR was conducted initially. Subsequently, samples, in which only one allele was amplified, were further analyzed by ATTTT RP-PCR-CE that differentiated homoallelism from large-sized allele carriers. If samples exhibited positive results by ATTTT RP-PCR-CE analysis, long-range PCR-based sequencing was employed to determine the size and composition of large alleles. According to the studied control population, 71% of the samples only needed standard PCR to complete the negative diagnosis. 82% of the total samples provided negative results by combining standard PCR with ATTTT RP-PCR-CE analysis. Long-range PCR followed by Sanger sequencing was simply required in the remaining 18% of the samples.

Figure 5: Tethering PCR-CGE-LIF [10].

11 and 12 CAG repeats heterozygous subjects as representatives to illustrate the scheme. Forward primer with a fluorochrome (green) anneals upstream of the CAG repeats, whereas reverse primer anneals within the repeat and downstream simultaneously. The whole repeat could be amplified to generate two main peaks (higher, heterozygous subject), as well as a set of discrete stutter peaks with a 3-bp interval, which could be produced by annealing inside the repeat (lower). A group of smaller peaks starting from five CAG repeats, followed by two main peaks representing 11 and 12 CAG repeats, respectively, are illustrated by the CE electropherogram.

Autosomal recessive inherited disease

The mutations in the phenylalanine hydroxylase (*PAH*) gene result in impaired *PAH* activities, causing PKU. To investigate and study the genetic variations in Iranian family PKU, sequencing combined with PCR-STR/VNTR-CE-LIF was respectively employed to determine the mutations in the *PAH* gene and mutant-associated polymorphisms (minihaplotype analysis) [11]. The results showed that 95% of alleles harbored identified causative mutations (154/162), and c.1066-11G > A was the most frequent mutation (20.37%), which correlated with CE-determined minihaplotype of 7/250. c.1066-11G > A mutation. Furthermore, the association between c.1066-11G > A mutation and minihaplotype 7/250 was also found in the Mediterranean population, which strongly suggested a mutual interaction in historical and geographical aspects [11]. Sequencing combined with PCR-STR/VNTR/*PAH*STR-CE-LIF was also applied to identify the causative mutations with linked haplotypes in the other classical PKU

studies [39, 40]. Together, sequencing and PCR-CE on the specific mutations and minihaplotypes provided useful information for PGD and PND in affected families.

Mutations in the cystic fibrosis transmembrane regulator (*CFTR*) gene lead to cystic fibrosis (CF). To study E1104X mutation-related CF haplotypes, multiplex PCR-CGE-LIF utilizing the intragenic and extragenic microsatellite polymorphisms around E1104 mutations combined with PCR-RFLP analysis revealed that haplotypes 112 (35%) and 221 (45%) accounted for 80% of extragenic haplotypes, and the 16-31-13 haplotype encompassed a majority of 50% in intragenic haplotype analysis. These results indicated that CE-based multiplex PCR analysis is a valuable tool in mutation-related haplotype analysis [36].

Homozygous or heterozygous mutations in the *COL4A3* or *COL4A4* gene account for ARAS. To study the correlations between clinical phenotype and genotype, a modified mutation analytical strategy (PCR-sequencing-CE), in which genomic PCR-sequencing was used first to detect mutations in the exon and exon-intron borders,

followed by RT-PCR-sequencing to detect RNA transcription abnormalities and PCR-CE to detect large heterozygous deletions, was exploited. A 100% mutation detection rate, as well as normal $\alpha 5(\text{IV})$ expression (X-linked characteristics), newly found in 20% ARAS patients, not only highlighted that genetic analysis rather than phenotype analysis provided precise diagnostic information but also demonstrated the reliability and effectiveness of this combined PCR-sequencing-CE diagnostic technique for promoting the clinical phenotype-genotype study [6].

The deletion of HBA1/HBA2 loci results in α -thalassemia. To develop a high-efficient genotyping method for clinical diagnosis of α -thalassemia, a two-tube multiplex-PCR-CGE-LIF was designed to detect the variants of one α -globin gene deletion ($\alpha^{3.7}$ and $\alpha^{4.2}$) and two α -globin gene deletion [$(\alpha)^{20.5}$, - $_{\text{SEA}}$, - $_{\text{MED}}$ and - $_{\text{FIL}}$]. Also, clinical samples previously assessed by additional lab tests were used to validate this novel technique. A concordance of 100% was achieved between the two institutes, which suggested that this novel multiplex PCR-CE is an alternative and effective tool for the diagnosis of α -thalassemia [41].

Autosomal dominant/recessive inherited disease

Gilbert's syndrome is an inherited bilirubin metabolic disorder. UGT1A1(TA) $_n$, TA repeats in the promoter region of the UDP-glucuronosyltransferase1A1 (*UGT1A1*) gene that decreases the enzyme activity (30%) was regarded as the main cause of the disease. To study the (TA) $_n$ polymorphism, devising reference materials for UGT1A1(TA) $_n$ genotyping is the prerequisite. Thus, multiple techniques encompassing DNA recombinant technology, sequencing, CE and denaturing HPLC were utilized to produce plasmid-based alleles (TA) $_{4-8}$ for routine genotyping of UGT1A1(TA) $_n$ [42]. Subsequently, PCR-CGE-LIF was used to explore the correlation between Gilbert-related hyperbilirubinemia and polymorphisms of UGT1A1(TA) $_n$ and heme oxygenase (HO) promoter (GT) $_n$ with normal or defective glucose-6-phosphate dehydrogenase (G6PD) in neonates. Surprisingly, the HO GT repeats, UGT1A1(TA) $_n$, and G6PD wild-type or defective, were found irrelevant to heme catabolism, underlining that studying a large population including various ethnic groups was warranted for further clarification [43].

DBA, an inherited BMFS disease correlated with mutations in ribosomal proteins (RPs) of the 40S and 60S ribosomal subunits, was identified in 60% of the patients. Thus, evaluating the ratio of 28S/18S rRNA may help

diagnose this haploinsufficiency disease. Based on this assumption, MCE-based rRNA analysis was explored in 53 Italian DBA patients. The results showed that RP mutations in the 40S ribosomal subunit decreased the amount of 18S rRNA, thereby producing a higher 28S/18S ratio in DBA patients as compared to normal controls, and vice versa, a lower 28S/18S ratio was found in 60S-defective DBA patients, which coincided with the hypothesis [15] (Figure 6). This principle was further applied to diagnose suspected DBA infants to result in the identification of a novel DBA mutation (RPL31) [16]. Therefore, this MCE-based rRNA analysis is an amenable diagnostic method for preliminary screening of DBA patients.

X-linked dominant inherited disease

The CGG repeat expansion in the 5'UTR of the *FMRI* (fragile X mental retardation 1) gene at chromosome region Xq27.3 causes fragile X syndrome (FXS). Traditional SB and sequencing are inappropriate to cost-effectively and routinely diagnose the familial inherited FXS [21, 26, 27, 29]. To establish a practical and effective tool for diagnosing FXS in the public health system, a TP (triplet repeat-primed)-PCR-CGE-FD was developed and standardized to routinely diagnose FXS [19], which effectively distinguishes affected patients with full mutations and asymptomatic carriers of premutation from normal individuals; it also detected the CGG repeat number up to 1380 with a precision deviation within 1 CGG [26], thereby emphasizing the availability, accuracy, robustness and cost-efficiency of the FXS diagnostic analysis. Furthermore, a methylation-specific duplex TP-PCR-CE-LIF (MS-dTP-PCR-CE-LIF) was exploited to characterize the AGG interruptions within CGG repeats for assessing the risk of FXS transgenerational, which not only uncovered the information regarding CGG repeats with methylation status and AGG interruptions but also detected female X-inactivation and male FXS mosaicism [21]. The accuracy and robustness of MS-dTP-PCR-CE-LIF were tested and validated by 131 genotype-known clinical samples. Considering the extremely low morbidity of abnormal CGG expansions in population, and with the aid of high-throughput, high-speed, high-sensitivity and low cost of PCR-CE-based sizing electrophoretogram in large-scale preliminary screening, a StairCase (SC) group testing was designed to lessen the number of samples needed to be retested independently by sequencing. Using this approach, only 21 assays were required to classify 210 samples into diverse genotypes (full mutation, premutation, intermediate and normal) with a 10-fold reduction in costs [25]. Identically,

Figure 6: MCE-based rRNA analysis in DBA diagnosis [15].

(A) The 28S/18S ratio between DBA patients and normal controls was investigated and compared. The 40S-defective DBA patients had a distinctly higher 28S/18S ratio as compared to that in 60S-defective DBA patients and normal controls. Vice versa, 60S-defective DBA patients showed a remarkably lower 28S/18S ratio. (B) MCE electropherograms (Agilent Bioanalyzer 2100) corresponded to DBA patients with RPS mutations, DBA patients with RPL mutations and healthy control, respectively. Two protruding peaks corresponding to 18S and 28S rRNA, a lower 18S peak in 40S-defective DBA patients, and a lower 28S RNA peak accompanied by a minor 32S pre-rRNA peak in 60S-defective DBA patients, were observed in CE electropherograms corresponding to normal, 40S-defective and 60S-defective samples, respectively, highlighting the usefulness and effectiveness of this MCE-based rRNA diagnostic approach.

in the light of an excellent correlation between T_m in MCA and CGG-repeat size determined by CE [27], a one-step TP-PCR-MCA was firstly utilized to preliminary screen the expanded positive CGG repeat samples (full mutation, permutation and intermediate) with 100% sensitivity and 99.6% specificity [20, 27, 29]. Subsequently, a post-MCA-TP-PCR-CE combined with MS-PCR and SB [20] or a post-MCA-TP-PCR-CGE-LIF with SB [27] was employed to characterize these selected positive results with respect to the CGG repeat length, methylation status and fragment size. These multi-technical integrated approaches capitalizing the merits of each technique exhibited a 100% sensitivity with 100% specificity, and were in agreement with the gold-standard techniques, such as sequencing, with a capability of detecting mosaicism as low as 1–4% [27]. Thus, these were applied to diagnose 850 symptom-suspected children to conclude that 2.2% of the suspected

group carried an expanded CGG repeat, which comprised 1.3% FXS full mutation, 0.8% pre-mutation and 0.1% intermediate [20].

X-linked recessive inherited disease

Mutations in the coagulation factor VIII (*F8*) gene, localized at the X chromosome, causes hemophilia A (HA). Inversion mutations from intron 22, including intron 22 inversion type 1 (Inv22-1) and type 2 (Inv22-2), account for almost 50% of HA cases. Thus, quickly identifying and genotyping Inv22-1 and Inv22-2 mutations are essential for clinically diagnosing HA [33, 38, 44]. However, common long-distance PCR is unable to differentiate between Inv22-1 and Inv22-2, although it can detect Inv22 mutations in *F8*. An inverse-shifting (IS)-multiplex PCR-CGE-LIF

was designed to resolve this dilemma: *BclI* restriction endonuclease, recognizing 5-T↓GATCA-3 sequence, was used to digest double-stranded DNA to produce DNA fragments with 5'cohesive termini for self-ligating into circular DNAs. Subsequently, these circularized DNAs were subjected to MPCR to generate specific PCR products followed by CGE-LIF analysis. Finally, these products were translated into distinctive and interpretable CE-based electrophoretograms to genotype *Inv22* [33, 44] (Figure 7). Using this technique, CGE substituted agarose gel electrophoresis for fast DNA analysis (12-fold faster than

slab gel electrophoresis [SGE] in analytical time), and CE short-end detection (distance between detector and outlet) replaced the long-end detection, which saved the total analytical time of 10 h with only a 5-min CGE detection [33, 44]. Then, a total of 57 HA clinical samples were successfully discriminated and genotyped [44], demonstrating that optimized PCR strategies coupled with high-efficiency CE analysis are powerful diagnostic tools for clinical applications.

In addition, universal (U) multiplex (M) PCR-CGE-LIF based on CE short-end detection was used to diagnose

Figure 7: Mechanism of *Inv22*, IS-PCR of *Inv22* and CGE-LIF analysis of characteristic IS-PCR products from *Inv22* [33].

(A) *Inv22* in the *F8* gene. Green, yellow and red squares indicate three homologous regions in intron 22: *int22h-1*, *int22h-2* and *int22h-3*, respectively. Arrows represented *F8* transcriptional orientation. T indicates telomere; C indicates centromere. (B) Characteristic DNA fragments generated by genotyping intron 22-related rearrangements using IS-PCR. Feature fragments were produced from wild-type, intron 22 inversion type 1 (*Inv22-1*), and intron 22 inversion type 2 (*Inv22-2*) were indicated as a, b and c, respectively. (C) CGE-LIF analysis translated these individualized DNA fragments into characteristic electrophoretograms by peaks. Optimal CGE separating conditions: 1XTBE buffer solution containing 0.4% HPMC, 8 kV separation voltage, injection at 10 kV for 20 s, temperature, 25 °C. Peaks: 1. 333 bp; 2. 385 bp; 3. 405 bp; 4. 457 bp; 5. 512 bp; 6. 584 bp; IS. 759 bp.

Duchenne muscular dystrophy (DMD), which mainly arises from exon deletions in the *dystrophin* gene. This novel UMPCR-CGE-LIF method designed a universal primer sequence within the forward primer to concurrently amplify all exons, followed by direct short-end CGE-LIF analysis, thereby eliminating the demand of post-PCR purification that would remove the impurities in common PCR-MLPA, which could identify DMD within 9 min [45] (Figure 8).

PGD and PND

Diagnosis for couples, who are at potential risk of transmitting a specific inherited disease to their offspring, is

defined as PGD or PND [46]. However, conventional molecular diagnostic workflows used by different institutes are easily interfered with disease, sample and known information involving mutations and proband. Furthermore, the procedures constituting of different separate steps are time-consuming, cumbersome and low cost-efficient. To optimize the diagnostic flowchart, a multiplex PCR-STR-CE-LIF approach (SEeMORE, single-tube electrophoresis analysis-based genotyping to detect monogenic diseases rapidly and effectively from conception to birth) was developed [47]. This method utilized specific intragenic and extragenic STRs around disease-related mutated genes to produce a characteristic post-PCR CE electrophoretogram that was interpretable irrespective of the

Figure 8: Universal multiplex PCR [45].

Forward and reverse primers were designed for amplifying genomic DNA, while the forward primers contained a small fraction of universal non-human sequence. Subsequently, eight cycles of PCR produced intermediate amplicons acquiring universal sections. Subsequently, the uni-FAM primer was hybridized with the complementary non-human sequence, and each exon was amplified in the following 25-cycle UMPCR. Consequently, a fluorophore was labeled in all DNA fragments.

mutations in the family and integrated multiple independent steps including linkage and mutation analysis, DNA contamination, and parenthood testing to obtain multiplex reports concurrently. Little consumed samples (a few cells), consolidated and streamlined procedures to rapidly arrive at results (1 day), curtailed costs that are only one-third of the traditional technique, and reliable, accurate, robust results irrelevant to the disease, sample, mutation and proband, are the advantages of this novel technique. The successful application of SEeMORE for diagnosing CF (autosomal recessive inherited disease) and DMD (X-linked inherited disease) demonstrated that this optimized CE-based diagnostic methodology is suitable for routine PGD and PND [47] (Figure 9).

As only a little of genetic sample could be collected from the embryo, SB, which consumes a lot of DNA, was inappropriate for PGD. Thus, a multiplex-PCR-CGE-LIF in a single tube with only a single run, which utilized 13 microsatellite markers around *FMR1* CGG repeat

(<1 Mbp) and the *Amelogenin* gene, was developed to determine the gender and PGD of FXS. In addition, the successful application of the method of PGD in 272 females offers an alternative, rapid and high-efficient tool [31].

Traditional karyotype analysis and sequencing for PND are time-consuming, labor-intensive and expensive. Furthermore, potential iatrogenic abortion in conventional PND (0.5–1%), which is caused due to insertion of invasive probes or needles into the uterus to collect fetal cells, is an issue [28, 48, 49]. To address these concerns, a novel, non-invasive technique with high-throughput, fast turnaround time, cost-effectiveness and automation is obligatory. Thus, a non-invasive genome-plex whole genome amplification (WGA)-quantitative fluorescent PCR (QF-PCR)-CE-LIF, which used microdissection to separate the circulating free fetal cells from maternal blood as diagnostic raw materials, was developed. WGA fragment products from a non-enzymatic randomly amplified genomic DNA (preparing DNA fragment molecular

	Sample-dependent	Mutation-dependent	Workflow (steps)	Instruments	Identification of amplification artifacts	Results	Costs in per case
SEeMORE	No	No	1	1	Yes	24 h	≤100
Traditional technique	Yes	Yes	3	2	No	7–15 days	200–500

C CE-based SEeMORE versus traditional technique

Figure 9: The workflows and comparisons between traditional technique and CE-based SEeMORE [47].

(A) The diagnostic flowchart of the traditional technique for PND and PGD. (B) The streamlined diagnostic processes of CE-based SEeMORE for PND and PGD. (C) The advantages of CE-based SEeMORE over the traditional technique.

library) were subsequently subjected to QF-PCR-CE-LIF analysis to obtain genetic information for the diagnosis of chromosomal abnormalities. Moreover, five fetal cells extracted from 2–3 mL of maternal peripheral blood were sufficient to gain detailed reports in >92% of cases within 24–48 h [28]. Furthermore, PCR-STR-CGE-LIF evaluation used to assess the percentage of maternal DNA contamination in fetal DNA reported that the adulterant probability was <6.25% [48]. This phenomenon suggested that non-invasive PND based on CE analysis of fetal DNA segregated from maternal blood is feasible and practical for a highly reliable and accurate PND result. In order to develop an NIPD (non-invasive PND) technique for diagnosing paternal CFTR p.Phe508del mutation, a MEMO-PCR-FLA-CGE-LIF approach, which utilized a blocking oligonucleotide to hamper the amplification of wild-type sequences while effectively amplifying and enriching the mutated sequences, followed by CGE-LIF analysis of PCR products, was developed. This MEMO-PCR-FLA-CGE-LIF could detect mutant DNA as low as 2% in a mutant/wild-type admixture, and this sensitivity exceeded the 10% of fetal DNA contained in total free circulating DNA. These results were validated and found to be in agreement with those from the multiplex PCR method and invasive techniques using chorionic villus sampling or amniocentesis [49]. Thus, NIPD MEMO-PCR-FLA-CGE-LIF was deemed as a sensitive, robust, practical and alternative approach for PND of CF.

Hereditary cancer

Splicing error in susceptibility genes resulting in germline variants is one of the reasons inducing hereditary cancers. To study BRCA2 splicing abnormality in inherited breast/ovarian cancer (HBOC), a pSAD (splicing reporter vector)-based minigene with BRCA2 exons 2–9 was constructed, which produced 53 (64%) aberrant transcripts among a total of 83 transcripts. RT-PCR-CE, capable of detecting small variations (1 bp) or rare isoforms by utilizing high-resolution CE mode, was combined with sequencing to determine and analyze these diverse transcript variants, which disclosed that most abnormal splicing transcripts were caused by exon skipping, and the majority of these splicing anomalies might contribute to HBOC development (pathogenicity); thus, PCR-CE was a useful tool in aiding research in hereditary cancer [50]. A *PALB2* (Partner and Localizer of BRCA 2) variant c.3201+5G>T in breast cancer (BC) family may affect BC pathogenesis by disrupting splicing motifs to result in abnormal mRNA transcripts. To investigate and study this genetic variant,

RT-PCR-CE, coupled with sequencing, was adopted to quantitatively determine and characterize the mRNA splicing landscape caused by c.3201+5G>T. The results showed that the broken splice site in intron 11 donor led to an altered amount of alternative transcripts ($\Delta 11$, $\Delta 12$ and $\Delta 11,12$), and the expression of these aberrant mRNA isoforms that originated from haploinsufficiency and homologous recombination deficiency may be responsible for BC susceptibility [51]. *BRCA1* c.4964_4982del19, one of the founder effects causing BRCA pathogenic variants, is prevalent in Italian hereditary breast and ovarian carcinoma syndrome. Conventional Sanger sequencing/NGS for population screening is expensive and unaffordable, and hence, to develop a practical, rapid and standardized technique, the PCR-MCE approach was tentatively tested. The results showed that PCR-MCE could detect mutated alleles as low as 2% in an admixture, which is more sensitive than the LOD of 10% using Sanger sequencing. Furthermore, 100% concordance was obtained between two MCE platforms (Agilent and Experion) and between MCE and Sequencing, respectively, demonstrating that this CE-based diagnostic technique is a robust, powerful and alternative tool instead of traditional techniques for diagnosing genetic diseases [34] (Figure 10). In the diagnosis of neurofibromatosis type 1 (NF1) derived from

Figure 10: CE electropherograms of detecting different percentages of c.4964_4982del19 mutant/wild-type tumor samples [34].

CE electropherograms of c.4964_4982del19 mutant allele of different percentages in an admixture, 50% (red), 30% (blue), 10% (green), 5% (light blue) and 2% (black), were magnified 20-fold to be placed in the upper-right. The results showed that less than 2% could be detected by PCR-MCE. The solid line in the middle indicates a 190–220 bp interval.

neurofibromin gene mutations, NGS combined with quantitative CE revealed that among 96% of pathogenic variants of the total variants, 51% were novel mutations. Thus, CE was used to quantitatively determine the percentage of mosaicism [32]. The cases aforementioned suggested that the use of multiple consolidated types of techniques, including CE, advanced diagnosis.

Limitations, challenges and perspective of PCR-CE used for the diagnosis of inherited diseases

The gold-standard techniques (sequencing, SB and karyotyping) exhibited some limitations and were not practical for extensive analysis of numerous clinical samples. Furthermore, some pathogenic mutated alleles are relatively simple and clear that the simple PCR-CE method (such as simplex PCR-CE) is sufficient for the identification, not requiring the expensive and heavy-workload gold-standard approaches [25]. Moreover, CE-based PCR analysis for diagnosing complicated clinical cases with hereditary disease is comparable to the traditional standard techniques in accuracy and reliability [27, 34], while showing advantages of flexibility, individuality, practicability, cost-efficiency, short turnaround time and automation over those cumbersome and expensive classic approaches, which make PCR-CE potential to be developed as an alternative high-efficient diagnostic technology instead of classic methods. Thus, PND of Down's syndrome using CE was explored as early as 1995 [52]. Nonetheless, PCR-CE for diagnosing genetic diseases still lingers on in the laboratory tentatively rather than extensively being used in clinical diagnosis; some intrinsic limitations are as follows: (1) CE technique belongs to analytical chemistry and designing suitable PCR strategies for diagnosing characteristically defective genetic genes correlate the disciplines of medicine and molecular biology. Also, flexible manipulating and integrating these two subjects is the prerequisite of developing novel PCR-CE diagnostic techniques. However, most researchers do not have such an interdisciplinary background; (2) Low-sensitivity using CE with ultraviolet-visible (UV-vis) detection originates from short light absorption dimension due to limited CE inner diameter (50–100 μm) and small available injection sample volume because of confined CE tube length (<100 cm) [1]. Furthermore, the conjugate double bond formed by the base group of DNA has a low UV-vis absorption, which further aggravates the dilemma of low

sensitivity using CE-UV. These drawbacks impede the use of CE-UV for DNA detection. Therefore, FD as an alternative solution is employed by most researchers to resolve the low-sensitivity predicament. However, it demands to label the sample with a fluorescence marker or intercalating dyes [33, 37, 38, 40, 41, 43, 45, 53, 54], which is time-consuming, expensive [14, 27, 37], labor-intensive, not suitable for automation and is toxic. As hundreds to thousands of clinical samples need to be tested in a clinical setting daily, fluorescence labeling cannot be used routinely. Moreover, fluorescence detectors are expensive, and CE-FD electropherograms confront the interchange challenge to integrate with the data from the most widely used UV electropherograms. As a result, FD is not a radical settlement for low sensitivity in CE, and thus has not been routinely applied despite that some commercial instruments are equipped with an FD detector; (3) Additionally, despite CE performances being improved significantly as compared to the initial results, the repeatability and reliability of the technique still need to be carefully validated and confirmed by traditional methods before actual applications [33, 41, 43]; (4) The conjunction between PCR and CE analysis needs to be improved, especially post-PCR management, including purification, extraction and sample labeling before CE detection, which adds costs, hampers automation and reduces efficiency in the high-throughput analysis [14, 26, 29, 38]; (5) Standardization of diagnostic processes, involving data integration and analysis and accuracy and reliability of method and results, especially the compatible difficulty when coupled with other clinical auxiliary devices to develop automatic diagnostic systems, is also a pending issue before routine application [47]; (6) CE equipment with matched reagents is yet relatively expensive [27, 29, 37]; (7) Although MCE was developed many years ago, the elegant design to integrate sample preparation, PCR and CE analysis within a miniature chip still confronts many practical issues that need to be addressed [34, 35].

Although CE has limitations, more advantages are as follows: (1) SGE [7, 9, 12, 23, 37, 55] or affinity chromatography, which is usually used to separate PCR fragments in common molecular diagnosis, is time-consuming, is labor-intensive, has low resolution, and has a significant pollution level, toxicity of dyes or harmful organic solvents. Furthermore, it can only provide approximate qualitative results. However, CE is a high-speed, automated, high-resolution, microscale, clean, digitization method which provides qualitative and quantitative results simultaneously [28, 32, 33, 44, 51] (Table 1); (2) CE can analyze multiple samples in parallel in several to dozens of minutes [44] and complete hundreds of analyses in 1 day. MCE can further enhance

Table 1: CGE analysis of PCR products as compared to SGE analysis.

Item	SGE for DNA	CGE for DNA
Procedures	Gel preparation → gel fixation → sample loading → electrophoresis → developing with dyes or ethidium bromide → computer scanning → electrophoretogram analysis, every step is independent	All the above steps are automatically and consecutively carried out by a computer program
Time-cost	Separation voltage: 5–8 V/cm. About 2 h is required for the whole process	Separation voltage: 200–300 V/cm. Automatic execution. Several to dozens of minutes to complete the analysis.
Efficiency	In the SGE electrophoretogram, bands are smeared, diverging and low-resolution, obstructing the accurate decoding of information.	High-resolution, clear electrophoretogram, millions or 10 millions of plates in peak efficiency could be obtained. Using sequencing gel, 1 bp difference could be detected [37].
Quantitative analysis	Incapability.	Quantitation by calculating peak area.
Profile and data processing	Manual and subjective analysis according to smeared bands, which does not meet the demand of large-scale screening clinical samples and establishing a large, standardized, clinical database.	During CE separation of DNA, CE-equipped operational software (such as Beckman's Karat software) in parallel and objectively analyze the parameters from peaks in the electrophoretogram. The results are of high accuracy and high reliability. Furthermore, the generated digitized data facilitate the establishment of a clinical database
Consumption and pollution	Dozens to hundreds of mL of gels are consumed in a single SGE run, which produce abundant waste with heavy pollution. In particular, use of toxic ethidium bromide or organic dyes is harmful to operators and surrounding people. Therefore, SGE must be performed in specially isolated rooms.	Only a few materials (μL) that are mostly water, inorganic salt and a handful of gel are consumed. It is cost-effective and clean. Furthermore, most reagents are non-toxic or insufficiently toxic (μL) to operators
Automation	All steps from gel preparation to final panel analysis are carried out by the operator stepwise and independently. It is labor-intensive, time-consuming and cumbersome.	The whole analytical process is automatically executed by the computer. With the development of MCE, which is more miniature, automated, high-throughput and integrated. CE-based analysis will analyze multiple samples more rapidly and in parallel, significantly simplifying and streamlining the diagnostic process.
Sample loading	Several to dozens of μL of sample is required. However, clinically collected or extracted samples are usually volume-restricted and precious; heavy sample loading affects the subsequent analyses.	Only nL of sample is needed. Generally, sample is far more enough for analyzing several times as well as the subsequent analysis.

the high-throughput ability which would largely meet the demand for rapid analysis of a large number of samples in the clinical setting; (3) Although CE equipment with paired reagents is expensive, the running fee is inexpensive because of the low consumption of materials (μL of water,

inorganic salt and some gel) [1]. Furthermore, most of the reagents are non-toxic or insufficiently toxic (μL) to operators. Thus, it is cost-effective and safe for massive analysis of clinical samples; (4) Most sequencing techniques are also CE-based Sanger sequencing, and the majority of

inherited genetic mutations involve only a small proportion of the genome. Hence, sequencing every base sequence is unaffordable, arduous and fruitless work. Conversely, CE-based PCR fragment analysis saves time and is versatile to fit the customized diagnostic requirements in clinics. Furthermore, it is likely to automatize the diagnostic process and save labor [1].

Taken together, although novel PCR-CE approaches are not yet perfect for clinical diagnosis of inherited diseases, optimized PCR strategies to amplify known and unknown mutated genes according to the principle that circumstances alter cases [54], coupled with the powerful PCR analytical tool-CE, exhibit a huge potential in revolutionizing future clinical diagnosis, thereby gaining increasing attention for continuous improvement and investigation of the PCR-CE technique. It is also speculated that none of the techniques possess all the advantages, and technical consummation costs time with a cycling process of trouble-feedback-improvement. Thus, in our view, PCR-CE encounters the aforementioned challenges, and hence will be developed in the future. (1) Interdisciplinary studies are strengthened to confront the practical concerns in the clinics directly. Delicate PCR design, lessened post-PCR handling and enhancement of CE performance, which bridge the medical biology and analytical chemistry, are the focus. (2) To settle down the low sensitivity of CE-UV and further promote its application, CE combined with other techniques, such as CE-MS [2] or stacking techniques employed during sample injection [56–62], was further explored. (3) Standardization of PCR-CE for clinical application [19, 34, 47]. (4) PCR-CE collaborated with other techniques [42], such as sequencing/NGS [6, 32, 40, 51] and CE-MS [2] to enhance the advantages to serve the purpose. (5) State-of-the-art MCE, which is more automatic, parallel and a miniature form of CE, integrating two independent steps of PCR with CE into a successive course, was further designed, developed, improved and applied [35]. (6) Multiplex PCR-MCE is given priority for development. (7) Versatile, accurate, customized, individualized, simplified, standardized, affordable, beforehand and preventive diagnoses are the fundamental guidelines for developing diverse PCR-CE innovation [49, 63]. (8) A clinical database quantified, digitized and standardized based on PCR-CE or PCR-CE integrated with related techniques should be established [40].

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