Ligation-Dependent Cas14a1-Activated Biosensor for One-pot Pathogenic Diagnostic

Xiao Tanb#, Xiufen Yanga#, Yuefeng Qiaoa, Yangdao Weia, Wenkai Shanga, Huiying Cia, Xidan Luoa, Hongwei Houc,d, Fengge Song*a, Yi Wan*a, Jinghong Lib

aState Key Laboratory of Marine Resource Utilization in South China Sea, Hainan University, Haikou 570228, China.
bDepartment of Chemistry, Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology, Tsinghua University, Beijing, 100084, China
cChina National Tobacco Quality Supervision & Test Center, Zhengzhou, China
dKey Laboratory of Tobacco Biological Effects, Zhengzhou, China

# These authors contributed equally to this work and are listed in alphabetical order of family name.

*Corresponding Author: songfg@hainanu.edu.cn (Prof. Fengge Song);
993602@hainanu.edu.cn (Prof. Yi Wan)
Abstract: Pathogenic identification requires nucleic acid diagnosis with simple equipment and fast manipulation. Our work established an all-in-one strategy assay with excellent sensitivity and high specificity, Transcription-Amplified Cas14a1-Activated Signal Biosensor (TACAS), for the fluorescence-based bacterial RNA detection. The DNA as a promoter probe and a reporter probe directly ligated via SplintR ligase once specifically hybridized to the single-stranded target RNA sequence, with the ligation product transcribed into Cas14a1 RNA activators by T7 RNA polymerase. This forming sustained isothermal one-pot ligation-transcription cascade produced RNA activators constantly and enabled Cas14a1/sgRNA complex to generate fluorescence signal, thus leading to a sensitive detection limit of 1 CFU/mL E.coli within 2-3 h of incubation time. TACAS was applied in contrived E.coli infected fish samples, and a significant signal differentiation between positive (infected) and negative (uninfected) samples was reached. Meanwhile, E.coli colonization and transmit time in vivo were explored and the TACAS assay promoted the understanding of the infection mechanisms of the E.coli infection, demonstrating an excellent detection capability.

Key words: Cas14a1, E.coli, Isothermal one-pot assay, Pathogenic RNA detection
1. Introduction

Currently, the technology of clustered regularly interspaced short palindromic repeats (CRISPR) has prompted progression in genetic editing and genome engineering\(^1\)-\(^4\). The platforms detecting nucleic acid that are based on a CRISPR-associated protein (Cas) system have caused an increasing attention\(^5\)-\(^7\). Some CRISPR-Cas systems can recognize the target to activate collateral nonspecific cleavage such as fluorescence-quench pair (FQ) label probe fragment, which can quantify target nucleic acids. Based on a pre-amplification method, a CRISPR-Cas-dependent nucleic acid platform potentially achieve point-to-care detection with high sensitivity and specificity\(^8\)-\(^10\). A compact CRISPR-Cas14 detection platform has been discovered to be successfully applied for RNA detection based on its \textit{trans}-ssDNA cleavage ability with outstanding specificity and high activity in the diagnostic of nucleic acid without the requirement of a restrictive substrate sequence\(^11\)-\(^16\). However, the CRISPR-Cas-dependent detection platform with an isothermal and PCR-free system is needed during a public health emergency or in resource-limited regions\(^17\)-\(^19\).

The nucleic acid detection based on ligation reaction is a sequence-specific approach that initially depends on the ligation reaction of two individual probes to achieve a hybridization of the target sequence on their adjacent sites\(^20\),\(^21\). Own to the specificity, the ligation-based strategies are applied to detect pathogen bacteria\(^22\),\(^23\), subsequently combined with amplification of the nucleic acid and the generation of signal to show results. Particularly, the SplintR ligase can specifically ligate two separate DNA probes
via a target RNA as a splint, allowing the RNA molecule-specific detection. And a transcription reaction via T7 polymerase can amplify the ligation products without PCR process in an isothermal condition.

Herein, we developed a detection of pathogenic strategy via combination of the detection relying on ligation, amplification based on transcription and signal activation via Cas14a1 protein. Accordingly, the biosensor contains following components: the target pathogen RNA, a pair of probes with specific sequence and three enzymes including ligase enzyme, T7 polymerase and Cas14a1 enzyme. Via the target pathogen RNA, the two probes can be ligated to form a hybrid single strand DNA and subsequently amplified by T7 polymerase via transcription reaction. Finally, the transcription products with the sgRNA sequence activate the Cas14a1/sgRNA complex to allow the cleavage of the ssDNA fluorophore quencher reporter (FQ reporter) and produce a fluorescent output signal. By contrast, without the pathogen RNA, the system reaction is muted because the ligation products can’t activate the Cas14a1/sgRNA complex. This method requires simple nucleic acid (mainly RNA) extraction and PCR-free amplification. Compared to the traditional PCR-based amplification methods that require repeated processes of heating and cooling via a certain temperature, our study TACAS developed a new isothermal nucleic acid amplification strategy that provided unique benefits for point-of-care identification of pathogen RNA. TACAS can be achieved via the simple equipment such as a water bath or heat block, as it only requires the single and constant temperature to maintain the whole reaction.
2. Materials and Methods

2.1 SplintR Ligation of probes

10 μM promoter probe (PP), 10 μM reporter probe (RP) and 10 μM target RNA were added in 8.5 μL reaction buffer, which contains 500 mM KCl and 100 mM Tris-HCl (pH 8.0) for probes annealing. Heat the mixture to 95 °C for 3 mins, then let it cool to room temperature. Next, the reaction solution was added with 1 μL SplintR ligase (25 U·μL⁻¹) and 2.5 μL 10× SplintR buffer (NEB), and then incubated at 37 °C for 30 min for ligation reaction. The ligation was terminated by 95 °C heating for 5 min. To verify that the ligated product was obtained, PCR reactions were performed using LigEC_F and LigEC_R primers (Supplementary Table S1) and then analyzed via agarose gel.

2.2 T7-Transcription

The ligated products were directly applied as templates for the in-vitro transcription. The transcription was performed in a mixture containing 2 μL T7 RNA polymerase (Hiscope T7 High Yield RNA Synthesis Kit (New England Biolabs Inc., NEB), 10 μL NTP mix buffer (Hiscope T7 High Yield RNA Synthesis Kit (New England Biolabs Inc., NEB), 1 μL Recombinant RNase Inhibitor (20 U/μL), 8 μL of the ligated product and 9 μL RNase-free water, which was incubated at 37 °C for overnight. And the products of the reaction were treated with 1 μL DNase I (RNase-free) for 30 min at 37 °C. The transcription product RNAs were purified using Monarch® RNA Cleanup Kit (NEB) and then analyzed via agarose gel.
2.3 sgRNA preparation

The plasmid, containing a T7 promoter at upstream of the sgRNA framework in the pUC-57 vector, was extracted. Then functional fragment that could be transcribed into sgRNA was PCR amplified using a pair of primers gRNA-A-F and gRNAT7-R (Supplementary Table S2) by the initial degeneration at subsequential 95 °C for 4 min, subsequently, 30 cycles of the degeneration step at 95 °C for 30 sec, annealing step at 55 °C for 30 sec and extension at 72 °C for 40 sec, lastly, extension step at 72 °C for 10 min. The PCR product was purified using the DNA Fragment Purification Kit (Takara) and transcribed by the Hiscribe T7 High Yield RNA Synthesis Kit (New England Biolabs Inc., NEB) after 12 h incubation at 37 °C in vitro. Then, 2 μL DNase I (RNase-free) was added into the transcription mixture and then incubated at 37 °C for 30 min. Finally, the sgRNA was purified by the Monarch RNA Cleanup Kit (NEB) and preserved at -80 °C for further use. The sgRNA sequences are listed in Table S2.

2.4 TACAS one-pot strategy

The isothermal one-pot reaction mixture consists of the following components: 5 μL NTPs mix (each 25 mM), 1 μL T7 RNA polymerase (50 U·μL⁻¹), 1 μL Ligation Buffer (NEB), 1 μL Annealing Buffer, 0.54 μL Reporter Probe (10 μM), 0.5 μL Promoter Probe (10 μM), 0.5 μL SplintR Ligase (25 U·μL⁻¹, NEB), 0.5 μL Recombinant RNase Inhibitor (20 U·μL⁻¹)
and 8 μL Reaction Buffer (10 mM MgCl₂, pH 9.0, 50 mM NaCl and 500 mM Tris-HCl).

The reaction mix was toped up with 0.5 μL target RNA and RNase-free water to produce a final volume of 20 μL. The mixture solution was then incubated at 37 °C for 2-3 h. Then, the reaction mix was added with 5 μL Cas14a1/sgRNA complex and 5 μL FQ Reporter, and loaded in a 384-well microplate for measurement of fluorescence at 37°C over a period of 120 min using fluorescence measurements (λex: 492 nm; λem: 520 nm) via microplate reader (BioTek H1).

3. Results and discussion

3.1 Transcription-Amplified Cas14a1-Activited Biosensor cascade

As shown in Figure 1, the Transcription-Amplified Cas14a1-Activited Signal biosensor (TACAS) is composed of three core reactions, including ligation for detection, transcription for amplification and FQ probe cleavage activation via Cas14a1 for visible fluorescent signal output. The ligation reaction occurs only in the presence of the target RNA and a pair of functionally complementary oligonucleotide probes that consists of tag-specific parts, ensuring specificity of detection. The promoter probe (PP) contains a sequence at the 5’ end (crimson) to match with downstream sequence of target RNA for ligation reorganization and stem-loop T7 promoter at 3’end (red) to enable the transcription for ligation products amplification. For ligation, the 5’ end of the promoter DNA probe needs to be phosphorylated. The Report probe (RP) includes upstream sequence of target
RNA for ligation reorganization at 3’ end (red) and a specific guide RNA (sgRNA) sequence as a complementary activator sequence at 5’ end (indigo) to initial the fluorescent signal activation. The ligation products are transcribed via T7 polymerase. Once both promoter DNA probe and reporter DNA probe hybridize correctly with the target RNA, ligation reaction connecting the two probes can be initiated by SplintR ligase. After that, T7 RNA polymerase uses the ligated probes as a template to synthesize the RNA. Once the amplified ligation products are enriched enough, they are mixed into reaction solution containing incubated Cas14a1-sgRNA complex, fluorophore quencher (FQ) reporter and reaction buffer (Tris-HCl, NaCl and MgCl₂). The tag sequence of transcription products binds to the sgRNA and triggers the collateral activity of Cas14a1, which subsequently cleaves the FQ reporter to produce fluorescent signal immediately. This method with high sensitivity and specificity could potentially be used as a powerful diagnostic biosensor for pathogenic diagnostic.
Figure 1. The schematic diagram of TACAS: after adding pathogen RNA, detection, amplification and signal generation can be carried out through the reaction processes of ligation, transcription and FQ cleavage via Cas14a1, respectively. The two designed single-stranded DNA probes were named as T7 promoter probe and reporter probe. The T7 promoter probe is composed of an upstream hybridization sequence hybridized with the 5-terminal half sequence of target RNA and a stem-loop T7 promoter formed by a stem-loop
structure. The reporter DNA probe consists of downstream hybridization sequences that is complementary to the other half part of the target RNA sequence and the region of partial sgRNA that can active Cas14a1 to cleavage FQ probe. Once the two probes hybridize with the target RNA, SplintR ligase ligates the promoter probe to the reporter probe. Consequently, T7 RNA polymerase synthesizes a new RNA utilizing full-length ligated probes as DNA templates that finally activate Cas14a1 to cleavage FQ and produce fluorescence signal. TACAS enables highly sensitive detection of RNA without any pre-amplification steps.

3.2 Feasibility of the TACAS strategy

In this study, TACAS strategy was applied to identify the pathogenic bacteria *Escherichia coli* (*E*.coli). First, to validate all reaction steps of the TACAS strategy for *E*.coli detection, we designed promoter probe and reporter probe that target the *E*.coli 16s rRNA according to the steps mentioned in the previous chapter, and tested the ligation between the two DNA probes. The SplintR ligase was applied to ligate two probes with or without the presence of target RNA via the amplification of the products using PCR primers (Method 2.2). Once the target RNA and two probes mixed and were added into the ligation solution, the corresponding size of the PCR product could be analyzed (Figure 2a). The ligated probe was then applied as the template for the transcription testing. The ligation products were added into a transcription reaction solution containing T7 RNA polymerase and NTPs mix.
The ligation and full transcription only occur in the existence of target RNA. By agarose gel analysis, 98 nt nucleotides transcription products were observed, which confirmed both transcription and target-dependent ligation (Figure 2b). We verified, finally, that the transcription from the ligated probe could generate fluorescence signal upon activation of Cas14a1 (Figure S1 and S2) to cleave the FQ reporter probe. An equivalent amount of RNA (500 ng/mL) from each mixture was added into the Cas14a1 reaction mixture. As results show, only the transcription product RNA that was from the mixture containing two probes, target RNA and the ligation factors generated strong fluorescent signal, while the other mixture produced almost none signal (Figure 2c and S3). Thus, by performing each component reaction via TACAS, we were able to detect the target RNA using a pair of probes.
Figure 2. The feasibility of the TACAS strategy. (a) Gel electrophoresis result of ligation reaction products in the existence of E.coli RNA after PCR confirmation. (b) Gel electrophoresis analysis of ligation and transcription reaction products in the presence of E.coli RNA. (c) Cas14a1 fluorescence analysis of ligation and transcription reaction products in the presence of E.coli RNA. (n=3 technical replicates; bars represent the mean ± s.d.; *P<0.0332, **P<0.0021, ***P<0.0002, ****P<0.0001; Δ represents background subtracted fluorescence).
3.3 TACAS strategy for one-pot assay

After all component reactions of TACAS cascade were validated respectively, we then attempted to combine all reactions with a single reaction buffer at an optimized temperature, where the ligation, transcription and the Cas14a1 activated fluorescence signal output described previously can occur in one-pot solution to simplify the TACAS detection system.

To achieve this, we combined buffer composition suitable for all reaction procedures (including ligation, transcription and fluorescence signal reaction buffers) to configure a single mixture as a basis for the optimization. The reagents were added as described, with a variety of reaction conditions having been optimized. These included the reaction time, temperature, concentrations of the involved enzymes and components to enhance the fluorescence signal. We first investigated a series of factors that affect the enzyme (ligase, T7 polymerase and Cas14a1) reactions directly, including buffer type (PBS, Tris-HCl, and HEPES buffer including 20 nM Na\(^+\)), metal ions (Mg\(^{2+}\), K\(^+\), Zn\(^{2+}\), Li\(^{2+}\) and Fe\(^{2+}\)) and pH of reaction buffer (5.0 to 11.0) (Figure 3a, b and S4a-c). Next, we optimized the concentrations of Cas14a1 and FQ probe which effected on the collateral activity that was vital for TACAS system signal output (Figure 3c, d and S4d, e). Additionally, we sought to figure out the incubation time influence on transcription that produce the amplified RNA to activate Cas14a1-sgRNA complex cleave reaction, and investigated an ideal temperature for all reactions within the applicable temperature range (35-40 °C) for the enzymes involved (Figure 3e, f and S4f-h). Figure 3 and Supplementary S4 suggested that the entire
reaction buffer could be established in Tris-HCl containing 15 nM MgCl$_2$ (pH=9.0). With the optimized detection conditions, *E.coli* RNA could be detected in a one-pot at 37 °C after 2-hour isothermal reaction using 2500 nM Cas14a1 and 1000 nM FQ (Figure 3).

![Figure 3. Reaction condition optimization (a) Metal ion types. (b) Buffer pH. (c) Cas14a1 concentration. (d) FQ probe concentration. (e) Reaction time. (f) Reaction temperature. (n=3 technical replicates; points represent individual measurements; bars represent mean ± s.d.; Δ represents background subtracted fluorescence).](image)

3.4 Specificity and sensitivity of TACAS system

Once the isothermal one-pot reaction condition was established and optimized, we investigated detection specificity and detection limitation of TACAS system. For a further confirmation of stable *E. coli* detection using TACAS strategy, independent replicate
experiments of the *E. coli* detection were also tested. A certain amount of *E. coli* (10^6 CFU/mL) were cultured and RNA extracted to implement six test using TACAS strategy under an identical condition. As shown in Figure 4a and S5, TACAS showed a high reproducibility, with the RSD of six measurements at 7.28%.

To validate the detection specificity, a concentration of five model pathogenic bacteria, including *E. coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus* and *Salmonella typhi*, were cultured in medium LB. The RNA of these bacteria subsequently was extracted and tested using the TACAS system individually or in mixture (Figure S6). We also added these bacteria into fresh blood collected from live healthy tilapia fish with a same concentration (10^8 CFU/mL) and investigated the detection specificity of *E. coli* in blood. All animal experiments have been approved by Animal Care and Use Committee of Hainan University. As Figure 4b showed, high fluorescence signal was only observed in the solution containing *E. coli* RNA. By contrast, low fluorescence signal was observed from the other single bacteria or their mixture without presence of *E. Coli*, which directly indicated that the TACAS sensor provided an excellent detection specificity of *E.coli* from the other bacteria in both LB and blood (Figure S7, 8).

We next assessed the detection sensitivity of TACAS cascade by testing fluorescence signal from the 4 hours reactions respectively containing the *E.coli* in the range of 1
CFU/mL to $10^{10}$ CFU/mL. The detection limitation of *E.coli* RNA reached as low as 1 CFU/mL level *E.coli* where the fluorescence signal was starting to be detected significantly, implying the excellent sensitivity of the TACAS (Figure 4c and S9). In addition, the linearity of the fluorescence intensity with a wide range of concentrations (coefficient of determination ($y = 44518 \ln(x) + 9356.3$, $R^2 = 0.9407$) indicates that TACAS is suitable for the quantification of target RNA (Figure 4d). Collectively, with the 2-hour reaction time, TACAS strategy was capable of specifically detecting the *E.coli* with a detection limit of 1 CFU/mL.
Figure 4. The TACAS as a specific and high sensitive detection tool for *E.coli*. (a) The detection reproducibility of *E.coli* in blood. (b) The detection specificity for the pathogenic bacteria *E.coli* cultured in medium LB or the blood collected from live fish. (c) TACAS detections for serial dilutions of *E.coli*. (d) Limit of detection (LOD) of pathogen detection using TACAS. (n=3 technical replicates; points represent individual measurements; bars represent mean ± s.d.; *P<0.0332, **P<0.0021, ***P<0.0002, ****P<0.0001; Δ represents background subtracted fluorescence).

3.5 Identification of *E.coli* colonization in contrived fish samples

Since we could detect *E.coli* via TACAS strategy in blood, we employed TACAS to detect *E.coli* in real complex samples to provide data of pathogenic bacteria diagnostics for clinical application (Figure 5a). A certain volume (800μL) of *E.coli* washed and suspended by PBS solution (10^{10} CFU/mL) were injected into the pectoral fin of healthy tilapia. We first investigated the *E.coli* infection situation of fish. The blood was collected from fishtail after 6, 12, 24, 48 and 72 hours, and then performed bacteria RNA extraction for the TACAS test and the fluorescence single from results showed that *E.coli* could be obviously detected after 6 hrs and reached stable level at 72 hrs (Figure 5b and S10). We injected 800μL of *E.coli* solution (10^{10} CFU/mL) to twelve healthy tilapia and thereafter collected blood from the 12 injected fish and 6 healthy fish as control after three days. The total 18
samples including 12 positives and 6 negatives were bacteria RNA extracted and detected using TACAS. Results from Figure 5c showed a significant fluorescence signal from positive samples and contrast signal in negative samples, indicating a feasibility of *E.coli* detection in live fish. The result of the Real-time PCR with cDNA from reverse transcription (RT–PCR) for RNA extracted from 9 fish (6 positives and 3 negatives) was well corresponding to the results from TACAS test, corroborating the validity of TACAS applied in fish samples (Figure 5d and Table S3). Additionally, the organs, including heart, liver, intestine, spleen, bile and head-kidney, were collected from random infected tilapia and then frozen using liquid nitrogen immediately. The result from detection for *E.coli* using TACAS method (Figure 5e and S11) indicated that after the *E.coli* injection of organs of fish. Based on these results, it appears that TACAS is a potential powerful tool with high sensitivity and specificity and a simple procedure for clinical microorganism diagnosis.
**Figure 5.** TACAS analysis of *E. coli* in vivo colonization in fishes. (a) Schematic illustration for the contrived *E. coli* infected fish sample preparations. (b) The fluorescence signal intensity detected on different time after *E. coli* injection. (c) A total of 18 fish samples (12 positives and 6 negatives) were diagnosed by the TACAS detection. (d) A total of 9 fish samples (6 positives and 3 negatives) were diagnosed by qPCR. (e) Different organs of *E. coli* injected fish including heart, liver, intestine, spleen, bile and head-kidney were diagnosed using TACAS. (n=3 technical replicates; points represent individual
measurements; bars represent mean ± s.d.; *P<0.0332, **P<0.0021, ***P<0.0002, ****P<0.0001; P: positive, N: negative; Δ represents background subtracted fluorescence)

The most conventional bacteria detection methods lack one or more of the following characteristics: rapidity, simplicity, economy, and sensitivity. Compared with methods based on PCR require a more complex procedure and repeated cooling and heating steps under precise temperature control, TACAS requires only a single fixed temperature instrument such as a water bath or a heat block. Additionally, the effective hybridization between the pair of two functional probes carrying specific sequence that recognize the target RNA and initial the cis/trans-ssDNA cleavage activities of Cas14a1 enables high specificity of the detection and quantifies the concentration of the observed target bacteria. Furthermore, the hybridization sequences were systematically designed via the nucleic acid design system Primer-BLAST, which allows the probe selection simple. In conclude, the TACAS processes prominent potential in food safety, environmental monitoring, and pathogenic bacteria diagnosis with excellent performance.

4. Conclusions

In this study, we reported a one-pot isothermal system for pathogenic bacteria RNA detection named as Transcription-Amplified Cas14a1-Activated Signal Biosensor
TACAS) with high specificity and sensitivity. TACAS method contained two core mechanisms to achieve amplification without PCR process: (1) binding response of target RNA sequence via a pair of functional probes; (2) transcriptional reactions involving full-length ligated probes amplified directly in reaction using T7 RNA polymerase. The output signal can be achieved via the Cas14a1-sgRNA complex cleaving the fluorophore quencher (FQ) reporter once the amplified ligation products were enriched enough. As a result, TACAS enabled highly sensitive detection of target RNA without any pre-amplification steps.

As an isothermal one-pot RNA detection strategy, TACAS provided an excellent performance for easy-to-use detection of pathogenic bacteria E. coli over traditional PCR-based methods that require complicated process and professional knowledge, particularly in resource-limited settings. The lowest detection limit of the E. coli could reach the level of 1 CFU/mL. It also indicated significant fluorescent signal output in detection of E. coli from contrived samples. We believe that the ability of TACAS can rapidly measure many different pathogens as a system for allowing simple manipulation and fewer equipment demands in future.
CRediT authorship contribution statement

Xiao Tan: Experimental, Methodology, Data curation, Writing - review & editing. Xiufen Yang: Experimental, Investigation, Methodology, Data curation, Writing - review & editing. Yuefeng Qiao: Methodology, Formal analysis. Yangdao Wei: Investigation, Methodology. Wenkai Shang: Investigation, Methodology. Huiying Cai: Formal analysis. Xidan Luo: Formal analysis. Hongwei Hou: Conceptualization, Writing – review & editing. Fengge Song: Conceptualization, Methodology, Data curation, Writing – review & editing. Yi Wan: Methodology, Data curation, Writing – review & editing. Jinghong Li: Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online.

References


