Optimization of a genotyping screening based on hydrolysis probes to detect the main mutations related to Leber hereditary optic neuropathy (LHON)

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Purpose: Leber hereditary optic neuropathy (LHON) is a mitochondrial inherited disease characterized by bilateral vision problems, such as reduced visual acuity, dyschromatopsia, and central or centrocecal scotoma. Of these cases, 95% are caused by three mutations in mitochondrial DNA (mtDNA): m.G11778A, followed by m.T14484C and m.G3460A. The remaining 5% of cases of LHON are caused by rare mutations also present in mtDNA. Although conventional molecular tools for molecular screening of LHON are becoming popular, in most cases these tools are still expensive and time-consuming and are difficult to reproduce. Therefore, to meet the need for more accurate, faster, and cheaper techniques for molecular screening, as well as make it more accessible, we used the high-throughput method TaqMan® OpenArray™ Genotyping platform for developing a customized high-throughput assay for the three main mutations related to LHON.
Methods: The assay was performed for 87 individuals diagnosed with LHON or acquired optic neuropathy of unknown origin. The three main mutations were screened using the customized assay with the TaqMan® OpenArray™ Genotyping platform, and all reactions were performed in triplicate. The positive and negative results were revalidated with restriction fragment length polymorphism PCR (RFLP-PCR) and Sanger sequencing.
Results: The main mutations related to LHON were detected in 34 patients with genotyping reactions, of which 27 cases had the m.G11778A mutation, and seven had the m.T14484C mutation.
Conclusions: The TaqMan® OpenArray™ Genotyping platform was shown to be an effective tool for molecular screening of the most common mutations related to LHON without presenting false positive or negative results for the analyzed mutations. In addition, this tool can be considered a cheaper, faster, and more accurate alternative for molecular screening of LHON mutations than PCR and Sanger sequencing, as 94 genotyping reactions can be performed within 6 h and specific TaqMan probes are used.

Leber hereditary optic neuropathy (LHON) is a maternally inherited dystrophy characterized by painless loss of vision, resulting from the degeneration of retinal ganglion cells, more specifically the papillomacular bundle and optic nerve axons. LHON affects three subunits of complex I in the mitochondrial respiratory chain [1] and the oxidative phosphorylation pathway, causing apoptosis of the ganglion cells [2,3]. This leads to decreased visual acuity, scotomas, dyschromatopsia, and atrophy with subacute or acute central vision loss and results in irreversible blindness in most cases. Vision problems seem to be the only clinical manifestation in patients with LHON, but some other changes, such as skeletal or neurologic disorders and cardiac abnormalities, can also be present [4,5]. Usually, the vision loss starts in one eye, and in more than 95% of the cases, the vision loss spreads to the second eye within 1 year, with the rate of progression varying between the eyes [6].

This optic neuropathy was the first clinically characterized mitochondrial disorder and remains the most common disorder related to mitochondria [7]. In a recent wide study of the Danish population, LHON was considered to affect one in 54,000 individuals, and similar numbers can be seen in the Finnish population [8]. The Dutch population presented a higher frequency, with one affected individual in every 39,000 individuals [9]. The highest frequency was reported in northeast England, where LHON reaches one in every 31,000 individuals [10], while a meta-analysis estimated the LHON frequency in Europe to be one in every 45,000 individuals [11]. Despite the high frequency, LHON is classified as a rare and complex disorder that typically affects young men (80–90% of the cases) with the age of onset between 15 and 35 years old in one eye and becoming bilateral after some months [12-17].

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Yu-Wai-Man and collaborators [5] reported 21 important mutations related to LHON, and the number has continued to increase. Three of the mutations are known as the main mutations because at least one of them is present in 90–95% of the individuals with LHON. The most common mutation is m.G11778A in the MT-ND4 gene (Gene ID: 4538; OMIM: 516003) [18], followed by m.T14484C in the MT-ND6 gene (Gene ID: 4541; OMIM: 516006) [19] and m.G3460A [20] in the MT-ND1 gene (Gene ID: 4535; OMIM: 516000). Recently, a new mutation, m.A3395G in the MT-ND1 gene, was associated with LHON after being found in one Danish patient and about 1% of other patients with LHON [1].

The frequency of the main mutations varies according to geographic location: The m.G11778A mutation is most common among Danish (75%), Finnish (69%), and Asian populations (95%), while it causes 50% of the cases in Europe [2,21,22]. The second most common mutation, m.T14484C, is found in approximately 14% of all cases [22,23], while the least frequent mutation, m.G3460A, occurs in 13% of the affected people in the world [21,24]. Spontaneous recovery of the clinical picture is rare but has been documented, although mainly for individuals with the m.T14484C and m.G11778A mutations [6].

The remaining genetically caused cases of LHON are classified as rare mutations and are reported in only 5–10% of all cases. The main rare mutations are present in the MT-ND1, MT-ND4, MT-ND4L (Gene ID: 4539; OMIM: 516004), MT-ND5 (Gene ID: 4539; OMIM: 516004), MT-ND6, and MT-CYB genes (Gene ID: 4519; OMIM: 516020) [5,22,25]. How these rare mutations can cause the LHON phenotype and their mechanism of action remain to be elucidated. For example, some rare mutations have been suggested to play a role in the penetrance and phenotype of LHON, but the significance of the variants is still unclear and sometimes claimed to be associated with other syndromes and symptoms [5,25,26].

Some important points must be considered in the expression of the LHON phenotype. First, the amount of variant or wild-type mitochondrial DNA (mtDNA) in each cell or tissue must be determined. In heteroplasmy, the phenotype is shown only when the mutation reaches a critical threshold for that tissue or cell. Second, it is known that LHON is an incompletely penetrant disorder, which means that not all individuals presenting the genotype will have visual problems [7]. This is exemplified by the prevalence of mutations related to LHON, which is around 1:8,500 people, while the disorder is seen in around 1:40,000–50,000 people [9]. The penetrance is also higher in men than in women (50% against 10%) [27]. Furthermore, a research group showed that the penetrance in men with a smoking habit can reach around 93% [28]. Other environmental factors that can increase the penetrance of LHON are excessive alcohol consumption, increased amounts of reactive oxygen species, antiretroviral therapies, and toxins [7].

In Brazil, the first molecular studies related to LHON were conducted for a large family of about 300 people, where most individuals exhibited only the m.G11778A mutation [29,30]. The first reported frequencies of the three main mutations in Brazil came from 13 unrelated individuals, of whom 60% had the m.G11778A mutation, 30% the m.T14484C mutation, and 10% the m.G3460A mutation [31]. Moreover, an analysis of 26 patients with a clinical diagnosis for LHON revealed that two had the m.G11778A mutation and two the m.T14484C mutation, while no patients were positive for the m.G3460A mutation or any of the 14 rare mutations studied [32]. Thus, there is no information regarding a large number of affected individuals and the frequency of LHON rare mutations in Brazil.

As the Brazilian population is ethnically heterogeneous, it is possible that the frequencies of the main and rare mutations could be different from those documented in other parts of the world. Furthermore, molecular analysis of LHON mutations could be an important tool to confirm the diagnosis in typical clinical cases of LHON, because other hereditary optic neuropathies, such as Wolfram's syndrome and dominant optic atrophy (DOA), can present the same pathological features as LHON. The use of conventional molecular tools to detect LHON mutations, such as PCR and Sanger sequencing, or even next-generation sequencing (NGS), is becoming popular, but in most cases, the tests are expensive, time-consuming, and hard to reproduce [33,34]. High-throughput technologies have been important tools for advancing the molecular diagnosis of genetic disorders. These robust techniques offer possibilities to generate larger sets of results simultaneously at a lower cost and within a shorter time. Among them, the TaqMan® OpenArray™ Genotyping (Thermo Fisher Scientific, Waltham, MA) platform is a promising candidate for use as a tool for diagnosis. This tool is a high-throughput method based on real-time PCR that allows checking of up to 3,072 genotyping reactions simultaneously in each genotyping plate. The work presented here set out to customize and optimize a panel based on the TaqMan® OpenArray™ Genotyping technique for the molecular screening of the main mutations (m.G11778A, m.T14484C, and m.G3460A) related to LHON.

METHODS

Patients: Clinical analyses were performed at the Center for Rehabilitation Research Studies (CEPRE), Faculty of Medical Sciences and at the Ophthalmology Outpatients Unit of the Clinical Hospital, both located at State University of Campinas (UNICAMP). A total of 91 patients with a previous clinical diagnosis of LHON or acquired optic neuropathy of unknown origin were selected for the study and classified into two groups: Group 1 consisted of 63 individuals (20 women and 43 men) with a diagnosis of LHON, and group 2 contained 28 individuals (six women and 22 men) with optic neuropathy of unknown origin. The individuals included in the study had clinically diagnosed optic neuropathy with painless and bilateral vision loss (acute or subacute), visual acuity under or equal to 20/50 (far) and J3 (close), dyschromatopsia, and central or centrocecal scotoma. Computed tomography (CT) or magnetic resonance imaging (MRI) was used to confirm the absence of neurologic abnormalities. Individuals who had had eye surgery or any other systemic disorder causing visual problems were excluded from the study.

All the individuals agreed to and signed informed consent. This study adhered to the tenets of the Declaration of Helsinki, as well as the Association for Research in Vision and Ophthalmology (ARVO) statement on human subjects. The study was approved by the Research Ethics Committee of the Faculty of Medical Sciences of UNICAMP (Process no. 690/2004).

DNA extraction and preparation: Five milliliters of peripheral blood were collected from each patient in a Lavender Vacutainer®. The samples were sent for molecular analysis at the Human Molecular Genetics Laboratory, Center for Molecular Biology and Genetic Engineering (CBMEG), UNICAMP, where the genomic DNA was extracted from the blood leukocytes using standard phenol-chloroform extraction [35].

For the genotyping reactions, each sample should have a concentration of 50 ng/µl and an $A_{260/280}$ ratio between 1.8 and 2, which were obtained using the Qubit® dsDNA BR Assay Kit with Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific), respectively.

TaqMan® OpenArray™ Genotyping: The TaqMan® OpenArray™ Genotyping platform was used to screen for the three primary mutations. Each assay amplification fragment had around 350 bp, including the variant site. All assays were analyzed using the Repeat Masking tool to identify repetitions and low complexity DNA sequences. Repetitive sequences were discarded, while masked sequences were added to the Custom TaqMan® Genomic Assays File Builder Version 3.1 software (Applied Biosystems, Foster City, CA) and sent for customization. The manufacturer selected the optimal primers and probes for each assay (Table 1).

Three TaqMan® OpenArray™ Plates (one for the experiment itself and two as technical repeats) were used to analyze all 91 patients. Additionally, three negative controls for the main mutations and two no-template controls (NTC) were used. The plates had 48 subarrays with 64 through-holes, and each agreed with the standard layout of 32 single nucleotide variants (SNVs) for 96 samples proposed by the manufacturer. The fluorophores selected for the probes of this assay were the VIC® fluorophore (normal allele) and the FAM® (mutant allele).

In a 384-well plate, 2 µl of each sample was mixed with 2 µl of TaqMan® OpenArray™ Master Mix. The loading of the mix into the TaqMan® OpenArray™ Plate was made with the OpenArray™ AccuFill® software and OpenArray™ AccuFill® system (Applied Biosystems). The plate was sealed in a glass

TABLE 1. CUSTOMIZED OPENARRAY™ ASSAYS.		
Assay	**Primer (5′ >3′)**	**Amplified sequence**
m.G11778A	**F:** TGCCTAGCAAACTCAAACTACGAA **R:** GGGAGTAGAGTTTGAAGTCCTTGAG	TGCCTAGCAAACTCAAACTACGAACGC**ACTCACAGTC[G/A]CATCAT**AATCCTCTCTCAAGGACTTCAAACTCTACTCCC
m.T14484C	**F:** TCCTCAATAGCCATCGCTGTAGTAT **R:** CGGGTGTGTTATTATTCTGAATTTTGGG	TCCTCAATAGCCATCGCTGTAGTATATCCAAAG**ACAACCA[T/C]CATTCCC**CCTAAATAAATTAAAAAAACTATTA-AACCCATATAACCTCCCCCAAAATTCAGAATAATAACA-CACCCG
m.G3460A	**F:** CCCCTACGGGCTACTACAAC **R:** GATGGTAGATGTGGCGGGTTT	CCCCTACGGGCTACTACAACC**CTTCGCTGAC[G/A]CCATA-A**AACTCTTCACCAAAGAGCCCCTAAAACCCGCCACATCTAC-CATC

F: Forward; R: Reverse. Underline/bold sequence: Customized TaqMan® probe. Inside the brackets, the first nucleotide indicates the normal sequence, dyed with VIC, and the second, the mutant allele, dyed with FAM. For each assay were customized a pair of primers and a pair of probes (bold and underlined).

box (provided with the OpenArray™ kits) and submitted to the amplification reaction for 4 h in a Dual Flat Block GeneAmp® PCR System 9700 thermocycler (Applied Biosystems). The fluorescence emitted by the fluorophores was measured with the OpenArray™ NT Cycler (Biotrove, Pleasanton, CA), and the results were analyzed with TaqMan® Genotyper software (Life Technologies) [36].

RFLP-PCR and Sanger sequencing: The three main mutations were previously screened using restriction fragment length polymorphism PCR (RFLP-PCR) [37]. All the details of the primers and endonuclease used in this work are described in Table 2. All the reactions were performed using the Veriti® 96-Well Thermal Cycler (Applied Biosystems) with a common cycle, taking into account the melting temperature for each primer set [38,39]. The digestion reactions were performed using 1 µg of DNA, 1 µl of the endonuclease and 5 µl of buffer (final volume of 50 µl). The reactions using the endonucleases *Sfa*NI, *Bsa*BI and *Bsa*HI were incubated for 2 h at 37 °C, 60 °C and 37 °C, respectively, followed by the enzyme inactivation at 80 °C for 20 min (New England Biolabs, Ipswich, MA). The results were analyzed on a 3% UltraPure™ Agarose 1000 gel and stained with ethidium bromide immersion [40,41].

Sanger sequencing was performed using the same primers as in the TaqMan® OpenArray™ Genotyping (Table 1), and the fragments were purified according to the EDTA/ethanol standard protocol. For the reactions, the ABI PRISM® 3700 DNA Analyzer and the BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) were used. The sequencing results were aligned with reference sequences (from the Genome Browser) in the CLC Sequence Viewer 7.6 software (Qiagen, Hilden, Germany), and the peak qualities were verified with the 4Peaks program (Nucleobytes B.V., Aalsmeer, The Netherlands). All results obtained with the TaqMan® OpenArray™ were revalidated with RFLP-PCR and Sanger sequencing.

RESULTS

Four samples (two from each group) did not fulfill the minimum requirements for purity ($A_{260/280}$ between 1.8 and 2.0) and concentration (50 ng/µl) requested by the manufacturer. Despite this, these four samples were included in the experiment, because the aim of this work was to check the efficiency of the customized layout. As a result, these genotyping reactions failed, and the samples of these individuals were excluded from the other analyses.

The m.G11778A, m.T14484C, and m.G3460A mutations were analyzed with the TaqMan® OpenArray™ Genotyping platform for the remaining 87 patients clinically diagnosed with LHON (group 1) or optic neuropathy of unknown origin (group 2). All genotyping results (Table 3) were revalidated using RFLP-PCR and Sanger sequencing.

In group 1, the visual loss was simultaneous in 83.60% of the cases, while the remaining patients had a sequential vision loss in the other eye within 7 to 365 days. Of 61 patients, 22 were positive for one of the main mutations associated with LHON. Ten women presented the most common mutation, m.G11778A, while one had the m.T14484C mutation. Similar numbers were seen for the men, of whom nine patients had the m.G11778A mutation and two had the m.T14484C mutation.

About 73.08% of the patients in group 2 had simultaneous vision loss, while for 26.92% the loss was sequential and affected the opposite eye within 16 to 970 days. Twelve primary mutations were identified: eight cases of the m.G11778A mutation (one woman and seven men) and four cases of the m.T14484C mutation, which were all in men.

In general, the m.G11778A mutation was the most common variant detected by the genotyping plates. In total, 27 patients (79.41% of all variants found) presented this mutation (Figure 1A), while seven patients (20.59%) had the m.T14484C mutation (Figure 1B), which was the second most common variant found by the customized plate. We also customized a TaqMan® OpenArray™ Genotyping platform for

TABLE 2. DETAILS OF THE PREVIOUS MOLECULAR SCREENING OF LHON BY RFLP-PCR.

Mutation	Primers (5′ >3′)	Endonuclease	Fragments	
			Wild Type	**Mutant**
m.G11778A	**F:** AACTACGAACGCACTCACAG **R:** GAAGTCCTTGAGAGAGGATTA	*Sfa*NI	17/32bp	49bp
m.T14484C	**F:** GTAGTATATCCAAAGATAACCA **R:** CTTCTAAGCCTTCTCCTA	*Bsa*BI	19/135bp	154bp
m.G3460A	**F:** GGCTACTACAACCCTTCGC **R:** GGCTCTTTGGTGAAGAGTTTT	*Bsa*HI	22/27bp	49bp

Customized PCR for the main mutations for LHON screenings. **F:** Forward strand; **R:** Reverse strand.

TABLE 3. RESULTS OF SCREENING FOR THE MAIN MUTATIONS RELATED TO LHON BY TAQMAN® OPENARRAY™ GENOTYPING.

Mutation	Group 1		Group 2		Total (%)	Technique used for re-validation
	Screening with OpenArray™	Age range*	Screening with OpenArray™	Age range*		
m.G11778A	19/61		8/26		31.03% (27/87)	RFLP-PCR and Sanger sequencing
m.T14484C	3/61	11–52 years	4/26	13–43 years	8.05% (7/87)	
m.G3460A	0/61		0/26		0% (0/87)	

*Age rate: Age where the individual started to present vision problems in, at least, one eye.

the m.G3460A mutation, but we did not manage to optimize this assay due to the absence of a positive control. Nevertheless, all individuals genotyped using this assay showed negative results as expected (Figure 1C).

For the 87 individuals who were genotyped, the platform proved to be efficient with 100% sensitivity and specificity for the mutations. The validation of the genotyping results with RFLP-PCR and Sanger sequencing corroborated the results obtained with the TaqMan® OpenArray™ Genotyping platform.

DISCUSSION

LHON is the first clinically classified mitochondrial disorder, and its molecular diagnosis is fundamental for the correct clinical diagnosis. This work used the TaqMan® OpenArray™ Genotyping platform as a method to produce a cheap, accurate, and fast test for the main mutations of LHON.

Among all the studied individuals, 34 had been previously diagnosed with the main mutations: 27 with the m.G11778A mutation (31.03%) and seven with the m.T14484C mutation (8.05%). The m.G3460A mutation was not found in any individual.

Regarding the m.G3460A mutation, additional studies should be performed to check whether the mutant sequence can be detected with the present customized assay. This test could not be validated due to the lack of a positive control for the m.G3460A mutation, but the test did not present any false positive results in this assay, as all genotyping reactions showed only binding of the probe for the normal sequence.

The values related to the mutations studied in this work differ from those reported in the literature, but nonetheless, they follow the same pattern of frequency with the m.G11778A mutation as the most common mutation and m.G3460A as the least common mutation [1,5,18,21,24,42]. All the different frequencies of the main mutations noted in these samples compared to previously published data could be explained by the fact that Brazil has a very ethnically heterogeneous population compared to many other countries.

In total, 91 affected individuals and three negative controls were genotyped, totaling 282 genotyping reactions performed for each mutation with the TaqMan® OpenArray™ Genotyping platform. When the two technical repeat plates is considered, the number of reactions is 846. Each plate showed the same genotyping results. The three negative controls were present in the normal cluster for all reactions (nine genotyping reactions). Among the samples, four did not present the minimal requirements (purity and concentration) for the genotyping, which led to a failed genotyping reaction. The 12 genotyping reactions related to these samples were used only to check the possibility of using conditions different from the ones requested for the technique, and thus, the failed genotyping reactions were not used to calculate the technique statistics.

The remaining 261 genotyping reactions presented the highest score for specificity and sensitivity, with the absence of false positive or negative results. Such accurate results are due to the use of TaqMan® probes for this platform, which ensure an exact and robust method for detecting the main mutations of LHON. Furthermore, these tests were performed in less than 1 day, which illustrates the high efficiency of the technique. Additionally, the method showed high reproducibility, as the two technical repeats presented the same results. The technique also follows a well-established protocol with easy handling for a trained person. If only the kit and plates are considered, the genotyping cost for the three main mutations was USD 5.32 per genotyped individual.

One patient with the m.T14484C mutation presented a special case. Revalidation with RFLP-PCR presented a smooth fragment of 135 bp, suggesting the presence of normal sequence mtDNA and thus, a possible case of heteroplasmy. In addition, the TaqMan® OpenArray™ Genotyping experiment indicated a difference because this patient was not placed in either the mutant or the normal cluster but in between (the black circle in Figure 1B). Thus, even though the TaqMan® OpenArray™ Genotyping technique cannot be considered a highly recommended technique for detecting heteroplasmy,
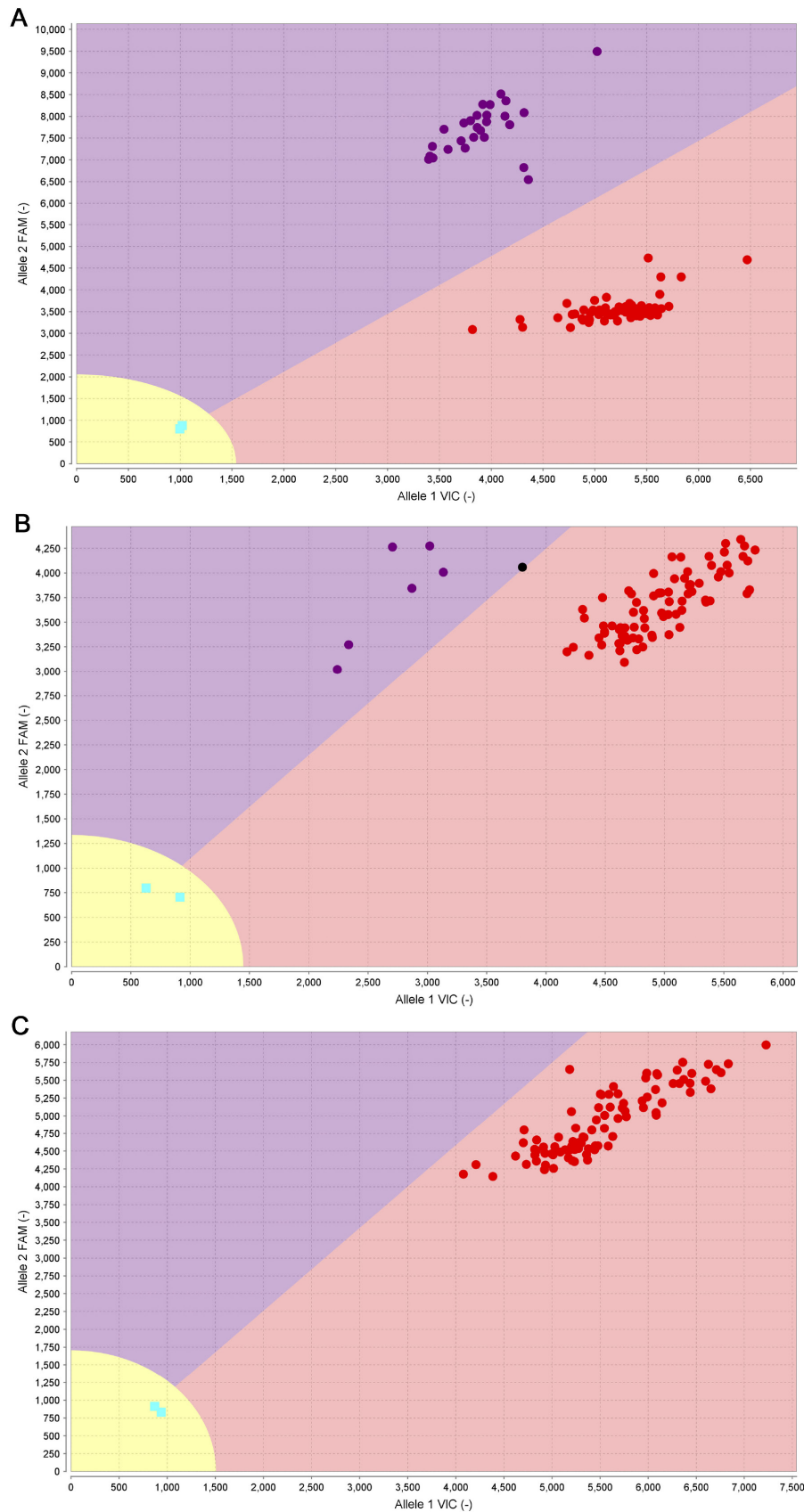
Figure 1. Results for the TaqMan® OpenArray™ Genotyping using the TaqMan® Genotyper software. The red clusters show the individuals with the probe marked with the VIC® fluorophore (normal sequence), while the purple clusters present the individuals with probes marked with the FAM® fluorophore (mutated sequence). **A**: The m.G11778A mutation was present in 27 individuals, while 60 individuals did not have the mutation. **B**: Seven individuals presented the m.T14484C mutation. The black dot was not autoclassified by the software, probably indicating heteroplasmy (checked with another technique). **C**: No mutant cases were found for the m.G3460A mutation as expected.

this technique can find indications of this, allowing further studies to detect the amount of mutated mtDNA.

This study did not aim to discover new variants but instead, to standardize and optimize a high-throughput technique for fast and efficient detection of the main mutations related to LHON. Despite the high success rate for finding mutations with the described setup, 54 individuals with LHON or optic neuropathy of unknown origin, but without any of the main or rare mutations, were left with an unclear diagnosis. The high percentage of individuals without a LHON molecular diagnosis could be due to many factors: (i) individuals with the wrong clinical diagnosis, whose symptoms resemble LHON; (ii) a novel gene or mutation not investigated in this study; (iii) a low rate of mtDNA containing the mutations (a low heteroplasmy rate); (iv) preferential amplification of a wild-type sequence; or (v) the amount of mutated mtDNA in the peripheral blood could be different because of differences in expression between tissues [43]. Of these factors, the hypotheses of a novel gene or mutation not investigated in this study, a low rate of mtDNA containing the mutations (a low heteroplasmy rate), and preferential amplification of a wild-type sequence can be considered technical limitations of the TaqMan® OpenArray™ Genotyping platform. Another drawback is the inability to change the customized assay, as the primers and probes are attached to the genotyping plates. However, it is important to highlight that the absence of mutations does not exclude that the individuals have LHON. Extensive research concerning neuropathy remains to be done, as well as in-depth studies of the nuclear and mitochondrial genes and their pathways, to obtain a better understanding of the pathology, and the development of new therapies and strategies to minimize the effects of the mutations.

To increase the screening strength of the TaqMan® OpenArray™ Genotyping platform, a new set of plates that also contain rare LHON mutations should be designed. However, as this study shows, this technique is highly promising for fast, money-saving, and accurate diagnosis of LHON. Conclusively, with the results presented here, the TaqMan® OpenArray™ Genotyping platform can be a valuable tool for routine detection of the main LHON mutations.

ACKNOWLEDGMENTS

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