Crispr-cas 12a combination to alleviate the false-positive in loop-mediated isothermal amplification-based diagnosis of *Neisseria meningitidis*

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Short Report

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Abstract

Loop mediated Isothermal amplification (LAMP) was recently suggested as a diagnostic tool for the identification of *Neisseria meningitidis*. However, this isothermal amplification is challenged by the fact its amplification leads to risks of obtaining false-positive results. Whereas, with abilities to accurately recognize specific sequence, the CRISPR/Cas12a can form complexes with cognate RNA sensors and cleave pathogen's DNA targets complementary to its cognate RNA and acquires collateral activity to unbiasedly cut nearby off-target fragments. Therefore, if relevant fluorescent-quencher-nucleic probes are present in the reaction, the non-specific cleavage of probes releases fluorescences and establish diagnostic read-outs. In this study, we demonstrate a proof-of-concept that in relevant biochemical conditions, CRISPR/Cas12a and LAMP can work synchronously to identify genetic materials of *Neisseria meningitidis* at the level of 0.00004% in less than 2 h. Additionally, our clinical data also showed that the combinatory of CRISPR/Cas12a help to alleviate false positive result therefore enhance the specificity gained by the LAMP assays.

Introduction

Neisseria meningitidis (NM) is a Gram-negative bacterium that causes severe meningitis and sepsis. These diseases require fast and accurate diagnostics to indicate proper antimicrobial therapies [1, 2]. So far, together blood culture, polymerase chain reaction (PCR) is recommended as a routine technique for the diagnostic confirmation [3, 4]. PCR requires laboratories with sophisticated infrastructures and well-trained personnel, therefore makes challenges for deploying in limited-resource areas. Loop-mediated isothermal amplification (LAMP) based approaches have been used to detect pathogens [5]. LAMP-based assays are faster and require no sophisticated instruments or/and skilled personnel, therefore have the advantage to use as on-site diagnostic device [6].

LAMP can gain PCR's sensitivity without complicated thermocycling, some LAMP assays can complete within 10 minutes. However, LAMP detection step acquired non-specific indicators (such as Mg²⁺, intercalating dyes, labelled primers) that cannot distinguish spurious amplicons [7–9]. We documented several phenomena that real-time PCR protocols [4, 10] could not recapitulate positive results gained by LAMP reactions and some LAMP positive cases lacked meningitidis specific clinical symptoms [1].

We suspected that LAMP assay might acquire given false positive potentials [7–9]. With abilities to accurately recognize specific sequence, the CRISPR/Cas system holds promising potentials. In this system, the DNase cas12a forms a complex with their cognate CRISPR RNAs to cleave pathogen's RNA or DNA in a sequence-specific manner, afterwards, the collateral transcleavage activity is induced to cut unbiasedly the nearby off-target fragments. If relevant fluorescent-quencher-nucleic probes are present in the reaction, the non-target cleavage of probes will release fluorescent signals and establish diagnostics read-outs [11–13].

Results

We first performed isothermal amplification assay using Bst DNA Polymerase with primers specific for *MetA* gene of *N. meningitidis*. The reaction products were resolved against 1.5% agarose gel. It is impossible to distinguish the electrophoretic banding pattern between human DNA, *E. coli* DNA or *N. meningitidis* DNA (Fig. 1 upper-left panel). However, once these products were treated with Crispr-cas12a with gRNA sequence complementary to *MetA* gene of *N. meningitidis*, only fluorescent signals from samples with *N. meningitidis* DNA was recorded. Thus, treatment of CRISPR-Cas12a helps alleviate false-positive results by single-use of LAMP assay.

To evaluate the detection limit of LAMP/ CRISPR-Cas12a combination for the detection of *N. meningitidis* DNA, we spiked series of 40, 400, 4,000 and 40,000 copies of *N. meningitidis* PCR amplicon into 25 mM Tris-EDTA pH 8 containing the background of 10^8 copies of *E. coli* PCR amplicon/ul (suppl. information). These dilution points were used as the templates for Bst DNA Polymerase isothermal amplification at 55°C for 45 minutes then treated with CRISPR cas12a (1.0 µM Cas12a per reaction), in the presence of 0,25 µM guide RNA and 0,25 µM fluorescence labelled reporter at 55°C for 30 minutes; the fluorescent signal was recorded in 510 nm in Roche light cycler 480. The fluorescence was detected at all prepared dilution points even at the lowest level of 4×10^1 copies (0.00004%) of *N. meningitidis* (Fig. 2).

To validate the clinical performance, we applied the newly established procedure to identify *N. meningitidis* from 51 CFS samples from *N. meningitidis* suspected patients. The standard conventional realtime PCR assay with *MetA* genes as a molecular target was also used to confirm the presence of *N. meningitidis* DNA. Realtime PCR identified *N. meningitidis* DNA from 13 out of 51 recruited CFS samples. Whereas, single-use of LAMP assay identified 18 cases positive with *N. meningitidis*, in which, five cases did not match to either result acquired by real-time PCR or patients' clinical symptoms and were considered as false positive. However, when LAMP reaction mixtures from 51 CFS samples were treated with Crispr cas12a, only 13 cases were positive. Importantly, all of these 13 cases were matched to the results gained by conventional PCR (Fig. 2).

Discussion

Single-use of LAMP embeds high risk of false-positive signals that challenge the employing LAMP-based assays into clinical practices [7, 9]. However, LAMP has strong intrinsic amplification potential and simple to operate, hence would benefit the communities. Our data revealed that the sequential treatment LAMP products by Crispr cas12a under the guidance of specific gRNA sequence can abolish un-specific signals. This technical integration of two enzymes, in one side help to sustain the strong amplification potential of LAMP on the other side, significantly enhances the specificity of diagnostic procedures.

We found that trehalose helps Crispr cas12a and Bst DNA Polymerase to sustain their activity in a single trehalose containing buffer at 55°C. This condition omits buffer replacement from the LAMP into Crispr assay thereby reducing sample handling and contamination risk. However, in various tested biochemical environments, Crispr Cas12a strongly inhibits isothermal DNA polymerases (Bst and Bsu), therefore, we were not successful to couple isothermal enzymes and Crispr cas12 into single reaction tubes. Further

studies are needed to mitigate the inhibitory effect of CRISPR Cas12a to Bst or Bsu DNA polymerase, thereby combining Crispr cas12a with isothermal amplification into single tube diagnostic device.

In conclusion: The sequential combination of LAMP amplification and Crispr cas12a treatment can alleviate false-positive acquired by single use of LAMP performance.

Declarations

Ethics statement: The study was submitted for regulatory approval to the Institutional Review Board of the 108 Military Central Hospital in Hanoi and was approved. The Ethical Committee of the 108 Military Central Hospital, Hanoi, provided ethical approval for the study. Informed written consent was obtained from all study participants or from their parents/guardians if the study participant was in an unconscious condition

Availability of data and materials: Data and supporting materials associated with this study will be shared upon request

Competing interests

The authors declare no conflict of interests.

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Author contributions

NTT, LHS, MHB designed and supervised the studies. LHPS and TXH, DTQ conducted the experiments. NTT, LHS, MHB, LHPS, TXH analysed the data and wrote the manuscript. All authors approved the manuscript.

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Tables

Table 1

Oligonucleotides used as primers to loop-mediated-isothermally amplify the MetA target of *N. meningitidis*

Oligo names/concentration	Sequences (5'-3')	Volume uses for one reaction
Tr-Hien-metA-F3(10 pmol/ul)	GCAGTTCCTAATTTACCATGA	0.5 µl 0.5 µl
Tr-Hien-metA-B3(10 pmol/ul)	GCAACGAAAATTGCAACTGTA	
Tr-Hien-metA-FIP(40 pmol/ul)	GGTGAATTTGTTCCCATTATTGCGCACCATGATACCCCCATG	0.75 µl 0.75 µl
Tr-Hien-metA-BIP(40 pmol/ul)	TTCACATTTTGGCTGTCAAAGGCTATGATGATTACACCTGT	
Tr-Hien-metA-LF(10 pmol/ul)	GCTGCTTTTGGCGGTGCATT	1 µl 1 µl
Tr-Hien-metA-LB(10 pmol/ul)	CTTGGCTGTCTAAATTTTGCGC	

Figures

Non-template control
Human DNA 1
Human DNA 2
E coli DNA
N. meningitis DNA

Figure 1

Addition of CRISPR/Cas12a help to alleviate false positive acquired by single use of LAMP performance: (upper left panel) the product mixture of LAMP assay was colometric indicated by addition of 100 μ M Hydroxy naphthol blue (HNB Sigma – Singapore) or (downer left panel) resolved against 1.2% agarose gel electrophoresis. Right panel - the same product mixture of LAMP was treated with CRISPR/Cas12a.

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Figure 2

Detection limit and diagnostics performance of LAMP/ CRISPR-Cas12a for identification of N. meningitis DNA

Left panel: Detection limit of LAMP/ CRISPR-Cas12a for identification of N. meningitis DNA: the fluorescent signals acquired by Bst DNA Polymerase based isothermally amplifying at 55oC for 30 minutes on pseudo-samples of 0 copy (0%), 40 copies (0.00004%), 400copies (0.0004%), 4000 copies (0.004%), 40000 copies (0.04) and 40000 copies (0.4%) of N. meningitis PCR amplicon spiked into 25 mM Tris-EDTA pH 8 containing the background of 108 copies E. coli PCR amplicon. Right panel: Van diagram to show diagnostic agreement of LAMP/ CRISPR Cas12a combination with conventional real-time PCR in identification of patients infected with N. meningitis: 51 CSF samples were equally subjected to conventional real-time PCR MetA genes as molecular target or LAMP assay or LAMP/Crispr cas12a combination. Data revealed that LAMP assay detected 18 positive cases, whereas LAMP/Crispr cas 12 identified only 13 cases that are fully matched to results acquired by real-time PCR.

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