Short Communication

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

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# Materials and methods

**Ethic approval:** 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.

**Sample processing and DNA extraction:** A total of 0.3 ml CSF samples from 14 *N. meningitidis* confirmed patients and 37 control subjects were mixed with 300 μl universal lysis solution (50mM Tris-HCl pH 7.5, 0.5% Triton-X100) and heated for 5 minutes at 65°C and centrifuged at 13,000 g for five minutes. The upper aqueous supernatant was collected into an Eppendorf tube and 5ul of collected supernatant was used as template for Loop-mediated-isothermal amplification described before[1].

## Loop-mediated-isothermal amplification (LAMP) was designed as described previously [1] in which oligonucleotides below (table 1) were used to isothermally amplify the MetA target of N. meningitidis at 55oC for 45 minutes in buffer containing (20mM TrisHCl pH 8.3, 10mM CH3COONH4, 2mM(CH3COO)2Mg2, 1% Tween20, 0.5mg/ml Casein, 320mMTrehalose) with the catalysis by 8 units of Bst DNA Polymerase (New England Biolabs) per reaction. The product mixture of LAMP assays was colormetric indicated by addition of 100 uM Hydroxy naphthol blue (HNB Sigma- Singapore) or resolved against 1.2% agarose gel electrophoresis.

***CRISPR-Cas12 assay:*** 3ul of the LAMP product was mixed with 2ul buffer containing (20mM TrisHCl pH 8.3, 10mM CH3COONH4, 2mM(CH3COO)2Mg2, 1% Tween20, 0.5mg/ml Casein, 320mMTrehalose) and 1.0µM EnGen® Lba Cas12a (Cpf1 - New England Biolabs), 0,25 µM guideRNA (UAA UUU CUA CUA AGU GUA GAU AGC CUG UGA UAA UUG AAU UGC) and 0,25 µM fluorescence labelled reporter (FAM-TTATT-IABkFQ). The reaction mixture was incubated at 55oC for 30 minutes and fluorescent signal was recorded in 510nm in Roche light cycler 480.

## Real-time PCR conditions: The real-time PCR assay mixtures consisted of 7.5 µl Taqman real-time PCR master mix (Qiagen, Hilden, Germany), 5 µl of DNA template, 5 pmol of primers TR-H-VapA-NM-F: GCA GTT CCT AAT TTA CCA TG/ TR-H-VapA-NM-R: GCG AAT TTG CTA ATC CTA TTT ATG TGC and 0.2 pmol of probes TR-H-VapA-NM-Probe: FAM-AAC CAG CGC AAC GAA AAT TGC AA. Reactions were run in the Roche light cycler 480 device with a pre-incubation step at 50 °C for 15 min, initial denaturation at 95 ° C for 5 min, followed by 45 cycles of 95 ° C for 15 sec and 60 ° C for 60 sec.

**Serial dilution formulation:** To make pseudo-dilution series of *N. meningitidis* gemonics DNA against the control pathogens, we first generated 500bp PCR amplicon amplified from *N. meningitidis* genomics DNA (we called *N. meningitidis* PCR amplicon) using primer pairs **tgc att aat tat tag cag cat tgg cac caa/ aat tat tga ctg aca agg gta aat tca aac** targeting MetA gene and PCR amplicon amplified from *E coli* genomics DNA using primer pairs acc tct ctt acc tgc gct gca tac cgg aaa/ggc gcg cat ttc ttc ctg agt tcc ttg tgg that targets Dx gene (we called *E coli* PCR amplicon). Afterward, *N. meningitidis* PCR amplicon and *E. coli* PCR amplicon were purified separately (using PCR purification kit from Thermofisher) equalized in molar. From these, the pseudo-dilution series of *N. meningitidis* against *E coli* was made by formulating series 40 copies, 400copies, 4000 copies and 40000 copies of *N. meningitidis* PCR amplicon into 25 mM Tris-EDTA at pH 8 containing the background of 108 copies *E. coli* PCR amplicon /ul. These dilution points were later used to validate the detection limits of LAMP and LAMP/Crispr combined assays.

# References

1. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, et al. (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28: E63.

## Table 1: Oligonucleotides used as primers to loop-mediated-isothermally amplify the MetA target of N. meningitidis

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| Oligo names/concentration  | Sequences (5’-3’) | Volume uses for one reaction |
| Tr-Hien-metA-F3(10pmol/ul) | GCAGTTCCTAATTTACCATGA | 0.5 µl0.5 µl |
| Tr-Hien-metA-B3(10pmol/ul) | GCAACGAAAATTGCAACTGTA |  |
| Tr-Hien-metA-FIP(40pmol/ul) | GGTGAATTTGTTCCCATTATTGCGCACCATGATACCCCCATG | 0.75 µl0.75 µl |
| Tr-Hien-metA-BIP(40pmol/ul) | TTCACATTTTGGCTGTCAAAGGCTATGATGATTACACCTGT |  |
| Tr-Hien-metA-LF(10pmol/ul) | GCTGCTTTTGGCGGTGCATT | 1 µl1 µl |
| Tr-Hien-metA-LB(10pmol/ul) | CTTGGCTGTCTAAATTTTGCGC |  |
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