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Identifying multiple bacterial pathogens by loop-mediated isothermal amplification on a rotate & react slipchip

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ABSTRACT

A rotate & react SlipChip (RnR-SlipChip) was developed for simultaneous visual detection of multiple bacterial pathogens by loop-mediated isothermal amplification (LAMP). The SlipChip system consists of two round PDMS-glass hybrid chips that are assembled coaxially by a plastic screw-nut suite. After sample loading, one-step rotation allowed immediate mixing and reaction of multiple bacteria samples with LAMP reagents on the chip. A home-built peltier heating device was used to implement the isothermal control by an advanced RISC machines (ARM) processor. Under the optimized LAMP conditions, a fluorescent signal-to-noise ratio of about 5-fold and a detection limit of 7.2 copies/μL genomic DNA were achieved. Furthermore, five common digestive bacterial pathogens including *Bacillus cereus, Escherichia coli, Salmonella enterica, Vibrio fluvialis and Vibrio parahaemolyticus*, were visually identified using the established method in 60 min with a success rate of 100%. The developed RnR-SlipChip provided a rapid, simple-to-use and cost-effective solution for pathogenic bacteria detection, which holds high potential for clinical diagnosis especially in resource-limited areas.

1. Introduction

Many bacteria-induced infectious diseases exhibit similar symptoms, such as common digestive, respiratory and blood-borne ailments. It is difficult to determine the pathogenic bacteria solely based on clinical presentations, resulting in delayed treatment and risk of deterioration of the condition. Thus, rapid and accurate diagnostic method is essential for effectively identifying bacterial pathogens to facilitate medical workers in selecting appropriate treatments. Conventional approaches for detecting bacterial infections include cell culture, immunoassays and polymerase chain reaction (PCR). However, these methods usually rely on the use of sophisticated and bulky equipment, involving multistep procedures and trained personnel, which hamper the widespread applications of these methods especially in resource-limited areas.

Loop-mediated isothermal amplification (LAMP) was first proposed by Notomi in 2000 [1]. Since then, it has been widely employed in one-step isothermal amplification and detection of pathogenic genes with sensitivity and specificity comparable to quantitative PCR and immunoassays [2]. With the *Bst* DNA polymerase and 4∼6 primers specific to the target gene, LAMP allows DNA magnification over billion-fold at 65 °C within 1∼1.5 h and results can be observed through various means, including naked eyes [3,4], fluorescence [5], turbidity [6], colorimetry [7], pH-value [8] and electrochemistry [9]. Thanks to its tolerance to crude samples and critical reaction settings [10], extraction of pathogenic genes or sample pretreatment can be sometimes bypassed for direct LAMP reactions [11]. Furthermore, LAMP offers a higher DNA synthesis rate and a milder reaction condition than PCR [6], which is ideal for rapid point-of-care detections with minimal setups.

Because of its efficiency and simplicity, coupling of LAMP reaction with microfluidic systems opened up a new avenue for developing pathogenic gene detection devices. Previously, a variety of microfluidic methods have been reported for LAMP reactions, demonstrating advantages of microfluidic approaches, such as small reagent consumption, multiplex compatibility, high throughput and capability of miniaturization and integration [12–17]. Among these methods, the SlipChip platform provided an attractive solution, which encodes a program for manipulating fluids into an array of wells and ducts imprinted in two contacted glass plates [18–20]. However, current SlipChip platforms are more suitable for laboratory operations. Innovation on the SlipChip system

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holds high potential for rapid on-site detection and point-of-care diagnosis.

In this study, we described a hybrid rotational SlipChip system, the rotate & react SlipChip (RnR-SlipChip), for rapid bacteria detection by LAMP. The RnR-SlipChip was composed of a pair of round PDMS-glass hybrid chips that were assembled coaxially by a plastic screw-nut suite. After reagents and samples were loaded into the reaction chambers on the chip through inlets and microchannels, one-step rotation allowed immediate mixing and reaction of multiple samples with reagents. The proposed method was evaluated by the mixing of two different food dyes and the quenching reaction of fluorescein by potassium iodide. Results indicated a good linearity ($\gamma = 0.993$) and reproducibility using two different chips (relative standard deviation 4.7%, $n = 6$). A miniaturized peltier heater was custom-built for the on-chip LAMP reactions. After optimization, the optimal LAMP conditions were determined to be 0.125 mM calcein, 1.4 mM dNTPs, 2.4 μM inner primers, 0.4 μM outer primers, 8 U *Bst* 2.0 DNA polymerase, 1 M betaine and 8 mM $MgSO_4$. Under the optimized conditions, a fluorescent signal-to-noise ratio of about 5-fold and a detection limit of 7.2 copies/μL genomic DNA of *Vibrio fluvialis* were achieved. Simultaneous detection of five common digestive bacterial pathogens was further demonstrated in 60 min with a success rate of 100%.

2. Materials and methods

2.1. Chemicals and reagents

Analytical grade chemicals such as ethanol, acetone, isopropanol, diacetone alcohol, hydrogen peroxide, sulfuric acid, hydrochloric acid, sodium hydroxide, sodium tetraborate, potassium iodide, ethylene diamine tetraacetic acid (EDTA) and manganese (II) chloride were purchased from Sinopharm Chemical Reagent (Shanghai, China). Tris-base and lysozyme were purchased from Biosharp Co., Ltd. (Hefei, China). Fluorescein, agar, betaine and Triton X-100 were purchased from Sigma–Aldrich (MO, USA). Agarose G-10 was purchased from Gene Co., Ltd. (Hongkong, China). Proteinase K (recombinant), calcein, uracil-DNA glycosylase (UDG), and five dNTPs including dUTP were purchased from Sangon Biotech (Shanghai, China). All LAMP primers were synthesized by Sangon Biotech (Shanghai, China). Tris-acetate-EDTA buffer premix powder was purchased from Dycent Biotech (Shanghai, China). Gel loading blue dye buffer, 100 bp DNA ladder plus and DNA extraction kit for bacterial genomic DNA were purchased from Dongsheng Biotech (Guangzhou, China). Magnesium sulfate, ThermoPol II buffer and *Bst* 2.0 DNA polymerase were purchased from New England Biolabs (MA, USA). SU-8 (GM 1070) was purchased from Gersteltec Sarl (Switzerland). PGMEA was purchased from JIMCEL Electronic New Material (Suzhou, China) and polydimethylsiloxane (Sylgard 184, PDMS) was purchased from Dow Corning (MI, USA). Aquapel was purchased from PPG Industries (PA, USA). Fluorinated oil (Fluorinert™ Electronic Liquid FC-40) was purchased from 3 M (MN, USA). Water was purified by the Millipore-Q system (Millipore, USA) before use.

2.2. Microchip fabrication

The RnR-SlipChip consisted of two round PDMS-glass hybrid chips as shown in Fig. 1, which were brought together coaxially by a plastic screw-nut suite. In both the upper and the lower chips, the PDMS layer contains microchannels and the glass layer contains the reaction chambers (Fig. S1). The PDMS replica was produced according to the rapid prototyping method as previously reported [21]. Briefly, the SU-8 mold was fabricated on a 3-inch silicon wafer using standard soft lithography technique. The PDMS base and the curing agent was thoroughly mixed in a weight ratio of 10:1 and slowly poured onto the trimethylchlorosilane treated SU-8 mold, which was further degassed for 15 min to get rid of the air bubbles and baked at 85 °C for 2 h. The cured PDMS replica was then gently peeled off from the mold. Twenty inlets and twenty outlets holes (0.8 mm in diameter) were punched though the upper PDMS layer according to the design of the microchip. The glass layer with reaction chambers were fabricated by micromachining on a benchtop computer numerical control (CNC) machine. The drilling patterns were designed with 91 holes including 1 center hole of 3.0 mm in diameter. A G coding program was then generated in the ArtCAM software for automatic CNC micromachining. After the drilling process, the glass substrates were rinsed in acetone ultrasonic bath and thoroughly decontaminated with piranha clean (98% H_2SO_4/30% H_2O_2 = 3:1, v/v). The fabricated PDMS replica and its glass counterpart were treated with oxygen plasma for 1 min and then carefully aligned and irreversibly bonded together under a stereo microscope. The glass surfaces of both the upper and the lower chips were treated with a hydrophobic coating reagent (Aquapel, PPG Industries, USA), and subsequently flushed with N_2 [22]. Both chips were then rinsed thoroughly with ethanol and dehumidified in a vacuum dryer. A screw-nut suite (one 12-mm-length screw, one hexagonal nut and two flat washers) was used to bring the two PDMS-glass hybrid chips together coaxially to form the final device.

2.3. Bacteria maintenance and the preparation of DNA templates

Five common digestive pathogenic bacteria strains were used in this study. *Bacillus cereus* (BC) was donated by the Institute of Environmental Resource & Microbial Technology, Huazhong University of Science and Technology (Wuhan, China). *Escherichia coli* (EC) DH5α was maintained in our own lab. Other three experimental strains including *Salmonella enterica* (SE), *V. fluvialis* (VF) and *Vibrio parahaemolyticus* (VP) were provided by China General Microbiological Culture Collection Center (Beijing, China). All strains were revived after incubation for about 12 h at 37 °C (30 °C for VF), and a single colony was picked up from the agar plate for culturing in liquid culture medium. After incubation for another 18 h, the proliferation of bacteria reached the logarithmic phase. All bacteria genome DNA were then collected and purified from the cultured strains using a rapid DNA extraction kit. The quantities of extracted genomic DNA were calculated using a UV–vis spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA). Detailed information of culturing conditions for these bacteria strains are listed in Table S1.

2.4. Pathogenic gene targets and the corresponding LAMP primers

Five target genes specific to the bacteria strains were selected according to literatures and NCBI nucleotide database (Supplementary information). The five LAMP target gene fragments are given in Table S2. The LAMP primers described previously [23–26], were used for the detection of *B. cereus*, *E. coli*, *S. enterica*, and *V. parahaemolyticus* respectively. The LAMP primers for *V. fluvialis* targeting the toxR gene were designed using PrimerExplorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html). Each set of LAMP primers has a pair of inner primers and a pair of outer primers. The 20 outer and inner primers are shown in Table S3.

2.5. On-chip LAMP reaction

The constitution of the LAMP reaction components was optimized and results were presented in the following section. Typically, LAMP reagent mixture of 25 μL was prepared in tube before use, containing 1× ThermoPol II buffer, 1 M betaine, 8 mM $MgSO_4$, 1.2 mM $MnCl_2$, 0.25 mM calcein, 2.8 mM dNTPs, 0.8 μM