Evaluation of Multiplex Ligation-dependent Probe Amplification Technique for Detecting Mitochondrial DNA Mutations in Leber Hereditary Optic Neuropathy Patients

การประเมินประสิทธิภาพของเทคนิค Multiplex Ligation-dependent Probe Amplification สำหรับตรวจหาการกลายพันธุ์ของไมโทคอนเดรียดีเอ็นเอ ในกลุ่มผู้ป่วย Leber Hereditary Optic Neuropathy

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ABSTRACT

Leber Hereditary Optic Neuropathy (LHON) is one of an important painless vision loss which causes by mitochondrial DNA mutation. For increasing the accessibility to the genetic testing an accurate with cost-effective assays are required. The purpose of this study was to evaluate the accuracy of Multiplex Ligation-dependent Probe Amplification (MLPA) technique for detecting the three primary mutations (m.3460G>A, m.11778G>A and m.14484T>C) within mitochondrial DNA in LHON Patients. The validation results from direct DNA sequencing technology proved that MLPA technique has 100% accuracy as the same as direct DNA sequencing method. In conclusion, this technique might be used as routine genetic testing for mitochondrial disorder or other genetic diseases with highly accurate, less expensive and make it more accessible.

บทคัดย่อ

โรค Leber herediaty Optic Neuropathy (LHON) เป็นหนึ่งในสาเหตุสำคัญของการสูญเสียการมองเห็น เนื่องมาจากการกลายพันธุ์ของไมโทคอนเดรียดีเอ็นเอ เพื่อเพิ่มการเข้าถึงการตรวจหาความผิดปกติทางพันธุกรรมจึงมีความจำเป็นที่จะต้องค้นหาเทคนิคที่ใช้ในการตรวจหาความผิดปกติของพันธุกรรมที่มีความแม่นยำและมีราคาที่เหมาะสม ดังนั้นจุดประสงค์ของการศึกษาครั้งนี้คือการประเมินประสิทธิภาพของเทคนิค Multiplex Ligation-dependent Probe Amplification (MLPA) ในการตรวจหาการกลายพันธุ์หลักในไมโทคอนเดรียดีเอ็นเอ ได้แก่ m.3460G>A, m.11778G>A และ m.14484T>C ในกลุ่มผู้ป่วย LHON ซึ่งผลจากการประเมินประสิทธิภาพโดยเปรียบเทียบกับเทคโนโลยีการถอดรหัสพันธุกรรม พบว่าเทคนิค MLPA ให้ความถูกต้องร้อยละ 100 ซึ่งให้ผลตรงกันกับเทคโนโลยีการถอดรหัสพันธุกรรม จึงสรุปได้ว่าเทคนิค MLPA อาจจะสามารถนำไปใช้ในงานบริการสำหรับการตรวจหาความผิดปกติของพันธุกรรมในกลุ่มผู้ป่วยที่มีความผิดปกติของไมโทคอนเดรียหรือโรคพันธุกรรมอื่น ๆ ด้วยความแม่นยำของเทคนิคที่สูงและราคาถูก ซึ่งจะทำให้เกิดการเข้าถึงการบริการได้มากยิ่งขึ้น

Keywords: Multiplex Ligation-dependent Probe Amplification Technique, Mutation, LHON

คำสำคัญ: เทคนิค Multiplex Ligation-dependent Probe Amplification การกลายพันธุ์ โรค LHON

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Introduction

Leber Hereditary Optic Neuropathy (LHON) is a rare maternally inherited disease that causes sudden painless central vision loss, usually found in the young adult with a peak of onset between 15 – 30 years old (Kim et al., 2018). It is bilateral disease that presents with vision loss in one eye before the other eye in the several months, and both eyes are simultaneously affected in about 25% of the patients (Newman, 2012). The prevalence of LHON has been estimated to be about 1 in 31,000 in North East of England (Yu-Wai-Man et al., 2003) and in Thailand, there has not been the report of prevalence. LHON caused by the mutation within mitochondrial DNA (mtDNA) and around 90% of LHON patients have one of three point mutations in mtDNA (m.3460G>A, m.11778G>A, and m.14484T>C), about 50% of patients have m.11778G>A mutation. Due to mutation in mtDNA causes impairment ATP production via alteration of oxidative phosphorylation (OXPHOS) and accumulation of reactive oxygen species (ROS) in mitochondria triggers apoptosis of retinal ganglion cells (RGCs) result in a clinical sign of painless, progressive unilateral or bilateral visual acuity loss and color vision deficiencies (Pilz et al., 2017).

Diagnosis of LHON mainly diagnosed by clinical diagnosis and patient's history (Man et al., 2002). However, only the clinical diagnosis has not enough accuracy and might lead to misdiagnosis because clinical manifestations in mitochondria disorders are very diverse and there have some diseases that share clinical features as LHON. Genetic testing plays an important role to solve the problems by confirming the mutation. Nowadays, there are many molecular techniques for detecting the mutation but conventional techniques which are widely used are direct DNA sequencing and Restriction Fragment Length Polymorphisms Polymerase Chain Reaction (RFLP-PCR) (Martins et al., 2017). Otherwise, these two techniques are suitable for finding only 1 or 2 mutations and need expert staff to interpret the results. Moreover, direct DNA sequencing technology is still expensive (Carrasco Salas et al., 2016; Miranda et al., 2016).

The technique called Multiplex Ligation-dependent Probe Amplification technique (MLPA) is one of the alternative techniques for detecting qualitative and quantitative structural reorganization in DNA (Mayorga et al., 2016). When compared to the conventional techniques, this assay is less expensive, easier to interpret and does not require high-experienced staff to perform. On the other hand, the accuracy of this assay has not been clarified and also lack of evaluation by the human sample.

In this study, we aim to evaluate the accuracy of MLPA for detecting mutations within mtDNA in Thai LHON patients by using direct sequencing technique as a validation method. As the LHON cannot completely cure, the appropriate medication is supportive treatment by giving anti-oxidant drugs and vitamins to the patients (Carelli et al., 2001; Mashima et al., 2000). The accurate and less expensive diagnostic test could help the patient access to the treatment quickly for reducing blindness that may occur in the future.

Objectives of the study

The aim of this study was to evaluate the accuracy of MLPA technique for detecting commonly mitochondrial DNA mutations in Leber Hereditary Optic Neuropathy Patients.

Methodology

Population and Specimen collection

Twelve Thai patients who have been diagnosed and suspected as LHON by clinical diagnosis based on ophthalmological findings at Department of Ophthalmology, Faculty of Medicine Ramathibodi Hospital, Mahidol University. Peripheral blood samples were collected from each patient, a 6 ml of whole blood are preserved in EDTA tubes for detecting mutations in mtDNA by Multiplex Ligation-dependent Probe Amplification (MLPA) technique. This study was considered and approved by the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University, based on the Declaration of Helsinki. The medical record data such as clinical presentation, patient's history, sex and age of onset would be used in this study.

DNA extraction and quality assessment

Genomic DNA samples from peripheral EDTA blood were extracted by using QiAamp DNA Blood Mini Kit (Qiagen, Germany) following the manufacturer's protocol. All DNA samples were determined quantity and quality using NanoDrop™ 2000 Spectrophotometers (Thermo Scientific, USA), each sample should have a 50 ng/µl concentration of DNA and an $A_{260/280}$ ratio between 1.8 and 2.

Multiplex Ligation-dependent Probe Amplification (MLPA)

Multiplex Ligation-dependent Probe Amplification (MLPA) technique was used to detect the three primary mutations, using SALSA MLPA probemix P125-B1 Mitochondria kit (MRC-Holland, Amsterdam, Netherlands) following the manufacturer's protocol. The P125-B1 Mitochondria kit contains 37 MLPA probes which are specific for mtDNA and include 5 mutation-specific probes (included three primary mutations) that will generate a signal when the mutations are present. The samples were adjusted concentration of DNA to at least 50 ng in a final volume of 5 µl and then adjusted samples were denatured at 95 ºC for 5 min and added probemix in order to hybridize with target sequences, incubation at 60 ºC for 16-18 hours. To ensure whether the probes completely hybridized, the Ligase-65 mix was added to ligate a minute gap of probes at 54 ºC for 15 min. Subsequently, ligated products were amplified by 10 µl of SALSA PCR master mix with only single primer pairs which have fluorescently labeled on a forward primer. The PCR fragments were separated using capillary electrophoresis 3130 Genetic Analyzer (Applied Biosystems) and all results were going to be analyzed through Coffalyser.net software (MRC-Holland, Amsterdam, Netherlands) for mutations detection.

Validation by direct sequencing

Direct sequencing was performed using the specific primers to three primary targeted sequences (Table 1), the PCR reaction was performed in a total volume of 25 µl. The PCR reaction was conducted in GeneAmp® PCR System 9700 thermocycler (Applied Biosystems) with initial denaturation at 95 ºC for 10 min and then 35 cycles of denaturation at 95 ºC for 30 s, annealing at 57 ºC for 30 s (59ºC for only the ND1 primers), extension at 72 ºC for 30 s and a final extension step at 72 ºC for 10 min. The reactions were held at 4 ºC until collection. The PCR products were confirmed by agarose gel electrophoresis and purified with ExoSAP-IT™ PCR Product Cleanup (Thermo Fisher Scientific, USA) in accordance with manufacturer's protocols. The sequencing reactions were prepared by using BigDye™ Terminator v1.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems). Subsequently, the

cycle sequencing products were purified according to the ethanol precipitation standard protocol. All of the products were sequenced with 3130 Genetic Analyzer (Applied Biosystems) in accordance with manufacturer's instructions. The sequencing results were aligned with the Human mtDNA reference sequence (accession number NC_012920) in the BioEdit v7.0.5 Biological sequence alignment editor (Tom Hall, Ibis Therapeutics, Carlsbad, CA) for confirming the mutations.

Table 1 Primers used for PCR amplification and sequencing of the three common mutations

Mutation	Primer sequences (5'-3')	Gene	Product size (bp)
m.3460G>A	F: CGAAAGGACAAGAGAAATAAGG R: ATAGTAGAAGAGCGATGGTGAG	*MT-ND1*	437
m.11778G>A	F: CAGCCATTCTCATCCAAACC R: GTCGTAAGCCTCTGTTGTC	*MT-ND4*	540
m.14484T>C	F: CATACTCTTTCACCCACAGCA R: TCATGCGGAGATGTTGGA	*MT-ND6*	491

Results

Five DNA samples from volunteers who have no mutations in the interesting locations (m.3460G>A, m.11778G>A, and m.14484T>C) that were confirmed by whole mitochondrial genome sequencing will be marked as normal samples. The normal DNA samples were also performed MLPA technique and direct DNA sequencing and the results show that no any mutations found in both techniques (Table 2).

All samples from twelve patients who were clinically diagnosed and suspected as LHON were performed MLPA technique. The twelve patients included 10 (83%) male and 2 (17%) female with an age of onset between 12 and 34 years old (Table 2). In this study, the m.11778G>A mutation was the most common mutation detected by MLPA technique. Eight patients (67%) presented the m.11778G>A mutation, while one patient (8%) had the m.14484T>C mutation and three patients (25%) were not found for the three primary mutations. The MLPA test kit also provides a specific probe for m.3460G>A mutation, but we did not detect the patients who had m.3460G>A mutation in this study (Table 2).

For the 12 samples that were performed by the MLPA technique, this technique proved that it is efficient with 100% accuracy for detecting the three common mutations. The validation of MLPA results with direct sequencing confirmed the results obtained with MLPA technique (Figure 1).

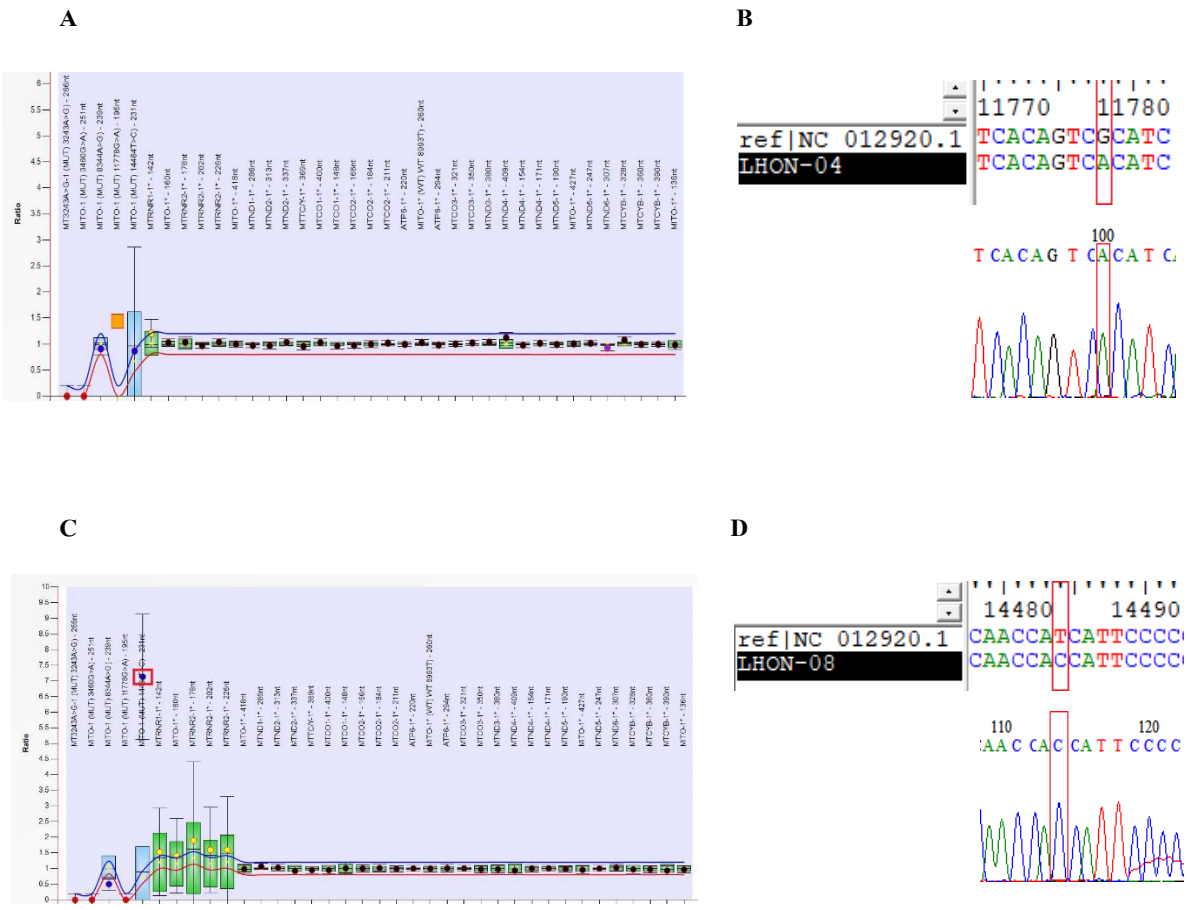

Figure 1 shows the example positive results by MLPA technique and validated results from direct sequencing.

A: The m.11778G>A mutation (orange box) that found on the MLPA technique and was analyzed by coffalyser.net software.

B: The validated result from direct sequencing found the m.11778G>A mutation when analyzed by BioEdit 7.0.5 Biological sequence alignment editor software.

C: The m.14484T>C mutation (the ratio is higher than average ratio) that found on MLPA technique.

D: The m.14484T>C mutation result that found on direct sequencing.

Table 2 Result of the three common mutations related to LHON by MLPA and validated by direct sequencing

Patient No.	Gender	Age of onset	Mutations detected by MLPA technique			Validated by
		(year)	m.3460G>A	m.11778G>A	m.14484T>C	Direct sequencing
NC-1*			-	-	-	-
NC-2*			-	-	-	-
NC-3*			-	-	-	-
NC-4*			-	-	-	-
NC-5*			-	-	-	-
LHON-01	M	17	-	√	-	m.11778G>A
LHON-02	M	12	-	√	-	m.11778G>A
LHON-03	M	12	-	√	-	m.11778G>A
LHON-04	M	18	-	√	-	m.11778G>A
LHON-05	M	26	-	-	-	-
LHON-06	M	14	-	√	-	m.11778G>A
LHON-07	M	17	-	√	-	m.11778G>A
LHON-08	M	19	-	-	√	m.14484T>C
LHON-09	M	16	-	-	-	-
LHON-10	M	34	-	-	-	-
LHON-11	F	20	-	√	-	m.11778G>A
LHON-12	F	12	-	√	-	m.11778G>A

NC: Normal control, M: Male, F: Female, -: No mutation detected, *: Already confirmed by Whole mitochondria sequencing

Discussion and Conclusions

According to the clinical diagnosis of the mitochondrial disorder has insufficient accuracy to diagnose, the genetic testing is a key to correct the clinical diagnosis. This study used MLPA technique as a method to simultaneously detect main mutations and deletion or duplication within mtDNA with high throughput, accurate, cost-effective and easy to reproduce.

In total, twelve patients who were clinically diagnosed and suspected as LHON: eight patient had the m.11778G>A mutation (67%), one with the m.14484T>C mutation (8%) and none of the m.3460G>A mutation was detected in any patient. In this study, we found that the m.11778G>A is the most common mutation which found in LHON patient and the proportion is similar to the previous Thai LHON patient and other studies that were reported (Chuenkongkaew et al., 2005) Moreover, the ratio between male and female who had the mutation is 3.5:1 and it might prove that LHON is also characterized by marked gender bias which was found in many studies (Macmillan et al., 1998; Mashima et al., 1998; Newman et al., 1991).

The MLPA technique also has several limitations, the m.14484T>C probe will not be flagged an orange box in the coffalyser.net software and the mutation will be considered by the ratio on Y-axis which is higher than mean ratio. If the

m.14484T>C ratio is near to the mean ratio, we suggest that direct sequencing should be performed in order to confirm this mutation. Even though this MLPA test kit covers all main mutations for LHON but it cannot detect other mutations that are not included in probemix leads to the fact that the novel mutation will not be found in this study. This could be a reason that three patients (25%) were not found for the main mutations despite having the symptoms, or the patients may have been misdiagnosed with LHON since the beginning.

In conclusion, The MLPA technique might be used as routine genetic testing for mitochondrial disorder or other genetic diseases with highly accurate and absence of false positive. This can detect point mutations and aberrant of copy number at the same time and cost effective. However, the large number of clinical samples will be required in order to further confirm its sensitivity, specificity, PPV, and NPV.

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