Multiplex MALDI-TOF MS detection of mitochondrial variants in Brazilian patients with hereditary optic neuropathy

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Purpose: Leber hereditary optic neuropathy (LHON) is a mitochondrial disease characterized by bilateral vision loss. More than 95% of LHON cases are associated with one of the three main mtDNA mutations: G11778A, T14484C, and G3460A. The other 5% of cases are due to other rare mutations related to the disease. The aim of this study was to identify the prevalence and spectrum of LHON mtDNA mutations, including the haplogroup, in a cohort of Brazilian patients with optic neuropathy and to evaluate the usefulness of iPLEX Gold/matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) technology in detecting LHON mutations.
Methods: We analyzed a total of 101 patients; 67 had a clinical diagnosis of LHON and 34 had optic neuropathy of unknown etiology. Direct sequencing and iPLEX Gold/MALDI-TOF MS were used to screen for the most common pathogenic point mutations in LHON, together with the rare mutations G3733A, C4171A, T10663C, G14459A, C14482G, A14495G, C14568T, and C14482A.
Results: We identified mutations in 36 patients, of whom 83.3% carried the G11778A mutation and 16.7% carried the T14484C mutation. In individuals with mutations, the haplogroups found were L1/L2, L3, C, R, U, D, and H. Rare mutations were not detected in any of the patients analyzed.
Conclusions: The frequencies of the main LHON mutations were similar to those previously reported for Latin America. A different frequency was found only for the A3460G mutation. The most frequent haplogroups identified were of African origin. The iPLEX Gold/MALDI-TOF MS technology proved to be highly accurate and efficient for screening mutations and identifying the haplogroups related to LHON. The MassArray platform, combined with other techniques, enabled definitive diagnosis of LHON in 36% (36/101) of the cases studied.

Leber hereditary optic neuropathy (LHON) is a mitochondrial disease characterized by bilateral vision loss caused by degeneration of retinal ganglion cells and axons of the optic nerve, which leads to acute or sub-acute loss of central vision [1]. LHON is a maternally transmitted disease that mainly affects young adult men, with age at onset ranging from 18 to 35 years (average age of 22 years). Although vision loss is usually the only clinical manifestation in patients with LHON, other changes can include cardiac abnormalities and skeletal and neurologic disorders [2,3].

In 1988, Wallace and colleagues identified the first LHON mutation in humans, and since then, a growing number of mtDNA missense mutations have been associated with the disease. Among all the mutations reported, three are known as the main causes of LHON, with at least one present in 95% of cases. These mutations, G11778A [4], T14484C [5], and G3460A [6], can be found in the genes *mitochondrially encoded NADH dehydrogenase 4* (*MT-ND4;* Gene ID: 4538, OMIM: 516003), *mitochondrially encoded NADH dehydrogenase 6* (*MT-ND6;* Gene ID: 4541; OMIM: 516006), and *mitochondrially encoded NADH dehydrogenase 1* (*MT-ND1;* Gene ID: 4535, OMIM: 516000), respectively. These genes encode the subunits of complex I of the mitochondrial respiratory chain [7].

The frequencies of the three main mutations can vary considerably between populations. The frequencies of the G11778A mutation are 50% in Europe, 69% in Finland, and almost 95% in Asian populations [8,9]. The T14484C mutation is the second most frequent, described in approximately 14% of cases [10,11]. The G3460A mutation is the least frequent of the three main mutations, occurring in 13% of patients globally [8,12].

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The first Brazilian LHON molecular studies involved a large family totaling about 300 individuals. In this family, only the G11778A mutation was found to segregate with LHON [13,14]. However, the first Brazilian report concerning the relative frequencies of the three main mutations identified frequencies of 60% for G11778A, 30% for T14484C, and 10% for G3460A [15].

In addition to the three main mutations, many other mtDNA nucleotide changes have been associated with LHON [16]. However, these mutations are considered rare and have been reported in only 5% of LHON cases. Moreover, the interpretation of the alterations caused by these point mutations is complex, and the mechanisms of action have not been fully elucidated [17,18]. The main rare mutations are present in the genes *MT-ND1*, *MT-ND4*, *mitochondrially encoded NADH dehydrogenase 4L* (*MT-ND4L;* Gene ID: 4539, OMIM: 516004), *mitochondrially encoded NADH dehydrogenase 5* (*MT-ND5;* Gene ID: 4540, OMIM: 516005), *MT-ND6*, and *mitochondrially encoded cytochrome b* (*MT-CYB;* Gene ID: 4519, OMIM: 516020) [9,17,19]. Previous studies of the prevalence and spectrum of mitochondrial mutations in a large cohort of Han Chinese subjects with LHON have suggested that the *MT-ND4* and *MT-ND6* genes are hotspots for mutations associated with LHON [20,21].

The mtDNA mutations associated with LHON commonly occur in homoplasmy, while the degree of heteroplasmy (the number of mitochondria that possess mutant alleles) can influence the development of signs and symptoms of this disorder. It has been suggested that heteroplasmy may be associated with incomplete penetrance; according to this theory, 60% or more of the mitochondrial DNA molecules must be mutated to constitute a risk of blindness [22]. The incomplete penetrance observed in LHON pedigrees carrying mtDNA mutation(s), together with the predominance in men, suggests that the presence of the mutation alone is not sufficient to cause the pathogenesis, and that additional nuclear and mitochondrial genetic modifiers, or environmental factors, are involved [23]. In addition, it has been suggested that mitochondrial haplogroups play a role in the expression of LHON by increasing the penetrance of the three main mutations. However, the roles of these genetic modifiers in phenotypic expression of the mutations remain poorly defined. These factors reflect the complex etiology of this disease [24-26].

Other hereditary optic neuropathies, such as dominant optic atrophy (DOA), Wolfram's syndrome, or other neuropathies associated with neurologic diseases, can share the same pathological features as LHON. However, clinical data, family history, and neurologic signs can help differentiate these neuropathies [27,28]. Definitive diagnosis of LHON can be achieved by finding the change in the mitochondrial gene. Molecular diagnosis is useful for confirming the clinical diagnosis in cases with classic clinical signs and for establishing the diagnosis in cases of optic neuropathy of unknown cause. Nevertheless, in most cases, the molecular diagnosis is complex and expensive. Molecular diagnosis techniques have been improved by the use of high-throughput platforms that offer reduced costs and increase the number of tests that can be performed simultaneously. Such methods are attractive for the screening of mutations in the case of genetic diseases.

In the present study, we investigated the potential use of a novel single nucleotide polymorphism (SNP) genotyping technology, called iPLEX Gold (Sequenom Inc., San Diego, CA), followed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis for the simultaneous identification of the 11 main LHON mutations and mitochondrial haplogroup markers. Thus, the aims of this study were to evaluate the usefulness of the iPLEX Gold technology in detecting LHON mutations, to assess the frequencies of LHON mutations in patients with clinical diagnosis of LHON or acquired optic neuropathy of unknown origin, and to identify the frequency and spectrum of the mitochondrial haplogroups in the patients studied.

METHODS

Patients: We evaluated 101 unrelated Brazilian patients from the Center for Rehabilitation Research Studies (CEPRE) at the Faculty of Medical Sciences of the State University of Campinas (UNICAMP), as well as from the Ophthalmology Department of the UNICAMP Central Clinical Hospital, and from other organizations. The inclusion criteria were as follows: patients older than 18 years, with a history of bilateral sub-acute or acute visual loss (less than 20/40), together with central or cecocentral scotomas, without any distinction of race. The patients were divided into two groups. Group I comprised 67 patients with clinical and ophthalmological characteristics compatible with diagnosis of LHON. Group II comprised 34 patients with optic neuropathy of unknown etiology.

The study was approved by the Research Ethics Committee of the Faculty of Medical Sciences of UNICAMP, and informed consent was obtained from all participants. The consent was based on the Declaration of Helsinki and the guidelines provided for collection of human genetic disease samples, according to National Plan 863 and the Research Ethics Committee of the Faculty of Medical Sciences of UNICAMP (Process no. 690/2004).

DNA extraction: Genomic DNA was obtained from peripheral blood leukocytes using standard phenol-chloroform extraction [29]. The molecular analysis was performed at the Human Molecular Genetics Laboratory of the Center for Molecular Biology and Genetic Engineering (CBMEG) at UNICAMP.

Screening of LHON mutations: The three most frequent LHON mutations were investigated with PCR-restriction fragment length polymorphism (PCR-RFLP), using the following restriction enzymes: *Sfa*NI for G11778A, *Bsa*BI for T14484C, and *Bsa*HI for G3460A, as described previously [30,31]. The other eight common LHON mutations, G3733A, C4171A, T10663C, G14459A, C14482G, A14495G, C14568T, and C14482A, were screened with direct sequencing using an ABI PRISM® 3700 DNA Analyzer and the BigDye™ Terminator Ready Reaction Cycle Sequencing Kit v. 3.0 (ABI Prism; AplliedBiosystems, Foster City, CA). The results obtained after sequencing were analyzed with the Chromas Lite 2.01 and CLC Sequence Viewer 6.0.1 software packages. All sequences were analyzed against mitochondrial reference sequence NC_012920.

iPLEX Gold kit/MALDI-TOF MS: The presence of the 11 main LHON mutations was investigated in all patients using the iPLEX Gold/MALDI-TOF MS technology (Sequenom). The data were compared with the results obtained using other techniques (PCR-RFLP and direct sequencing). This technology was also used for the simultaneous analysis of 32 primary haplogroup markers. These SNP markers can be used to determine 25 different haplogroups. All SNP sequences were previously selected based on the Human Mitochondrial Genome Database (MITOMAP) [32]. The primer sequences used to screen the main LHON mutations and haplogroup markers are shown in Table 1 and Appendix 1.

The iPLEX Gold technology consists of an initial locus-specific PCR reaction, followed by single base extension (SBE) using mass-modified dideoxynucleotide terminators of an oligonucleotide primer that anneals immediately upstream of the polymorphic site of interest. MALDI-TOF MS is used to identify the SNP and mutation allele according to the mass of the extended primer.

Genomic DNA samples were diluted to concentrations between 2.5 and 5 ng/µl in TE buffer (the UV A260/A280 ratio should be between 1.7 and 2.0). Based on the sequence data available in the MITOMAP database, the PCR and SBE primers for the 11 LHON mutations and 32 haplogroup markers were designed using MassArray Assay Design software (Sequenom Inc.).

The optimal amplicon size was set to 80 to 120 bp. A 10-mer tag (5′-ACG TTG GAT G-3′) was added to the 5′ end of each PCR primer to avoid confusion in the mass spectrum, and the SBE primers were 5′ tailed with non-homologous sequences varying in length to create sufficiently large mass differences between the different SBE products to enable detection with MALDI-TOF MS. To avoid interaction among the primers of the 42-plex, MassArray Assay Design divided the PCR amplification and the SBE into five multiplex reactions.

The whole process was performed according to the manufacturer's instructions for the multiplex reactions, including the PCR amplification, the shrimp alkaline phosphatase (SAP) treatment, and the primer extension reactions using the iPLEX Gold assay. The reaction products were then dispensed on a 384-SpectroCHIP using the MassArray Nanodispenser and analyzed on the MassArray platform. The mass signals for the different alleles were captured with high accuracy by MALDI-TOF MS. Typer v. 4.0 software (Sequenom, San Diego, CA) was used to process the raw data obtained from the assays.

A parallel experiment was conducted to determine the sensitivity of the iPLEX Gold/MALDI-TOF MS technology in screening LHON mutations at low rates of mutant mtDNA relative to normal mtDNA (heteroplasmy). First, we selected positive and negative controls that had been previously screened for LHON mutations. The mtDNA positive controls were mixed in low proportions (5%, 10%, 20%, 30%, 40%, and 50%) with mtDNA from negative controls. The experiment was conducted with the G11778A and T14484C mutations.

RESULTS

The sample group with clinical LHON (Group I) consisted of 46 men and 21 women. The age at onset ranged from 11 to 54 years (average of 36 years). Vision loss occurred simultaneously in both eyes in 81.6% and sequentially in 18.4% of the patients. All patients showed central or cecocentral scotomas in the visual field, and only one patient was unable to perform the exam. Group II consisted of 23 men and 11 women. The age at onset ranged from 13 to 60 years (average of 24 years). Vision loss occurred simultaneously in both eyes in 74% and sequentially in 26% of the patients. All patients showed central or cecocentral scotomas in the visual field, and six patients were unable to perform the exam.

The results of the visual acuity tests were highly variable in both groups, ranging from 20/50 to less than or equal to 20/400. Most patients carrying LHON mutations have visual acuity below 20/200. Among all the patients carrying LHON

mutations (36), 23 (64%) were male, and 13 were female (36%). In addition, 13 had familial recurrence, five were sporadic cases, and for 18, no information was available.

Mutations were identified in 24 of the 67 unrelated patients of Group I (24/67, 35.8%). The G11778A mutation was found in 21 patients (31.3%) and the T14484C mutation in three cases (4.5%). No mutations were found in 43 patients (64.2%). In individuals with mutations, the haplogroups found were L1/L2 (13), L3 (7), C (2), R (1), and H (1). The results are compiled in Table 2.

In Group II (34 samples), nine patients (26.5%) had the G11778A mutation, and three (9%) had the T14484C

mutation. In these cases, the haplogroups L1/L2 (4), L3 (4), U (2), C (1), and D (1) were identified. The results for Group II are shown in Table 3.

Direct sequencing and the iPLEX Gold assay were also used to screen the mutations G3733A, C4171A, T10663C, G14459A, C14482G, A14495G, C14568T, and C14482A. These mutations were not detected in any of the cases analyzed in this study.

During the analyses of the samples with direct sequencing, the C4167T variant in the *MT-ND1* gene was found in one patient, and the T11697C alteration in the *MT-ND4* gene was detected in another subject. The G11719A

Table 1. Primer sequences used for the screening of mutations related to LHON by MALDI-TOF platform.		
Mutations		**Primers**
G3733A	Extension	gGCAGTAGCCCAAACAATCTCATAT
	Forward	ACGTTGGATGGAGCAGTAGCCCAAACAATC
	Reverse	ACGTTGGATGAAGGGTGGAGAGGTTAAAGG
G3460A	Extension	tGGCTCTTTGGTGAAGAGTTTTATGG
	Forward	ACGTTGGATGAGAGGGTGATGGTAGATGTG
	Reverse	ACGTTGGATGACGGGCTACTACAACCCTTC
C4171A	Extension	GAGTGGTAGGAAGTTTTTTCATA
	Forward	ACGTTGGATGAATGCTAGGGTGAGTGGTAG
	Reverse	ACGTTGGATGAATTCGAACAGCATACCCCC
T10663C	Extension	aAATATTGTGCCTATTGCCATACTAG
	Forward	ACGTTGGATGGCCAATATTGTGCCTATTGC
	Reverse	ACGTTGGATGGAGATTGAGACTAGTAGGGC
G11778A	Extension	aTCCTTGAGAGAGGATTATGATG
	Forward	ACGTTGGATGAGCTATTAGTGGGAGTAGAG
	Reverse	ACGTTGGATGCTCAAACTACGAACGCACTC
G14459A	Extension	agACTCCTCAATAGCCATC
	Forward	ACGTTGGATGACACTCACCAAGACCTCAAC
	Reverse	ACGTTGGATGGGAATGATGGTTGTCTTTGG
C14482A / C14482G	Extension	gCGCTGTAGTATATCCAAAGACAAC
	Forward	ACGTTGGATGGCCATCGCTGTAGTATATCC
	Reverse	ACGTTGGATGGGGAGGTTATATGGGTTTAA
T14484C	Extension	AGTATATCCAAAGACAACCA
	Forward	ACGTTGGATGGCCATCGCTGTAGTATATCC
	Reverse	ACGTTGGATGGGGAGGTTATATGGGTTTAA
C14568T	Extension	AACACACCCGACCACAC
	Forward	ACGTTGGATGAAACCCATATAACCTCCCCC
	Reverse	ACGTTGGATGAGCCTTCTCCTATTTATGGG
G15927A	Extension	CTAATACACCAGTCTTGTAAACC
	Forward	ACGTTGGATGAAATGGGCCTGTCCTTGTAG
	Reverse	ACGTTGGATGCTGATTTGTCCTTGGAAAAAG

Mutation	Cases	Percentage	Haplogroups
		TABLE 2. LHON MUTATIONS AND HAPLOGROUPS FOUND IN GROUP 1.	
G11778A	21/67	31.4%	L1/L2 (12) - L3 (5) - C (2) - R(1) - H (1)
T14484C	3/67	4.5%	L1/L2 (1) - L3 (2)
G3460A	0/67	-	-
G3733A	0/67	-	-
C4171A	0/67	-	-
T10663C	0/67	-	-
G14459A	0/67	-	-
C14482G	0/67	-	-
C14482A	0/67	-	-
A14495G	0/67	-	-
C14568T	0/67	-	-
No mutation	43/67	64.1%	L1/L2 (6) - L3 (15) - R (11) - X (2) C (4) - H (3) - T (1) - K (1)

mutation in the *MT-ND4* gene was found in 29 patients, and the G14560A mutation in the *MT-ND6* gene was detected in two individuals. However, according to the literature, these changes are not related to LHON.

The 101 samples tested with the iPLEX Gold assay yielded allelic data fully concordant with those obtained previously with PCR-RFLP and direct sequencing. Moreover, the iPLEX Gold/MALDI-TOF MS technology was able to detect a minimum heteroplasmy rate of 10%. In both tests with different proportions of G11778A or T14484C mutations, the minimum rate of mutant mtDNA detected was similar (Figure 1 and Figure 2).

DISCUSSION

We analyzed 101 unrelated patients with acute or sub-acute, bilateral, persistent optic neuropathy characterized by central vision loss. Of these patients, 30 (29.7%) had the G11778A mutation, and six patients (5.95%) had the T14484C mutation. The G3460A mutation was not found in any individual.

Group I consisted of patients with a clinical diagnosis of LHON, and mutations were identified in 24 of the 67 samples analyzed. Therefore, the diagnosis of LHON could be confirmed in approximately 36% of patients. Of the 34 individuals analyzed in Group II, 12 were carriers of LHON mutations. In these cases, molecular tests were important to

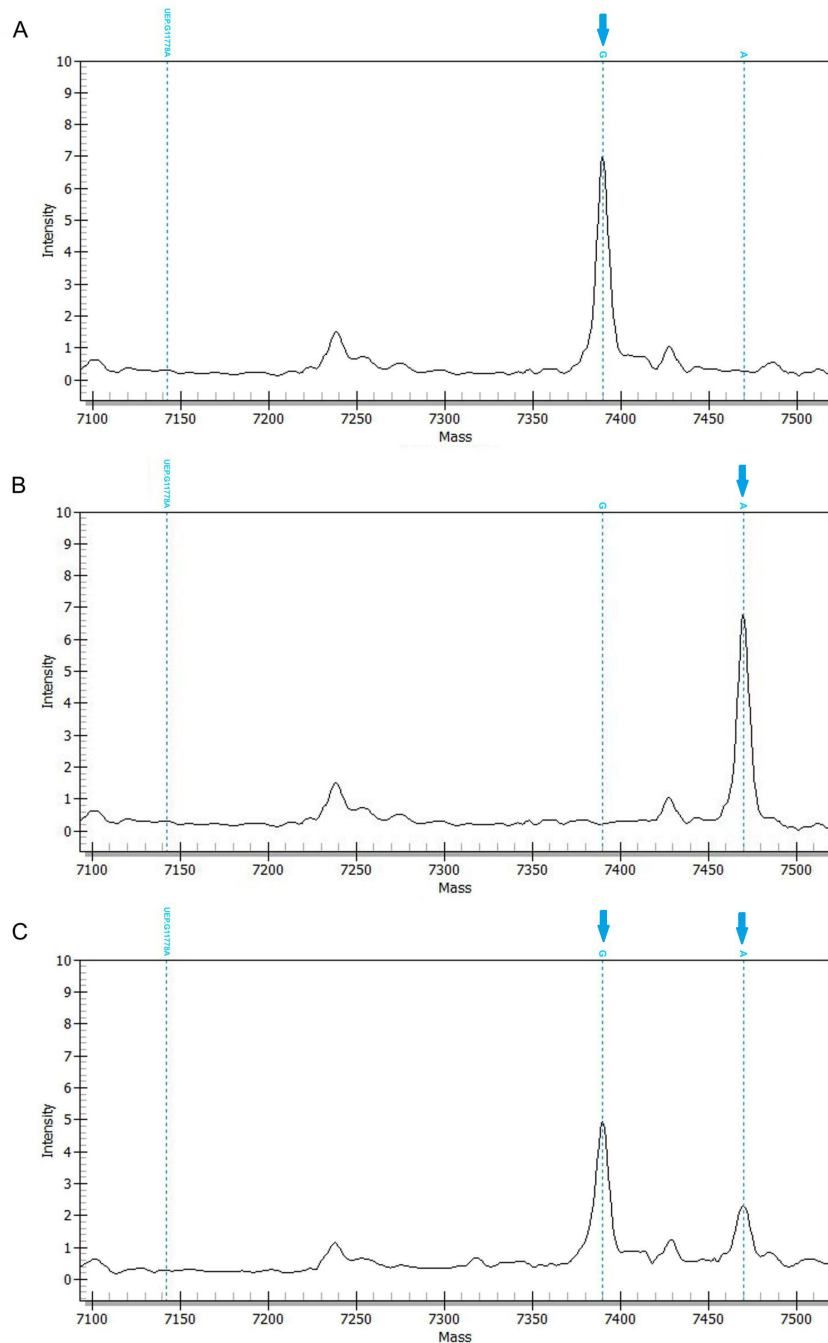| Mutation	Cases	Percentage	Haplogroups
		TABLE 3. LHON MUTATIONS AND HAPLOGROUPS FOUND IN PATIENTS WITH OPTIC NEUROPATHY OF UNKNOWN ETIOLOGY (GROUP2).	
G11778A	9/34	26.4%	L1/L2 (3) - L3 (3) - U (1) - C (1) - D (1)
T14484C	3/34	8.8%	L1/L2 (1) - L3 (1) - U (1)
G3460A	0/34	-	-
G3733A	0/34	-	-
C4171A	0/34	-	-
T10663C	0/34	-	-
G14459A	0/34	-	-
C14482G	0/34	-	-
C14482A	0/34	-	-
A14495G	0/34	-	-
C14568T	0/34	-	-
No mutation	22/34	64.7%	L1/L2 (5) - L3 (9) - R (4) - H (2) T (1) - K (1)

Figure 1. Typical raw data obtained using the Sequenom MassArray system for G11778A mutation screening. The blue arrows above the wave peak indicate the resulting genotypes. **A**: 100% wide type mtDNA. **B**: 100% mutant mtDNA. **C**: Heteroplasmic (G/A) mtDNA.

explain 35.3% of the cases with optic neuropathy of unknown etiology.

The relative frequencies of the different LHON-causing mtDNA mutations vary globally. Overall, the G11778A mutation is the most prevalent, accounting for 70% of cases among Northern Europeans [7,22] and approximately 90% of

cases in Asian populations [33,34]. Thus far, few studies have investigated the frequency of these mutations in the Brazilian population. In the present study, analysis of the frequencies of the three most common LHON mutations among patients with identified mutations revealed that the frequency of the G11778A mutation was 83.3% (30/36), while that of the T14484C mutation was 16.7% (6/36). The frequency obtained
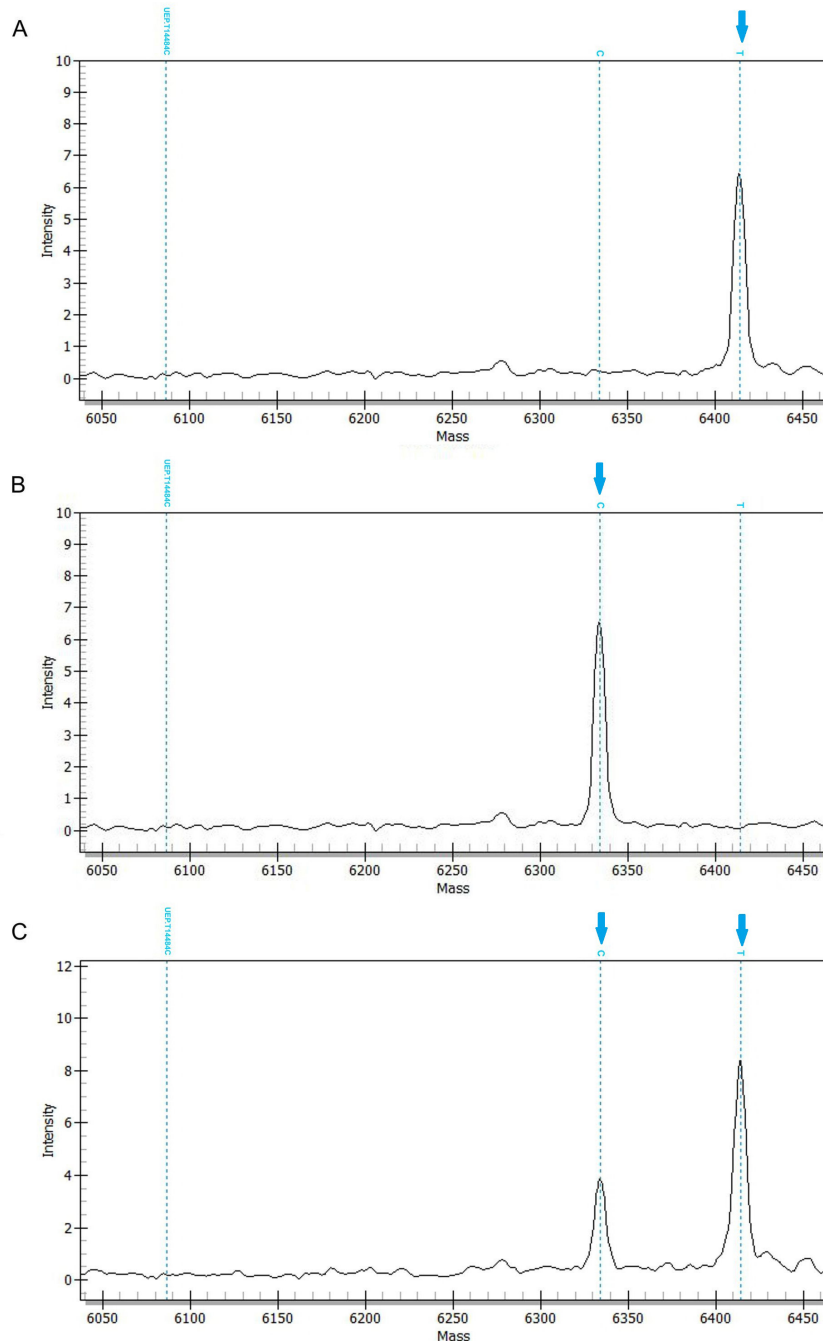
Figure 2. Typical raw data obtained using the Sequenom MassArray system for T14484C mutation screening. The blue arrows above the wave peak indicate the resulting genotypes. **A**: 100% wild-type mtDNA. **B**: 100% mutant mtDNA. **C**: Heteroplasmic (C/T) mtDNA.

in the present study for the G11778A mutation differed from the value of 60% reported previously in 2010 [15], which could have been due to the small number of patients studied in the earlier work.

The T14484C mutation is the most common cause of LHON among French Canadians, due to the founder effect

[35], but is much less frequent in Northern European populations [7,22]. In contrast, the T14484C frequency identified in this study (16.7%) was comparable to the value of 14% reported previously, although there are significant differences among data from other Brazilian studies [15,36].

In the case of the G3460A mutation, a clinical and molecular profiling study of a Brazilian sample conducted in 2010 identified only one case with this mutation [15]. Furthermore, in another investigation of the frequency of LHON mutations in Brazilian patients with optic neuropathy, the G3460A mutation was not observed in any of the patients analyzed [36], as in the present work. Therefore, these results suggest that the G3460A mutation is not common in Brazil. Interestingly, the Brazilian population is one of the most heterogeneous in the world, with interethnic crosses between individuals of Caucasian, African, and Amerindian origin [37]. It is likely that this important characteristic could contribute to differences in the observed frequencies of the mutations when comparison is made with other populations or even within the Brazilian population.

Previous studies conducted with Latin American families affected by LHON have reported similar results, with different frequencies found only for the A3460G mutation. In Chile, the frequencies obtained for the G11778A, T14484C, and G3460A mutations were 78.7%, 16.0%, and 5.3%, respectively [38]. In Cuba, 80% of the families studied carried the A117789 mutation, while the A3460G mutation was detected in 20% [39].

In the present study, none of the other eight mutations investigated was found in the patients analyzed, consistent with previous studies reporting that these mutations are rare in various populations [17,19]. Thus far, the presence of these point mutations in patients with LHON has not been reported in the Brazilian population.

In this study, other changes were identified from direct sequencing of the regions in which the mutations were screened. The C4167T change in the *MT-ND1* gene and the T11697C change in the *MT-ND4* gene were found in the homoplasmic state. However, these mutations are considered silent, because the replacement of their bases is not predicted to lead to amino acid residue change. In addition, 29 patients had the G11719A alteration, and two had G14560A. These changes are considered polymorphisms [40], and to date, there have been no reports suggesting these polymorphisms are involved in LHON.

After screening for LHON mutations, 65 patients (64%) remained mutation negative. In these cases, mutations in different regions of mtDNA not assessed in this work, as well as epigenetic or nuclear-encoded factors, could be involved in the phenotypic expression of LHON. Therefore, further studies are required to confirm the diagnosis of LHON in these patients.

Comparison of the two groups of patients revealed no statistically significant differences in the type of vision loss (sequential or bilateral) or visual acuity. However, all the patients carrying LHON mutations presented profound vision loss, with visual acuity less than or equal to 20/200. It is likely that such intense perturbation of visual function could be used to assist in the clinical diagnosis of LHON. Leber hereditary optic neuropathy has a severe negative impact on quality of life and has the worst score in the visual function 14 (VF-14) [41] questionnaire, compared to other ophthalmic disorders that have been studied previously [42].

Most of the patients whose molecular diagnosis was confirmed by the presence of LHON mutations were male. The predominance of affected men in LHON cannot be explained by mitochondrial inheritance. Several studies have provided evidence for an X-chromosome-linked disease modifier [43,44]. However, the causative gene(s) on the X chromosome remain to be identified.

In the 36 patients who carry LHON mutations, the L1/L2, L3, C, L, R, D, and H haplogroups were identified. Carriers of the G11778A mutation belong to haplogroups L1/L2, L3, C, L, R, D, and H. Patients with the T14484C mutation belong to one of haplogroups L1/L2, L3, and U. According to the literature, individuals with the G11778A and T14484C mutations have a higher risk of vision loss when associated with the J haplogroup. However, this haplogroup was not identified in either of the groups studied here. Although European (H and U), Asian (C, R, and D), and Oceanic (R) haplogroups have been found, most cases are associated with haplogroup L of African origin (in the present study, there were 17 cases of L1/L2 and 11 of L3) [24,32,45,46]. Even in patients who present no LHON mutation, the most frequent haplogroups were L1/L2 and L3. This result is not surprising, considering the high contribution of Africans in the Brazilian mtDNA pool, which can be up 40% in some regions [37]. To date, no studies have shown the involvement of L1/L2 and L3 haplogroups in increased risk of LHON expression.

In South America, few studies have correlated mitochondrial haplogroups with clinical manifestations in patients with LHON [13,38]. In the last decade, several studies have evaluated the genetic variability and evolutionary processes of the Brazilian population [47-49]. However, no studies have found any specific mtDNA background associated with the clinical expression of LHON due to increased penetrance of the primary mutations. Comparison of the results obtained for patients with one of the LHON mutations and those for individuals without any mutations did not enable establishment of any correlation between the mutations and haplogroups found in this study.

The usefulness of the iPLEX Gold/MALDI-TOF MS platform in detecting LHON mutations was also evaluated. Perfect agreement was found when the data generated by this technique were compared with the results obtained previously using the PCR-RFLP technique and direct sequencing. According to the manufacturer, the iPLEX Gold platform can detect low levels of heteroplasmy (up to 10%), as confirmed in the present work in the experiments performed to assess the sensitivity of this technology. However, it was also observed that the platform should be used only for qualitative testing, because although the platform presented absolute accuracy in detection of low levels of mtDNA (minimum 10%) in all the experiments, it was not possible to establish a quantitative relation between the peak intensity and the amount of mutant mtDNA present. As there are rare cases in which patients with LHON mutations manifest the disease with less than 50% mutant DNA, this method seems to be highly efficient in detecting LHON disease [22,50]. In the present study, all the mutations were found in the homoplasmic state. Therefore, the iPLEX Gold/MALDI-TOF MS platform can be considered a highly accurate analytical method for detecting mutations and identifying haplogroup markers related to LHON. Although the major drawback of the iPLEX assay lies in the requirement for specific equipment, investment in a MassArray platform could appeal to laboratories that analyze large numbers of samples. This technique is easy to use and produces data that are easy to interpret, as it follows a well-established protocol. Despite the limitation of identifying only known mutations, the method offers a high-throughput, flexible, and cost-effective option for use in genetics laboratories.

Definitive diagnosis of LHON can be established with molecular genetic testing. As there are many similarities in the clinical manifestation of LHON and other optic neuropathies, the molecular genetic investigation of LHON mtDNA mutations is essential for differential diagnosis. In particular, molecular genetic testing can facilitate clinical diagnosis in cases with atypical clinical features and no clear maternal history of blindness. A simple blood test for LHON can determine whether an individual carries one of the main LHON mutations. Once a LHON mutation has been identified in a proband, molecular genetic testing should be offered to other family members. It is important for LHON carriers to be made aware that it is currently not possible to predict accurately whether, or when, they might become affected. Only approximately 50% of male and approximately 10% of female mutation carriers develop symptoms, which indicate that additional genetic or environmental factors are required for the phenotypic expression of LHON [51]. It is suggested that heteroplasmy can cooperate with incomplete penetrance,

and according to this theory, 60% or more of the mitochondrial DNA molecules would need to be mutated to constitute a risk of blindness [52,53].

In summary, it was possible to establish a definitive diagnosis of LHON in 36% (36/101) of the cases studied. The results highlight the importance of molecular genetic analysis for more accurate diagnosis, which is fundamental in determining the most appropriate genetic counseling and treatment strategies. The frequencies of the most common pathogenic point mutations in LHON were similar to those reported for other LHON populations. A different frequency was found only for the A3460G mutation. The most frequent haplogroups were of African origin; however, further studies correlating mitochondrial haplogroups with clinical manifestations in patients with LHON are necessary to confirm the influence of these haplogroups in clinical expression of LHON. The iPLEX Gold/MALDI-TOF MS technology seems to be a suitable tool for investigation of the mitochondrial alterations involved in LHON disease.

APPENDIX 1. PRIMER SEQUENCES USED FOR THE SCREENING OF HAPLOGROUPS BY MALDI-TOF PLATFORM.

To access the data, click or select the words "Appendix 1."

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