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Original Article

Molecular Design Real Time Loop-Mediated Isothermal Amplification Method for Rapid Detection of *Neisseria Meningitidis*

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Abstract

Introduction: Detecting *Neisseria meningitides* using conventional methods is time consuming and laborious. Development of a reliable, rapid method for prompt control and prevention of meningococcal disease is required. Although PCR and real time PCR methods have been developed, they require electrophoresis or expensive devices. Loop-mediated isothermal amplification (LAMP) method is a simple gene amplification method which can be performed at a single temperature without the need for thermal cycling.

Aim: We aimed to develop a quantitative real-time LAMP assay for detection of *N. meningitides* and accurate quantification of the bacterial load in patients with meningococcal disease.

Materials and methods: LAMP reaction was set up and optimized by four primers. Amplification results were assessed by obtaining real time turbidity graphs from each LAMP reaction tube using real time turbidimeter apparatus. A standard curve was generated from turbidity graphs corresponding to ten-fold serial dilution of *crgA* gene containing recombinant plasmid.

Results: LAMP assay could isolate only *N. meningitides*, whereas no amplification was obtained with negative control isolates, which indicates 100% specificity. The limit of detection (LOD) of our LAMP assay was found to be ~ 5 copies of *crgA* gene per reaction. REAL LAMP analysis of the standard curve revealed excellent linear correlation between gene copy number and time threshold, with a correlation coefficient equal to 0.92.

Conclusions: The REAL LAMP assay is a rapid, simple, cost-effective, sensitive, specific method for detection of *N. meningitides*. It has the potential of finding application in epidemiological studies and biodefense situations.

Keywords

crgA gene, detection, *N. meningitides*, real time LAMP

INTRODUCTION

Diagnosis and treatment of meningitis because of associated complications and adverse outcomes is one of the important diseases in emergency medical services.[1] Bacterial and viral factors are involved in causing the disease and any of these factors are detected by separate diagnosis and treatment protocols.[2] In the meantime, according to

the most recent studies, the three most common factors of bacterial meningitis - *Streptococcus pneumoniae*, *Hemophilus influenza*, and *Neisseria meningitides* - are the most important causes of morbidity and mortality.[3,4] *Neisseria meningitidis* among the bacterial meningitis factors is considered as one of the most important one and the most common cause of illness and death in developing countries. This is an important epidemic factor in Asian and African countries. It is a specific pathogen of meningitis in humans.[5] Each year about 2,000 people with the disease are reported through health centres and about 10% of them are meningococcal.[6] The bacteria can be detected by microbiologic and molecular methods. However, using spinal fluid culture is considered as a confirmatory test but this technique is usually reliable and very time consuming.[7] In addition, test sensitivity depending on the type of medium, use of antibiotics and test taking and transfer of sample lead to death of bacteria. In some cases, if left without diagnosis and treatment, a patient can suffer damage and die within 12 hours.[7] Some researchers also have made great efforts to use serological and immunological techniques to detect the bacterium but there was no acceptable results to identify this bacterium.[8] Molecular techniques such as PCR and Real Time PCR possessing high sensitivity and specificity in the detection and differentiation of bacterial and viral causes of meningitis are used. However, it is worth noting that although the technique is rapid and accurate, we should also consider the high cost of the equipment and the need of qualified personnel skills.[9-11] The use of laboratory diagnostic techniques to quickly and accurately identify *Neisseria meningitidis* in previous years in a very short time and accurately as well as the cost for most diagnostic centres are very important factors.[12]

LAMP technique is a new method for detection of pathogens using 4 to 6 primers of specific gene in target DNA samples. In this technique, the reaction is done at a constant temperature between 60 to 65 degrees for 45 minutes to an hour without the need for special equipment. Therefore, by this method simply by using two or three pairs of primers four or six areas in target DNA or RNA can be identified, which means the high specificity of the method.[13] In LAMP test, results can be detected by the naked eye without the need for electrophoresis, colorimetric and there is a haze because of the great proliferation that occurs during the process. All of the bacterial, fungal, viral and parasitic pathogens simply are identified.[13,14]

AIM

The aim of this study was to launch and develop a REAL LAMP technique as a rapid and highly sensitive method for detection of *Neisseria meningitidis* and specific detection based on *crgA* gene and to carry out REAL LAMP sensitive set up technique (quantitative LAMP) for quantitative analysis of bacteria. The study is the first study to investigate and diagnosis Neisseria meningitides by REAL LAMP techniques.

MATERIALS AND METHODS

In this study, the *crgA* gene was used for the intended purpose, because this gene has more specificity and is more sensitive than other genes. This gene exists in all strains of *Neisseria* and can be used in classifying the bacteria. This gene is responsible for coding and transcriptional regulation. A sequence of bp850 and primers were designed.[15] To design primers, sequences of the gene was used from the NCBI database (National Center for Biotechnology Information) and by related software (CLC bio, Aarhus, Denmark) CLC Sequence viewer version 6, sequence was arranged. Based on protected areas for gene sequences in target bacteria using Primer Explorer V4 software, Eiken-specific primers to identify the bacteria Neisseria meningitidis were designed (http://primerexplorer.jp/e/). Later primers (after design) are examined by thermodynamic and BLAST in PRIMER BLAST section of the NCBI site as well as the regulations and general specifications for primers used in LAMP, which are the steps associated with primers intervals during the target sequence. After final approval, primers in **Table 1** were sent to Bioneer (Daejeon, Korea) to be synthesized.[13]

LAMP Reaction

The reaction was performed in a final volume of 25 µl containing 40 pmol of internal primers (FIP and BIP), 5 pmol external primers (F3 and B3), 1.4 mM of deoxy nucleotide triphosphatase (dNTPs), 1M betaine, 1x ThermoPol Reaction Buffer, 8 mM MgSo4, 8 units of *Bst* DNA polymerase enzyme (New England Biolabs, Ipswich, MA, USA) and 2 µl of the DNA.[13] In addition, 25 micromolar concentration of fluorescent calcein compound (Dojindo Molecular Technologies, Inc., Tokyo, Japan) is added to the mix that

Table 1. Primer sequences of *CrgA* gene for *Neisseria* meningitidis in LAMP reaction

Primer name	Sequence	Reference
F3	CGCCGCAATCTACAGAAGA	This study
B3	GCGARGRCGRRGRCAACCAA	This study
FIP	GCGCSTCTAAAACCGCCCATGTGCTTGCCGGTCACCAATG	This study
BIP	CACTTTACCGCCAGCAGCGGAATACCGCAACCTGAAAGGC	This study

indicated that the reaction was performed. Mixture was incubated at 65°C for 1 hour and finally for 5 min at 80° on - Real time turbidimeter (LA-320C; Teramecs, Kyoto, Japan), and turbidity in the reaction mixture is checked every 6 seconds at a wavelength of 650 nm. To examine the specificity of LAMP reaction, effect of restriction enzyme on products was analyzed with the restriction enzyme *AluI* (Fermentas, Vilnius, Lithuania) at 37°C for 1 hour and then the reaction product was analyzed on 2% agarose gel.[13]

LAMP reaction specificity

To examine specificity of reaction and primers in addition to *Neisseria*, the reaction is done for 11 genomic DNA of other bacterial species as shown in **Table 2**. The turbidity reaction is done for them. The results for final confirmation were run on the 2% gel.[13]

Table 2. List of genomes of negative bacteria in specificity test reaction

Shigella sonnei	ATCC 9290
Klebsiella pneumoniae	ATCC 7881
E. coli	ATCC 25922
Bacillus subtilis	ATCC 6051
Staphylococcus aureus	ATCC 25923
Enterococcus faecalis	ATCC 29212
Enteropathogenic Escherichia coli (EPEC)	ATCC 43887
Escherichia coli O157:H7	ATCC 43895
Yersinia enterocolitica	ATCC 23715
Streptococcus pneumonia	ATCC700669
Coxiella burnetii	(Nine Mile strain, ATCC VR-615)

Cloning and standard preparation

After PCR reaction with the aid of external primers (F3-B3), PCR product was purified and TA cloning was done. Given that there was only one band on electrophoresis gel on 200 bp, PCR purification (Bioneer Co.) was performed.[13] To achieve this goal, a *CrgA* gene with a length of 196 bp from the *Neisseria* was cloned in the pTZ57R/T vector (Fermentas) according to instruction kit. Then it was transformed into susceptible bacteria of JM107. Transformed bacteria was incubated at 37°C for 24 hours on Luria Bertani medium (Merck, Darmstadt, Germany), which contains 38.4 micrograms per ml of IPTG (isopropyl-beta D-thio galactopyranoside) (Sigma, St. Louis, MO, USA), 40 micrograms per ml X-gal (5-bromo-4-chloro-3-indolyl beta dgalactoside) (Sigma), 50 micrograms nalid oxic acid, 100 micrograms of ampicillin (Merck).[15] Recombinant clones are diagnosed by blue and white colonies screening. White colonies as recombinant clones, which have vector are selected and then the plasmid is extracted from the desired clone using the kit AccuPrep Plasmid Mini Extraction (Bioneer). For final approval, PCR was performed using external primers (F3-B3). Digestion reaction with AluI enzyme was performed as a confirming reaction for cloning. Then the concentration of extracted plasmid to do sensitivity reactions and positive control of LAMP reactions was measured at a wavelength of 260 to 280 nm.[15]

Sensitivity and a standard curve based on moment turbidity of LAMP reaction

To examine sensitivity, serial dilutions of 10/1 were prepared from the plasmids extracted with OD = 640 ng (from 10^{-1} to 10^{-11}) and the reactions were carried out. In addition, LOD (Limit of Detection) of LAMP reaction is calculated. By REAL LAMP method, the number of bacteria in the sample can be estimated. It is done by drawing a standard curve of standard serial concentrations of genes in reaction tube in terms of reaction time to reach the threshold of turbidity, which was recorded by moment turbidimeter.[13] At first, LAMP reaction for standard serial concentrations of genes was performed on moment turbidimeter device and judgment graph is achieved, which represents the turbidity threshold time of each sample by measuring the OD. Then standard curve was drawn using Microsoft Excel with the standard serial concentrations data and time to reach the threshold (Tt) and linear regression was calculated. In addition, to evaluate the reaction, Calcein was added as a fluorescent indicator. The reaction products were electrophoresis on 2% gel.[15]

RESULTS

LAMP reaction and cloning

In positive sample tubes due to the presence of magnesium pyrophosphate, after the reaction, noticeable turbidity was seen. In addition, in cases where there was calcein in a mixture, after reaction it was mixed with magnesium ions and led to green color under UV light **(Fig. 1D)**. Electrophoresis on 2% agarose gel showed ladder mode for DNA. So results due to AluI I reaction enzymes digest LAMP amplified products and the created 20 and 194 products. In the cloning process, F3, B3 primers was used for PCR. Then plasmid containing the desired gene from white colonies on 2% agarose gel create bp200, *AluI* enzyme results also confirmed the authenticity of plasmid cloning.

Sensitivity and specificity of LAMP

The sensitivity of this method was evaluated using primers designed using serial dilution. The lowest detected concentrations were 7^{-10} (180×10^{-7}), which is shown in graph

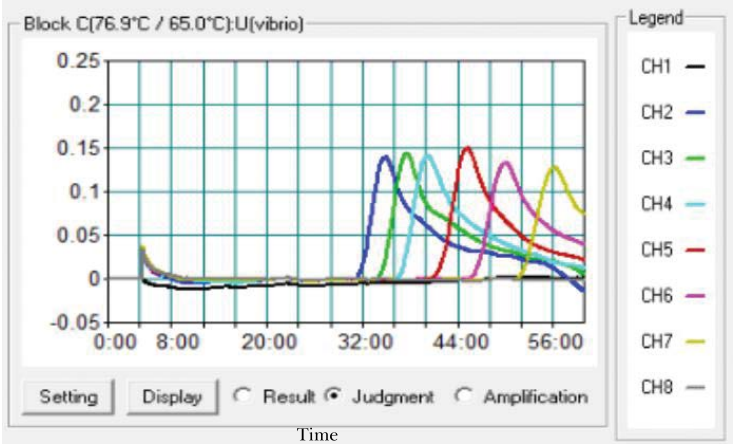

Figure 1A

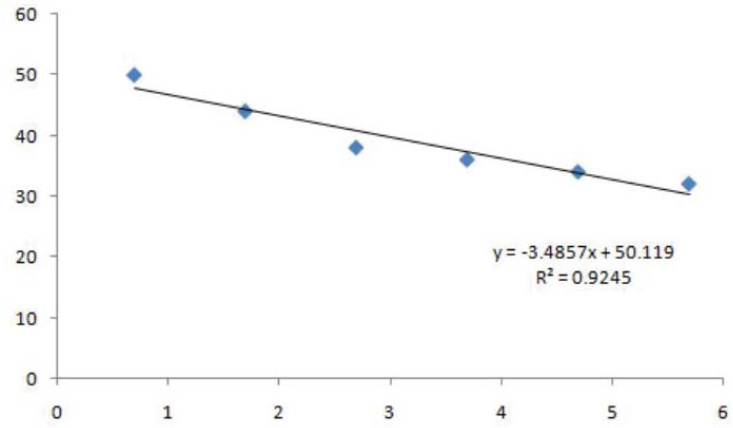

Figure 1B

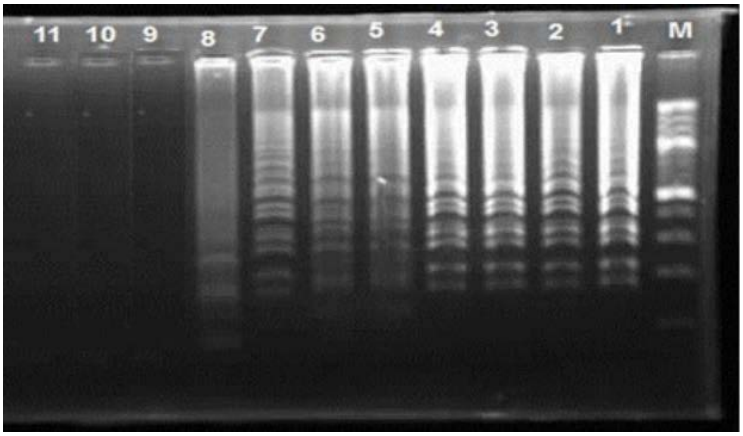

Figure 1C

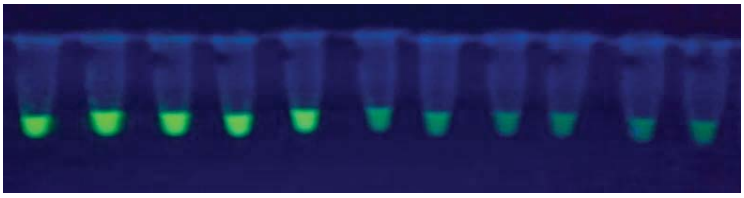

Figure 1D

Figure 1. Real-time LAMP sensitivity based on turbidimetry by serial dilution of DNA. **A.** Graph judgment of turbidimetry. (CH 2 and CH 8: dilution of 10^{-1} to 10^{-7} and CH1: negative control. **B.** A standard curve based on graph judgment. **C.** M marker 100 bp, column 1 to 11, serial dilution 10^{-1} to 10^{-11} and columns 1: negative control. **D.** review of calcein fluorescent under UV light in the positive reaction.

judgment. The number of copies/reaction (Copy number/ Reaction) was determined to be approximately 5, which indicated the high sensitivity of reaction and was able to determine the number of pathogens in low samples **(Fig. 1C)**. LAMP reaction specificity analysis on gel electrophoresis showed that (**Fig. 2)**. In addition, based on the standard curve drawn based on turbidity-based real time LAMP assay correlation coefficient (r^2) was 92% **(Figs 1A, 1B)**. These results show that the number of copies of the reaction is significantly associated with proliferation. This means that in higher concentration of the target gene, proliferation will happen in a short time. In our study, the first tube with a 180×10^7 copies/reaction showed turbidity in 32 minutes. The results on the gel electrophoresis determined up to the dilution of 180×10^7. In addition, discoloration caused by Calcein, in accordance with the results graph Judgment is 10^{-7}.

DISCUSSION

Meningitis caused by bacteria is still considered a major health problem.[15] Bacterial meningitis can be caused by *Hemophilus influenza*, *Streptococcus pneumoniae,* and *Neisseria meningitidis*.[16] Meningococcal disease can be an important disease threat of all ages.[17] The serological, culture methods, and mucus analysis used to detect these bacteria cannot be used successfully as an approved technique because they are time-consuming, have low sensitivity and specificity, yield false positive and negative results and are inefficient in cases in which antibiotics are used.[18] In the past 10 years there has been much progress in the field of rapid disease diagnosis using molecular techniques based on PCR. Due to disadvantages of the traditional methods, this technique can be used as a suitable technique for detecting *Neisseria*.[19] The LAMP technique as a reliable method, with high-sensitivity, high detection rate and reasonable cost is a very reliable in detecting each organism. This technique due to many advantages can be used as a suitable technique for the detection of different pathogenic factors.[20] This technique requires no special equipment to detect and can be done without the need for special procedures and the results can be examined with the naked eye. Another advantage of this technique is the use of specific *Bst* DNA polymerase enzyme for amplification in LAMP that compared to Taq DNA polymerase has the least inhibitor for this enzyme.[21] Therefore, we can say this technique can be used as a reliable diagnostic method.[22] In this study, required primers are designed based on *CrgA* gene to detect Neisseria, as a fixed gene, stable with high specificity in all strains of this organism to other genes.[22] This gene, of *LysR* family, is the largest family of bacterial regulators that are bound to epithelial cells of humans and acts as activator and inhibitor on promoter and the controls the operon.[23] In this study REAL TIME LAMP technique was able to identify five copies of the DNA of *Neisseria meningitidis* in reaction compared with other methods. This result demonstrates the high sensitivity of this method and the lack of false reaction with DNA samples with 4 primers can be an advantage of this method. In this condition, breaking the amplified fragment by *AluI* restriction enzyme with the size of the desired product indicates the specificity of the reaction and amplification of desired fragment. In this study calcein was used to evaluate proliferation that is visible by turbidity in the reaction tube. The use of fluorescent compounds in reaction leads to analysis of proliferation of positive samples with the naked eyes. So, using this method in laboratory settings can greatly reduce electrophoresis contamination in the laboratory.[24] After the reaction, results of the study with turbidity growth and graph judgment of loop amp REAL TIME device and proliferation based on calcein were examined. The lowest detected concentration was 7^{-10}

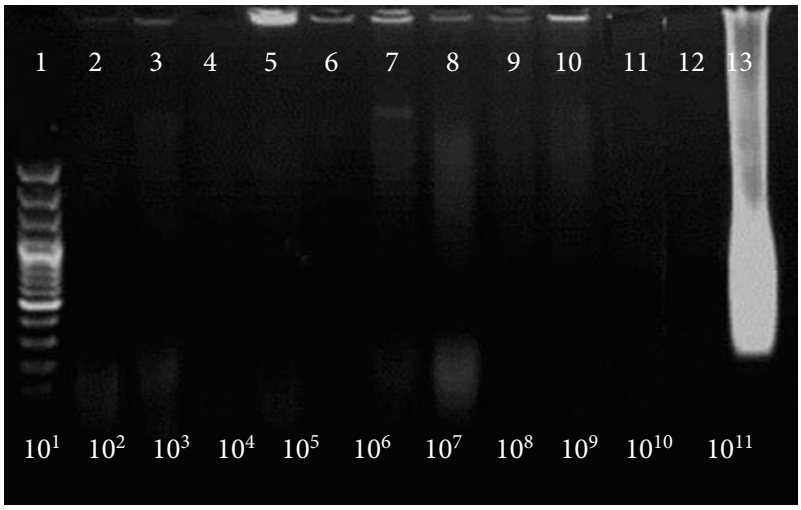

Figure 2. LAMP reaction specificity analysis on gel electrophoresis. Gel electrophoresis of LAMP reaction with other organisms. M marker 100 bp. Column 1: negative control. Column 2: *Shigella sonnei*. Column 3: *Klebsiella pneumoniae*. Column 4: *E. coli*. Column 5: *Bacillus subtilis*. Column 6: *Staphylococcus aureus*. Column 7: *Enterococcus faecalis.* Column 8: Enteropathogenic *E. coli*. Column 9: *E. coli* 0157: H7. Column 10: *Yersinia enterocolitica.* Column 11: *Streptococcus pneumoniae*. Column 12: *Coxiella burnetii*. *Column 13: Neisseria meningitidis*

(180×10^{-7}) that is obvious in graph Judgment. Based on the standard curve drawn based on turbidity-based real time LAMP assay correlation coefficient (r^2) was 92%. These results show that the number of copies of the reaction is significantly associated with proliferation. This means that the higher the concentration of target gene, the shorter time it takes for proliferation to occur. In our study, the first tube with a 180×10^7 copies/reaction showed turbidity in 32 minutes. The results on the gel electrophoresis determined up to the dilution of 180×10^7. In addition, discoloration caused by calcein in accordance with the results of graph judgment is 7^{-10}. Mckenna et al. used LAMP technique in a study to identify bacteria based on *CtrA* that had the ability to detect six copies of this bacterium in 48 minutes, which is comparable to our study that could detect five copies in 32 minutes.[25] In another study by Dokyung et al, a study was done based on *CtrA* gene and was compared with PCR, which was solely for the qualitative detection of this method.[26]

CONCLUSIONS

LAMP technique due to easiness, low cost, no need for sophisticated equipment to carry out the reaction, can be an appropriate technique for quantitative analysis with high sensitivity and specificity. Also because of the reaction type, it can be used as a quick and efficient diagnosis techniques of *Neisseria* in different areas.

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Conflict of Interest

Authors declare no conflict of interest.

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Метод молекулярного конструирования петлевой изотермической амплификации в реальном времени для быстрого обнаружения *Neisseria Meningitidis*

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Резюме

Введение: Выявление *Neisseria meningitides* традиционными методами занимает много времени и является трудоемким. Необходимо разработать надёжный и быстрый метод своевременной борьбы и профилактики менингококковой инфекции. Хотя были разработаны методы ПЦР и ПЦР в реальном времени, они требуют электрофореза или дорогостоящих инструментов. Циклическая петлевая изотермическая амплификация (LAMP) – это простой метод амплификации гена, который может выполняться при той же температуре без необходимости тепловых циклов.

Цель: Наша цель заключалась в разработке количественного метода LAMP в реальном времени для обнаружения *N. meningitides* и точного определения количества бактериальной нагрузки у пациентов с менингококковой инфекцией.

Материалы и методы: LAMP-ответ был получен и оптимизирован с помощью четырёх праймеров. Результаты амплификации оценивали путём построения графиков мутности для каждой пробирки с реакцией LAMP с использованием турбидиметра в реальном времени. Стандартная кривая была построена из графиков мутности, соответствующих последовательным десятичным разведениям гена crgA, содержащего рекомбинантную плазмиду.

Результаты: Метод LAMP позволяет изолировать только *N. meningitides* до тех пор, пока не будет достигнута амплификация в изолятах отрицательного контроля, что является показателем 100% специфичности. Предел обнаружения (LOD) нашего метода LAMP был установлен как ~ 5 копий гена crgA на ответ. LAMP-анализ стандартной кривой в реальном времени выявил отличную линейную корреляцию между количеством скопированных генов и временным порогом коэффициента корреляции, равным 0.92.

Заключение: Метод LAMP в реальном времени – это быстрый, простой, экономичный, чувствительный и специфический метод обнаружения *N. meningitides*. Он имеет потенциал для применения в эпидемиологических исследованиях и в ситуациях биобезопасности.

Ключевые слова

ген crgA, обнаружение, *N. meningitides*, LAMP в реальном времени