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A microfluidic lab-on-a-disc integrated loop mediated isothermal amplification for foodborne pathogen detection

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ABSTRACT

Salmonellosis is one of the most common foodborne diseases caused by the genus *Salmonella* and is considered to be a significant global health concern. Therefore, the development of effective techniques for rapidly detecting *Salmonella* is fundamental in preventing foodborne outbreaks. This paper presents a lab-on-a-disc integrated loop mediated isothermal amplification (LAMP) on a microfluidic platform for *Salmonella* detection. We have developed a centrifugal microfluidic platform device in which the main steps in pathogen detection, reagent preparation, LAMP, and detection can be integrated onto a single microfluidic compact disc (CD). A forced convection heating source was used for wax valve actuation and temperature heating for the LAMP amplification, leading toward compactness and system miniaturization. The detection limit was 5×10^{-3} ng/μL DNA concentration when tested on tomatoes spiked with *Salmonella*. The whole procedure, from sample preparation (on the microfluidic CD) up to detection, was completed within 70 min using a fully automated process. The final detection step was performed via direct visual observation of the color change of the SYBR Green I dye. Our system offers a fast and automated molecular diagnostic platform, thereby reducing the need for skillful operators and expensive instrumentation. This developed portable device would have a wide range of potential biomedical applications, including foodborne pathogen detection and molecular diagnostics in developing countries.
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1. Introduction

Foodborne illnesses remain the major cause of morbidity and mortality worldwide [1]. The common etiologic agents of foodborne outbreaks are *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Campylobacter* and *Salmonella* [2,3]. *Salmonella enterica* are Gram-negative facultative anaerobes that cause acute gastroenteritis and are responsible for nearly half of foodborne disease outbreaks worldwide [4]. Failure to detect these foodborne pathogens in contaminated food could cause significant losses in the food industry and health sectors [5]. Current conventional methods for detecting and identifying *Salmonella* are mostly based on cell culture and colony counting, which require a minimum of 3–4 days to obtain presumptive results and approximately 7 days to obtain definitive positive results [6]. To overcome these drawbacks, several methods for rapid detection have been introduced [7], such as, (1) Polymerase Chain Reaction (PCR) [8–10], (2) enzyme-linked immuno assay (ELISA) [11] and (3) microarray immunoassays [12–14]. However, immunoassay methods have limited usage because of their low specificity, particularly for *Salmonella* detection [15]. Furthermore, these methods are labor intensive, time consuming, costly for meeting food safety control standards and require bulky as well as sophisticated instruments. All these obstacles have limited the usage of these methods and make rapid detection difficult. Therefore, there is a crucial need for alternative methods that provide rapid, automated, easy and accurate detection of foodborne pathogens. Point-of-care (POC) devices are promising alternatives to rapidly identify pathogens in clinical and food samples, resulting in immediate and accurate diagnoses.

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Microfluidic devices, referred to as "lab-on-a-chip" (LOC), have played a significant role in the analysis and diagnosis of biological, clinical and chemical samples, including foodborne pathogens [16]. The automation of the operation (reduction of manual steps), shortened time-to-result, reduced reagent consumption, low cost, miniaturized size and precise volume control are the key factors that make LOC devices powerful tools in food forensics [17–19]. Therefore, rigorous research has been performed on foodborne pathogen detection using LOC devices [20,21]. The Xingyu Jiang Group has developed a lab-on-a-chip called μLAMP for the detection of Pseudorabies virus by integrating loop mediated isothermal amplification (LAMP) on an 8 channel microfluidic chip [22]. The Reddy Jr Group has introduced a silicon chip for the multiplexed detection of foodborne pathogens utilizing LAMP [23]. Another group has developed a microfluidic CD for the detection of *Salmonella* using recombinase isothermal amplification (RPA) [24]. Although the multiple functions for foodborne pathogen detection, such as DNA extraction, amplification and detection, can be performed on a single microfluidic chip, there is a need for microfluidic control, such as valving and mixing. Thus, lab-on-a-disc platforms are considered stronger contenders than lab-on-a-chip platforms because they eliminate the requirement for tubing and external pumping devices, as they require only a simple electric motor for fluid control [25,26]. Furthermore, the centrifugal force generated from the rotation of the microfluidic CD can be used for microfluidic propulsion [25,27], mixing [27–29], metering [27,30,31], decanting and calibration [27] of samples for diagnosis. Moreover, valving techniques are important for all microfluidic analysis platforms, as samples must be properly reserved until needed [17,27,30,32]. Many biological and biochemical assays requiring multistep processes, such as nucleic acid extraction, amplification and detection, also need valving techniques.

A recent method in nucleic acid detection is isothermal nucleic acid amplification, which generates a large number of target copies, thus significantly increasing assay sensitivity. Several isothermal nucleic acid amplification techniques, such as Nucleic Acid Sequence-Based Amplification (NASBA) [33], Strand Displacement Amplification (SDA) [34], Recombinase Polymerase Amplification (RPA) [35], and Rolling Circle Amplification (RCA) [36], have been used for pathogen detection. Another recent powerful analytical tool for pathogen detection is LAMP, which is the technique used in our study. LAMP is a nucleic acid amplification method that amplifies DNA/RNA under isothermal conditions (temperature between 60–65 °C) with high sensitivity and specificity using a set of six primers and a *Bst* DNA polymerase [37]. LAMP does not need a sophisticated heating block system that requires a high temperature (90–100 °C), as the amplification take place at a constant temperature of 63 °C for 60 min. LAMP method has sensitivity and specificity as the conventional PCR [22]. Some isothermal amplification methods use complicated and time consuming detection techniques, such as a strip lateral flow sensor, which needs to first absorb the liquids, followed by a period of waiting for the results to be confirmed as reported by Kim et al. [24], who developed a microfluidic CD platform using the RPA isothermal technique to detect *Salmonella*. In contrast, LAMP is considered to be a powerful and easy tool for detection process because it requires an easier and faster method when detecting the target. For example, monitoring the turbidity of LAMP reactions resulting from magnesium–pyrophosphate precipitation can be seen with the unaided eye as a white precipitate. For better visualization, SYBR Green I is added to the reaction to visualize the results by monitoring the color change which would give a more confirmed result. Liang et al. [38] developed a closed tube LAMP detection of using a Wax Sealed Fluorescent Intercalator to detect several viruses and pathogens (HBV, 2009H1N1), and SYBR Green I dye was added to this system to provide more definitive results.

In this paper, we present a microfluidic lab-on-a-disc integrated loop mediated isothermal amplification (LAMP) platform for automated foodborne pathogen amplification and detection. This proof-of-concept system demonstrates fully automated centrifugal functions, including pumping, mixing, metering and sealing integrated with LAMP to detect a foodborne pathogen target with higher sensitivity and specificity. The practical effectiveness of the system was validated through the detection of *Salmonella*, a major foodborne pathogen, in spiked tomato samples. Thus, reduction of manual steps and manpower, shortened time-to-result, low cost, portable and miniaturized size (miniaturize all laboratory equipment into a single microfluidic CD) will make our microfluidic CD more useful and practical.

2. Materials and methods

2.1. Cell culture and DNA preparation

A strain of confirmed *Salmonella* Enteritidis previously isolated from food samples was used for method development. The DNA was extracted using boiling method and a 2.5×10^{-3} ng/μL was used in each LAMP reaction (for more details about the extraction of the DNA, please refer to the "Supplementary material").

To determine the specificity of the assay, the experiment was carried out with DNA from *Salmonella* and non-*Salmonella* strains (*E. coli*, *Shigella sonnei*, *Listeria monocytogenes*, *Vibrio cholerae*, *Yersiniae* spp. and *Acinetobacter baumannii*). A 2.5×10^{-3} ng/μL DNA concentration from *Salmonella* and non-*Salmonella* strains was used for each LAMP reaction (pre-loaded to the amplification chambers).

To determine the sensitivity of the assay, a 10-fold serial dilution of DNA extracted from a tomato spiked with *Salmonella* was used. A 2.5×10^{-3} ng/μL DNA concentration was also used in this method (pre-loaded to each amplification chamber, more details will be discussed in Section 3). The results obtained were compared with the conventional LAMP method and PCR assay. Each assay was repeated at least twice to ensure reproducibility. For more details about extraction of the DNA from *Salmonella* spiked tomato, please refer to "Supplementary material".

2.2. Reagents and sample

A set of 6 primers was designed (unpublished work) using the LAMP Primer Explorer V4 software to specifically target *Salmonella* in the food sample. The primer set consists of 2 outer primers (F3 and B3), 2 inner primers (FIP and BIP) and 2 loop primers (LF and LB). All primers were commercially synthesized. One reaction of LAMP was performed in a total volume of 25 μL composed of 12.5 μL reaction mixture, 1 μL *Bst* DNA polymerase (Eiken Chemical Co., Ltd., Tokyo, Japan), 2 μL deionized water, 2.5 μL crude DNA extracted as discussed in previous section, 2 μL each of F3 and B3, 0.5 μL each of FIP and BIP and 1 μL each of LB and LF. The LAMP reaction was set at 63 °C for 60 min prior to the inactivation of *Bst* polymerase for 2 min at 80 °C.

2.3. Design and fabrication of the microfluidic CD

A microfluidic CD for automated *Salmonella* detection was designed using a computer aided design software (AutoCAD), as shown in Fig. 1. The microfluidic CD consists of top and bottom polymethyl methacrylate (PMMA) layers bonded with a pressure-sensitive adhesive (PSA) material, as shown in Fig. 1A. The microfluidic CD operations were engraved in the PMMA bottom layer using a Computer Numerical Control (CNC) machine as shown in Fig. 1B. The microfluidic CD operations and features in the PSA layer were cut using a cutter plotter machine. The microfluidic

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