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A microdevice for rapid, monoplex and colorimetric detection of foodborne pathogens using a centrifugal microfluidic platform

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ABSTRACT

Outbreaks of foodborne diseases have become a global health concern; hence, many improvements and developments have been made to reduce the risk of food contamination. We developed a centrifugal microfluidic automatic wireless endpoint detection system integrated with loop mediated isothermal amplification (LAMP) for monoplex pathogen detection. Six identical sets were designed on the microfluidic compact disc (CD) to perform 30 genetic analyses of three different species of foodborne pathogens. The consecutive loading, mixing, and aliquoting of the LAMP primers/reagents and DNA sample solutions were accomplished using an optimized square-wave microchannel, metering chambers and revulsion per minute (RPM) control. We tested 24 strains of pathogenic bacteria (*Escherichia coli*, *Salmonella* spp *and Vibrio cholerae*), with 8 strains of each bacterium, and performed DNA amplification on the microfluidic CD for 60 min. Then, the amplicons of the LAMP reaction were detected using the calcein colorimetric method and further analysed via the developed electronic system interfaced with Bluetooth wireless technology to transmit the results to a smartphone. The system showed a limit of detection (LOD) of 3×10^{-5} ng μL^{-1} DNA by analysing the colour change when tested with chicken meat spiked with the three pathogenic bacteria. Since the entire process was performed in a fully automated way and was easy to use, our microdevice is suitable for point-of-care (POC) testing with high simplicity, providing affordability and accessibility even to poor, resource-limited settings.

1. Introduction

Foodborne disease remains a threat to human health worldwide. According to the WHO (2015), 600 million foodborne diseases were caused by 31 types of foodborne bacteria and 420,000 deaths in 2010 worldwide. The transmission of foodborne pathogens to humans is via the oral-faecal route when contaminated food is consumed. Therefore, early detection of foodborne pathogens is of the utmost importance (Velusamy et al., 2010). Such a challenge and demand have provided a caveat for researchers to develop innovative point-of-care (POC) techniques for foodborne pathogen detection using precision, rapidity and user friendliness (Foudeh et al., 2012).

Molecular diagnostic methods are essential to clearly confirm a pathogen's identity. Recently, isothermal DNA amplification methods have replaced conventional polymerase chain reaction (PCR) to make POC diagnosis possible due to their simplified setting (Asiello and Baeumner, 2011; Craw and Balachandran, 2012; Mori and Notomi, 2009). Isothermal amplification techniques have the advantage that the target gene can be amplified at a constant temperature using specifically designed primers, strand-displacing DNA polymerases, recombination enzymes, etc. with high sensitivity and specificity. LAMP is a nucleic acid amplification method that proceeds at a constant temperature between 60 and 65 °C by amplifying the DNA, which leads to high sensitivity and specificity. LAMP DNA amplification uses a set of six primers and a *Bst* DNA polymerase and takes 30–60 min to complete (Notomi et al., 2000). Furthermore, a diversity of detection methods for LAMP products (amplicons) have been developed including fluorescence monitoring using calcein (Tomita et al., 2008),

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electrochemical detection (Hsieh et al., 2012), and colorimetric detection using SYBR Green 1 (Sayad et al., 2016). Several recent studies have shown the suitability of LAMP integration with a POC diagnostic lab-on-a-chip technology (Dou et al., 2014; Fang et al., 2010; Lee et al., 2016; Liu et al., 2011; Luo et al., 2014).

Centrifugal microfluidic lab-on-a-disc (LOD) is the most recent innovative tool in the microfluidic field (Madou et al., 2006). Centrifugal microdevices have the shape of a compact disc (CD). These devices offer a combination of microfluidic unit operations, such as liquid mixing, metering, aliquoting, switching, valving and storage, which are controlled by the rotational speed of the disc (Gorkin et al., 2010; Mark et al., 2010). Centrifugal microfluidic devices have been developed for many applications due to their versatile and simple design and operation. These applications include immunoassay analysis (Park et al., 2012), blood analysis (Nwankire et al., 2014), cell analysis (Burger et al., 2015), nanoparticle synthesis (Park et al., 2015) and molecular diagnostics (Focke et al., 2010a, 2010b; Keller et al., 2015).

Recently, research on molecular pathogen detection using centrifugal microfluidic platforms has been reported. The works varied, and some showed the whole analysis process including nucleic acid extraction, DNA amplification and detection on a single disc, while in others DNA extraction procedures were performed off chip. Kim et al. (2014) developed a fully centrifugal microfluidic system integrated with recombinase polymerase amplification (RPA) for *Salmonella enterica* analysis utilizing a single laser diode for ferrowax valve actuation and DNA amplification. The novelty of the system is that it can perform a complete automated process from DNA extraction to the final detection in one single platform. The disadvantage of this system is that the isothermal amplification could start at any point if the reagents are mixed due to the low cardinal temperature of the RPA (37 °C), which is close to room temperature. To overcome this problem, Oh et al. (2016) have improved the platform by detecting 3 pathogens and replaced the RPA amplification with LAMP amplification, which began at only 65 °C. In addition, this technique performs multiplex pathogen detection for 25 tests at once compared to work by Kim et al. (2014) in which 4 tests were performed at once. The drawback of this system is that DNA extraction and LAMP amplification were performed using different platforms, which limits the potential use as point-of-care device. Most researchers are trying to have one complete system (platform) covering DNA extraction to the final detection, but the application varies. Roy et al. (2015) have developed a sample-to-answer centrifugal microfluidic system for nucleic acid analysis of *Bacillus atrophaeus* spores using cell lysis, PCR, amplicon digestion and analysis with a microarray in the same platform. Again, the isothermal amplification technique used in this method was an issue compared to Kim et al. (2014) and Oh et al. (2016) as PCR operates at different temperature ranges and takes a longer time. Jung et al. (2015). presented a centrifugal microdevice for H1N1 analysis using microbead-assisted RNA purification, reverse transcription LAMP and real-time optical monitoring as the detection method. Czilwik et al. (2015) reported a fully automated LOD system for pathogen detection that used the DNA extraction, consensus multiplex PCR pre-amplification and geometrically multiplexed species-specific real-time PCR. Again, most researchers have been competing to have a complete system (platform) from DNA extraction to final detection (Kim et al., 2014; Roy et al., 2015; Czilwik et al., 2015) and have neglected the isothermal amplification technique used in such systems. Others have been investigating the other features of the platform, such as having more tests, multiplicity, and simplicity more than taking the isothermal amplification method into consideration as Oh et al. (2016) did. Overall, all these systems have demonstrated high-performance DNA detection except it is difficult to find a system having all features in one platform. Therefore, it is recommended to have the maximum number of tests, the best isothermal amplification technique embedded in the same platform, simplicity, and multiplicity and should be a time-saving technique in one automated microfluidic lap-on-a-disc platform.

Herein, we present a microfluidic LOD device integrated with LAMP, which can conduct rapid foodborne pathogen detection identifying three types of pathogens. The microfluidic CD device demonstrated a fully microfluidic operations and functions including pumping, mixing, metering, amplification and detection on a single microfluidic disc unit on the same platform overcoming most of the drawbacks reported above. A simple colorimetric detection method using calcein dye is implemented in this study for visual detection rather than lateral strip flow as by Kim et al. (2014), which is time consuming or as by Jung et al. (2015), which used complicated optical system for detection. Furthermore, the amplicons are further analysed by an electronic digital system for accurate, confirmed analysis of results utilizing the emitted florescence from calcein and displays the results on a mobile phone, which, to our best of knowledge, no such technique exists. Consequently, reduction of manpower, sample-to-answer capability, low cost, portable, and miniaturized size will make the microfluidic CD more valuable.

2. Materials and methods

2.1. Cell culture and sample preparation

A total of 24 foodborne bacterial strains (*Salmonella* spp, n = 8; *Vibrio cholerae* n = 8); *E. coli* (n = 8) were used for the method development. Crude DNA from these bacterial strains were prepared off chip using the boiling method and the concentration of DNA was measured by the spectrophotometer at 260 nm. Approximately 3×10^{-3} ng/μL DNA concentration was used in each LAMP reaction for *Salmonella*, *Vibrio cholerae* and *E. coli* (refer to the Supplementary material for DNA extraction details, section 1.1). The detection limit of the LAMP assay based on the microdevice was determined using a 10-fold serial dilution of DNA extracted from the chicken meat (refer to the Supplementary material for the extraction steps) spiked with *Salmonella* spp, *Vibrio cholerae* and *E. coli*. The specificity test of the assay-based microdevice was carried out with DNA from *Salmonella* spp, *E. coli* and *Vibrio cholerae* strains and other bacterial spp (*Shigella sonnei*, *Listeria monocytogenes, Yersiniae enterocoliticus* and *Acinetobacter baumannii*). The DNA was pre-loaded to the microdevice in all steps (to the amplification chambers, more details will be discussed in Section 3). The microdevice results were validated and compared to the results obtained using the conventional LAMP method and PCR assay. Each assay was repeated at least twice to ensure reproducibility.

2.2. LAMP assay and colorimetric detection

Four types of LAMP primers were designed for each target pathogen using Primer Explorer V4 software (http://www.primerexplorer.jp/e/). All primers were commercially synthesized by a commercial company. One individual LAMP reaction contained a total volume of 12.5 μL of each target pathogen and was set at 63 °C for 60 min prior to the inactivation of *Bst* polymerase for 2 min at 80 °C.

Calcein was used as an indicator and results can be visualized directly by the change in colour. It is a synthetic fluorescein that emits a bright fluorescence that causes colour change. In visible light, the positive calcein is indicated by a yellow to green colour while negative sample retains a light orange colour. This happens as the amplification of the target gene. The calcein was mixed with the LAMP mixture during sample preparation. Then, to quantitate the degree of colour change, the amplicons were analysed by the designed electronic system (see Section 2.4).