

Author



Richard Jimenez became fascinated with the mitochondrial genome through a forensics biology seminar in the Spring of 2004. His interest in the inheritance, importance and applications of mitochondrial DNA took him to the lab of Dr. Procaccio, where he has been conducting research since the Fall of that year. Over that time, he has enjoyed building personal relationships with others in the lab, especially his mentors. He hopes to use the critical skills he has learned through research to pursue his goal of becoming a veterinary scientist. In his spare time, Richard enjoys boogie boarding, running, and spending time with friends and family.

Key Terms

- ◆ 3243A>G Mutation
- ◆ Diabetes
- ◆ Heteroplasmy
- ◆ High Performance Liquid Chromatography (HPLC)
- ◆ Mitochondrial Disorders
- ◆ Mitochondrial DNA (mtDNA)

A New Strategy for the Detection of Low Levels of Mitochondrial DNA Mutations Using Blood Derived from Diabetic Patients

Richard A. Jimenez

Biological Sciences and Chemistry

Abstract

Mitochondria are organelles that produce essential energy for cellular function. Each mitochondrion contains multiple copies of a small circular piece of maternally inherited DNA called mitochondrial DNA (mtDNA). MtDNA mutations cause many known mitochondrial diseases, which can be diagnosed from a muscle biopsy. A common mtDNA mutation at position 3243A>G can cause diabetes at low levels of mutation, and a severe neurological disease at higher levels. Conventional techniques used to detect this 3243A>G mutation overlook its presence at low levels, and we sought to assess an alternate technique that might improve the detection sensitivity and throughput of mtDNA mutations. Our technique is based on the use of the Transgenomic WAVE System for the HPLC-mediated analysis of mutation-specific restriction fragments derived from PCR products. This was derived through analysis of blood samples from patients who possibly carried the mutation. Our technique found that several patients carried the 3243A>G mutation, while conventional techniques, such as sequencing, overlooked its presence. Our study suggests that this sensitive technique can accurately diagnose a carrier of a mtDNA mutation without the use of a painful muscle biopsy, even when the mutation is present at low levels. The use of this technique may help reassess the prevalence of the 3243A>G mutation in the diabetic population.

Faculty Mentor



Energy is provided to cells by structures known as mitochondria, each of which contains multiple copies of a piece of DNA called mitochondrial DNA. Individuals may suffer from a condition known as mosaicism or heteroplasmy, in which normal and abnormal mitochondria can exist within the same cell or tissue. Richard Jimenez's project in our laboratory was to improve the detection level of mitochondrial DNA mutations in blood samples from diabetic patients. A more sensitive detection tool might be an effective method to screen populations for mitochondrial disorders, with significant potential clinical implications. It could also be used to examine the presence of mitochondrial DNA mutations in cancer cells. Richard's work shows the opportunities faculty-mentored undergraduate research offers for students to gain experience and apply the theoretical concepts they learned in class.

Vincent Procaccio

School of Medicine

Introduction

Mitochondria, often referred to as the “powerhouses of the cell”, are responsible for the production of 90% of the energy needed for cells to function (Wallace et al., 2002; Lamson and Plaza, 2002). The energy generated by mitochondria is in the form of a chemical carrier called adenosine triphosphate (ATP). Oxidative phosphorylation (OXPHOS) produces ATP by a process that involves five different complexes located within the inner mitochondrial membrane.

Each mitochondrion contains multiple copies of a small circular piece of maternally inherited DNA called mitochondrial DNA (mtDNA). The thirteen essential proteins that form complexes I, III, IV, and V needed for oxidative phosphorylation are encoded by the mtDNA, while the remaining proteins that form the five complexes are encoded by the nuclear genome. The mitochondrial genome also encodes 22 transfer RNAs (tRNAs), and the 12S and 16S ribosomal RNAs (Wallace et al., 2002). Since mitochondria serve as a center for energy production, more mitochondria are found in cells, such as muscle or brain cells, that have higher energy needs.

More than 100 mutations in the mitochondrial genome have been identified and associated with a variety of human disorders that arise from mitochondria’s failure to provide sufficient energy to meet the cellular demands (Brandon et al., 2005). Diabetes has been linked to mtDNA mutations and has become one of the leading causes of premature death in countries throughout the world. In the United States, more than 16 million people have type II diabetes, and the World Health Organization projects that more than 300 million people worldwide may be affected with diabetes within the next 20 years (Lowell and Shulman, 2005). One of the most common mtDNA pathogenic mutations, found in the mitochondrial genome, which plays an important role in the development of type II diabetes, is the 3243 A>G mutation (Moraes et al., 1993). This mitochondrial mutation accounts for 1 to 3% of the type II diabetes population (Lamson and Plaza, 2002). The mutation is caused by an adenine-to-guanine transversion change in the mtDNA-encoded tRNA^{Leucine(UUR)} gene at position 3243 of the mitochondrial genome (Lamson and Plaza, 2002). The 3243A>G mutation was first identified in patients with a mitochondrial disease called MELAS syndrome (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) (Goto et al., 1990). This mutation is usually present in heteroplasmic form, which means that a mixture of normal mtDNA and mutated mtDNA exists in the same cell or tissue (Wallace et al., 2002). Therefore, the diversity

or severity of the clinical phenotype seen in individuals is due to the mutant load and the tissue distribution of the mutation. Higher levels of mutant mtDNA cause more severe clinical phenotypes.

To assess the prevalence of the 3243A>G mutation associated with diabetes, a sensitive, high-throughput technique must be used to analyze a larger number of diabetic patients. A variety of genetic techniques have been developed for the analysis of mtDNA, such as the Surveyor nuclease-mediated heteroduplex digestion (Bannwarth et al., 2005) and the denaturing High Performance Liquid Chromatography (dHPLC) that detects heteroduplex formations (Biggin et al., 2005; Wong and Boles, 2005). All of the current methods available, including DNA sequencing, screen for unknown and known mutations, or score for specific known mutations in the human mitochondrial genome. Although DNA sequencing is time consuming, costly and inadequate in determining the mutant load, it is the technique many investigators prefer for detecting homoplasmic mtDNA mutations.

We have developed a new strategy that can effectively detect and quantify the presence of the 3243A>G mtDNA mutation. This method is based on the use of the WAVE System (Transgenomic, Inc., Omaha, NE) for the HPLC-mediated size-based separation of restriction fragments derived from specific PCR products, and is hereafter referred to as the PCR Amplicon Restriction Fragment Analysis by HPLC (PARFAH) method. The aim of this study is to assess the sensitivity, usefulness and reproducibility of the PARFAH method for the routine detection and quantification of mtDNA mutations, including the 3243A>G mtDNA mutation found in patients with type II diabetes. It will be shown that this new method can reliably detect and quantify mtDNA mutations with a higher degree of sensitivity than is available in conventional techniques, such as sequencing and gel electrophoresis.

Materials and Methods

Patients and Cell Lines

Total DNA was extracted from each patient’s sample of blood cells and from lymphoblastoid cell lines using the Puregene DNA Isolation Kit (Gentra System, Minneapolis, MN). Blood samples were obtained from 13 consenting patients in accordance with the guidelines set by the local institutional review boards. Two patients (P1 and P2) suffering from mitochondrial diseases were known to harbor the 3243A>G mtDNA mutation, while the remaining 11 patients, ranging from 7 to 73 years of age, were suspected

to harbor the 3243A>G mutation based on their pedigree information. Four of these patients were asymptomatic (not affected) while the remaining seven patients were affected with mitochondrial diabetes. This study was approved by the Institutional Review Board (IRB) of UCI, under Protocol #2002-2608.

Cell lines harboring various mutant levels of the 3243A>G mutation were obtained by fusing rho⁰ (ρ⁰) cells depleted of mtDNA with lymphoblastoid cells that harbor both wild type and mutant mtDNA. The 143B TK- ρ⁰ cell line was grown in Dulbecco's modified Eagle's medium with high glucose (4.5 mg/ml). A lymphoblastoid cell line harboring the heteroplasmic 3243A>G mutation was cultured in RPMI 1640 (Invitrogen, Carlsbad, California) medium with high glucose (4.5 mg/ml). Both of the cells' media were supplemented with 10% fetal bovine serum, 50 μg/ml of uridine, and 1mM of pyruvate. We then enucleated the lymphoblastoid cell line from a patient who was heteroplasmic for the 3243 A>G mutation, and fused the mitochondria-containing cytoplasts to a mtDNA-deficient ρ⁰ cell line, 143B TK. This ρ⁰ cell line had been deprived of its own mtDNA by prolonged incubation with ethidium bromide, a chemical known to inhibit DNA replication. The fusion of these two cells produces a cell line called cybrids, or hybrid eukaryotic cells, that combines the nuclear genome from one source with mitochondrial genomes from other sources. These individual cybrid clones were then isolated and the percentage of mutant DNA was determined (Trounce et al., 1996).

Molecular Analysis of Restriction Fragments

A 364 base pair (bp) mtDNA fragment containing the 3243A>G mutation site was amplified by polymerase chain reaction (PCR) using two oligonucleotide primers corresponding to nucleotide positions 3007–3023 (forward) and 3370–3351 (reverse). The thermal cycling conditions consisted of one cycle at 94 °C for 2 min, followed by 30 cycles at 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 20 s, and a single cycle at 72 °C for 5 min. The presence, purity and quantity of the PCR products were checked by electrophoresis on a 1% agarose gel, and the remaining volume of PCR products was then digested completely at 37 °C with the restriction enzyme *Hae*III (Roche Applied Science, Indianapolis, IN) added directly to the PCR amplification mixture as outlined in Figure 1. The *Hae*III enzyme cleaves the normal 364-bp fragment differently than the fragment containing the 3243A>G mutation. The normal, wild-type fragment contains two *Hae*III recognition sites, which allows the enzyme to generate three fragment sizes of 169, 141, and 54 bp. In the mutant fragment, the A to G mutation at nucleotide position 3243 provides an additional

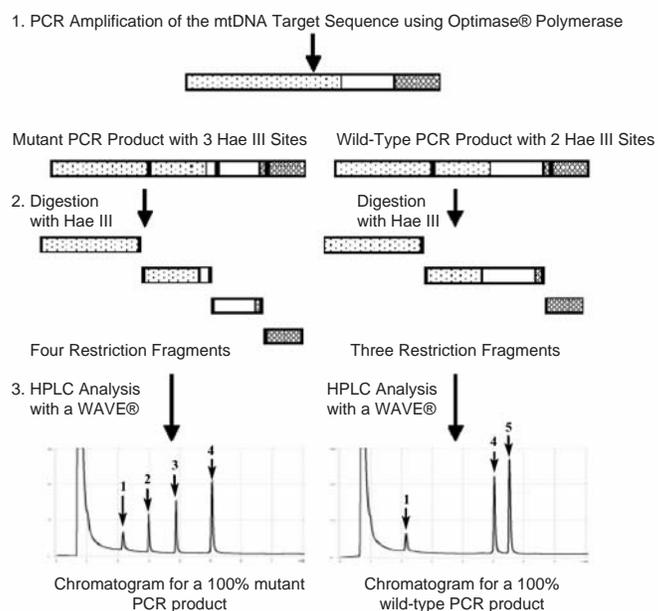


Figure 1

A flowchart of the PARFAH Method. The distinct chromatogram patterns for the mutant and wild-type PCR products provide the basis for the detection and quantification of the 3243A>G mtDNA.

*Hae*III recognition site, which splits the 169-bp fragment into two smaller fragment sizes of 97 and 72 bp. Restriction fragments in the digested PCR products were later separated and analyzed by the PARFAH method (Transgenomic, Omaha, NE). In most cases, 10 μl samples of the digests were injected by the WAVE System's autosampler. In others, the restriction fragments were analyzed by polyacrylamide gel electrophoresis. A volume of 10 μl of the digested PCR products derived from plasmid templates, and 20 μl of digested PCR products derived from two patients (P1 and P2) were separated on an 8% polyacrylamide gel stained with ethidium bromide.

Assessment of the Sensitivity of the PARFAH Method

We prepared and analyzed a series of PCR product mixes that were known to contain various percentages of mutant mtDNA to test the sensitivity of the PARFAH method and to compare the new method with standard polyacrylamide gel electrophoresis methods. PCR products for these mixes were from plasmid clones containing either mutant or wild-type amplicons derived from a patient harboring the 3243A>G mitochondrial mutation. The mutant and wild-type amplicons were cloned into the plasmid vector pCR2.1-TOPO according to the manufacturer's conditions (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA). Aliquots of pooled mutant and wild-type PCR products were combined to prepare a series of standard mixes in

which the percentage of mutant mtDNA varied from 100 to 0% (i.e., 100, 50, 40, 30, 20, 10, 5, 1 and 0%). PCR products in these standard mixes were digested with *Hae*III. The digests obtained were analyzed by the PARFAH method and, for comparison, by polyacrylamide gel electrophoresis.

Sequencing of mtDNA

PCR products derived from patients or cell lines, or present in the standard mixes, were purified using ExoSAP-IT following the recommendations of the manufacturer (USB Corporation, Cleveland, OH). The purified PCR products were cycle-sequenced using the same primers that were used for the initial amplification, purified according to standard protocols, and analyzed on an automated ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence data were analyzed using a software program called Sequencher (version 4.0.5, Gene Codes Corporation, Ann Arbor, MI).

Quantification of Mitochondrial Heteroplasmy Level

Patient DNA samples and the cybrid cells with different levels of mutant mtDNA were analyzed for their percentages of the 3243A>G mutation. The relative mutant loads of the samples were calculated by dividing the sum of the areas under chromatogram peaks 2 and 3 by the sum of the areas under peaks 2, 3 and 5 (Figure 2B). All quantifications were done by analyzing at least four independent amplifications of each patient or cell line DNA. The values are given as mean \pm standard deviation (SD).

The quantification of mitochondrial heteroplasmy levels of the cybrid cells with different levels of mutant mtDNA was also assayed using a radioactive PCR-RFLP "Last Cycle Hot" method. This assay included a series of procedures that involved PCR amplifications in which radioactive (α^{32} P) dCTP was added to the reaction just before the last PCR cycle, followed by a restriction enzyme digest with *Apa*I, polyacrylamide gel electrophoresis, autoradiography, and densitometry of the autoradiographs (Violettes et al., 1995).

Results

Detection of the 3243A>G Mutation

Samples with mutant 3243 A>G mtDNA can be identified by the presence of two extra peaks (peaks 2 and 3) in the chromatograms (Figures 1 and 2B) or two extra fragments in the polyacrylamide gel (Figure 2A). The two mutation-specific restriction fragments and peaks were detected in plasmid DNA controls and in DNA samples derived from patients by the PARFAH method, and by polyacrylamide gel electrophoresis.

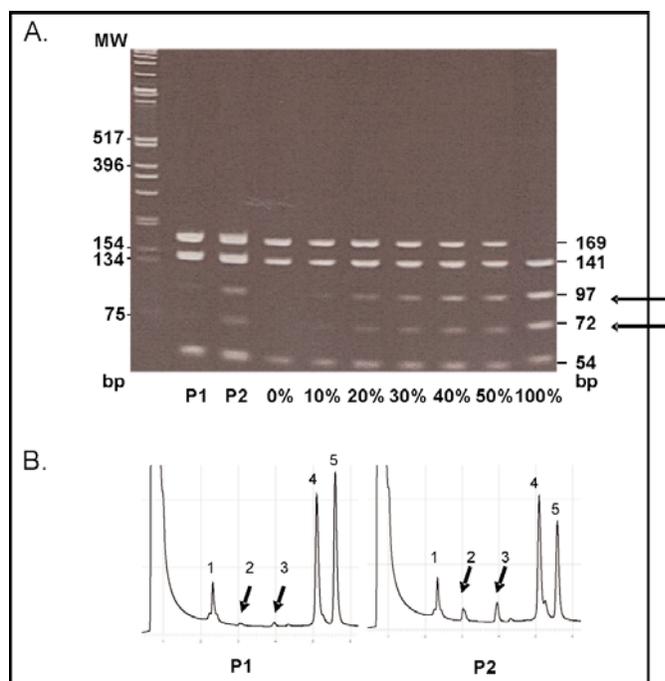


Figure 2

PCR Amplicon Restriction Fragment Analysis: (A) *Hae*III restriction fragments analysis on an 8% polyacrylamide gel by electrophoresis for DNA samples. Arrows indicate bands that indicate the presence of the mutation. MW: Molecular Weight DNA ladder; P1: Patient 1; P2: Patient 2; Different 3243 A>G mutant loads from 0 to 100%. (B) Chromatograms of *Hae*III restriction fragments derived from both patients using the PARFAH method. Five peaks are identified corresponding to 54, 72, 97, 141 and 169-bp fragment sizes; arrows indicate mutant-specific peaks 2 and 3 of 72-bp and 97-bp fragment sizes, respectively. P1: Patient 1 (mean: $4.8 \pm 0.87\%$ mutant load); P2: Patient 2 (mean: $26.1 \pm 0.94\%$ mutant load).

Sensitivity of the PARFAH Method Compared to Conventional Techniques

Patient DNA and a series of template stocks containing varying amounts of the mutant mtDNA ranging from 0% to 100% were analyzed by three methods: (1) the PARFAH method; (2) sequencing; and (3) a conventional restriction fragment analysis technique, such as a polyacrylamide gel electrophoresis, coupled with ethidium bromide staining. The results of these experiments showed that neither polyacrylamide gel electrophoresis nor sequencing were as sensitive as the PARFAH method (Figures 2 and 3). The sequencing results showed that automated base calling sequencing software fails to detect a mutation present at less than 30% (Figure 3). However, mutant percentages as low as 20% can be detected by sequencing if the results are manually analyzed. Levels of mutants as low as 10% were detected by gel electrophoresis (Figure 2A), while mutant loads as low as 1% could be detected by the PARFAH method (Figure 3).

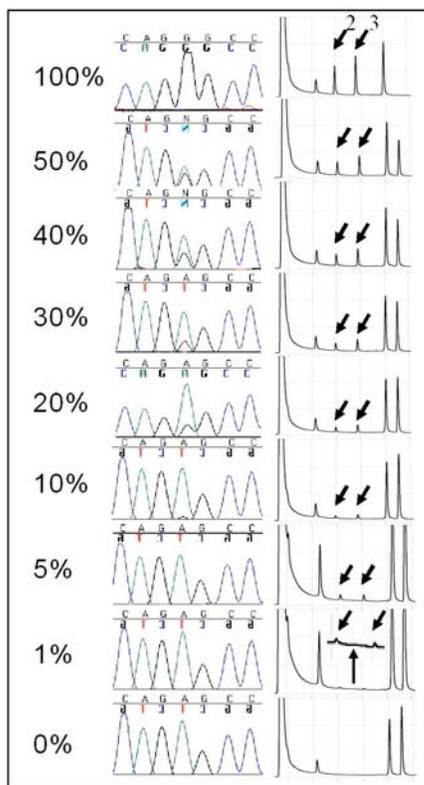


Figure 3
A comparison of the sequencing (left) and PARFAH (right) methods. Analyzed samples had varying mutant load of the 3243A>G mutation ranging from 100% to 0% mutant. Note that PARFAH was able to detect a mutant load as low as 1% mutant (indicated by the two arrows), while sequencing failed to detect that level of mutation.

Quantification of Percentages of Mutant DNA

Estimates of the percentage of mutant mtDNA were performed by using the peak area calculation feature of the WAVE System's Navigator software to obtain area values for peaks 2, 3 and 5 (Figure 2), and substituting these values into Equation 1:

$$\text{Percentage Mutant DNA} = \frac{\text{Peak 2 Area} + \text{Peak 3 Area}}{\text{Peak 2 Area} + \text{Peak 3 Area} + \text{Peak 5 Area}} \quad (1)$$

Using the PARFAH method and the above equation, patients P1 and P2 were found to carry mutant loads of $4.8 \pm 0.87\%$ and $26.1 \pm 0.94\%$, respectively.

Analysis of Various Levels of Mutant DNAs from Cell Lines

To check the reproducibility of the PARFAH method, four independent amplifications and analyses of each DNA sample from the cybrid cell lines were conducted. The results of this experiment showed that the calculated percentages of mutant mtDNA from the PARFAH method were very

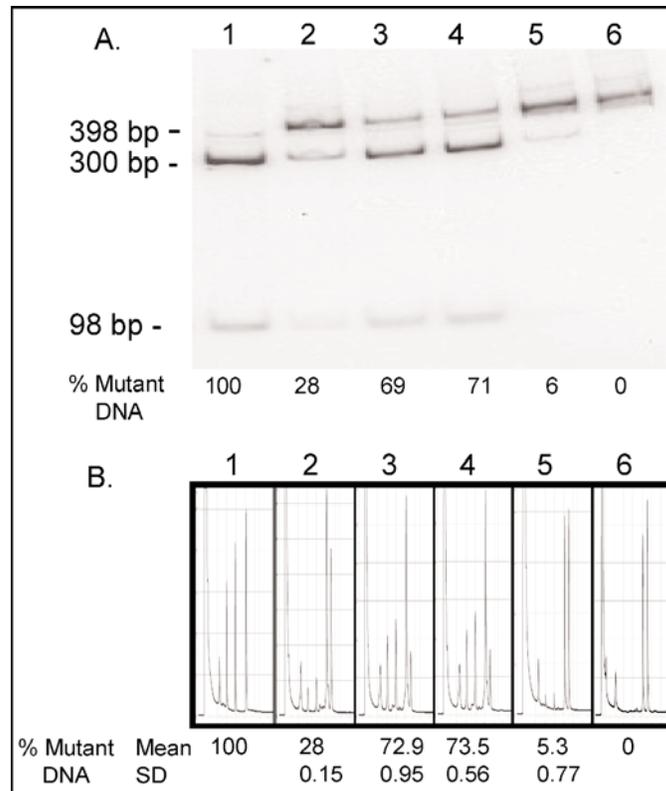


Figure 4
(A) Percentages of mutant mtDNA were determined by densitometric analysis of autoradiographs. (B) Percentages of mutant mtDNA were determined by the PARFAH method. Mean and SD are indicated.

reproducible, and that they ranged from 5.3% to 73.5%, with standard deviations below 0.95 (Figure 4B). The percentages of mutant for these samples were also assessed using a radioactive PCR-RFLP "Last Cycle Hot" method. The results obtained with this method were found to be comparable to the data obtained with the PARFAH method (Figure 4A).

Analysis and Estimation of the 3243A>G Mutation in Patients with Mitochondrial Diseases

We next reassessed the presence and the mutant level of the 3243A>G mutation in the blood of 11 patients. Previous analysis of the DNA extracted from blood by conventional techniques using gel electrophoresis reported that the patients did not harbor the 3243A>G mtDNA mutation, even though several other relatives in their pedigrees expressed a variety of clinical phenotypes (Figure 5 and Table 1). However, after these patients' blood samples were reanalyzed, using our PARFAH method to detect the 3243A>G mutation, the results showed that the 3243 A>G mutation was present in the blood samples of at least five of the eleven patients who had been previously reported to

be negative for the mutation in the same samples (Table 1). In family A, patient 8 carried the mutation with a load of 2.6%. In family B, patient 1 harbored the mutation at a load of 6.4%. This female patient, originally reported to be negative for the mutation, was affected and was also an obligate carrier, since her daughter and her grandchildren were also found to be positive for the mutation (Figure 5). In family C, several generations were affected with diabetes, and in patient 6 the mutation load was found to be at a level of 1.2%. Two other patients found to carry the mutation did not have an informative pedigree, but the levels of heteroplasmy detected were determined to be 0.2% and 8.8%,

respectively. The remaining patients were confirmed to be negative for the 3243 A>G mtDNA mutation.

Discussion

Diabetes has been associated with various mtDNA mutations, including mtDNA rearrangements and nucleotide base substitutions (Ballinger et al., 1992). The mtDNA 3243A>G mutation in the mitochondrial tRNA^{L_{eu}(UUR)} gene is a common cause of type II diabetes, which initially causes a defect in glucose-induced insulin secretion by the pancreas, resulting in a decrease in ATP production (Lamson and Plaza, 2002). Generally, patients manifesting type II diabetes due to the 3243 A>G mutation have been found to have around 10% heteroplasmy levels in their blood (Wallace and Lott, 2002). These same individuals can have much higher levels of mutant mtDNA in post-mitotic tissues such as skeletal muscle. Mutation levels of 70% or more in muscle or brain tissue can lead to MELAS syndrome or other life threatening brain and muscle disorders. However, a patient may harbor the 3243 A>G mtDNA mutation and be asymptomatic, as seen for patients 6 and 8 (Figure 5 and Table 1). This phenomenon is due to the fact that the patient's mutant load does not exceed a critical threshold level of mutation to cause the onset of diabetes (Maassen et al., 2004; Moraes et al., 1993; Lamson and Plaza, 2002). The asymptomatic patient who harbors the mutation may transmit it to her offspring and have affected children, or show symptoms later in life due to an increase in mtDNA mutations over time (Lamson and Plaza, 2002).

The association between the level of mutant mtDNA and clinical symptoms has led to a need for a mutation detection method that combines increased sensitivity, high throughput, cost effectiveness, and the ability to quantify the level of mutant mtDNA. We have developed the PARFAH method, which is suitable for a sensitive high throughput patient screening of families with type II diabetes. The presence of peaks 2 and 3 in the chromatograms of the PARFAH method indicates that the 3243 A>G mtDNA mutation is present in the sample. In addition to the detection of low levels of the 3243 A>G mutation in patients' blood samples, as seen in patients 2 and 6 (Table 1), the PARFAH method also permits an estimation of the

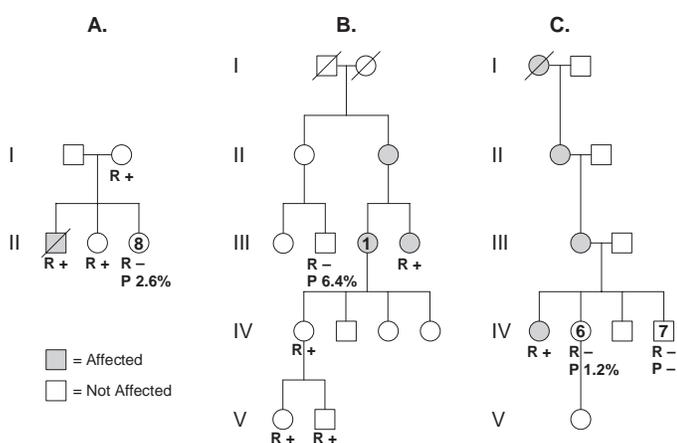


Figure 5
Pedigrees of families harboring the 3243 A>G mutation. A) with MELAS, B) and C) with diabetes and deafness. R: indicates RFLP/gel analysis and P: PARFAH analysis; Symbols + or - indicate positive or negative results for the 3243 A>G mutation using the two techniques. Percentages of mutant loads indicate the individuals previously negative by RFLP/gel analysis but found to be positive for the 3243 A>G mutation using the PARFAH method.

Table 1
Clinical features of 11 patients studied. Age (in years) when blood samples were drawn; Patients 6 and 7 are related; Pedigree phenotype of patients, Mitochondrial Diabetes, MELAS: Myopathy, Encephalopathy, Lactic acidosis, and Stroke-like, Stroke, Epilepsy; Status Affected or Not Affected.

Patient Number	Age	Pedigree Phenotype	Status	Detection in Blood by RFLP/gel analysis	Detection in Blood by PARFAH (Mean % ± SD)	Detection in Muscle by PARFAH and RFLP/gel analysis
1	57	Diabetes	Affected	Not Detected	6.4 ± 0.35	Not Tested
2	73	Diabetes	Affected	Not Detected	0.2 ± 0.03	Not Tested
3	49	Diabetes	Affected	Not Detected	Not Detected	Detected
4	54	Stroke	Affected	Not Detected	8.8 ± 0.48	Detected
5	55	Epilepsy	Not Affected	Not Detected	Not Detected	Not Tested
6	48	Diabetes	Not Affected	Not Detected	1.2 ± 0.13	Not Tested
7	45	Diabetes	Not Affected	Not Detected	Not Detected	Not Tested
8	7	MELAS	Not Affected	Not Detected	2.6 ± 0.54	Not Tested
9	36	MELAS	Not Affected	Not Detected	Not Detected	Not Tested
10	53	MELAS	Not Affected	Not Detected	Not Detected	Not Tested
11	51	MELAS	Not Affected	Not Detected	Not Detected	Not Tested

percentage of mutant mtDNA present in the sample being analyzed. The entire semi-automated procedure, starting with PCR amplification and ending with chromatogram analysis, can be completed within a single day. Hence, the PARFAH method could replace the electrophoresis methods currently used in clinical molecular laboratories, and significantly increase the throughput and sensitivity of sample analyses looking for specific mitochondrial DNA mutations.

The results of this study have established that the PARFAH method can reliably detect as little as 1% mutant 3243A>G in a sample. This is a considerable clinical improvement over present methods (Figure 3). The results show that the sensitivity of the PARFAH method is also dependent on the sample volume injected into the HPLC column. The method was able to detect and quantify a mutant load as low as 0.2% for patient 2 and a mutant load of 1.2% for patient 6 (Table 1) if the sample volume injected into the HPLC column was increased to 30 μ l. The use of a proofreading DNA polymerase such as Optimase Polymerase, in lieu of Taq DNA polymerase, resulted in cleaner chromatograms and enhanced the sensitivity of the method, especially for the detection of low percentages of mutant DNA. The use of Optimase Polymerase simplifies the assay further since the restriction enzyme *Hae*III is active in the amplification buffer itself and no buffer exchange is required. Not only is this new PARFAH method sensitive and able to quantify the level of heteroplasmy, it also simplifies the process of detecting the 3243 A>G mtDNA mutation.

Because heteroduplexes are not digested by restriction enzymes, the formation of heteroduplexes during the amplification process is known to decrease the sensitivity of mutation detection methods based on restriction fragment analysis (Moraes et al., 1992). The four independent amplifications and digestions performed for each DNA sample addressed this issue and showed that the results obtained from the PARFAH method were very reproducible (Table 1). Furthermore, the data obtained from the cybrid cells with different mutant loads showed that the radioactive PCR-RFLP "Last Cycle Hot" method (Moraes et al., 1992; Vialettes et al., 1995) and the PARFAH method gave comparable results (Figure 4). These results suggest that the PARFAH method can be a reliable way to quantify the level of mutant mtDNA in patients harboring a mitochondrial mutation without the use of radioactive materials. Although other techniques have been developed and used to quantify mtDNA mutations, none are as cost effective as the PARFAH method. For example, pyrosequencing has been recently shown to be a sensitive and reproducible technique

for mtDNA mutation quantification, but it is expensive (Biggin et al., 2005). Peptide Nucleic Acid-directed PCR clamping has also been successfully used to detect low levels of the 3243A>G mutation (Murdock et al., 2000); however, the data obtained from this method does not provide information about the mutant load. DNA sequencing is the technique most commonly used for the detection of mtDNA mutations (Wong and Boles, 2005); however, it is time consuming and costly. It fails to detect mutations that may be present at a mutant load level of less than 30%, as shown in our study (Figure 3) and others (Biggin et al., 2005).

Although the DNA analyzed in this study was obtained from patients' blood samples, many studies have observed that the mutant load is usually higher in affected post-mitotic tissues such as skeletal muscle. With these findings, molecular genetic analysis of skeletal muscle is considered to be the best tissue for an accurate diagnosis of patients with suspected mtDNA mutations (Shanske et al., 2004). However, muscle samples were not used in this study because muscle biopsies are invasive and painful. Instead, only easily accessible tissue samples such as blood, hair follicles, cheek mucosa, or urine epithelial cells were used. Of the tangential tissues described above, blood is the most commonly used tissue source for noninvasive analysis of mtDNA. Recent studies have observed that the mutant loads in other tissues, such as urinary sediments or buccal swabs, were present in higher levels than found in blood (Shanske et al., 2004). Furthermore, it has been shown that the level of the 3243A>G mutation decreases with aging in mitotic tissues, such as blood, due to the preferential selection of wild-type mtDNA in dividing blood cells over mutant forms of the mitochondrial genome. The lower levels of mutant mtDNA become progressively more difficult to detect in blood of older patients (Olsson et al., 2001). As seen in this study, the 3243 A>G mutation in the blood sample of Patient 3, who was 49 years old and affected with diabetes, was not detected by PARFAH or by gel electrophoresis (Table 1). However, when a muscle sample was obtained from Patient 3, the 3243 A>G mtDNA mutation was detected by both methods.

The detection of potential carriers of the 3243A>G mutation in relatives of affected individuals is important for genetic counseling. Furthermore, having the capability to detect this mutation at an early stage is of crucial importance. If the mutation is detected earlier, the patient can take preventative strategies that may manage or delay the onset of the disease, or even avoid common drugs that may cause the disorder to worsen. For example, one of the common drugs given to patients with type II diabetes is metformin, which

has been shown to be a mild inhibitor of mitochondrial respiratory chain complex I (Guigas et al., 2004). Since metformin therapy is known to induce lactic acidosis, other types of drugs should be recommended to patients with impaired mitochondrial function, because they are already predisposed to having higher levels of lactic acid.

Conclusion

This study investigated the usefulness and sensitivity of the new PARFAH strategy in detecting and quantifying mutations in mtDNA. The results indicate that the PARFAH method provides a fast, sensitive and specific assay for the detection and estimation of the 3243A>G mutation. In theory, this method can be applied to other mtDNA mutations. It is anticipated that the implementation of this new diagnostic aid will improve the ability to manage patients with mitochondrial diabetes efficiently. In addition, the sensitivity of this method, combined with the use of peripheral tissues such as urinary epithelial cells, can help future studies reassess the prevalence of the 3243A>G mutation in the type II diabetes population.

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