CRISPR-based Detection for Identifying Snake Species from Snakebite Envenoming

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ABSTRACT

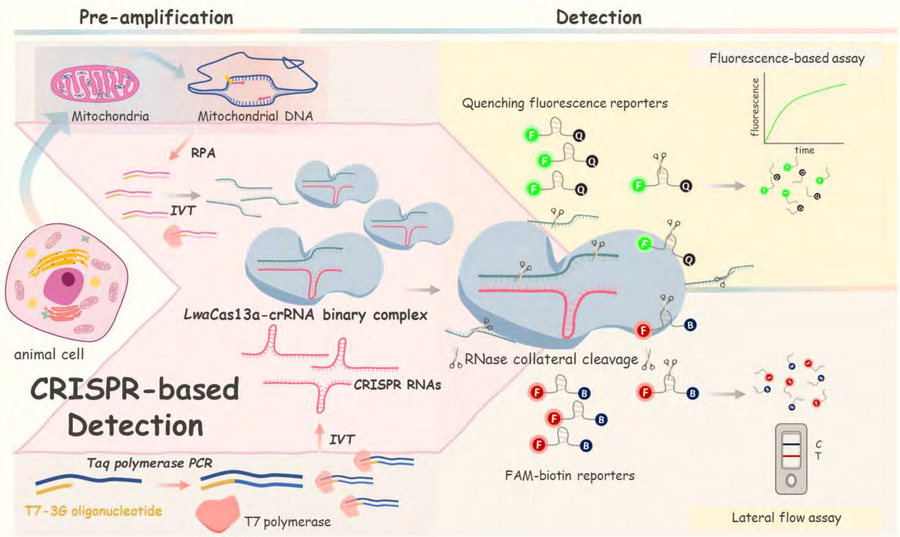
Snakebite envenoming is a neglected tropical disease which causes broad symptoms ranging from mild to high severity, affecting healthcare management and economy of tropical developing countries including Thailand. Multiple venomous snake species are distributed throughout different regions in Thailand, and it is not trivial to identify snake species upon getting bitten or threaten based on old-fashioned means, rendering timely administration of the antivenom difficult or impossible. Here, we propose the development of a point-of-care genotyping tool based on CRISPR for rapid and accurate identification of snake species from bite wounds. DNA regions within the mitochondrial *cytochrome b* (*cytb*) gene of eight venomous snake species endemic to Thailand—*O. hannah*, *N. kaouthia*, *C. rhodostromata*, *D. russelii*, *D. siamensis*, *T. albolabris*, *B. fasciatus*, and *B. candidus*—were chosen as targets for CRISPR-Cas13a-mediated detection, which utilizes recombinase-polymerase amplification (RPA) and *Leptotrichia wadei* (Lwa) Cas13a for maximal detection sensitivity and specificity. We successfully prepared all biomolecular components necessary for the RPA/LwaCas13-based detection of the *cytb* gene from different snake species, optimized their detection conditions, validated their specificity, and assessed their analytical sensitivity upon detection of surrogate DNA substrates. Detection modules with sufficiently high sensitivity will be used to detect the *cytb* gene from biological fluids obtained from snakes in the near future.

Keywords: CRISPR-Cas13, RPA, snakebite, tropical disease

INTRODUCTION

Key to the technology is an RNA-guided/RNA-targeted CRISPR-associated enzyme Cas13a from *Leptotrichia wadei* (LwaCas13a) which exhibits collateral cleavage activity of bystander RNA probes upon activation through recognition of target RNA, via a programmable CRISPR RNA (crRNA). Cas13a does not have a strict PAM requirement, allowing high flexibility in choosing the targeting site, and can be coupled to both DNA- and RNA-based detection via the use of enzymes that can interconvert RNA and DNA i.e., reverse transcriptase and RNA polymerase.

To maximized detection sensitivity and specificity, the LwaCas13a-based CRISPR detection reaction can be coupled to an isothermal amplification technique, such as Recombinase Polymerase Amplification (RPA) or loop-mediated isothermal amplification (LAMP). Here, we propose to apply a CRISPR-Cas13a based assay for snake species-specific detection of the mitochondrial *cytochrome b* gene (*cytb*). Previous studies suggested that *cytb* can be used as a genetic marker to differentiate both the genus and species level of snake type according to the intraspecific and interspecific variations. In this study, we examine venomous snake species distributed in Thailand and design the SHERLOCK fluorescence-based assay for the detection of these snake species. We took into consideration the most prevalent venomous snake species in Thailand, for which there is local production of antivenoms. In total, we selected eight snake species to develop the SHERLOCK-based genotyping technology: *Ophiophagus Hannah* (King cobra), *Naja kaouthia* (Cobra), *Calloselasma rhodostoma* (Malayan Pit Viper), *Daboia russellii* (Russell's Viper), *D. siamensis*, *Trimeresurus albolabris* (Green Pit Viper), *Bungarus fasciatus* (Banded Krait), and *B. candidus* (Malayan Krait). Venoms from all seven species can be neutralized by a selection from nine equine antivenoms: seven for species-specific monovalent antivenoms, and two polyvalent pan-species antivenoms.

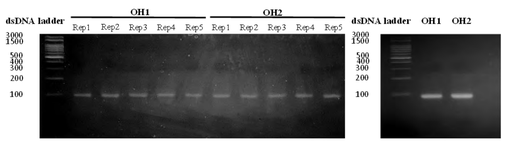


MATERIALS AND METHODS

1. Design of specific SHERLOCK: RPA primer pairs, target sites, and their specific crRNAs
2. Sample preparation
3. crRNA production by *in vitro* transcription and purification
4. Isothermal amplification via recombinase polymerase amplification (RPA)
5. Cas13-SHERLOCK nucleic acid detection via fluorescence-based detection assay
6. LoD determination and data analysis

RESULTS

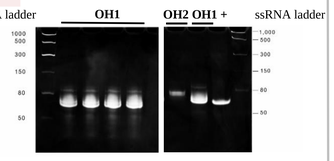
Double-stranded *cytb* DNA segments



We produced the double-stranded *cytb* DNA segments to be RPA templates as synthetic dsDNA templates to analyze the limit of detection (LoD). The partially complementary oligonucleotides (sense and anti-sense) were annealed then amplified by *Taq* DNA polymerase-mediated PCR.

CRISPR RNAs for LwaCas13a-based detection

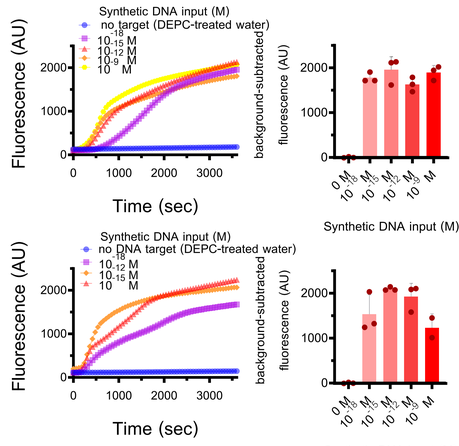
CRISPR RNAs (crRNAs) needed for LwaCas13a-based detection of the *cytb* gene of different snake species were prepared using the Ribomax Large Scale RNA Production System-T7 (Promega) and purified in two steps via magnetic bead enrichment and phenol-chloroform precipitation. Purified crRNAs were analyzed for correct size and sufficient purify using denaturing acrylamide gel electrophoresis.



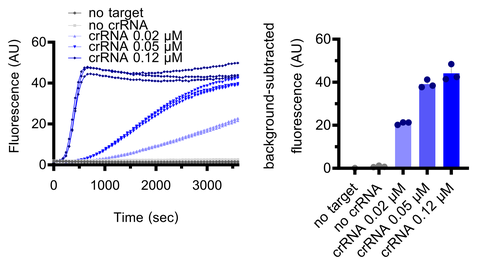
Limit of detection (LoD)

We first determined the analytical sensitivity, or the limit of detection (LoD) of SHERLOCK-based detection of *O. hannah cytb* gene segment. We observed that for the desired *cytb* gene segments of *O. hannah*, here referred to as targets OH1 and OH2, SHERLOCK detection was highly sensitive, capable of detecting both targets at attomolar levels

We expanded the limit of detection measurements of SHERLOCK-based detection to *ctyb* gene segments from seven additional snake species: *N. kaouthia*, *C. rhodostromata*, *D. russelii*, *D. siamensis*, *T. albolabris*, *B. fasciatus*, and *B. candidus*.



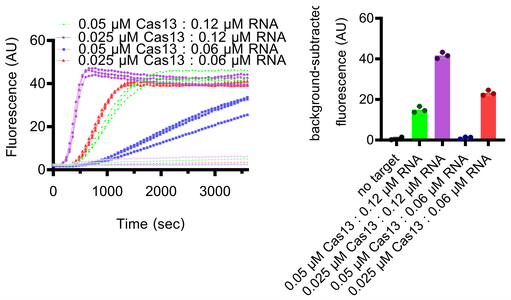
Optimization of crRNA amounts



We found that the reaction kinetics increased with increasing crRNA concentrations (from 0.5 ng/μL (0.02 μM) to 1.0 and 2.5 ng/μL (0.05, and 0.12 μM)) with 6.3 μg/mL LwaCas13a (0.05 μM), resulting in higher end-point fluorescence intensity at the high crRNA concentrations after 60 min of CRISPR reactions.

Optimization of LwaCas13a and crRNA amounts

We also concurrently optimized LwaCas13a concentrations to match the increased crRNA concentrations. We found the combination of 0.12 μM crRNA and 0.025 μM LwaCas13a to provide the fastest reaction kinetics, followed by 0.06 μM crRNA and 0.025 μM LwaCas13.



CONCLUSION

We currently used purified DNA surrogate substrates to validate the functionality of RPA primers and crRNAs. We observed varying limit of detection values upon detecting *cytb* genes from different snake species. We will dissect whether the low sensitivity of *cytb* gene detection, particularly with *N. kaouthia* and *B. fasciatus cytb*, originates from inefficient RPA or CRISPR-Cas13a, and will re-design faulty components (RPA primers, or crRNAs) accordingly. To extend the detection platform to real samples, we will first test the detection on DNA extracted from snake saliva. Such samples will be obtained with ethical approval from Queen Saovabha Memorial Institute (QSMI), in collaboration with Dr. Lawan Chanhome. We will also test the detection on DNA obtained from crudely lysed (through heating and detergent-induced cell lysis) snake saliva.

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